Elucidation of Cellular Mechanisms That Regulate the Sustained Contraction and Relaxation of the Mammalian Iris

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Purpose. In mammals, pupil constriction and dilation form the pupillary light reflex (PLR), which is mediated by both brain-regulated (parasympathetic) and local iris-driven reflexes. To better understand the cellular mechanisms that regulate pupil physiological dynamics via central and local photoreception, we have examined the regulation of the PLR via parasympathetic and local activation, respectively.

Methods. In this study, the PLR was examined in mouse enucleated eyes ex vivo in real-time under different ionic conditions in response to acetylcholine and/or blue light (480 nm). The use of pupillometry recordings captured the relaxation, contraction, and pupil escape (redilation) processes for 10 minutes up to 1 hour.

Results. Among others, our results show that ryanodine receptor channels are the main driver for iridal stimulation–contraction coupling, in which extracellular influx of Ca^{2+} is required for amplification of pupil constriction. Both local and parasympathetic iridal activations are necessary, but not sufficient for sustained pupil constriction. Moreover, the degree of membrane potential repolarization in the dark is correlated with the latency and velocity of iridal constriction. Furthermore, pupil escape is driven by membrane potential hyperpolarization where voltage-gated potassium channels play a crucial role.

Conclusions. Together, this study presents new mechanisms regulating synchronized pupil dilation and contraction, sustained pupil constriction, iridal stimulation-contraction coupling, and pupil escape.

Keywords: iris, pupil light reflex, acetylcholine, melanopsin, mouse

The pupillary light reflex (PLR) describes the photoactivation of pupil constriction and subsequent dilation in response to darkness, which both serve as a major determination of retinal image quality by regulating the intensity and scattering of light that reaches the retina.[1,2] The understanding of the neural circuits mediating the PLR has advanced significantly over recent years; however, the local mechanisms in the iridal sphincter smooth muscle that regulate a sustained pupil constriction versus dilation have not been fully determined.

Traditionally, it was thought that pupil constriction was solely a centrally mediated reflex, driven by rods and cones, but also intrinsically photosensitive retinal ganglion cells (pRGCs)[3–5] that express the G protein-coupled photopigment melanopsin (encoded by the *Opn4* gene).[6–8] Upon light exposure, the activation of rods, cones, and pRGCs trigger a signaling cascade that ultimately reaches the olivary pretectal nuclei (OPN) in the brain.[9,10] From the OPN, efferent pathway projections end up in ciliary post-ganglionic fibers that release acetylcholine (ACh), which activates the sphincter muscle of the iris, thus inducing pupil constriction.[9,10] However, irises that are intrinsically (i.e. locally) photosensitive have been reported in many species, including mammals, indicating a conserved mechanism.[11] In the mouse, the local PLR requires the activation of melanopsin,[12] whereas in fish and amphibians it appears to be mediated by the rod photopigment,[13,14] and in birds, cryptochromes have been implicated as the PLR mediator.[15] Thus, the PLR can be regulated by both a brain-regulated and a local iris-driven reflex. Nonetheless, the cellular mechanisms that regulate pupil constriction via central and local photoreceptive processes remain elusive.

Dilation of the pupil occurs through two integrated processes: (i) the parasympathetic innervation of the iris sphincter muscle, which is suppressed by supranuclear inhibition via central sympathetic neurons, that results in iridal relaxation,[16] and (ii) the excitation of the α_1-adrenergic-mediated sympathetic pathway, which is followed by contraction of the iris dilator muscle.[16,17] The dynamics of pupil dilation depend on both the brightness and duration of light, where dilation proceeds more slowly after prolonged exposure to bright light compared with dim light and/or a short duration of light stimulation.[11]

Both relaxation and contraction of mammalian cardiomyocytes, skeletal, and smooth muscles, like the iris, are driven by changes in the concentration of intracellular calcium ions $[Ca^{2+}]_i$. These changes involve the flux of Ca^{2+} ions between intra- and extracellular spaces, as well as intracellular stores that consist of mainly the sarcoplasmic reticulum (SR) and Ca^{2+} storage in mitochondria.[18,19] In addi-

tion, gap junctions (via connexins) coordinate cytosolic Ca^{2+} flux between nearby muscle cells. The processes of depolarization and repolarization/hyperpolarization, which regulate changes in the membrane potential via different concentration gradients and flux of potassium (K^+) and sodium (Na^+) ions, are also crucial for muscle cell function.[20] Determining the physiological dynamic mechanisms that modulate both relaxation and contraction in the iris sphincter muscle will also be important for understanding muscle cell kinetics in general.

In this study, physiological mechanisms regulating iris sphincter muscle relaxation and contraction were analysed ex vivo in mouse eye preparations in the absence of sympathetic innervation of the dilator muscle. The most important results observed were (i) Ca^{2+} efflux is the major mechanism that regulates pupil dilation, in addition to the contribution of the gap junctions for faster relaxation; (ii) ryanodine receptor (RyR) channels are the main driver for the stimulation–contraction coupling process, with the extracellular influx of Ca^{2+} being required for amplification of pupil constriction; (iii) the degree of membrane potential repolarization in the dark is correlated with the latency and velocity of iridal constriction; (iv) both melanopsin stimulation and ACh activation are necessary, but not sufficent for sustained pupil constriction; and (v) pupil escape (redilation) is driven by membrane potential hyperpolarization.

Methods

Mouse Eyes

Fresh mouse (*Mus musculus*) eyes from adult littermates of BL/6 wild-type animals, aged between 3 and 6 months, were used. All animals were kept under a strict photoentrainment cycle of 12 hours:12 hours light:dark (L:D) before harvesting the eyes for pupillometry studies and immunohistochemistry analyses. Animal studies were approved by the Institutional Animal Care and Use Committee of Umeå University (Dnr A38-19) and conducted in accordance with the guidelines for the care, good conduct, and use of laboratory animals.

Dissection and Preparation of Eyes

Eyes from photoentrained adult mice were rapidly enucleated in the light phase. Essentially as described before,[15] enucleated eyes were immobilized and positioned in a small (4 cm) petri dish in Tyrode's solution (134 mM NaCl, 3 mM KCl, 20 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 12 mM glucose; and pH 6.4) using acrylic super glue (Loctite), and dark-adapted for 1 hour, 2 hours, or overnight (ON; approximately 12 hours; see Supplementary Fig. S1).

Pupillometry of Isolated Eyes

Video recordings of the pupil diameter were conducted in real-time under dim light illumination, determined to be at a subthreshold level not triggering iridal contraction, using a Nikon SMZ25 stereomicroscope coupled to an Andor Zyla4.2P monochrome microscope camera (Supplementary Movie S1 and Supplementary Movie S2). Irradiance levels were measured using a PM400 Optical Power and Energy Meter (Thorlabs) and normalized to dark conditions. NIS-Elements BR-5.02 software was used to stimulate light sources and start recordings simultaneously. Stimulation of the eyes with blue light at 480 nm (herein referred to as 480 nm light) was conducted via a pE-300^{ultra} Illumination System (CoolLED) and the use of an ET480/20X (470 to 490 nm) narrow single bandwidth filter (Chroma). The 480 ± 10 nm light stimulus had a corrected irradiance of 49.78 ± 0.15 W/s/m2, which equated to a photon flux of $1.2 \times 10^{20} \pm 3.7 \times 10^{17}$ photons/s/m^2 at the level of the eye/pupil. The same intensity of 480 nm light was used throughout the study.

Iridal contraction was induced by 480 nm light stimulation and/or ACh activation. Recordings of the pupil diameter were analyzed by NIS-Elements BR-5.02 software and the equatorial diameter of the iris was measured using the same software. The pupil size was measured at different time points during various conditions and normalized using the equation: Pupil diameter during stimulation divided by the unstimulated pupil diameter, where the unstimulated and full constricted pupil normalized values were taken as 1 (or 100%) or 0 (or 0%), respectively, and multiplied by 100 to determine percentages. The maximal contraction was determined before the escape phase was initiated. All experiments were performed at room temperature (RT), with light stimuli varying from 5 minutes to 1 hour.

Pharmacological Agents and Solutions

Pharmacological agents used in the pupillometry recordings are listed in Supplementary Table S1. To confirm that the concentrations of the pharmacological agents used in this study were not toxic to the eye preparations, the pharmacological agents were washed away after recordings, and after ON dark incubation the iris contraction in response to 480 nm light or ACh were verified. The very few eye preparations that did not exhibit an iris contraction after washing away factors were not included in the study. In Ca^{2+}- and Na^+-free experiments, Ca^{2+} free Tyrode's solution (134 mM NaCl, 22 mM NaHCO3, 3 mM KCl, 1 mM MgCl2, 12 mM glucose, 1 mM EGTA; and pH 6.5) or Na^+ free Tyrode's solution (240 mM sucrose, 5 mM KHCO3, 3 mM KCl, 3 mM CaCl2, 1 mM MgCl2, 12 mM glucose, 1 mM EGTA; and pH 6.5) was used.

Immunohistochemistry

Fresh eyes were pierced in the posterior segment and fixed for 2 hours at 4 deg Celsius (°C) in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Gradual cryoprotection of the eyes in 3.25% to 25% sucrose in PB at 4°C was performed before embedding in NEG-50 (Cellab) and cryosectioning at 20 µm. Immunohistochemistry was performed using standard protocols,[21] including blocking with 10% fetal calf serum before primary antibody incubation for 48 hours at 4°C. Antibodies used in this study are listed in Supplementary Table S2, together with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) to counterstain nuclei. Slides were mounted with fluorescent mounting medium (Agilent Technologies, Santa Clara, CA, USA).

Imaging and Statistics

The pupillometry curves were created using GraphPad Prism 8.3 software (GraphPad Software, Inc., La Jolla, CA, USA). The graphs represent the mean ± the standard error of the mean (SEM). Student´s *t*-tests were used to determine significance and P values of < 0.001 were accepted as being statistically significant. Images were generated using a fully automated Nikon A1 confocal microscope,

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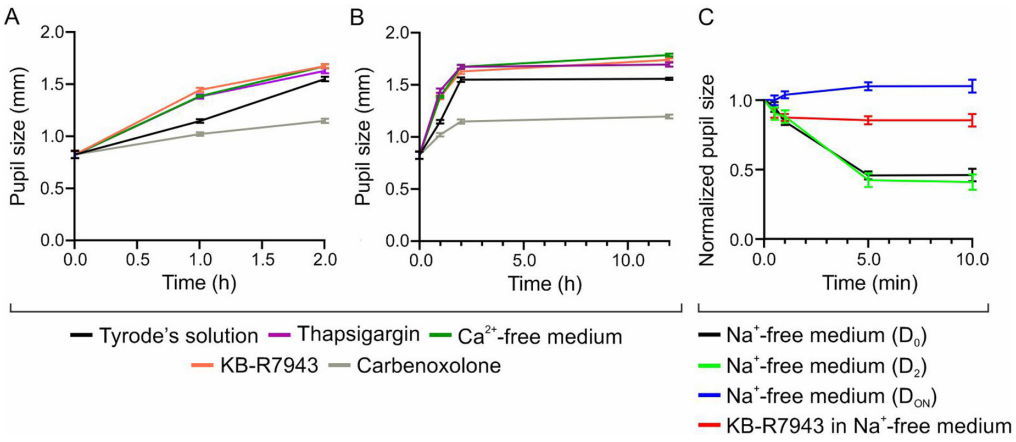

Figure 1. The role of Ca^{2+} efflux and Na^{+} influx in pupil dilation. **(A, B)** Measured pupil size of enucleated mice eyes at 0 hours, and after 1 hour, 2 hours **(A)**, and ON (12 hours) **(B)** of dark incubation incubated in Tyrode's solution alone or in addition to thapsigargin, carbenoxolone, KB-R7943, and Ca^{2+}-free medium. **(C)** Normalized pupil sizes in D_0, D_2, and D_{ON} enucleated mice eyes incubated in Na^{+}-free medium alone, and D_0 eyes in Na^{+}-free medium with KB-R7943. Data plotted as the mean ± SEM ($n = 5$). D_0, before dark adaptation; D_2, 2 hours in the dark; D_{ON}, overnight in the dark.

paired with NIS-Elements C for simultaneous epifluorescence/differential interference contrast (DIC) observations. Images were subsequently processed with Photoshop CC 2019 (Adobe, San Jose, CA, USA).

Results

Dark Adaptation is Required to Produce Repeatedly Strong Iris Contraction

To investigate pupil physiological dynamics at a local level, both iridal contraction and relaxation were studied in response to dark conditions, 480 nm (blue) light and/or ACh, in relation to specific ion channels (See Supplementary Fig. S2 for a schematic summary). Pupil dilation and contraction were studied ex vivo under different conditions in enucleated eyes from adult (3–6 months old) mice kept in a strict L:D cycle of 12 hours:12 hours. Mice were euthanized during the light phase and enucleated eyes were collected. To avoid any possible effect of stored noradrenaline in the sympathetic nerve endings that could affect the iridal dilator muscle, all eyes were incubated in Tyrode's solution in the presence of an α_1-adrenoreceptor antagonist (Tamsulosin, 10 mM) in the dark at RT. The health and stability of the eye preparations were verified by observing a continually functional iris in response to a series of light flashes/exposures at 480 nm, with both short (15 minutes) and long (6–24 hours) recovery times in the dark in between cycles of light/dark periods (Supplementary Fig. S3). It should be noted that a functional PLR was observed even after 36 hours of incubation (see Supplementary Fig. S2B). The results also revealed that iris responses declined with the number of light exposures in relation to the time spent in the dark (see Supplementary Fig. S2). Thus, a longer duration in the dark is required to produce repeatedly strong contractions in response to 480 nm light.

Calcium Efflux Plays a Significant Role During Pupil Dilation

The importance of dark adaptation for iris contractions prompted us to address pupil dilation during the transition from light to dark conditions. The size of the pupils from freshly enucleated eyes (i.e. no dark incubation [D_0]) was 0.82 ± 0.04 mm (Figs. 1A, 1B). After 1 hour in the dark (D_1), the pupil size increased to 1.15 ± 0.02 mm and after 2 hours (D_2) in the dark 1.55 ± 0.02 mm (Figs. 1A, 1B), which corresponded to the average size of fully dilated pupils in vivo after dark adaptation of similarly aged mice.[22,23] Consistently, dark incubation for 12 hours (i.e overnight; D_{ON}) did not increase the pupil size (1.55 ± 0.01 mm) of enucleated eyes when compared with the pupil size at D_2 ($P > 0.05$; Fig. 1A).

To define the mechanisms contributing to low intracellular calcium levels [Ca^{2+}_i] that is required for the relaxation of the iris in the dark,[24,25] eyes were first incubated in Ca^{2+}-free Tyrode's solution to inhibit any extracellular Ca^{2+} influx. During these conditions, a faster and increased relaxation of the iridal sphincter muscle was observed, resulting in a more dilated pupil after D_1 (1.4 ± 0.02 mm), D_2 (1.67 ± 0.018 mm), and D_{ON} (1.78 ± 0.012 mm; Figs. 1A, 1B). Ca^{2+} influx is increased by, among others, the reverse mode of the Na^{+}/Ca^{2+}-exchanger (NCX).[26] Consistently, a similar faster relaxation was observed when enucleated eyes were incubated with KB-R7943 (100 µM), an NCX reverse mode inhibitor to block Ca^{2+} influx (D_1, 1.4 ± 0.022 mm; D_2, 1.66 ± 0.023 mm; and D_{ON}, 1.73 ± 0.016 mm; Figs. 1A, 1B). Next, blocking the uptake of Ca^{2+} from the cytosol to the intracellular SR was studied by the addition of thapsigargin (30 µM) during dark incubation. Thapsigargin inhibits the specific SR Ca^{2+}-ATPase pump (i.e. Serca), and the use of this compound resulted in a more rapid relaxation during the first hour ($P < 0.001$), reaching a larger pupil size after D_1 (1.44 ± 0.02 mm), D_2 (1.67 ± 0.018 mm), and D_{ON} (1.69 ± 0.021 mm) compared with controls ($P = 0.0025$ and $P < 0.001$, respectively; Figs. 1A, 1B). These results indicate that relaxation of the iridal sphincter muscle is dependent on lowering the [$Ca^{2+}]_i$, in which cytosolic efflux mechanisms appears to play a more important role than uptake of Ca^{2+} to the intracellular stores.

Inhibition of the reverse mode of NCX resulted in faster pupil relaxation, which prompted studies of stimulating the reverse mode action of NCX by using Na^{+}-free Tyrode's solution (Fig. 1C). At D_0 and D_2, the change to Na^{+}-free

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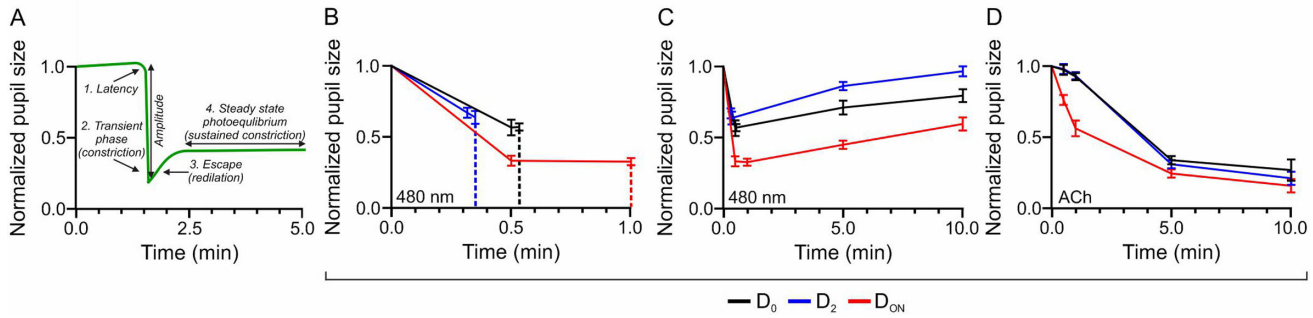

Figure 2. The pupil stimulus response (PSR) ex vivo at different time points of dark adaptation. **(A)** Schematic representation of an in vivo pupil light response (PLR) conserved between human and rodents. **(B, C)** Normalized pupil sizes in response to 480 nm light stimulation over 1 minute **(B)** and 10 minutes **(C)** of enucleated eyes dark adapted for different time points; D_0, D_2, and D_{ON}. **(D)** Normalized pupil sizes in D_0, D_2, and D_{ON} enucleated eyes in response to ACh (100 mM) measured over 10 minutes. Data plotted as the mean ± SEM (n = 5). D_0, before dark adaptation; D_2, 2 hours in the dark; D_{ON}, overnight in the dark; ACh, acetylcholine.

medium triggered pupil constriction, which reached a minimal normalized size of approximately 0.45 after 5 minutes (Fig. 1C), which remained constricted after 10 minutes (Fig. 1C). Consistently, by blocking the reverse mode of NCX with KB-R7943 (50 µM) at D_0, the use of Na^+-free medium failed to induce a significant pupil constriction after 10 minutes (0.85 ± 0.045 mm; Fig. 1C). By contrast, when Na^+-free medium was added to D_{ON} eyes, no pupil constriction was observed (Fig. 1C), suggesting a lower level of intracellular Na^+ compared to that present at D_0. Together, these data suggest that $[Na^+]_i$ is higher during light exposure promoting the activity of the NCX reverse mode and increasing the force of contraction, whereas in the dark $[Na^+]_i$ is lower, which shifts the NCX exchanger to its forward mode for additional Ca^{2+} removal.

To determine the role of gap junctions in the relaxation of the iris sphincter muscle, the presence of connexin 43 (Cx43) in the iris was confirmed by immunohistochemistry, together with smooth muscle α-actin 1 (Sma1; Supplementary Figs. S4A, S4B). Next, gap junction activity was inhibited by the addition of carbenoxolone (200 µM) to enucleated eyes during dark incubation ON. This treatment resulted in significantly slower relaxation in the first 1 to 2 hours (1.2 ± 0.02 mm) compared to the control (1.55 ± 0.01 mm; P < 0.001; Figs. 1A, 1B) and was sustained after up to 12 hours. Taken together, these data suggest that relaxation of the iridal sphincter muscle is dependent upon the removal of cytosolic Ca^{2+}, primarly via cellular efflux mechanisms that are mediated by gap junctions.

Latency, Velocity, and Amplitude of Contraction are Correlated with the Timing of Dark Adaptation

Iridal contraction is regulated by the central brain-mediated parasymphathetic pathway, via ACh as the activator, and by a locally mediated iridal response where melanopsin is stimulated by blue light. Consistently, and in agreement with previous findings,[27,28] immunohistochemistry confirmed that the mouse iris expresses both the muscarinic ACh receptor M3 (Chrm3) and melanopsin (Opn4m; Supplementary Figs. S4C, S4D). To analyze the pupil stimulus response (PSR), an ex vivo real-time pupillometry assay was used, where enucleated eyes were exposed to blue light at 480 nm (i.e. the λ_{max} of mouse Opn4m),[29,30] with a photon flux of 1.2×10^{20} photons/s/m^2 at the level of the eye/pupil, or ACh (1 mM, 10 mM, or 100 mM). Specifically, four phases in the PSR were studied: (i) the latency phase; (ii) constriction velocity and amplitude during the transient phase; (iii) escape (redilation); and (iv) sustained constriction (Fig. 2A).

At D_0, when eyes were freshly enucleated during the light phase, stimulation with continuous 480 nm light induced pupil constriction with a velocity of 18 ± 3 µm/s during the first 30 seconds (Figs. 2B, 2C). During this time interval, the pupil reached a minimal size of 57 ± 5% compared to the unstimulated iris (Figs. 2B, 2C). At 32 seconds, the stimulated pupil started to escape to reach 79 ± 4% compared to the control pupil after 10 minutes (Fig. 2C). In D_2 eyes, 480 nm light induced a faster constriction velocity (23 ± 6 µm/s) compared to D_0, reaching a similar minimal size of 67 ± 3% (P = 0.15) already at 19 seconds compared to 30 seconds in D_0 eyes (Fig. 2B). Moreover, pupil constriction started to escape earlier (after 21 seconds) to reach a baseline size (96 ± 3.5% compared with controls) after 10 minutes (Figs. 2B, 2C). By contrast, eyes that were dark-adapted ON and stimulated with 480 nmlight resulted in faster pupil constriction (37 ± 5 µm/s) that reached a minimal size of 33 ± 4% after 30 seconds (Fig. 2B), which was significantly smaller when compared to the contracted iris in both the D_0 and D_2 eyes (P < 0.0001 for both comparisons). The pupil maintained its contraction up to 60 seconds, before it started to slowly dilate, thus indicating that both the transient time and the amplitude differed between the D_0, D_2, and D_{ON} (Figs. 2B, 2C). After 10 minutes, the stimulated pupil of D_{ON} reached 59 ± 4% of the size of the unstimulated pupil, which was still significantly smaller compared to the D_0 (P < 0.0014) and D_2 (P < 0.0001) contracted iris at 10 minutes (Fig. 2B). No differences in latency were observed between D_0, D_2, or D_{ON} dark incubated enucleated eyes stimulated with 480 nm light (Figs. 2B, 2C). These data show that increased dark incubation increases both the velocity and amplitude of iridal contraction in response to 480 nm light and that dark incubation ON delays the escape phase.

To evaluate the role of ACh activation in pupil constriction, concentrations of 1 mM to 100 mM of ACh were tested, where 100 mM of ACh was observed to induce the strongest contraction of D_2 eyes (Supplementary Fig. S5). As such, 100 mM ACh was used throughout all subsequent experiments. Pupil constriction in response to 100 mM ACh was investigated in enucleated eyes at D_0, D_2, and D_{ON}. In D_0 and D_2 eyes, a latency of approximately 1 minute was observed

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before ACh triggered iridal contraction, compared to D_{ON} where contraction latency only lasted for a few seconds in response to the addition of ACh (Fig. 2D). Thus, when activating with ACh, pupil constriction latency is inversely correlated with the dark incubation time. In addition, pupil constriction was faster in D_{ON} eyes (12 ± 4 µm/s) compared to either D_0 or D_2 eyes (1.96 ± 0.4 µm/s; Fig. 2D). In all three settings (D_0, D_2, and D_{ON} eyes), a similar minimal pupil size of on average 16 ± 4% was reached at 5 minutes, which remained constricted at 10 minutes (Fig. 2D). Collectively, compared to no or 2 hours of dark incubation, incubation in the dark ON results in a faster and more transient amplitude of pupil constriction in response to either 480 nm light or ACh.

The 480 nm Light-Induced and ACh-Induced Contractions Require Membrane Potential Repolarization

Based on the above results, the following ex vivo experiments were conducted using eyes that were dark-adapted ON in combination with different pharmacological agents (see Supplementary Table S1) and/or ionic conditions. As the contraction of many mammalian smooth muscle cells are sensitive to membrane depolarization,[31,32] the impact of repolarization for iridal contraction was studied.

The Na^+/K^+-ATPase assists in generating the cellular resting potential by maintaining an intracellular low concentration of Na^+ $[Na^+]_i$ and high K^+ $[K^+]_i$, which are crucial for muscle relaxation and subsequent contraction.[33,34] To determine the contribution of Na^+/K^+-ATPase activity to pupil constriction, ouabain (100 µM), which suppress Na^+ efflux and K^+ influx, was added to Tyrode's solution before dark incubation ON. During these conditions, the normalized pupil sizes were similar to the control conditions (i.e. ON dark incubation in Tyrode's solution; $P = 0.12$). Subsequently, eyes were exposed to either 480 nm light or ACh. Under these conditions, 480 nm light induced a reduced pupil constriction that reached a minimal size of 58 ± 5.5% after 1 minute, compared with 33 ± 3.5% in controls after 30 seconds ($P = 0.0042$; Figs. 3A, 3B). Moreover, the inhibition of the Na^+/K^+-pump resulted in a complete escape to the baseline pupil size compared to the control eye after 10 minutes (Fig. 3B). In addition, increasing the extracellular concentration of K^+ ($[K^+]_o$) to 20 mM during dark incubation ON resulted in a slower contraction rate that reached a minimal size (55 ± 5.5%) after 5 minutes, followed by a continuous escape recorded up to 10 minutes (67 ± 5.5%; Figs. 3A, 3B). These results show that 480 nm light-induced iridal contration is sensitive to changes in the membrane potential.

Next, the role of the Na^+/K^+-pump in ACh-induced contraction was examined. Inhibition of Na^+/K^+-ATPase by ouabain during dark incubation ON, followed by stimulation with ACh, caused an increase in latency for 1 minute before pupil constriction was initiated (Figs. 3C, 3D). Furthermore, iridal contraction in the presence of ouabain was slower than control irises (Figs. 3C, 3D) and did not reach a similar minimal pupil size (19 ± 3.5%) when compared with control irises (15 ± 4.5%; $P = 0.51$) until after 20 minutes (see Supplementary Fig. S5). An increase in $[K^+]_o$ had a similar effect on latency, like ouabain, followed by slow iridal contraction (Figs. 3C, 3D). Collectively, it appears that iridal contraction induced by either 480 nm light or ACh both require repolarization of the membrane potential, where Na^+/K^+-ATPase seems to play a crucial role in maintaining the resting transmembranous gradient of Na^+ and K^+ necessary for subsequent propagation of Ca^{2+} waves and synchronized contraction.

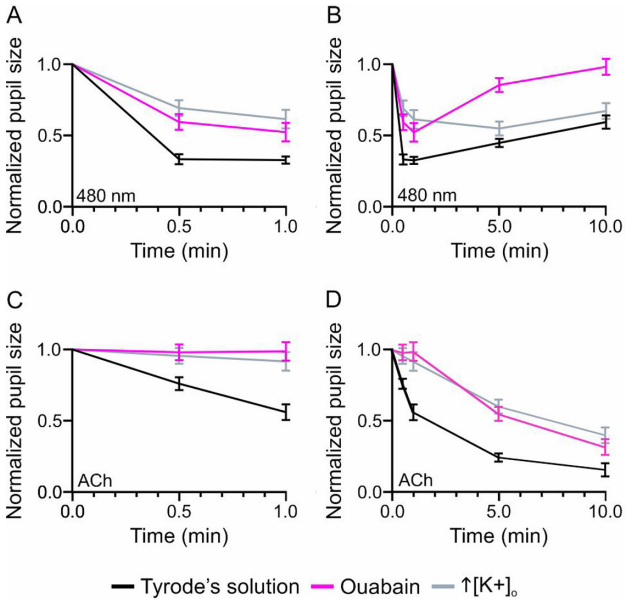

Figure 3. The contribution of the membrane potential to the PSR. **(A–D)** Normalized pupil sizes of enucleated eyes in response to 480 nm light stimulation over 1 minute **(A)** and 10 minutes **(B)**, or to ACh exposure over 1 minute **(C)** and 10 minutes **(D)**, after ON incubation in Tyrode's solution alone or together with ouabain or increased $[K^+]$, respectively. Data plotted as the mean ± SEM (n = 5). ACh, acetylcholine; ON, overnight; PSR, pupil stimulus response.

The 480 nm Light-Induced and ACh-Induced Contraction is Dependent on Similar Ca^{2+} Dynamics

To evaluate the role of specific ion channels in relation to Ca^{2+} homeostasis during iridal contraction, the inositol-1,4,5-triphosphate receptor (Itpr1, also known as IP_3R) and the RyR, two important channels for releasing Ca^{2+} from intracellular SR stores,[35,36] were studied. IP_3R and RyR were inhibited by using 2-aminoethoxydiphenylborate (2-APB and 1 mM) and 4-chloro-3-ethylphenol (4-CP and 1 mM), respectively. Each inhibitor was individually added 1 hour before 480 nm light stimulation of D_{ON} enucleated eyes, followed by 10-minute recordings. Compared with controls, iridal contraction was almost completely inhibited when either IP3R or RyR activity was suppressed (Fig. 4A). These results implicate that the release of Ca^{2+} from intracellular SR stores is required for iridal contraction, and that both IP_3R and RyR channels play crucial roles for this process.

To analyze the contribution of the influx of $[Ca^{2+}]_o$ to iridal contraction, L-type voltage-gated calcium channels (L-VGCCs) were blocked by the addition of verapamil (300 µM) 1 hour before 480 nm light stimulation. A significant decrease in iridal contraction was observed compared with control conditions ($P < 0.001$), reaching a pupil size that was 75 ± 5.5% compared with the unstimulated iris at approximately 30 seconds (Figs. 4B, 4C). Incubation ON in Ca^{2+}-free media, or changing to Ca^{2+}-free media 10 minutes before 480 nm light stimulation, resulted in similar constriction curves

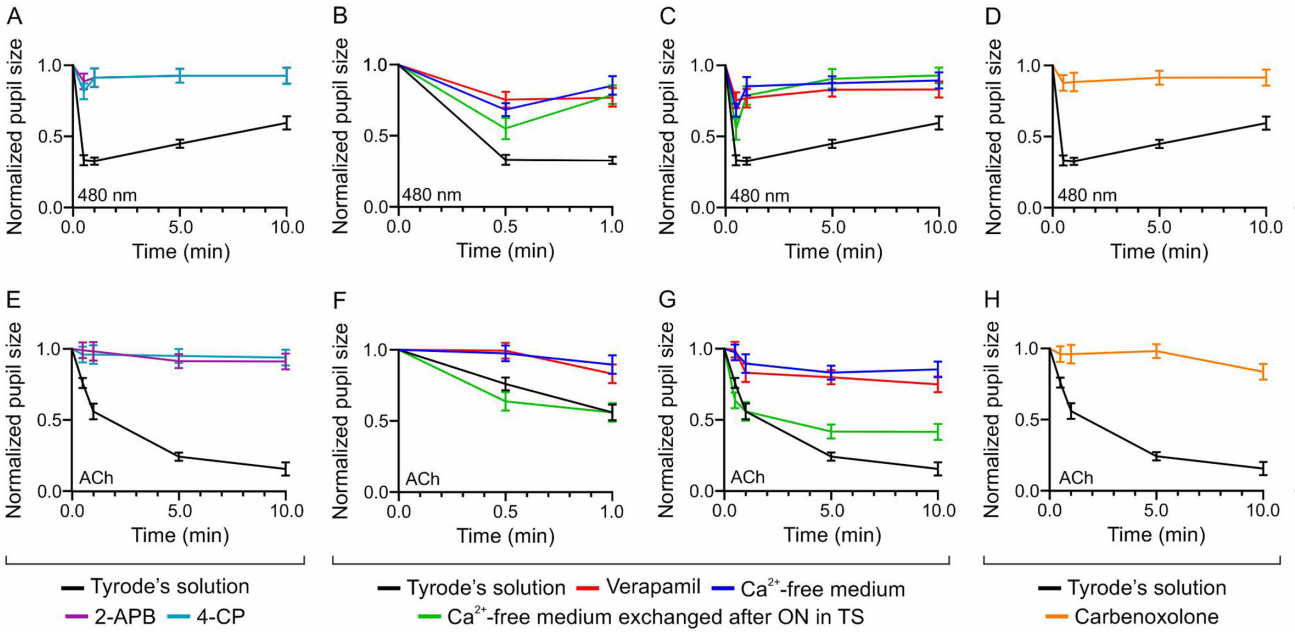

Figure 4. Determination of the Ca^{2+} dynamics that mediate the pupil stimulus response. **(A–E)** Normalized pupil sizes of enucleated eyes in response to 480 nm light stimulation **(A)** and ACh activation **(E)** after incubation ON in Tyrode's solution alone, or together with 2-APB (added 1 hour before stimulation), 4-CP (added 1 hour before stimulation), over 10 minutes. **(B, C, F, G)** Normalized pupil sizes in response to 480 nm light exposure **(B, C)** and ACh activation **(G, F)** after incubation ON in Tyrode's solution alone, or together with verapamil (added 1 hour before stimulation), Ca^{2+}-free medium (incubated ON), and Ca^{2+}-free medium (exchanged 1 hour before stimulation) over 1 min **(B, F)** and 10 minutes **(C, G)**. **(D, H)** Normalized pupil sizes in response to 480 nm light exposure **(D)** and ACh activation **(H)** after incubation ON in Tyrode's solution alone, or in addition to carbenoxolone (added 1 hour before stimulation) over 10 minutes. Data plotted as the mean ± SEM ($n = 5$). ACh, acetylcholine; ON, overnight; PSR, pupil stimulus response.

($P > 0.05$) with the minimal pupil size reaching 68 ± 5% and 55 ± 4% of the unstimulated iris at approximately 30 seconds, respectively (Figs. 4B, 4C). These data indicate that the influx of extracellular Ca^{2+} to the iris sphincter muscle also plays a significant role in iridal contraction.

To determine if ACh-induced iridal contraction is regulated in a similar manner as with melanopsin-dependent stimulation by 480 nm light, the release of Ca^{2+} from the SR was inhibited by 2-APB (1 mM) or 4-CP (1 mM) to inhibit IP_3R or RyR, respectively. When either IP_3R or RyR were blocked, ACh did not induce iridal contraction and the pupil size remained unaffected (Fig. 4E). Morover, blocking the influx of extracellular Ca^{2+} by verapamil via the L-VGCCs also inhibited iridal contraction, where a minimal pupil size of only 75 ± 5.5% compared to an unstimulated control eye was reached at 10 minutes (Figs. 4F, 4G). Similarly, D_{ON} eyes in Ca^{2+}-free Tyrode's solution exhibited a large reduction in iridal contraction when compared with controls, reaching 85 ± 5% of the size of an unstimulated iris after 1 minute ($P < 0.001$; Figs. 4F, 4G), and did not undergo further contraction over the following 10 minutes of incubation (Fig. 4G). Nevertheless, when Ca^{2+}-free medium was exchanged after incubation ON with Tyrode's solution containing 2 mM Ca^{2+}, ACh induced a reduced contraction that reached a minimal pupil size of 41 ± 4.5% after 5 minutes, which remained unaltered for 10 minutes compared to the continuous contraction observed in the control (Figs. 4E, 4G). Next, the importance of gap junctions in synchronizing iridal contraction was investigated by using the gap junction inhibitor carbenoxolone (200 µM). When carbenoxolone was added to D_{ON} eyes 1 hour before exposure to either 480 nm light or ACh activation, iridal contractions were clearly suppressed, in which the pupil size reached 87 ± 5.5% (with 480 nm light) and 83 ± 6% (with ACh) after 10 minutes (Figs. 4D, 4H). Together these results indicate that both the influx of $[Ca^{2+}]_o$ and Ca^{2+} release from the SR play synergistic roles for iridal contraction in response to 480 nm light and ACh. Moreover, gap junction-coupling is required to synchronize all sphincter muscle cells to contract as a single functional unit.

ACh-Induced and 480 nm Light-Induced Contractions are Mediated by the Same Intracellular Ca^{2+} Compartments

The observation that the iris contracted significantly ex vivo in response to 480 nm light, which reduced the pupil size up to 70% of its full constriction after approximately 30 seconds compared to an 85% constriction after 10 minutes in response to ACh (Figs. 4B, 4C, 4F, 4G), prompted further evaluation of how the local and central iridal dynamic pathways act together.

Simultaneous exposure to both ACh and 480 nm light to D_{ON} eyes induced a similar contraction as with 480 nm light alone in the first transient phase, but instead of escaping after approximately 1 minute, the pupil continued to constrict to reach a similar size as was observed with ACh alone (Fig. 5A). This result indicates an overlapping, but not a cumulative effect, when 480 nm light and ACh acted together. To determine the cause of pupil escape observed during 480 nm light stimulation, a regime of prestimulation with 480 nm light for 10 minutes before ACh activation was performed. Results showed that 480 nm light prestimulation did not abolish the response to ACh, which induced pupil constriction to reach a minimal size of 152 ± 21 µm (Fig. 5B).

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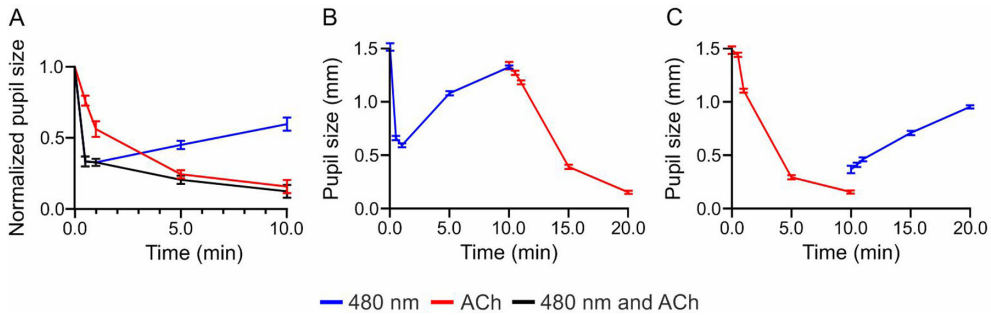

Figure 5. Intrinsic 480 light-induced and ACh-induced iridal contractions use the same intracellular Ca^{2+} compartments. **(A)** Normalized pupil sizes of ON dark-adapted enucleated eyes in response to separate 480 nm light exposure and ACh activation, and both stimuli added simultaneously over 10 minutes. **(B, C)** Sequential pupil dynamics in response to 480 nm light exposure for 10 minutes, followed by ACh activation for 10 minutes **(B)**, or in response to ACh exposure for 10 minutes, followed by 480 nm light exposure for 10 minutes **(C)**. Data plotted as the mean ± SEM ($n = 5$). ACh, acetylcholine; ON, overnight.

Together, this suggests that pupil escape during 480 nm light stimulation is not related to depletion of Ca^{2+} from SR stores.

To distinguish whether 480 nm light and ACh activate release of Ca^{2+} from the same or different intracellular stores, the opposite regime was adopted (i.e. pretreatment with ACh for 10 minutes before 480 nm light stimulation). The pretreatment with ACh abolished the normal pupil constriction effect of 480 nm light stimulation, and instead a pupil dilation was observed (Fig. 5C). Together, these results suggest that both 480 nm light and ACh induce Ca^{2+} release from the same SR compartments.

RyR Channels are Necessary for the Iridal Contraction-Coupling Process

To address if the Ca^{2+} intracellular receptors, IP_3Rs, and/or RyR channels, are essential for iridal contraction, experiments were conducted using caffeine. Caffeine sensitizes RyR channels to cytosolic Ca^{2+}, which locks them in an open state and thereby induces contraction in some smooth muscles.[37–40] On the other hand, caffeine can also induce relaxation in other smooth muscle types through the inhibition of IP_3Rs and reduced Ca^{2+} release.[41,42] To examine whether caffeine induces contraction or relaxation of the iridal sphincter muscle, eyes were incubated with caffeine at D_0, where $[Ca^{2+}]_i$ is high, and after dark incubation ON, where $[Ca^{2+}]_i$ is low. When caffeine (10 mM) was added at D_0, a latency period of 30 seconds was observed, which was followed by a faster rate of iridal contraction that reached a normalized pupil size of 30 ± 4.5% after 10 minutes (Figs. 6A, 6B). The addition of caffeine in D_{ON} eyes, with low $[Ca^{2+}]_i$, did not induce contraction during the first 10 minutes (Figs. 6A, 6B). Only after 20 minutes was a significant decrease in pupil size detected (85 ± 2.5%), which continued to decrease to 41 ± 4.5% after 1 hour (Fig. 6B), suggesting that caffeine-induced iridal contraction is sensitive to $[Ca^{2+}]_i$.

To analyze the contribution of extracellular Ca^{2+}, caffeine was added at D_0 in Ca^{2+}-free medium. A latency period was observed for 1 minute, followed by iridal contraction (67 ± 2.9%) up to 5 minutes, and with no further change up to and after 10 minutes (Figs. 6C, 6D). By contrast, D_{ON} eyes in Ca^{2+}-free medium, followed by the addition of caffeine, did not contract even after 1 hour (Fig. 6D). These results indicate that caffeine-induced contraction is mediated by intracellular stores and that this Ca^{2+} release is sensitive to the influx of extracellular Ca^{2+}.

Finally, to study whether caffeine induces iridal contraction via activation of RyR channels and the subsequent release of Ca^{2+} from the SR, the RyR inhibitor 4-CP (1 mM) was added to D_0 eyes 10 minutes prior to adding caffeine. Under these conditions, caffeine was unable to induce a significant contraction (Fig. 6E). Together, these data indicate that RyR channels are the main mediators of iridal contraction in response to caffeine and that Ca^{2+} influx influences channel open state probability.

The 480 nm Light and ACh Stimulation is Necessary, but not Sufficent for Sustained Pupil Constriction

To understand pupil dynamics under more physiological conditions, where exposure to light is prolonged, continuous subjection to 480 nm light and/or ACh for 30 minutes was studied. Enucleated D_{ON} eyes that were stimulated with 480 nm light for 30 minutes exhibited a continuous escape from approximately 1 minute until 30 minutes, when the pupil size reached 67 ± 2.5% compared to an unstimulated iris at D_{ON} (Fig. 7A). When incubated with ACh for 30 minutes, pupil escape was also observed, but only after 10 minutes to reach a pupil size of 50 ± 3.6% after 30 minutes (Fig. 7A). A similar pattern was observed with simultaneous exposure to 480 nm light and ACh, where the pupil size was 57 ± 3% after 30 minutes (Fig. 7A). Taken together, these results indicate that neither continuous high concentration of ACh nor 480 nm light exposure, or a combination of ACh and 480 nm light, are sufficient to maintain full pupil constriction under longer exposure periods.

Pupil Escape is Driven by Membrane Potential Hyperpolarization

Presently, the molecular mechanisms that mediate pupil escape in the presence of various stimuli are unclear. However, it is known that K^+ currents, also called spontaneous transient outward currents (STOCs), play an important role in the relaxation of different smooth muscles.[43–47] Two channels that affect the membrane potential are the large conductance Ca^{2+}-activated potassium channel (BKCa), where K^+ efflux can be inhibited by iberiotoxin,[48,49]

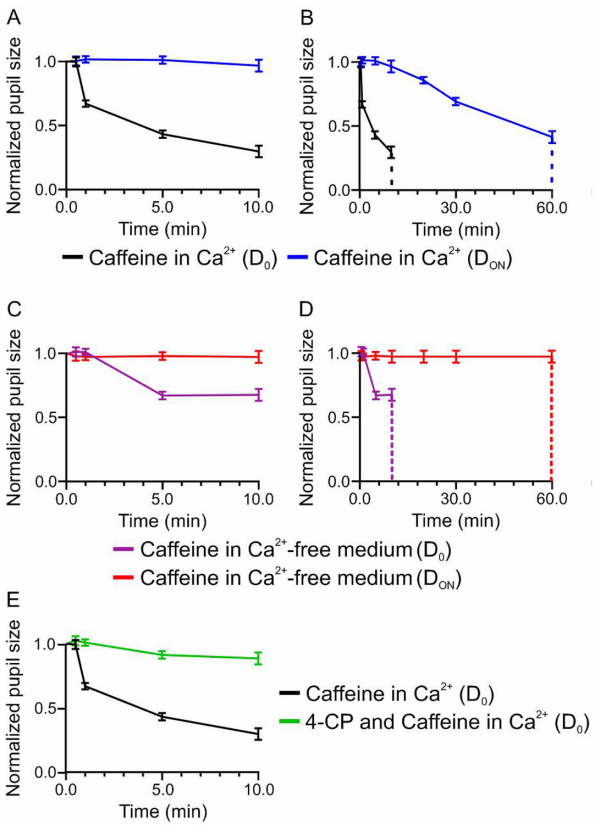

Figure 6. The contribution of RyR channels to pupil constriction. **(A, B)** Normalized pupil sizes of D_0 and D_{ON} enucleated eyes in response to caffeine in Tyrode's solution over 10 minutes **(A)** and 60 minutes **(B)**. **(C, D)** Normalized pupil sizes of D_0 enucleated eyes in response to caffeine in Ca^{2+}-free medium, and of D_{ON} enucleated eyes exposed to Ca^{2+}-free medium after ON dark incubation, over 10 minutes **(C)** and 60 minutes **(D)**. **(E)** Normalized pupil responses of D_0 enucleated eyes to caffeine in Tyrode's solution alone, or in Tyrode's solution with 4-CP for 10 minutes before caffeine was added. Data plotted as the mean ± SEM ($n = 5$). D_0, before dark adaptation; D_{ON}, overnight in the dark; ON, overnight.

and the voltage-gated potassium channels (K_v) that are inhibited by 4-aminopyridine (4-AP).[44,49]

To examine the contribution of BKCa and K_v to pupil escape, D_{ON} enucleated eyes were incubated with either iberiotoxin or 4-AP for 1 hour before 480 nm light stimulation or ACh activation. Iberiotoxin did not show an effect, but whether this was due to insufficient drug penetration cannot be excluded. When adding 4-AP (1 mM) 1 hour before stimulation with 480 nm light, the pupil constriction and escape progression curve was similar to the control eyes for the first 10 minutes (Fig. 7B). However, at 20 minutes, eyes treated with 4-AP started to diverge from controls ($P = 0.0063$) and reached a significantly smaller pupil size of 55 ± 3% compared with controls (82 ± 4.5%) at 30 minutes ($P = 0.0014$; Fig. 7B). Furthermore, when 4-AP (1 mM) was added 1 hour before ACh activation, the latency of ACh-induced iridal contraction was reduced (Fig. 7C). After 10 minutes, instead of detecting pupil escape, a sustained pupil constriction was observed that continued and reached 10 ± 2.5% at 20 minutes, which was followed by a small escape reaching 23 ± 3% after 30 minutes (Fig. 7C). This redilation was significantly smaller compared with eyes activated by ACh (50 ± 3%; $P = 0.002$; Fig. 7C). Thus, K_v channels that are important for hyperpolarization of the iris muscle appear to be crucial for pupil escape.

The Replenishment of Intracellular Ca^{2+} Stores During Prolonged Stimulation is Independent of Ca^{2+} Influx

During prolonged exposure to stimuli, intracellular Ca^{2+} stores would be depleted if they were not replenished through a combination of extracellular influx and intracellular uptake. To examine this, Ca^{2+}-free medium was added to D_{ON} eyes just before ACh activation for 60 minutes. Under these conditions, ACh induced iridal contraction, but only 60% of the full pupillary effect observed in D_{ON} eyes in control conditions with 2 mM Ca^{2+} (Fig. 7D). Furthermore, no significant escape was observed after 10 minutes and up to 1 hour of incubation (Fig. 7D). A similar pattern was observed when caffeine was added to D_0 eyes in Ca^{2+}-free medium, where pupil constriction reached 67 ± 3% after 5 minutes, with no change after 10 minutes (Fig. 7E). Moreover, after incubation in Ca^{2+}-free medium with caffeine ON, the pupil partially dilated to 76 ± 5%, but did not reach baseline levels (Fig. 7F). Finally, the addition of caffeine to D_0 eyes in control Tyrode's solution containing Ca^{2+} resulted in a similar iridal contraction profile as that observed in D_0 eyes in Ca^{2+}-free medium, although pupil constriction reached 30 ± 4.5% after 10 minutes with no change ON (Fig. 7F). Together, these results indicate that the influx of extracellular Ca^{2+} is not required for SR replenishment, but primarily plays a role in amplifying iridal contraction. Furthermore, it appears that the uptake of intracellular Ca^{2+} is sufficient for SR replenishment.

Discussion

Calcium [Ca^{2+}] is ubiquitously used as an intracellular second messenger signaling molecule that regulates many fundamental cellular processes, but it has become increasingly apparent that Ca^{2+} signaling is far more complex than first thought. This study is the first ex vivo investigation to highlight Ca^{2+} signaling mechanisms under different ionic conditions in the iris sphincter muscle, as one type of smooth muscle cells (SMCs) in mammals. Specifically, this study assayed the PLR as a functional output to explore Ca^{2+} signaling during both iridal contraction and subsequent redilation at both cellular and physiological levels. In particular, ACh activation and exposure to blue light (at 480 nm) were used to investigate centrally mediated and intrinsically mediated pupil reflex dynamics.

It is accepted that in the dark, eyes require a certain refactory time period before they respond to subsequent light stimulation, so called photoreceptor adaptation, which increases the electroretinogram amplitude and modulates PLR kinetics.[50] Our results show that dark adaptation, in addition to regulatory mechanisms that are centrally mediated by the retina, relies on the repolarization of the iris sphincter muscle through the Na^+/K^+-pump by maintaining the resting membrane potential, thus priming SMCs to respond to subsequent light stimulation. Restoring the resting negative membrane potential leads to inhibition of Ca^{2+} influx and a decrease in $[Ca^{2+}]_i$. Our results show that the cytosolic efflux mechanisms appears to play a more important role than uptake of Ca^{2+} to the intracellular stores to

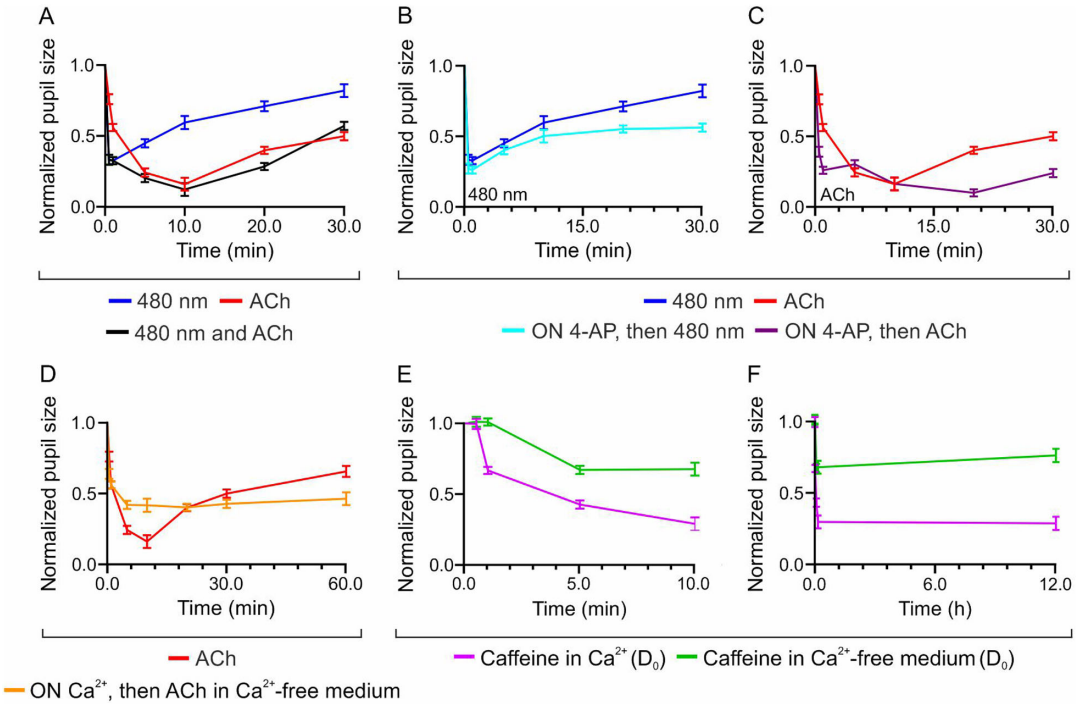

Figure 7. Pupil responses to prolonged exposure to ACh and 480 nm light. **(A)** Normalized pupil sizes of D_{ON} enucleated eyes in response to separate 480 nm light exposure and ACh activation, or given simultaneously, over 30 minutes. **(B, C)** Normalized pupil sizes of D_{ON} enucleated eyes in response to 480 nm light exposure **(B)** and ACh activation **(C)** in Tyrode's solution alone, or with 4-AP (added 1 hour before stimulation) over 30 minutes. **(D)** Normalized pupil sizes of D_{ON} enucleated eyes in Tyrode's solution in response to ACh stimulation only, or in combination with a switch to Ca^{2+}-free medium over 60 minutes. **(E, F)** Normalized pupil sizes of D_0 enucleated eyes in response to caffeine in Tyrode's solution, or in Ca^{2+}-free medium over 10 minutes **(E)** and ON **(F)**. Data plotted as the mean ± SEM (n = 5). D_0, before dark adaptation; D_{ON}, overnight in the dark; ACh, acetylcholine; ON, overnight.

lower the $[Ca^{2+}]_i$. This is supported by previous findings, in which either blocking intracellular Ca^{2+} uptake or removal of extracellular Ca^{2+} diminished iris contraction in response to 436 nm light.[12] During dark conditions, the restored resting negative membrane potential and decrease in $[Ca^{2+}]_i$ has a major impact on RyR channel activity. As a consequence, RyR channel sensitivity to cytosolic Ca^{2+} decreases, which results in RyR channel closure and efflux of cytosolic Ca^{2+} via the plasma membrane Ca^{2+}-ATPase (PMCA) and NCX forward mode,[24,25] which leads to relaxation of the iris sphincter muscle and replenishment of intracellular Ca^{2+} stores for subsequent stimulation. Thus, a possible explanation for a faster muscle relaxation in response to thapsigargin is that the blocking of sarco-ER calcium ATPase (SERCA) leads to decreased levels of Ca^{2+} in the intracellular stores, and thereby a decrease in RyR firing due to negative luminal feedback.[51] By consequence, the Ca^{2+}-calmodulin interaction, which stimulates contractile activity,[52] decreases and the relaxation of the myofilament complex is faster than in absence of thapsigargin. In this study, repolarization of the membrane potential was shown to have a direct impact on both the latency and transient phase amplitude of pupil constriction in mammals. These data suggest that dark adaptation also functions at the level of the pupil sphincter muscle itself, as well as via adaptation of retinal photoreceptors. Collectively, the photoresponse of both classical retinal photoreceptors and pupil sphincter muscles are sensitive to the duration of the dark period.

Compared with some types of SMCs, where membrane depolarization permits extracellular Ca^{2+} to flood into the cell and induce muscle contraction (e.g. bladder, uterus, and the vas deferens),[43] the contraction of the murine iris sphincter muscle is more similar to the SMCs of the vascular, lymphatic, and respiratory systems, as well as the corpus cavernosum, where muscle contraction depends on the periodic release of Ca^{2+} from internal cellular stores.[43] Two reports from the laboratory of King-Wai Yau have shown that in the iris sphincter muscle of mice, both ACh and melanopsin signal primarily through $G\alpha_{q/11}$, $PLC_{\beta 2}/PLC_{\beta 4}$, and IP_3R1 to elicit an increase in cytosolic Ca^{2+} and induce subsequent pupil constriction.[12,28] By blocking IP_3Rs and RyR channels, our data now show that the influx of Ca^{2+} alone is not sufficient to induce iridal contraction. This may be because cytoplasmic Ca^{2+} buffer capacity reduces contractile activation,[53,54] and the influx of Ca^{2+} plays a role in the steady release of Ca^{2+} from intracellular stores, a process that is similar to that determined for vascular SMCs.[53,55] RyR channels open when the concentration of local cytosolic Ca^{2+} increases, via the opening of IP_3Rs, a process known as Ca^{2+}-induced Ca^{2+} release (CICR).[56] Consistently, our data using 4-CP and caffeine, to block IP_3R mediated signaling and lock activated RyR channels in an open state, show that IP_3Rs does not play a direct role in iridal contraction, but predominantly functions to activate adjacent RyR channels that drive stimulation–contraction coupling. These results extend previous observations in the

Itpr1$^{-/-}$ mouse model, where the loss of IP_3R1 (encoded by the *Itpr1* gene) did not result in iridal contraction in response to either 436 nm (blue) light or ACh.[28] By contrast, RyR channels in other SMCs do not participate in IP_3R-mediated contraction.[57–61]

Our results indicate clear differences in the dynamics of pupil constriction in response to caffeine under Ca^{2+}-free conditions, in which freshly dissected eyes exhibited a slow, but distinct iridal contraction. By contrast, dark adaptation ON suppressed contraction of the iris smooth muscle. These results suggest that RyR channels are sensistive to the membrane potential, via the influx of Ca^{2+}, and that the open probability of these channels is increased when the membrane is depolarized to maintain a steady release of Ca^{2+}. Moreover, these data show that the influx of Ca^{2+} is not the major factor that replenishes iridal SR stores, as indicated by sustained pupil constriction during stimulation with either ACh or caffeine in Ca^{2+}-free medium, respectively. Furthermore, our results suggest that the turnover of intracellular Ca^{2+} predominantly contributes to the replenishment of Ca^{2+} in the SR. Similar observations have been reported in a mouse respiratory SMC study in cardiac mycocytes.[62]

In the wild, it is thought that the PLR is mediated by two pathways: (i) an initial transient, but rapid closure of the pupil that is orchestrated by rods and cones under dim or bright light intensities, respectively; and (ii) a maximal and sustained iris constriction that occurs under prolonged exposure to high intensities of illumination via pRGCs that compensate for the photobleaching of rods and cones due to the expression of bistabile melanopsin in a small subset of RGCs.[11] Thus, it has been postulated that melanopsin bistability underpins the continuous depolarization of pRGCs, which results in constant firing of olivary pretectal neurons and sustained pupil constriction.[63,64] This suggests that sustained iridal constriction is caused by the repeated depolarization of parasympathetic varicosities and the continuous secretion of ACh to maintain a minimal pupil size. These studies, however, only examined sustained pupil constriction with light exposure for up to 5 minutes, which does not reflect physiological conditions in the wild. We now provide strong evidence that neither prolonged stimulation with high concentrations of ACh nor photoactivation of iridal sphincter muscle cells that express endogenous melanopsin, seperately or acting together, are sufficient to mantain pupil constriction over a long time period. Moreover, our results indicate that membrane potential hyperpolarization mediates pupil escape as observed during prolonged exposure to ACh and/or 480 nm light. The membrane potential of the iridal sphincter muscle, like that of vascular myocytes,[47] is a major determinant of muscle tone, as it controls the magnitude of steady-state Ca^{2+} influx across the membrane through voltage-gated Ca^{2+} channels. However, persistent high levels of Ca^{2+} in the cytoplasm can be toxic to the cell, causing cell death through both necrosis and apoptosis.[25] Regarding the PLR, such effects might cause pupil closure and prevent light from reaching the retina and the loss of vital visual information. To prevent such cellular stress, sphincter muscles, like other types of SMCs, rapidly restore the basal resting concentration of cytosolic Ca^{2+} by inhibiting a sustained high level of cytosolic Ca^{2+} that relies on either individual spontaneous transient inward Ca^{2+}-activated chloride (Cl^-) currents (STICs) and outward (K^+) currents (STOCs) or mixed Cl^-/K^+ currents (i.e. STOICs), which leads to membrane potential hyperpolarization.[47,48,65] Our study, focused on STOCs, without excluding any possible contribution by STICs. K_v channels are indirectly modulated by increased $[Ca^{2+}]_i$, which cause a depolarized plasma membrane, which, in turn, cause the K_v channels (and possibly also KCa channels) to open.[66] Subsequently, K^+ ions leave the cell through their electrochemical gradient and hyperpolarize the membrane potential. As a consequence, the influx of Ca^{2+} is inhibited and relaxation of SMCs ensue. This process explains the partial escape (i.e. redilation) observed during prolonged ex vivo activation with ACh and 480 nm light stimulation. As sustained pupil constriction was observed with prolonged exposure to a high concentration of ACh in Ca^{2+} free medium, it appears that pupil escape, as observed during longer stimulation with ACh, is not due to G protein-coupled receptor uncoupling (i.e. homologous desensitization), which may occur after prolonged exposure to high concentrations of an agonist.[67,68] One possible alternative mechanism to maintain sustained pupil constriction would be a reduction in the sensitivity of the hyperpolarizing channels (e.g. K_v and BKCa), which are indirectly or directly activated/opened by high cytosolic Ca^{2+}. Moreover, inhibition of K_v channels did not fully prevent pupil escape during longer exposure to ACh, indicating that inhibition of STOCs is not sufficient to maintain the influx of Ca^{2+} via L-VGCCs. The L-VGCC channels are known to be negatively regulated by both the maintenance of membrane depolarization and by Ca^{2+}-dependent inactivation.[69,70] To overcome this situation, and as an additional mechanism for sustained pupil constriction, the influx of Ca^{2+} can be maintained via the reverse mode of the Na^+/Ca^{2+}-exchanger (i.e. NCX). It has been shown that when NCX is regulated allosterically by cytoplasmic Na^+ and Ca^{2+}, the contraction force of skeletal and smooth muscles is increased when NCX functions in its reverse mode.[71–73] Our data provide evidence that although the pupils were similar in size after dark adaptation for either 2 hours or ON, NCX only induced iridal contraction in D_2 irises in Na^+-free medium, but not in D_{ON} irises. Thus, it appears that during light exposure, Na^+ accumulates intracellularly, either via the melanopsin signaling pathway that results in activated Trpc6 and/or Trpc7, as shown in pRGCs[12,74] or by voltage-gated Na^+ channels (Na_v).[75] Whether Trpc or Na_v channels activate the reverse mode of NCX and facilitate the influx of Ca^{2+} when L-VGCCs are blocked, and thereby contribute to sustained pupil constriction at high irradiances, is not clear. The expression patterns of both Trpc and Na_v channels in the iris and their potential impact on iridal dynamics during prolonged light exposure, therefore, is a subject for future investigation.

Collectively, our results show that both ACh activation and 480 nm light stimulation of local iridal melanopsin results in the release of Ca^{2+} from the same intracellular compartments. These results suggest that at bright and prolonged light intensities, intrinsically expressed melanopsin, probably through the activity of Trpc channels, leads to activation of the reverse mode of NCX, thereby permitting the influx of Ca^{2+} that is necessary for the steady-state opening of RyR channels and sustained pupil constriction. Thus, our study presents new information that explains the intrinsic cellular mechanisms that are required for stimulation-contraction coupling, with a special focus on additional mechanisms that mediate sustained pupil constriction and the subsequent rapid repolarization of iridal smooth muscle cells. As a useful model system, these observations and conclusions may be highly relevant to the

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more widespread cellular processes that govern stimulation contraction/relaxation-coupling in other types of SMCs.

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Supplementary Material

Supplementary Movie S1. Iris contraction in response to 480 nm light. A representative fast motion video of an ex vivo pupil in response to continuous blue light (480 ± 10 nm) exposure over 10 min. The enucleated eye was kept in Tyrode's solution at room temperature (RT) in the dark overnight (ON) prior to the recording when subjected to 480 nm light exposure.

Supplementary Movie S2. Iris contraction in response to acetylcholine. A representative fast motion video of an ex vivo pupil response to 100 mM acetylcholine (ACh) over 10 min. The enucleated eye was kept in Tyrode's solution at room temperature (RT) in the dark overnight (ON) prior to the recording when subjected to ACh stimulation.

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