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

Soufien Sghari, Wayne I. L. Davies, and Lena Gunhaga

Umeå Centre for Molecular Medicine (UCMM), Umeå University, Umeå, Sweden

Correspondence: Soufien Sghari and Lena Gunhaga, Umeå Centre for Molecular Medicine (UCMM), Umeå University, 901 87 Umeå, Sweden; soufien.sghari@umu.se, lena.gunhaga@umu.se.

**Received:** April 20, 2020
**Accepted:** August 19, 2020
**Published:** September 3, 2020

Citation: Sghari S, Davies WIL, Gunhaga L. Elucidation of cellular mechanisms that regulate the sustained contraction and relaxation of the mammalian iris. *Invest Ophthalmol Vis Sci.* 2020;61(11):5. https://doi.org/10.1167/iovs.61.11.5

**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 $\alpha_1$-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-

Copyright 2020 The Authors


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

Downloaded from iovs.arvojournals.org on 01/20/2021

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,

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

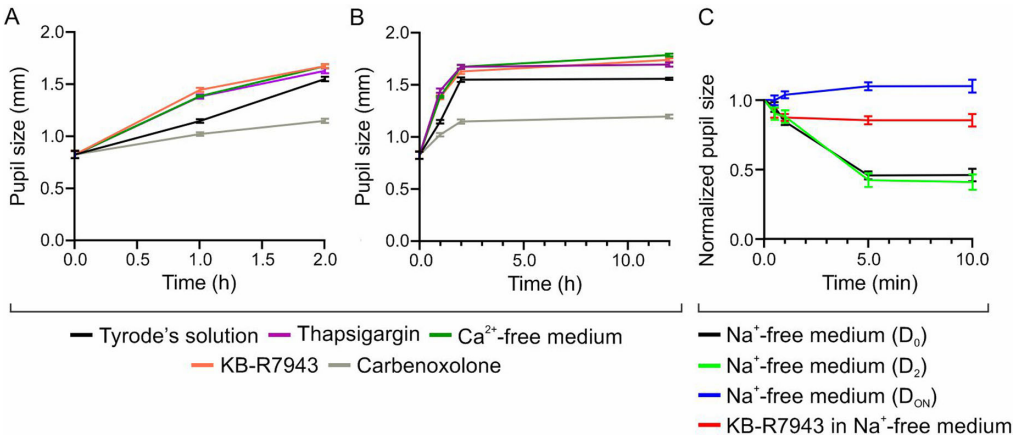



**Figure 1.** The role of $Ca^{2+}$ efflux and $Na^{+}$ influx in pupil dilation. **(A, B)** Measured pupil size of enucleated mice eyes at 0 hours, and after 1 hour, 2 hours **(A)**, and ON (12 hours) **(B)** of dark incubation incubated in Tyrode's solution alone or in addition to thapsigargin, carbenoxolone, 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 $\alpha_1$-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

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

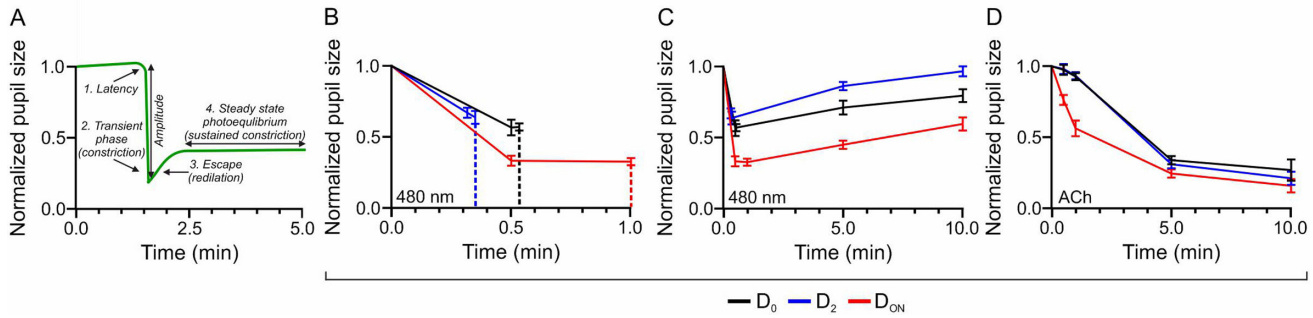



**Figure 2.** The pupil stimulus response (PSR) ex vivo at different time points of dark adaptation. **(A)** Schematic representation of an in vivo pupil light response (PLR) conserved between human and rodents. **(B, C)** Normalized pupil sizes in response to 480 nm light stimulation over 1 minute **(B)** 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 $Ca^{2+}$, primarily 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 $\lambda_{max}$ of mouse Opn4m),[29,30] with a photon flux of $1.2 \times 10^{20}$ 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

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

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.

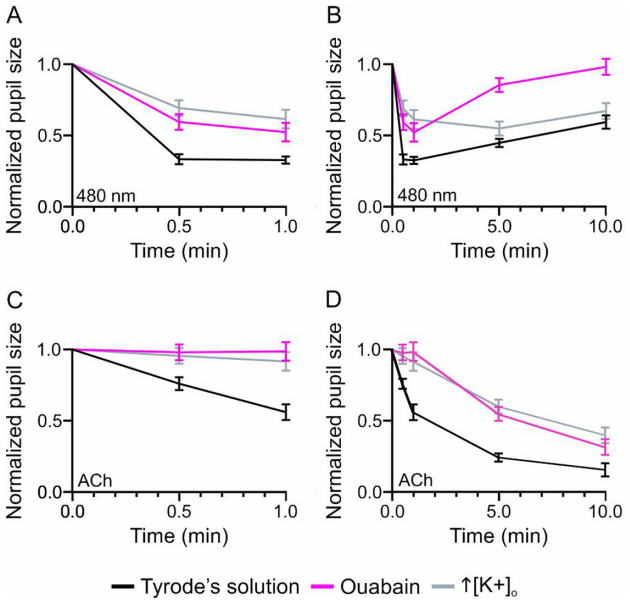



**Figure 3.** The contribution of the membrane potential to the PSR. **(A–D)** Normalized pupil sizes of enucleated eyes in response to 480 nm light stimulation over 1 minute **(A)** and 10 minutes **(B)**, or to ACh exposure over 1 minute **(C)** and 10 minutes **(D)**, after ON incubation in Tyrode's 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

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

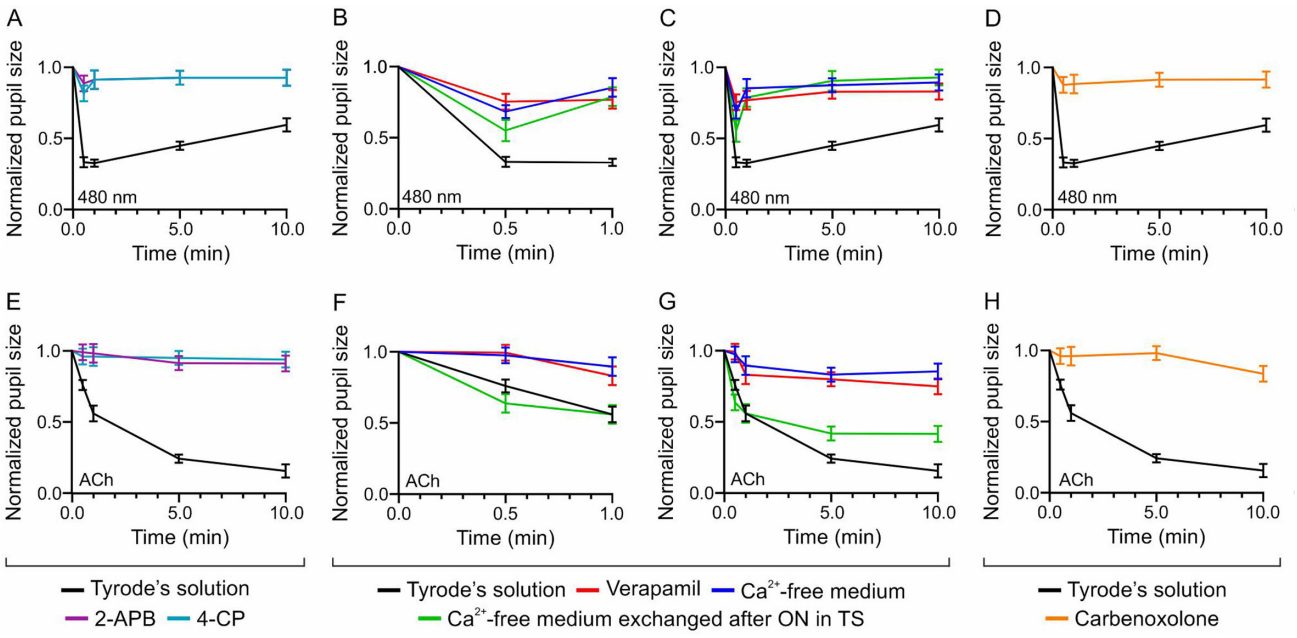



**Figure 4.** Determination of the $Ca^{2+}$ dynamics that mediate the pupil stimulus response. **(A–E)** Normalized pupil sizes of enucleated eyes in response to 480 nm light stimulation **(A)** and ACh activation **(E)** after incubation ON in Tyrode's solution alone, or together with 2-APB (added 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).

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

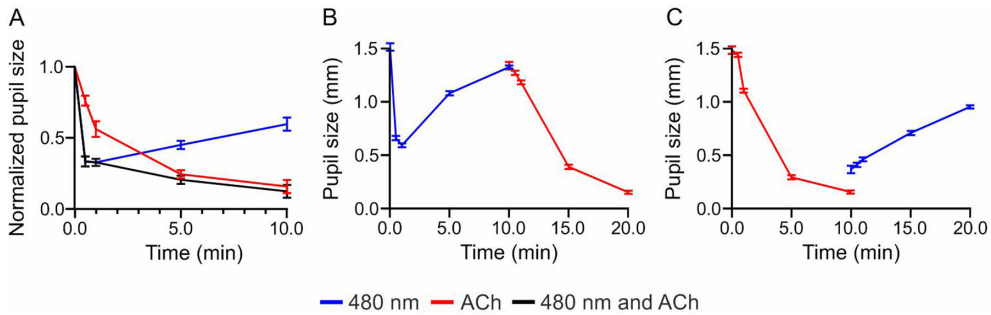



**Figure 5.** Intrinsic 480 light-induced and ACh-induced iridal contractions use the same intracellular $Ca^{2+}$ compartments. **(A)** Normalized pupil sizes of ON dark-adapted enucleated eyes in response to separate 480 nm light exposure and ACh activation, and both stimuli added simultaneously over 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]

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

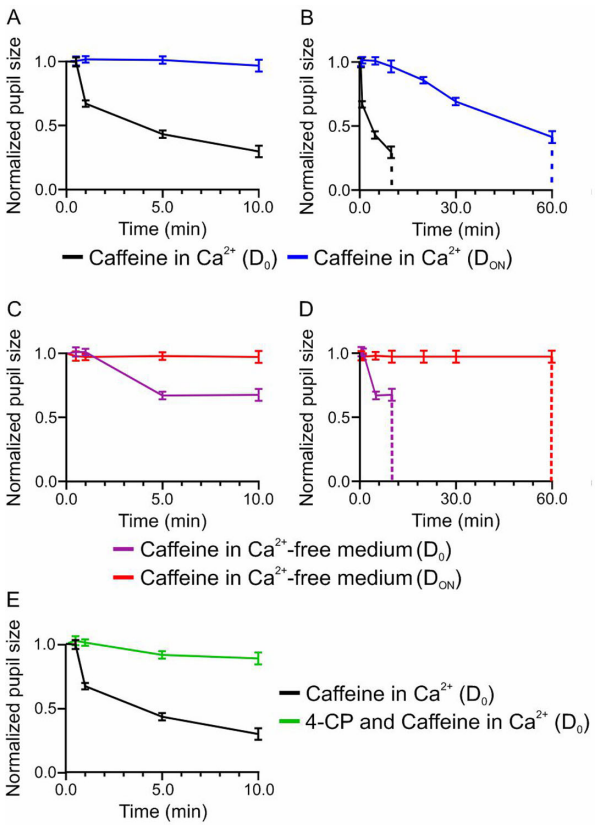



**Figure 6.** The contribution of RyR channels to pupil constriction. **(A, B)** Normalized pupil sizes of $D_0$ and $D_{ON}$ enucleated eyes in response to caffeine in Tyrode's solution over 10 minutes **(A)** and 60 minutes **(B)**. **(C, D)** Normalized pupil sizes of $D_0$ enucleated eyes in response 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

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

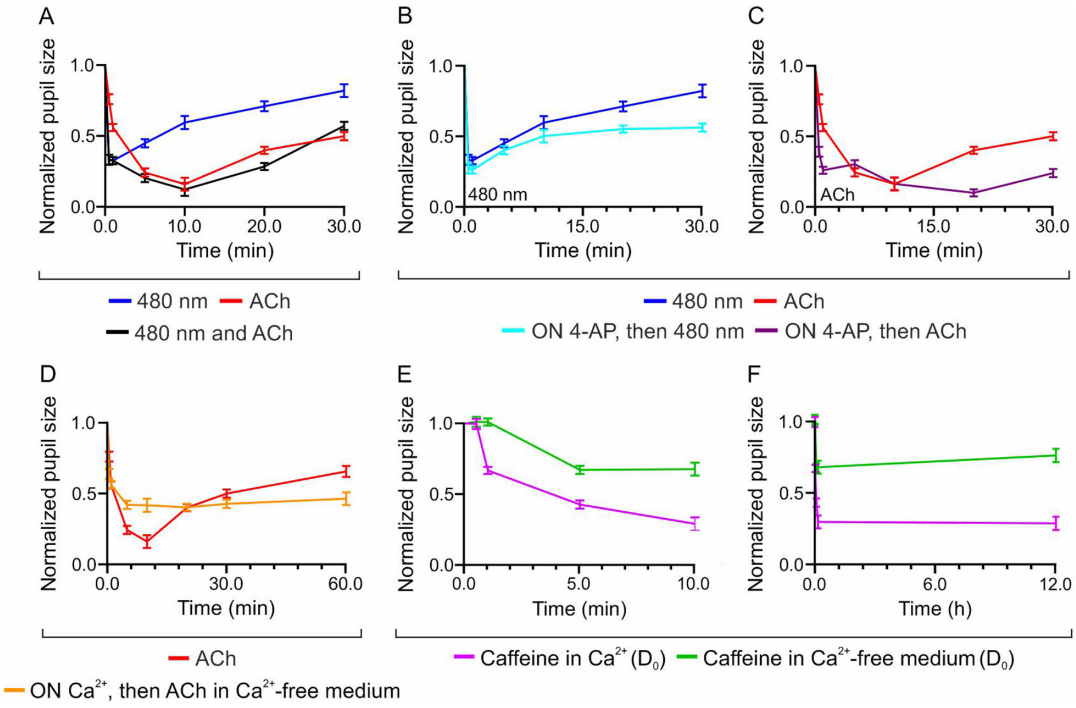



**Figure 7.** Pupil responses to prolonged exposure to ACh and 480 nm light. **(A)** Normalized pupil sizes of $D_{ON}$ enucleated eyes in response to separate 480 nm light exposure and ACh activation, or given simultaneously, over 30 minutes. **(B, C)** Normalized pupil sizes of $D_{ON}$ enucleated 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

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

*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

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

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

## *Acknowledgments*

The authors thank members of the research group of Professor Helena Edlund for their generosity in donating mouse eyes, Petter Lundberg and Professor Ludvig Edman for help with photometric advices, and Professor Richard Lang for initial discussions of the project. The authors would also like to thank Assistant Professor Ethan Buhr, Professor Helena Edlund, and Associate Professor Ludvig Backman for kindly providing aliquots of the melanopsin, Sma1, and Cx43 antibodies, respectively. Gratitude is offered to members of the Gunhaga research group for insightful general discussions.

Supported by the Swedish Research Council (2017-01430), The Medical Faculty at Umeå University, Kempestiftelserna, Ögonfonden, and Stiftelsen Kronprinsessan Margaretas Arbetsnämnd för synskadade (2018-108; 2019-015).

Disclosure: **S. Sghari,** None; **W.I.L. Davies,** None; **L. Gunhaga,** None

## *References*

1. Laughlin SB. Retinal information capacity and the function of the pupil. *Ophthalmic Physiol Opt*. 1992;12:161–164.
2. McDougal DH, Gamlin PD. Autonomic control of the eye. *Compr Physiol*. 2015;5:439–473.
3. Hayter EA, Brown TM. Additive contributions of melanopsin and both cone types provide broadband sensitivity to mouse pupil control. *BMC Biology*. 2018;16:83.
4. Keenan WT, Rupp AC, Ross RA, et al. A visual circuit uses complementary mechanisms to support transient and sustained pupil constriction. *Elife*. 2016;5:e15392.
5. Zele AJ, Adhikari P, Cao D, Feigl B. Melanopsin and cone photoreceptor inputs to the afferent pupil light response. *Front Neurol*. 2019;10:529.
6. Fu Y, Zhong H, Wang MH, et al. Intrinsically photosensitive retinal ganglion cells detect light with a vitamin A-based photopigment, melanopsin. *Proc Natl Acad Sci USA*. 2005;102:10339–10344.
7. Lucas RJ, Hattar S, Takao M, Berson DM, Foster RG, Yau KW. Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. *Science*. 2003;299:245–247.
8. Provencio I, Rodriguez IR, Jiang G, Hayes WP, Moreira EF, Rollag MD. A novel human opsin in the inner retina. *J Neurosci*. 2000;20:600–605.
9. Bouffard MA. The pupil. *Continuum (Minneap Minn)*. 2019;25:1194–1214.
10. Belliveau AP, Somani AN, Dossani RH. *Pupillary Light Reflex*. Treasure Island, FL: StatPearls; 2019.
11. Douglas RH. The pupillary light responses of animals; a review of their distribution, dynamics, mechanisms and functions. *Prog Retin Eye Res*. 2018;66:17–48.
12. Xue T, Do MT, Riccio A, et al. Melanopsin signalling in mammalian iris and retina. *Nature*. 2011;479:67–73.
13. Seliger HH. Direct action of light in naturally pigmented muscle fibers. I. Action spectrum for contraction in eel iris sphincter. *J Gen Physiol*. 1962;46:333–342.
14. Barr L, Alpern M. Photosensitivity of the Frog Iris. *J Gen Physiol*. 1963;46:1249–1265.
15. Tu DC, Batten ML, Palczewski K, Van Gelder RN. Nonvisual photoreception in the chick iris. *Science*. 2004;306:129–131.
16. Szabadi E. Functional organization of the sympathetic pathways controlling the pupil: light-inhibited and light-stimulated pathways. *Front Neurol*. 2018;9:1069.
17. Hall CA, Chilcott RP. Eyeing up the future of the pupillary light reflex in neurodiagnostics. *Diagnostics (Basel)*. 2018;8:19.
18. Drummond RM, Fay FS. Mitochondria contribute to $Ca^{2+}$ removal in smooth muscle cells. *Pflugers Arch*. 1996;431:473–482.
19. McCarron JG, Olson ML, Wilson C, Sandison ME, Chalmers S. Examining the role of mitochondria in $Ca(2)^{(+)}$ signaling in native vascular smooth muscle. *Microcirculation*. 2013;20:317–329.
20. Bueno-Orovio A, Sanchez C, Pueyo E, Rodriguez B. Na/K pump regulation of cardiac repolarization: insights from a systems biology approach. *Pflugers Arch*. 2014;466:183–193.
21. Wittmann W, Schimmang T, Gunhaga L. Progressive effects of N-myc deficiency on proliferation, neurogenesis, and morphogenesis in the olfactory epithelium. *Dev Neurobiol*. 2014;74:643–656.
22. Kircher N, Crippa SV, Martin C, Kawasaki A, Kostic C. Maturation of the pupil light reflex occurs until adulthood in mice. *Front Neurol*. 2019;10:56.
23. Kostic C, Crippa SV, Martin C, et al. Determination of rod and cone influence to the early and late dynamic of the pupillary light response. *Invest Ophthalmol Vis Sci*. 2016;57:2501–2508.
24. Oloizia B, Paul RJ. $Ca^{2+}$ clearance and contractility in vascular smooth muscle: evidence from gene-altered murine models. *J Mol Cell Cardiol*. 2008;45:347–362.
25. Uhlen P, Fritz N. Biochemistry of calcium oscillations. *Biochem Biophys Res Commun*. 2010;396:28–32.
26. Kraft R. The $Na^{+}/Ca^{2+}$ exchange inhibitor KB-R7943 potently blocks TRPC channels. *Biochem Biophys Res Commun*. 2007;361:230–236.
27. Tu DC, Zhang D, Demas J, et al. Physiologic diversity and development of intrinsically photosensitive retinal ganglion cells. *Neuron*. 2005;48:987–999.
28. Wang Q, Yue WWS, Jiang Z, et al. Synergistic signaling by light and acetylcholine in mouse iris sphincter muscle. *Curr Biol*. 2017;27:1791–800.e5.
29. Hattar S, Lucas RJ, Mrosovsky N, et al. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature*. 2003;424:76–81.
30. Lucas RJ, Douglas RH, Foster RG. Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nat Neurosci*. 2001;4:621–626.
31. Dora KA, Garland CJ. Properties of smooth muscle hyperpolarization and relaxation to $K^{+}$ in the rat isolated mesenteric artery. *Am J Physiol Heart Circ Physiol*. 2001;280:H2424–H2429.
32. Mekata F. The role of hyperpolarization in the relaxation of smooth muscle of monkey coronary artery. *J Physiol*. 1986;371:257–265.
33. Britton OJ, Bueno-Orovio A, Virag L, Varro A, Rodriguez B. The electrogenic $Na^{(+)}/K^{(+)}$ pump is a key determinant of repolarization abnormality susceptibility in human ventricular cardiomyocytes: a population-based simulation study. *Front Physiol*. 2017;8:278.
34. Sommer B, Flores-Soto E, Gonzalez-Avila G. Cellular $Na^{+}$ handling mechanisms involved in airway smooth muscle contraction (Review). *Int J Mol Med*. 2017;40:3–9.
35. Santulli G, Nakashima R, Yuan Q, Marks AR. Intracellular calcium release channels: an update. *J Physiol*. 2017;595:3041–3051.
36. Tykocki NR, Boerman EM, Jackson WF. Smooth muscle ion channels and regulation of vascular tone in resistance arteries and arterioles. *Compr Physiol*. 2017;7:485–581.
37. Hobai IA, O'Rourke B. Enhanced $Ca^{(2+)}$-activated $Na^{(+)}$-$Ca^{(2+)}$ exchange activity in canine pacing-induced heart failure. *Circ Res*. 2000;87:690–698.

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

38. Sato K, Ozaki H, Karaki H. Multiple effects of caffeine on contraction and cytosolic free $Ca^{2+}$ levels in vascular smooth muscle of rat aorta. *Naunyn Schmiedebergs Arch Pharmacol*. 1988;338:443–448.
39. Sims SM, Jiao Y, Preiksaitis HG. Regulation of intracellular calcium in human esophageal smooth muscles. *Am J Physiol*. 1997;273:C1679–C1689.
40. Murayama T, Ogawa H, Kurebayashi N, Ohno S, Horie M, Sakurai T. A tryptophan residue in the caffeine-binding site of the ryanodine receptor regulates $Ca^{(2+)}$ sensitivity. *Commun Biol*. 2018;1:98.
41. Tazzeo T, Bates G, Roman HN, et al. Caffeine relaxes smooth muscle through actin depolymerization. *Am J Physiol Lung Cell Mol Physiol*. 2012;303:L334–L342.
42. Kang SS, Han KS, Ku BM, et al. Caffeine-mediated inhibition of calcium release channel inositol 1,4,5-trisphosphate receptor subtype 3 blocks glioblastoma invasion and extends survival. *Cancer Res*. 2010;70:1173–1183.
43. Berridge MJ. Smooth muscle cell calcium activation mechanisms. *J Physiol*. 2008;586:5047–5061.
44. Nelson MT, Cheng H, Rubart M, et al. Relaxation of arterial smooth muscle by calcium sparks. *Science*. 1995;270:633–637.
45. Karlin A. Membrane potential and $Ca^{2+}$ concentration dependence on pressure and vasoactive agents in arterial smooth muscle: a model. *J Gen Physiol*. 2015;146:79–96.
46. Wulff H, Kohler R. Endothelial small-conductance and intermediate-conductance KCa channels: an update on their pharmacology and usefulness as cardiovascular targets. *J Cardiovasc Pharmacol*. 2013;61:102–112.
47. Thorneloe KS, Chen TT, Kerr PM, et al. Molecular composition of 4-aminopyridine-sensitive voltage-gated $K^{(+)}$ channels of vascular smooth muscle. *Circ Res*. 2001;89:1030–1037.
48. Satake N, Shibata M, Shibata S. The involvement of KCa, KATP and KV channels in vasorelaxing responses to acetylcholine in rat aortic rings. *Gen Pharmacol*. 1997;28:453–457.
49. Wu BN, Chen CF, Hong YR, Howng SL, Lin YL, Chen IJ. Activation of BKCa channels via cyclic AMP- and cyclic GMP-dependent protein kinases by eugenosedin-A in rat basilar artery myocytes. *Br J Pharmacol*. 2007;152:374–385.
50. Park JC, Cao D, Collison FT, Fishman GA, McAnany JJ. Rod and cone contributions to the dark-adapted 15-Hz flicker electroretinogram. *Doc Ophthalmol*. 2015;130:111–119.
51. Laver DR. $Ca^{2+}$ stores regulate ryanodine receptor $Ca^{2+}$ release channels via luminal and cytosolic $Ca^{2+}$ sites. *Biophys J*. 2007;92:3541–3555.
52. Hong F, Haldeman BD, Jackson D, Carter M, Baker JE, Cremo CR. Biochemistry of smooth muscle myosin light chain kinase. *Arch Biochem Biophys*. 2011;510:135–146.
53. Itoh T, Ueno H, Kuriyama H. Calcium-induced calcium release mechanism in vascular smooth muscles–assessments based on contractions evoked in intact and saponin-treated skinned muscles. *Experientia*. 1985;41:989–996.
54. Somlyo AP. Excitation-contraction coupling and the ultrastructure of smooth muscle. *Circ Res*. 1985;57:497–507.
55. del Valle-Rodriguez A, Lopez-Barneo J, Urena J. $Ca^{2+}$ channel-sarcoplasmic reticulum coupling: a mechanism of arterial myocyte contraction without $Ca^{2+}$ influx. *EMBO J*. 2003;22:4337–4345.
56. Fill M, Copello JA. Ryanodine receptor calcium release channels. *Physiol Rev*. 2002;82:893–922.
57. Boittin FX, Coussin F, Morel JL, Halet G, Macrez N, Mironneau J. $Ca^{(2+)}$ signals mediated by Ins(1,4,5)P(3)-gated channels in rat ureteric myocytes. *Biochem J*. 2000;349(Pt 1):323–332.
58. Iino M, Kobayashi T, Endo M. Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea-pig. *Biochem Biophys Res Commun*. 1988;152:417–422.
59. Pacaud P, Loirand G. Release of $Ca^{2+}$ by noradrenaline and ATP from the same $Ca^{2+}$ store sensitive to both InsP3 and $Ca^{2+}$ in rat portal vein myocytes. *J Physiol*. 1995;484(Pt 3):549–555.
60. Wang YX, Kotlikoff MI. Muscarinic signaling pathway for calcium release and calcium-activated chloride current in smooth muscle. *Am J Physiol*. 1997;273(2 Pt 1):C509–C519.
61. Flynn ER, Bradley KN, Muir TC, McCarron JG. Functionally separate intracellular $Ca^{2+}$ stores in smooth muscle. *J Biol Chem*. 2001;276:36411–36418.
62. Sato D, Bartos DC, Ginsburg KS, Bers DM. Depolarization of cardiac membrane potential synchronizes calcium sparks and waves in tissue. *Biophys J*. 2014;107:1313–1317.
63. Mure LS, Cornut PL, Rieux C, et al. Melanopsin bistability: a fly's eye technology in the human retina. *PLoS One*. 2009;4:e5991.
64. Zhu Y, Tu DC, Denner D, Shane T, Fitzgerald CM, Van Gelder RN. Melanopsin-dependent persistence and photopotentiation of murine pupillary light responses. *Invest Ophthalmol Vis Sci*. 2007;48:1268–1275.
65. Xin W, Li N, Cheng Q, Petkov GV. BK channel-mediated relaxation of urinary bladder smooth muscle: a novel paradigm for phosphodiesterase type 4 regulation of bladder function. *J Pharmacol Exp Ther*. 2014;349:56–65.
66. Shimizu S, Yokoshiki H, Sperelakis N, Paul RJ. Role of voltage-dependent and $Ca^{(2+)}$-activated $K^{(+)}$ channels on the regulation of isometric force in porcine coronary artery. *J Vasc Res*. 2000;37:16–25.
67. Osorio-Espinoza A, Escamilla-Sanchez J, Aquino-Jarquin G, Arias-Montano JA. Homologous desensitization of human histamine H(3) receptors expressed in CHO-K1 cells. *Neuropharmacology*. 2014;77:387–397.
68. Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev*. 2001;53:1–24.
69. Evans RC, Herin GA, Hawes SL, Blackwell KT. Calcium-dependent inactivation of calcium channels in the medial striatum increases at eye opening. *J Neurophysiol*. 2015;113:2979–2986.
70. Morales D, Hermosilla T, Varela D. Calcium-dependent inactivation controls cardiac L-type $Ca^{(2+)}$ currents under beta-adrenergic stimulation. *J Gen Physiol*. 2019;151:786–797.
71. Dan P, Lin E, Huang J, Biln P, Tibbits GF. Three-dimensional distribution of cardiac $Na^{+}$-$Ca^{2+}$ exchanger and ryanodine receptor during development. *Biophys J*. 2007;93:2504–2518.
72. Armoundas AA, Hobai IA, Tomaselli GF, Winslow RL, O'Rourke B. Role of sodium-calcium exchanger in modulating the action potential of ventricular myocytes from normal and failing hearts. *Circ Res*. 2003;93:46–53.
73. Liu B, Peel SE, Fox J, Hall IP. Reverse mode $Na^{+}/Ca^{2+}$ exchange mediated by STIM1 contributes to $Ca^{2+}$ influx in airway smooth muscle following agonist stimulation. *Respir Res*. 2010;11:168.
74. Perez-Leighton CE, Schmidt TM, Abramowitz J, Birnbaumer L, Kofuji P. Intrinsic phototransduction persists in melanopsin-expressing ganglion cells lacking diacylglycerol-sensitive TRPC subunits. *Eur J Neurosci*. 2011;33:856–867.
75. Saleh S, Yeung SY, Prestwich S, Pucovsky V, Greenwood I. Electrophysiological and molecular identification of voltage-gated sodium channels in murine vascular myocytes. *J Physiol*. 2005;568(Pt 1):155–169.


Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021

## 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.

Investigative Ophthalmology & Visual Science

Downloaded from iovs.arvojournals.org on 01/20/2021