**RESEARCH ARTICLE**

**Extraocular muscle function is impaired in ryr3<sup>−/−</sup> mice**

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Calcium is an ubiquitous second messenger mediating numerous physiological processes, including muscle contraction and neuronal excitability. Ca<sup>2+</sup> is stored in the ER/SR and is released into the cytoplasm via the opening of intracellular inositol trisphosphate receptor and ryanodine receptor calcium channels. Whereas in skeletal muscle, isoform 1 of the RYR is the main channel mediating calcium release from the SR leading to muscle contraction, the function of ubiquitously expressed ryanodine receptor 3 (RYR3) is far from clear; it is not known whether RYR3 plays a role in excitation–contraction coupling. We recently reported that human extraocular muscles express high levels of RYR3, suggesting that such muscles may be useful to study the function of this isoform of the Ca<sup>2+</sup> channel. In the present investigation, we characterize the visual function of ryr3<sup>−/−</sup> mice. We observe that ablation of RYR3 affects both mechanical properties and calcium homeostasis in extraocular muscles. These changes significantly impact vision. Our results reveal for the first time an important role for RYR3 in extracellular muscle function.

**Introduction**

RYRs are intracellular calcium channels mediating calcium release from ER/SR intracellular calcium stores (Takeshima et al., 1989; Zorzato et al., 1990). Three main isoforms of RYRs encoded by different genes sharing an overall amino acid identity of ~65% have been identified in vertebrates (Ogawa et al., 2000; Meissner, 2017). In skeletal muscle, RYR1 is mostly located on the terminal cisternae junctional face membrane, where it is involved in excitation–contraction coupling (ECC), the process whereby a change in membrane potential is converted into calcium release, leading to muscle contraction (Endo, 1977; Takeshima et al., 1989; Zorzato et al., 1990; Ríos and Pizarro, 1991). RYR2 is predominantly expressed in the cardiac muscle SR, where it is activated by a process called CICR, leading to repetitive contractions of the heart (Bers, 2002). RYR3 is ubiquitously expressed on ER/SR membranes, with high levels of expression in the central nervous system, smooth muscle cells, and developing muscles (Giannini et al., 1992, 1995; Clark et al., 2010; Vaithianathan et al., 2010).

In skeletal and cardiac muscles, RYR1 and RYR2 are either directly coupled to or in close proximity to the dihydropyridine receptor (DHPR; Ríos and Pizarro, 1991; Franzini-Armstrong and Jorgensen, 1994; Protasi et al., 2000; Bers, 2002). A coupling partner has not been identified for RYR3, though it has been suggested that RYR3 may act as an amplifier and release Ca<sup>2+</sup> by a CICR mechanism (Protasi et al., 2000; Yang et al., 2001). Lower vertebrates such as fish, frogs, and birds express both RYR1 (referred to as aRYR) and RYR3 (referred to as bRYR) in their skeletal muscles (Airey et al., 1990; Percival et al., 1994). In contrast to aRYRs, which are located on the junctional face membrane, bRYRs are expressed parajunctionally, where they cannot support ECC but are involved in the generation of Ca<sup>2+</sup> sparks (Perni et al., 2015).

From a functional point of view, the three RYR isoforms share several biophysical properties, including activation by submicromolar [Ca<sup>2+</sup>], calmodulin, ATP, and caffeine, as well as inhibition by micromolar [Mg<sup>2+</sup>], low and high calmodulin, ruthenium red, and procaine. They also share similar units of conductance (Ogawa et al., 2000). Nevertheless, there are important differences, such as RYR3’s increased resistance to inactivation by high concentrations of Ca<sup>2+</sup> (Ogawa et al., 2000).

From a physiological point of view, RYR3s are not essential, as reflected by the fact that ryr3<sup>−/−</sup> mice grow and reproduce...
normally and do not exhibit gross abnormalities in smooth muscle cell function (Takeshima et al., 1996). However, developing skeletal muscles from ryr3−/− mice exhibit reduced twitch amplitudes in response to electrical stimulation and caffeine addition, but the lack of RYR3 does not apparently impact the function of adult muscles (Bertocchini et al., 1997). Interestingly, ryr3−/− mice exhibit behavioral abnormalities, including increased locomotor activity, impaired performance in the water maze, and a mild tendency to circular running (Balschun et al., 1999).

Extraocular muscles (EOMs) are classified as a distinct muscle allotype when compared with limb and dorsal muscles. They are either selectively spared in certain congenital muscle diseases such as Duchenne muscular dystrophy or preferentially affected in conditions such as myasthenia gravis and mitochondrial myopathies, indicating that they are biochemically different from their limb muscle counterpart. EOMs have a unique embryonic origin, can be singly or multiply innervated, contain a large number of mitochondria, are highly vascularized, do not store glycogen, are fatigue resistant, and express distinct myosin heavy chain (MyHC) isoforms (Spencer and Porter, 1988; Hoh et al., 1989; Kaminski and Ruff, 1997; Porter et al., 2001; Ketterer et al., 2010; Zhou et al., 2010). Furthermore, they contain a population of preactivated satellite cells that continuously fuse into existing myofibers under “uninjured” conditions (McLoon and Wirtshafter, 2002a,b; Stuelatz et al., 2015).

While investigating the biochemical and physiological characteristics of human EOMs, we reported that such muscles contain a distinct toolkit component of the proteins involved in ECC, as they express both Ca1.1 and Ca1.2 (the skeletal and cardiac isoform of the α 1 subunit of the DHPR, respectively), calsequestrin 1 and 2, RYR1, and RYR3, resulting in distinct intracellular calcium homeostasis properties (Sekulic-Jablanovic et al., 2015). In the present study, we used the ryr3−/− mouse model to investigate the function of RYR3 in EOMs. Our data show that constitutive ablation of RYR3 significantly affects both the mechanical properties of mature EOMs and calcium homeostasis, and we propose that these changes are responsible for the impaired visual functions of ryr3−/− mice.

Materials and methods
Animal model and ethical permits
The ryr3−/− mouse model was generated by Takeshima et al. (1996). Experiments were performed on 8–12-wk-old mice. All experimental procedures were approved by the Cantonal Veterinary Authorities (BS Kantonaes veterinärart permit numbers 1728 and 2115 and BE Kantonaes veterinärart BE32/15). All experiments were performed in accordance with relevant guidelines and regulations.

Assessment of visual acuity by the Morris water maze task
Visual acuity was tested using the cued Morris water maze (Enzmann et al., 2006; Zulliger et al., 2011). A round gray tank of 1.7-m diameter filled to a height of 30 cm with water at room temperature (23 ± 1°C) was used. The water was made opaque by the addition of non-toxic white paint. A video camera fastened above the center of the pool recorded the swimming pattern of the mice using a video tracking system (Ethovision XT11; Noldus Information Technology). The water surface was virtually divided into four quadrants, and there were four entry zones to the pool. A white, round platform with a diameter of 20 cm was placed in one quadrant at a distance of 50 cm from the border and 1.5 cm above the water surface. Four entry zones were marked outside the pool. The room was illuminated at an intensity of <150 Lux. Animals were transferred to the experimental room 72 h before the experiments in order to acclimatize them. The light in the room was switched on at 6 a.m., and the mice were exposed to 12-h light/dark cycles. Mice were provided with water and food ad libitum. On day 0, mice performed a habituation run, swimming for 60 s in the water maze. On day 1, mice performed four trials with the platform at a fixed position and varying each of the four entry zones; the test duration was 60 s per run. If the mice did not find the platform after 60 s, then they were guided to it by hand and allowed to stay on it for 10 s. On day 2, the tests were repeated similarly to day 1, except that the platform was moved in the opposite quadrant. The following parameters were evaluated: swimming velocity, total distance moved, and time to reach the platform. The results are presented as average per experimental group, per day. Heat maps showing the relative combined traces of the mice for each group were generated using Ethovision.

Optokinetic reflex (OKR)
The Optomotry System from CerebralMechanics was used to measure the OKR as previously described (Prusky et al., 2004; Thomas et al., 2004). Briefly, a virtual cylinder comprising a vertical sine wave grating was projected in 3-D coordinate space on computer monitors (17” LCD computer monitors, model 1703FP; Dell) arranged in a square around and projecting to the testing area, consisting of a platform positioned 13 cm above a mirrored floor under a likewise mirrored lid. To record the behavior of the mice, a video camera (DCR-HC26; Sony) was positioned directly above the platform and connected to a computer, allowing live video feedback. The computer was also used to project the virtual cylinder on the monitors in a 3-D coordinated space, controlling the speed of rotation and geometry of the cylinder and spatial frequency of the stimuli. The image on the monitors was expanded by the mirrors on the floor and the ceiling, thereby creating a virtual 3-D world. To measure under scotopic conditions, light levels were attenuated with cylindrical neutral density filters (R211 0.9ND; LEE Filters) inside the tracking area, thereby reducing the ambient light in the testing area to a luminance level of ~4.5 log cd m−2.

During testing, the mice stood unrestrained on the platform tracking the 3-D pattern with a reflexive head movement. The movements of the mice on the platform were followed by the experimenter with a crosshair superimposed on the video image to center the rotation of the virtual cylinder with the x−y positional coordinates of the crosshair at the mouse’s viewing position, maintaining the pattern at a constant distance from the animal. Mice normally stopped moving when a grating perceptible to them was projected onto the cylinder wall and the
cylinder was rotated and began tracking the pattern with reflexive head movements in concert with the rotation. The decision of whether the mouse tracked the pattern or not was made by the experimenter based on evident head movement against the stationary arm of the crosshair. Animals that slipped or jumped off the platform during the test trial were returned to the platform, and testing was continued. The measurements were always made in the morning, and all animals were acclimated to the testing by placing them on the platform for a few minutes without testing. Two independent experimenters were blinded both to the genotype and to previous records.

Electroretinography

Mice were dark-adapted overnight. The animals were then anesthetized using 80 mg/kg ketamine (Ketalar 50 mg/ml; Pfizer AG) and 1 mg/kg medetomidine (Domitor; Orion Pharma). Pupils were dilated at the time of anesthesia by topical instillation of 2.5% phenylephrine + 0.5% tropicamide (MIX-Augentropfen; ISP!, and oxybuprocaine (Oxybuprocaine 0.4% SDU Faure; Thea Pharma) was used for additional local anesthesia. The anesthetized mice were positioned in the recording apparatus (Ganzfeld stimulator Q400; Roland Consult). The scotopic electroretinogram was elicited at different light intensities (−25, −20, −10, 0, +10, and +15 dB). Eight flashes (256-ms duration) were presented at 0.1 Hz with a 20-ms interval, and the responses were averaged. A- and B-wave amplitudes were quantified using the RETI-port/scan 21 analysis tool (Roland Consult). The anesthesia was revoked by injection of 2.5 mg/kg atipamezole (Antisedan; Orion Pharma), but never before 30 min after initiation of the anesthesia.

Isometric force measurements

Isolated EOMs were mounted intact on a MyoStation (Myotronic). The force transducer was equipped with small hooks. One of the rectus muscles, mainly medial, was tied with a prolene suture (EP8703H; Ethicon) by stitching through the eyeball close to the entry point of the muscle. The other side was ligated on the left over the orbital socket bone with a surgical suture (D50; Advansta) or the Chemiluminescence kit from Roche (18068–716; Millipore), mouse anti-MyHC (A2304; Sigma). The immunopositive bands were visualized by chemiluminescence using the WesternBright ECL-HRP Substrate (K-12045-50; Advansta) or the Chemiluminescence kit from Roche (11500694001; Roche) on a Vilber Fusion Solo S system. Statistical analysis of the intensity of the immunopositive bands was determined using ImageJ/FIJI (Schindelin et al., 2012). The intensity values were normalized to the intensity of the indicated skeletal muscle-specific protein.

Single-fiber isolation

Mice were sacrificed with a Pentobarbital overdose, their hearts were cannulated, and the circulatory system was washed with mammalian Ringer buffer (137 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 0.1% glucose, and 11.8 mM HEPES, pH 7.4). Subsequently, 17 ml of a collagenase cocktail prepared in modified mammalian Ringer buffer (137 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.1% glucose, and 11.8 mM HEPES, pH 7.4) containing 20 mg collagenase type I (ClOstridium histolyticum type I, C0130; Sigma), 6 mg collagenase type 2 (LSO04176; Worthington Biochemical Corporation), and 2.5 mg Elastase (LSO02294; Worthington Biochemical Corporation) was injected into the circulatory system. EOMs were removed under a dissecting microscope and further digested in a small Erlenmeyer flask.

Electrophoresis and immunoblotting

Total muscle homogenates were prepared from flash-frozen EOM samples in 10 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM EDTA, and anti-protease (1873580001; Roche) as previously described (Sekulic-Jablanovic et al., 2015, 2016). Protein concentration was determined using a Protein Assay Kit II (5000002; Bio-Rad Laboratories), and BSA was used as a standard. SDS-PAGE, protein transfer on to nitrocellulose membranes, and immunostaining were performed as previously described (Sekulic-Jablanovic et al., 2015), except for parvalbumin. In the latter case proteins were separated on a 10% Tris-Tricine gel (Schägger and von Jagow, 1987) and subsequently transferred onto nitrocellulose. The following primary antibodies were used for Western blotting: rabbit anti-RYR1 (81535; Cell Signaling), goat anti-Ca₉,1.1 (sc-8160; Santa Cruz), rabbit anti-Ca₉,1.2 (sc-25686; Santa Cruz) rabbit anti-calcequin-1 (C-0943; Sigma) and calcequin-2 (ab-3516; Abcam), goat anti-SERCA1 (sc-8093; Santa Cruz), goat anti-SERCA2 (sc-8095; Santa Cruz), mouse anti-MyHC (05–716; Millipore), mouse anti-MyHC13 (4A6; DSHB Iowa), rabbit anti-Desmin (sc-14026; Santa Cruz) and rabbit anti-parvalbumin (PV25; Swant), and rabbit anti-JP-45 (Zorzato et al., 2000). Secondary peroxidase conjugates were Protein G-peroxidase (P8170; Life Technologies) and peroxidase-conjugated goat anti-mouse IgG (A2304; Sigma). The immunopositive bands were visualized by chemiluminescence using the WesternBright ECL-HRP Substrate (K-12045-D50; Advansta) or the Chemiluminescence kit from Roche (11500694001; Roche) on a Vilber Fusion Solo S system. Statistical analysis of the intensity of the immunopositive bands was determined using ImageJ/FIJI (Schindelin et al., 2012). The intensity values were normalized to the intensity of the indicated skeletal muscle-specific protein.
containing 0.2% collagenase type 2 and 0.1% Elastase, in mammalian Ringer for 70 min at 37°C, 5% CO2. At the end of the digestion, fibers were gently separated from the tendons by passing them through progressively narrower pipette tips.

**Immunofluorescence**

Isolated EOM fibers were pipetted onto laminin and collagen-coated Ibidi μ-Slide 4 well or μ-Slide 8 well chambers (#80426 and #80826). The fixation and permeabilization steps depended on the antibody being used. For Ca,1.1 and Ca,1.2, 4% paraformaldehyde (made in PBS) was used as a fixative and 1% Triton in PBS for permeabilization and processed as previously described (Treves et al., 2011). For RYR1 and Ca,1.1 staining, fibers were fixed in ice-cold (-20°C) methanol for 15–30 min and permeabilized in 2% Triton in PBS for 20–30 min. After staining with the appropriate secondary antibodies, the fibers were incubated with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. The following primary and secondary antibodies were used: rabbit monoclonal anti-RYR1 (D4E1, #8153; Cell Signaling), mouse monoclonal anti-Ca,1.1 (IIC12D4 and IID5E1; Iowa Hybridoma bank), rabbit anti-Ca,1.2 (sc-25686; Santa Cruz), Alexa Fluor 568 donkey anti-mouse IgG (A10037; Invitrogen), and Alexa Fluor 488 chicken anti-rabbit IgG (A21441; Invitrogen). A Nikon AIR confocal microscope with a 60× oil-immersion Plan Apo VC Nikon objective (numerical aperture, 1.4) was used to make the images (Lopez et al., 2016).

**Single-fiber calcium measurements**

All measurements were performed at room temperature. The resting Ca2+ fluorescence in single fibers was measured on glass-bottom culture dishes (P35G-0-14-C; MatTek) using the ratiometric calcium indicator fura-2 AM (F1201; Invitrogen) and experiments were performed as previously described (Sekulic-Jablanovic et al., 2015, 2016). For measurements of myotube calcium oscillations and sparks, cells were loaded with 5 µM Fluo-4 as previously described (Sekulic-Jablanovic et al., 2015, 2016) and experiments were performed at room temperature.

**Analysis of calcium transients**

Linescans were saved as stacked images using the microscope software (NIS-Elements, version 4.60). A plug-in for Icy (de Chaumont et al., 2012) was written to concatenate the linescans and calculate their profiles in one step. The plugin has the possibility of adding regions of interest and performing calculations on the regions of interest. For analysis of the peak Ca2+ and Ca2+ kinetics in fibers and myotubes a Ca2+ analysis program in Microsoft’s .net Framework (https://visualstudio.microsoft.com) was designed. The analysis of the Ca2+ peaks for fibers and myotubes is similar but not identical; the fiber profile files contain only one transient, and the program requires information only on the number of header columns contained within the file. The myotube profiles are larger since they contain multiple transients. Therefore, the program requires an additional file (Peak-Summary) to analyze their profiles, containing, among other information, data relative to the beginning, the end, and the location of the maximum value of each peak within the profile. The Peak-Summary files were created in OriginPro 2018 by a batch peak search of the raw data.

For analysis of the Ca2+ transient, the baseline was calculated as the average of the first 5 ms of the profile. Once the maximum height of the transient was reached, the ΔF/F was calculated. The beginning of the peak was defined as the time where the transient increased by 5% of the maximum height, with respect to the baseline, for the first time. Time to peak (TPP) is the time from the beginning of the peak to the maximum height. Time to half peak (TTHP) is the time from the beginning of the peak to the time where the transient increases by 50% of the maximum height with respect to the baseline for the first time. Half relaxation time (HRT) is the time from the maximum height until the time where the transient is higher than 50% of the maximum height, for the last time. These parameters were calculated by the Ca2+ analysis program. For an automated kinetic analysis of the fiber transient, a Savitzky–Golay smoothing with polynomial order of 2 and window size of 12 was applied, while the ΔF/F calculations were done on the raw data. Savitzky–Golay smoothing is known for preserving the area, position, and width of peaks better than a moving average filter (Guiñón et al., 2007; Ostertagova and Ostertag, 2016).
Results

**Ryr3**−/− mice have a defect in their visual acuity, and isolated muscles exhibit altered kinetic parameters

Mouse EOMs are similar to their human counterpart in that they express high levels of RYR3 compared with limb muscles (Fig. 1 A); the ratio of RYR1 to RYR3 in WT mouse EOMs was assessed by qPCR and found to be 323 ± 65 to 1 (mean ± SEM, n = 4). We used ryr3−/− transgenic mice to evaluate the function of RYR3 in eye muscles. The total body ryr3−/− mouse line used in the present investigation was created by conventional gene targeting and was previously characterized by Takeshima et al. (1996). The presence of a cassette of ∼1,000 bp disrupting exon 1 of the genomic RYR3 DNA was confirmed by PCR amplification (Fig. 1 B), and the absence of RYR3 protein was confirmed by mass spectrometry analysis (Fig. 1 C).

To assess visual function in WT and ryr3−/− mice, we used complementary noninvasive parameters, namely the cued water maze and the OKR test (Prusky et al., 2004; Thomas et al., 2004; Zulliger et al., 2011). The Morris water maze is a standard method used to assess spatial learning in rodents (Thomas et al., 2004; Zulliger et al., 2011). In one of its versions, animals need to locate a visible platform and are subsequently scored according to the total swimming distance and time required to identify the platform. Thereby, the animal relies on visual information to locate a platform. This test is often used to control for visual ability (Brown and Wong, 2007). As shown in Fig. 2 A and Videos 1 and 2, ryr3−/− mice performed significantly worse than age-matched WT mice. In particular, the mean swimming distance necessary to find the platform was increased by ∼50%, and the mean time taken to find the platform was increased by ∼30% (Fig. 2 A). Qualitatively similar results were obtained when visual acuity was assessed using the OptoMotry detection system, a simple and precise method for quantifying mouse vision (Prusky et al., 2004). It assesses the OKR, an involuntary fixation on objects that are moving in relationship to the head. The eyes have the tendency to track moving objects for a distance and then subsequently saccade in the opposite direction to reacquire a target. Saccades rely on EOMs to move the eyes the correct distance in the appropriate direction (Purves et al., 2001). In the OptoMotry detection system, unrestrained mice are placed on a central platform inside a virtual 3-D cylinder, and the reflective head movements in response to changes in the spatial frequency movement of a grid are scored (Thomas et al., 2004). The top panel of Fig. 2 B shows a schematic representation of the test setup (reproduced with permission from Prusky et al., 2004), and the bottom panel shows the results obtained in individual male and female mice. As shown in the figure, the visual acuity of ryr3−/− mice was decreased by ∼20% compared with WT mice. The

**Online supplemental material**

Fig. S1 shows that retinal morphology and layer structure is unaltered in ryr3−/− mice. Fig. S2 shows photomicrographs of myotubes from EOM. Table S1 lists primers used for qPCR and for mouse genotyping. Table S2 lists kinetic properties of isolated EOM from WT and ryr3−/− mice. Video 1 shows a WT mouse swimming in a cued water maze. Video 2 shows a ryr3−/− mouse swimming in a cued water maze.

**Statistical analysis and graphical software**

Statistical analysis was performed using the unpaired two-tailed t test for equal variance and the unpaired two-tailed Welch’s t test for unequal variance and unequal sample size. Means were considered statistically significant when the P value was <0.05. Data were processed, analyzed and plotted using the software OriginPro 2018 (OriginLab Corporation). Images were assembled using Adobe Photoshop CS (version 8.0).

Figure 1. **RYR3 is expressed in murine EOM.** (A) Expression of ECC transcripts in mouse EOM muscles. Transcript levels were quantified by qPCR in muscles from three WT mice (performed twice in triplicate). Values are plotted as mean (SEM) change in EOM vs. hindlimb muscles, which was set as 1. ***, P < 0.001, Student’s t test. Primers used are detailed in Table S1. (B) Genotyping WT and ryr3−/− mice using the primers detailed in Table S1; PCR amplification of genomic DNA from the ryr3−/− mouse yields a DNA fragment that is >1,000 bp larger than that of WT mice, compatible with the insertion of a >1,000-bp cassette in exon 1. Amplification of genomic DNA from heterozygous mice shows the presence of the smaller WT allele and the larger allele containing the inserted cassette. (C) Mass spectrometry analysis of EOMs from two WT and two ryr3−/− mice shows the absence of RYR3. ND, not detectable.

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RYR3 ablation affects EOM function

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results shown in Fig. 2 were obtained by pooling male and female mice, which were similar in their response. The decrease in visual capacity of ryr3−/− mice was not caused by alterations of the retinal function, since the electroretinogram under scotopic conditions was similar in WT and ryr3−/− mice (Fig. 2, C and D). The decrease in visual capacity was also not caused by alterations in the structure of the retina as a consequence of RYR3 ablation, since no changes were observed in retinal thickness, spatial distribution, and appearance of the different layers constituting the retina (Fig. S1). Taken together, these results show that in vivo, ryr3−/− mice have an alteration of their visual capacity, but they do not discriminate whether the problem is of neuronal origin or relates to skeletal muscle function.
To answer this question directly, we investigated the mechanical properties of isolated EOM using a MyoStation-intact force transducer modified to measure twitch force in mounts of mouse EOMs as detailed in Materials and methods. In the experimental setup, we removed the oblique muscles and measured the force generated by electrical stimulation of medial rectal muscles. The force generated in response to a single pulse of 0.5 ms and of 24.6 V was significantly reduced and slower in EOMs from \( \text{ryr3}\)−/− mice (Fig. 3, A and B, light gray line; Table S2). The maximal peak force developed was \( \sim 38\% \) of that developed by EOMs from WT mice (WT: 0.328 ± 0.095 mN, \( n = 8 \); \( \text{ryr3}\)−/−: 0.126 ± 0.017 mN, \( n = 9 \); Fig. 3, A and B, black line), and the kinetics was significantly slower. The kinetic properties of the muscles were not uniform and could be classified into four groups: twitches with a single rapid peak and rapid relaxation time (WT group 1), a rapid peak and a two-phased relaxation time of which one was rapid and the other slower (WT group 2), a slow peak and a slow relaxation time (\( \text{ryr3}\)−/− group 3), and rapid twitch with a double peak and slow relaxation time (\( \text{ryr3}\)−/− group 4). To facilitate their direct comparison, the individual peak maximal forces were each set to 100%. The results plotted in Fig. 3 C and detailed in Table S2 show that there were significant differences in the kinetic properties of WT and \( \text{ryr3}\)−/− EOMs. The mean TTHP was 2.5 ± 0.2 and 8.8 ± 2.3 ms, the mean TTP was 10.1 ± 1.1 and 42.8 ± 8.8 ms, and the mean HRT was 42.3 ± 9.8 and 147.4 ± 8.4 ms in WT (\( n = 8 \)) and \( \text{ryr3}\)−/− mice (\( n = 9 \)), respectively (values are the mean ± SEM). Fig. 3 D shows the kinetic data analyzed according to the principal-component analyses. This statistical procedure allows the simultaneous comparison of multiple variables or parameters that are reduced to a smaller set of data, facilitating the comparison of data by grouping similar datasets (Hwang et al., 2013). Analysis of the twitch parameters of muscles from WT mice (empty dots, Fig. 3 D) indicates that the values are similar to each other and can all be grouped into one cluster (bottom left, black bordered circle, Fig. 3 D); on the other hand, the data from the \( \text{ryr3}\)−/−
muscles (gray dots, Fig. 3 D) fall not within the same cluster but within two completely separate clusters (gray bordered circles, Fig. 3 D). These changes in the mechanical properties were not caused by muscle atrophy, as the overall size distribution of fibers within the eye muscles (minimal Feret’s diameter) was similar in WT and 

ryr3−/− mice (Fig. 4, A and B), nor were the changes caused by gross alterations in the overall MyHC isoform composition (Fig. 4 C).

The content and subcellular localization of proteins involved in ECC in EOM from WT and 

ryr3−/− mice are similar

The changes in visual acuity and mechanical properties of the EOM from 

ryr3−/− mice could be due to alterations in the content of proteins involved in ECC and/or in their subcellular localization.

Analysis of the transcripts encoding the major proteins involved in calcium regulation revealed only small differences between WT and 

ryr3−/− mice, with the latter mice showing a reduction in 

RYR1 and CASQ1 transcripts (Fig. 5 A). The expression levels of the 

Cav1.1 (CACNA1S) and Cav1.2 isoforms (CACNA1C) of the α1 subunit of the DHPR were not affected by 

RYR3 ablation (Fig. 5 A). As to protein levels, quantitative Western blot analysis did not reveal significant changes in content of proteins involved in ECC, except for an increase in parvalbumin (Fig. 5, B and C). We tested all commercially available anti-RYR3 antibodies, but none appeared to be specific. The absence of the RYR3 protein was confirmed by mass spectrometry analysis (Fig. 1 C).

To verify whether the observed changes in the mechanical properties of isolated EOMs in 

ryr3−/− mice were due to
alterations in the subcellular localization of RYRs and/or of DHPRs, high-resolution confocal immunohistochemistry was performed on enzymatically dissociated EOM fibers stained with different antibody combinations. Fig. 6 shows a representative image of fibers from WT and ryr3−/− EOM stained for RYR1 (with rabbit anti-RYR1 mAb D4E1, whose epitope surrounds Arg830 of the human RYR1 protein; left panel, green color on the merged image) and of Cav1.1 (central panel, green fluorescence on merged image) do not share subcellular localization in EOMs. In fact, the fluorescent pattern of Cav1.2 showed a predominantly patchy appearance on the plasmalemma of the fibers, with low levels of fluorescence within the double rows that are positive for Cav1.1. The Cav1.2 antibodies are specific as they recognize a band of ~170 kD on Western blot (Fig. 5 C) and do not recognize any structure when tested on extensor digitorum longus (EDL) muscle fibers that lack the Cav1.2 isoform (Fig. 6 B, bottom central panel). There was no apparent difference in the subcellular localization of Cav1.2 between WT and ryr3−/− mice (Table 1).

**ECC in single EOM-muscle fibers and EOM-derived myotubes**

Ca2+ homeostasis and the ECC characteristics of fibers isolated from WT and ryr3−/− mice were analyzed by fluorescence microscopy. Enzymatically dissociated fibers were loaded either
with the Ca\textsuperscript{2+} indicator Mag-Fluo-4 (Fig. 7, A, B, D, and E) for measurements of electrically evoked Ca\textsuperscript{2+} transients or fura-2 (Fig. 7 C) for measurements of the resting cytoplasmic [Ca\textsuperscript{2+}]. A representative trace of a Ca\textsuperscript{2+} transient elicited by field stimulation in EOM fibers from WT and ryr3\textsuperscript{−/−} mice is shown in Fig. 7 B. Detailed quantitative analysis revealed that the peak ΔF/F was similar in WT and ryr3\textsuperscript{−/−} EOM fibers (Fig. 7 D and Table 2), as was the TTP (Fig. 7 E and Table 2); however, the mean TTHP and HRTs were significantly slower in fibers from ryr3\textsuperscript{−/−} mice (Table 2). We would like to point out that these experiments were performed at room temperature (23–25°C), and thus, the absolute kinetic values in vivo may be faster (at 37°C) than those reported here. Nevertheless, the impact of ambient temperature on the Ca\textsuperscript{2+} kinetics of fibers from WT and ryr3\textsuperscript{−/−} should be the same. No significant difference was observed in the resting [Ca\textsuperscript{2+}] (Fig. 7 C). Experiments were also performed to verify if EOM fibers exhibit sparks; 91 fibers from four WT mice were loaded with Fluo-4 (Lopez et al., 2016), but we failed to observe any spontaneous Ca\textsuperscript{2+} release events.

The results described so far indicate that RYR3 ablation causes specific alterations of intracellular calcium homeostasis and could be responsible for the reduced visual capacity of the ryr3\textsuperscript{−/−} mice. However, the differences in calcium homeostasis observed between WT and ryr3\textsuperscript{−/−} mice could potentially be caused by changes in muscle development brought about by the absence of RYR3 rather than to changes in calcium fluxes due to the specific absence of the Ca\textsuperscript{2+} channel. This point is especially relevant, since (a) the expression of the RYR3 isoform peaks during development and decreases in mature muscles (Conti et al., 2005) and (b) in EOMs, but not in other skeletal muscles, there is a continuous process of myonuclear addition into normal uninjured adult myofibrils. The myonuclei derive from activated satellite cells residing within uninjured EOMs (McLoon and Wirtschafter, 2002a,b; Stuelsatz et al., 2015).

To address the subject of abnormal calcium homeostasis directly and bypass issues linked to development or muscle adaptation due to the lack of RYR3 in innervating neurons, we isolated satellite cells from EOMs, cultured them, and analyzed Ca\textsuperscript{2+} homeostasis in the resulting multinucleated myotubes (Fig. S2). No gross change in the number of myotubes or in their fusing capacity was observed. Furthermore, no significant changes in the resting Ca\textsuperscript{2+} concentration
(86.6 ± 3.2 nM vs. 95.3 ± 5.1 nM in WT and ryr3−/−, respectively; n = 75 cells) or the size of the rapidly releasable intracellular stores (3,210.0 ± 539.6 vs. 2,548.5 ± 180.7 arbitrary units in WT and ryr3−/− respectively; n = 9) were observed. No Ca2+ sparks were detected in myotubes from WT or ryr3−/− as determined with the calcium indicator Fluo-4. Interestingly, however, EOM-derived myotubes exhibited extremely rapid, repetitive Ca2+ oscillations that were more frequent in cells from WT than ryr3−/− mice (Fig. 8 D and E). Additionally, the Ca2+ oscillations in ryr3−/− myotubes were significantly slower than those observed in myotubes from WT mice (Fig. 8 D and Table 2).

**Discussion**

In the present report, we investigated the role of RYR3 in skeletal muscle by studying the biochemical and physiological characteristics of EOMs from ryr3−/− mice. Knockout mice exhibited reduced visual capacity that is caused by a change in the physiological properties of the muscles, since (a) the mechanical properties of isolated muscles assessed in vitro were significantly different and (b) calcium homeostasis in single fibers and cultured myotubes explanted from EOMs was altered.

EOMs are subspecialized and highly complex muscles showing single and multiple innervations, as well as the presence of fibers with mixed fast- and slow-twitch contractile

**Table 2. Analysis of the kinetics of the calcium transients in EOM fibers and myotubes from WT and ryr3−/− mice**

| Type of Ca2+ response | Genotype and cell type | Peak Ca2+ (ΔF) | TTHP (ms) | TTP (ms) | HRT (ms) |
|-----------------------|------------------------|----------------|-----------|----------|----------|
| Electrically evoked transient parameters | WT EOM fibers | 0.458 ± 0.036 (n = 14) | 0.621 ± 0.019 (n = 12) | 1.042 ± 0.032 (n = 12) | 1.147 ± 0.125 (n = 12) |
| ryr3−/− EOM fibers | 0.424 ± 0.027 (n = 21) | 0.718 ± 0.034* (n = 19) | 1.310 ± 0.159 (n = 19) | 5.531 ± 0.877*** (n = 19) |
| Spontaneous Ca2+ transient parameters | WT myotubes | 0.506 ± 0.003 (n = 9,319) | 20.598 ± 0.192 (n = 9,319) | 29.885 ± 0.347 (n = 9,319) | 77.813 ± 0.8138 (n = 9,319) |
| ryr3−/− myotubes | 0.6111 ± 0.010***** (n = 1,368) | 27.527 ± 0.570***** (n = 1,368) | 46.878 ± 1.173***** (n = 1,368) | 87.586 ± 1.931***** (n = 1,368) |

Experiments were performed at room temperature. Values represent mean ± SEM. *, P < 0.05; ***, P < 0.001, Student’s t test; ***** P < 0.000001, Welch’s t test.
characteristics within a single contracting unit (Mayr, 1971; Ketterer et al., 2010). We found that the absolute maximum force developed by mouse EOMs is ∼10 times lower than that of other mouse limb muscles. In this study, because the size of mouse EOMs is very small (300–600 µm diameter) and a small error in the cross-sectional area measurements would have a large impact on the corrected developed force, we displayed the absolute and noncorrected force values. The mechanical properties of EOMs from adult (12–16-wk-old) ryr3−/− mice were different than those of WT mice. Indeed ryr3−/− EOM developed less force and had slower kinetics (but did not show changes in muscle cross-sectional area). Such data are consistent with a recent report (Bertocchini et al., 1997) showing that diaphragm strips from 15-d-old ryr3−/− mice stimulated at a frequency of 0.1 Hz developed less tension than their WT counterpart, though in muscles from adult mice, the differences disappeared.

The three- to fourfold slower half relaxation time observed in EOMs from ryr3−/− mice would significantly impact the speed of the eye movement necessary for the mice to perform the requested visual tasks, and we are confident that such dramatic changes in force account for the decreased visual acuity detected in vivo. It should be pointed out that neither the OKR nor the cued water maze directly assess ocular muscle function. The OKR represents an automatic compensatory eye movement without any conscious effort to stabilize the image and responds when animals move about in a visual scene. This reflex requires a visible retinal image and is particularly prominent in afoveate animals (i.e., animals lacking the fovea; Stahl, 2004). Performing the cued water maze task also requires intact eyesight and motor ability (swimming and eye movement) to focus the visible platform located at different locations in the respective trials (Vorhees and Williams, 2006). Therefore, although no direct conclusions can be drawn and we cannot totally exclude an effect caused by neuronal RYR3 ablation, our results point toward an impact of RYR3 ablation on eye muscle function, especially since (a) no morphological and electrophysiological retinal differences between WT and ryr3−/− animals were found, and (b) direct changes in the kinetics of the calcium signaling and velocity of contraction in isolated EOMs were found. We would also like to point out that ryr3−/− mice were reported to exhibit impaired spatial learning as assessed by the Morris water maze and increased spontaneous open-field activity (Balschun et al., 1999). In view of the present findings, it is also possible that the reported spatial memory defects observed in ryr3−/− mice were caused, at least in part, by altered ocular muscle function.

The reduced tension and speed of contraction observed in ryr3−/− EOMs are most likely not caused by differences in the expression levels of ECC proteins and/or contractile proteins, since the expressed MyHC isoforms were similar in WT and ryr3−/− EOMs and the ECC protein composition of total EOM extracts was similar in WT and transgenic mice. The small increase in the content of the calcium binding protein parvalbumin observed in ryr3−/− EOMs cannot account for the changes in
the kinetics of relaxation. Indeed, a higher content of parvalbumin would be expected to increase the speed of relaxation and not prolong it (Heizmann et al., 1982; Müntener et al., 1995), indicating that the changes in the kinetic properties must be due to a different mechanism. Of interest, we did observe a significant reduction in the expression of RYR1 and CASQ1 transcripts, but this did not result in an overall change in their level of protein content.

Although it is difficult to directly determine the cause of the slow relaxation times of ryr3−/− EOMs, they may relate to the location and physiological function of RYR3s. One possibility is that in murine EOMs, RYR3s are located parajunctionally, i.e., similar to their distribution in toadfish white swim muscle, frog sartorius, and 72 HPF zebrafish larvae (Fejer and Franzini-Armstrong, 2002; Forni et al., 2015). This parajunctional subcellular localization could place the RYR3s adjacent to some SERCAs, whereby Ca2+ release via the parajunctional RYR3 would rapidly activate the Ca2+ pump, leading to a faster rate of relaxation. The lack of RYR3 would fail to rapidly activate the calcium ATPase, leading to a slower relaxation rate. Of note, close subcellular distribution of RYR3 and SERCA2a and of RYR2 and SERCA2b have also been reported in other tissues (Greene et al., 2000; Clark et al., 2010; Dally et al., 2010).

Since release of Ca2+ from intracellular stores is the driving force of skeletal muscle contraction, we hypothesized that the mechanism resulting in the changes in the mechanical properties of EOMs in the transgenic mice were due to alterations of Ca2+ release. In mammalian cells, RYR3s have been reported to reinforce CICR in developing murine skeletal muscles (Yang et al., 2001) and play a role in the duration and amplitude of osmotic shock-triggered Ca2+ sparks in adult skeletal muscle fibers (Weisleder et al., 2007). On the other hand, in non-mammalian skeletal muscles, RYR3s contribute both to the generation of sparks and to the Ca2+ transients (Zhou et al., 2003, 2004; Forni et al., 2015). Surprisingly, no sparks were observed in mouse EOM fibers, indicating that in the latter muscles spontaneous Ca2+ release events do not occur, possibly because of their particular expression of ECC protein machinery and dissimilar calcium homeostasis (Zeiger et al., 2010; Stuelsatz et al., 2015; Sekulic-Jablanovic et al., 2016). Interestingly, the kinetics of the Ca2+ transients (TTP and TTHP) in EOMs from WT mice were approximately four times faster than those reported using the same Ca2+ indicator in rat and mouse fast-twitch fibers. Indeed, in rat and mouse EDL fibers, the reported TTP of the Ca2+ transient was 4.6 ± 0.42 ms (Delbono and Stefani, 1993) and 4.4 ± 0.1 ms (Hollingworth et al., 2008), respectively, and the TTHP was 3.2 ± 0.1 ms, while in mouse EOMs, we calculated a TTP of 1.04 ± 0.03 ms and TTHP of 0.62 ± 0.02 ms. Thus, it is not only the presence of MyHC13, which is responsible for the superfast characteristics of EOM muscle contraction, but also their particular ECC machinery. Nevertheless, the observed changes of the Ca2+ transients between EOMs from WT and ryr3−/− mice are clearly insufficient to account for the large changes in force development. The reduction in force may be due in part to the different preparations used to measure force and Ca2+ or to changes occurring during development. Indeed, EOMs from ryr3−/− mice may have physiologically adapted to the lack of RYR3 during muscle fiber maturation. To circumvent the possible alterations caused by modifications occurring during muscle fiber maturation, we studied Ca2+ homeostasis in myotubes obtained from satellite cells from adult EOMs and cultured and differentiated in vitro. Our results show that EOM-derived myotubes exhibit extremely rapid and repetitive Ca2+ oscillations, which occur in the absence of any exogenous stimulation and depend on extracellular Ca2+ (since they disappeared in Ca2+-free medium). More importantly, the velocity of the Ca2+ transients was 1.6 times slower in ryr3−/− myotubes. The spontaneous oscillations were not completely abolished in myotubes from ryr3−/− mice, but their number was substantially reduced, corroborating the role of RYR3 as a calcium signal amplifier. Though presently we do not know the function played by these Ca2+ oscillations, the lack of signals generated by their diminished activity potentially alters the myotubes, resulting in impaired regeneration of EOM.

In conclusion, our studies show that the lack of RYR3 has a significant impact on visual acuity by affecting the function of ocular muscles. It also suggests that in the future, mutations in RYR3 should be taken into consideration in patients with strabismus, ophthalmoplegia, and ptosis with or without involvement of the central nervous system.

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Author contributions: J. Eckhardt designed and performed the experiments and analyzed the results with guidance from F. Zorzato and S. Treves; C. Bachmann performed the immunohistochemistry experiments on isolated fibers; M. Sekulic-Jablanovic performed the initial experiments on RYR3 expression in mouse EOMs as well as some of the in vivo visual experiments. V. Enzmann supported J. Eckhardt and M. Sekulic-Jablanovic in the in vivo visual experiments. J. Ma, K. Ho Park, and H. Takeshima constructed and supplied the ryr3−/− mouse model. F. Zorzato and S. Treves designed the experiments on the mouse model, oversaw the project, and wrote the paper. All authors reviewed and helped elaborate the manuscript.

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Figure S1. **Retinal morphology and layer structure is unaltered in ryr3−/− mice.** (A) Overview staining of 5-µm paraffin sections using hematoxylin and eosin revealed no changes in the retinal structure of ryr3−/− mice compared with their WT controls. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars, 100 µm. (B) Furthermore, no significant difference in mean (±SEM) retinal thickness was detectable.

Figure S2. **Photomicrographs of myotubes from EOM.** Left: Low-magnification view of myotubes after 1-2 d in differentiation medium. Right: Cells were fixed with 3% paraformaldehyde 5 d after differentiation medium and stained with hematoxylin and eosin. Scale bar, 50 µm.
Table S1. Primers used for qPCR and mouse genotyping

| Gene   | Forward                                      | Reverse                                      |
|--------|----------------------------------------------|----------------------------------------------|
| ACTN2  | 5'-CATCGAGGAGGATTCAGGAAC-3'                 | 5'-CAATCTTGTTGGAACCGCATTTT-3'               |
| CACNA1C| 5'-TCCCGAGCAATCTCCTACTC-3'                  | 5'-ACTGACGGTAGATGGTTGC-3'                   |
| CACNA1S| 5'-TCAGATCGTGGAAATGGAAA-3'                  | 5'-GTTCAGATGTTTGTTCATCCT-3'                 |
| CASQ1  | 5'-ATGAGACTACCCAGAGGATG-3'                  | 5'-CACCCTCGTACCTAGGAAG-3'                   |
| CASQ2  | 5'-TGCTCATGGTGGGGTATTAC-3'                  | 5'-AGGTTCTGGTATAGGACAG-3'                   |
| JP-45  | 5'-GGGCTCAAGATGGGAAA-3'                     | 5'-CTTGTNCAGTTCTACAG-3'                     |
| KCNNMA1| 5'-GCACACAGAGAGCCCAT-3'                     | 5'-TCTTCAATAGATCGCCGGC-3'                   |
| PVALB  | 5'-ATCAAGAGCGCATAGGACC-3'                   | 5'-GGCCAGAGGTGTTTGT-3'                      |
| RYR1   | 5'-GCCCTTGCACTGCCATTAC-3'                   | 5'-CCCCAACCTGGAACCTTCTTCT-3'                |
| RYR3   | 5'-ACACAGAGAGCAGAT-3'                       | 5'-GGGGTCGTTCAAGATAGTCA-3'                  |
| RYR3 genotyping | 5'-ATGAAGTTGTAAGCCATGTCAT-3' | 5'-TCCAGGAATCTCGTATAGAG-3'                  |
| SLC8A3 | 5'-GCATATGGGAGCTTGGAG-3'                    | 5'-GCTTTCTGTCTGTCATTTCTGT-3'               |

Table S2. Kinetic properties of isolated EOM from WT and ryr3−/− mice

| EOM        | Peak force (ms) | TTHP (ms) | TTP (ms) | HRT (ms) |
|------------|-----------------|-----------|----------|----------|
| Twitch force kinetic parameters |                 |           |          |          |
| WT EOM     | 0.328 ± 0.095   | 2.53 ± 0.24| 10.09 ± 1.06| 42.28 ± 9.82 |
| (n = 8)    | (n = 8)         | (n = 8)   | (n = 8)  |          |
| ryr3−/− EOM| 0.126 ± 0.017*  | 8.81 ± 2.29*| 42.75 ± 8.78**| 147.44 ± 8.36**** |
| (n = 9)    | (n = 9)         | (n = 9)   | (n = 9)  | (n = 9)  |

Experiments were performed at 30°C. Values represent mean ± SEM. *, P < 0.05; **, P < 0.01; ****, P < 0.0001, Student’s t test.

Video 1. **WT mouse swimming in cued water maze.** Experiments were performed as described in the Materials and methods section.

Video 2. **ryr3−/− mouse swimming in cued water maze.** Experiments were performed as described in the Materials and methods section.