

The ryanodine receptor is essential for larval development in *Drosophila melanogaster*

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We have investigated the role of the ryanodine receptor in *Drosophila* development by using pharmacological and genetic approaches. We identified a *P* element insertion in the *Drosophila* ryanodine receptor gene, *Ryanodine receptor 44F* (*Ryr*), and used it to generate the hypomorphic allele *Ryr*¹⁶. An examination of hypodermal, visceral, and circulatory muscle showed that, in each case, muscle contraction was impaired in *Ryr*¹⁶ larvae. Treatment with the drug ryanodine, a highly specific modulator of ryanodine receptor channel activity, also inhibited muscle function, and, at high levels, completely blocked hypodermal muscle contraction. These results suggest that the ryanodine receptor is required for proper muscle function and may be essential for excitation-contraction coupling in larval body wall muscles. Nonmuscle roles of *Ryr* were also investigated. Ryanodine-sensitive Ca²⁺ stores had previously been implicated in phototransduction; to address this, we generated *Ryr*¹⁶ mutant clones in the adult eye and performed whole-cell, patch-clamp recordings on dissociated ommatidia. Our results do not support a role for *Ryr* in normal light responses.

The release of Ca²⁺ from intracellular stores is essential for the initiation and propagation of many Ca²⁺ signaling events (1). One of the channels that mediates this release from the endoplasmic reticulum is the ryanodine receptor (RyR), which, in vertebrates, is encoded by a family of three genes. The RyR channel is a ≈2-MDa homotetramer, and its activity is regulated by a diverse group of molecules and proteins, including Ca²⁺, cyclic ADP ribose, Ca²⁺-calmodulin, and FK506BP (2). All three RyR genes are expressed in brain, and one or more isoforms have been detected in a wide range of tissues (3), but mutations in these genes have largely reflected their roles in muscle contraction. RyR1 is essential for excitation-contraction coupling (ECC) in skeletal muscle (4), whereas RyR2 is important for cardiac muscle contraction (5). In contrast, RyR3-deficient mice showed few overt defects (6), although recent studies suggest a role for RyR3 in spatial learning and synaptic plasticity (7). Functional redundancies among the family members are possible, given their overlapping expression patterns.

The role of RyR in contraction appears to be conserved in invertebrates. The single *Caenorhabditis elegans* RyR gene is expressed and functions solely in muscles. The null mutant, *unc-68*, has diminished muscle function, resulting in decreased movement, slow growth, and low brood sizes (8). A single RyR gene, *Ryanodine receptor 44F* (*Ryr*), has been cloned in *Drosophila melanogaster* (this paper, and refs. 9 and 10), and the predicted protein shows ≈45% identity to mammalian family members. During embryogenesis, *Ryr* was shown to be expressed in developing body wall and head muscles (10), suggesting a role for *Ryr* in muscle contraction. In adults, *Ryr* transcript was found in muscles and the brain, and recently it was proposed that ryanodine-sensitive stores are essential for phototransduction (11).

Experimental Procedures

Isolation of *Ryr*¹⁶. The inverse PCR method of Jay Rehm [Berkeley *Drosophila* Genome Project (BDGP), unpublished observations] was used to obtain genomic sequence flanking the inser-

tion site of each *P* element that mapped to 44F (12). The resulting sequences were analyzed by using SEQUENCHER software. To generate deletions in *Ryr*, *l(2)k04913*, a *P*{*lacW*} that carries the miniwhite marker, was mobilized by introducing *T^{hs}.PRΔ2-3*, a stable source of transposase on the third chromosome. *l(2)k04913/CyO*; *T^{hs}.PRΔ2-3* males were crossed to *CyO/Adv* females, and 150 white *CyO* males were selected from the progeny and used to establish individual lines. Genomic DNA from each lethal excision line was screened by PCR using primers upstream of the insertion site and within *Ryr*. Roughly 10% of the lines yielded PCR products shorter than wild type, and these products were sequenced to determine the deletion boundaries.

Molecular Biology. Exons encoding the 5' untranslated region (UTR) of *Ryr* mRNA were identified by screening the HR library (Berkeley *Drosophila* Genome Project, unpublished observations) and by 5' rapid amplification of cDNA ends, which was performed by using the CLONTECH Marathon cDNA Amplification Kit. Exon designation and position within *Ryr* are as described (ref. 9; *Ryr* accession no. D17389). Two exons encoded the 154-bp 5' UTR: P1 and a new exon, designated P0, which was mapped relative to P1 by using Roche Molecular Biochemicals' Long Template PCR. The 5' boundary of exon P1 was reassigned to nucleotide 231, with the coding sequence starting at position 344.

RNA in Situ. *In situ* hybridization was performed on embryos as described (13) with modifications. Probes corresponding to exons within 18,216–25,477 bp and to 4,672–6,400 bp of the *Ryr* gene were derived from a partial cDNA or made by PCR amplification of genomic sequence, respectively. Digoxigenin (DIG) labeling (and subsequent detection) was carried out by using Roche Molecular Chemicals reagents and protocols with 50 ng of template. DIG probes were purified by ethanol precipitation or with Qiagen (Chatsworth, CA) nucleotide removal kit. To detect probe, embryos were incubated with preadsorbed anti-DIG-AP Fab fragments.

Larval Collection, Visualization, and Ryanodine Treatment. Assays were performed at room temperature (22°) on 0–2 h, i.e. newly hatched, larvae. For all experiments, *w*¹¹¹⁸ larvae are referred to as “wild type.” Adults laid embryos for 3 h at 25° on molasses agar plates seeded with a dab of yeast paste. To collect larvae, plates were cleared at 20–21 h and incubated for an additional 2 h in the absence of yeast. Larvae were placed on apple juice agar plates to detect the marked balancer *CyO-GFP^{prmm}*, in which green fluorescent protein (GFP) is expressed ubiquitously in

Abbreviations: RyR, ryanodine receptor; ECC, excitation-contraction coupling; DIG, digoxigenin; GFP, green fluorescent protein; BWC, body cell contraction; DV, dorsal vessel.

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embryos and larvae under the *armadillo* promoter (K.M.C.S. and M. Brodsky, unpublished results). GFP was detected with a Leica MZ8 stereomicroscope equipped with a fluorescence module and GFP-Plus filter set. The fluorescent images in Figs. 1 and 4 A and B were taken on a Leica confocal microscope, using an Argon 488-nm laser, GFP filter set, and the pseudocolor overglow, which gave the best contrast. Ethanol stocks of 1–10 mM ryanodine were diluted 1:100 in 5% yeast paste, which was then aliquotted onto glass microfibre filters (GF/C 2.4 cm, Whatman) placed on molasses agar plates. Larvae were allowed to ingest ryanodine for 30 min before performing muscle-contraction assays. Note that the effective concentration of ryanodine in larval muscles is unknown.

Muscle-Contraction Assays. Newly hatched larvae were placed on a molasses agar plate, and for those undergoing continuous, forward movement (80–100% of the sample), the number of body wall contractions (BWC) that occurred in a 30-s period was counted and converted to BWC/min. For ingestion assays, newly hatched larvae were placed directly on yeast paste dyed with bromophenol blue on a molasses agar plate. The dye is easily visible in the gut: the first section of the midgut appears as a slightly curving posterior extension from the pharynx, the hindgut as an elongated question mark or hook ending at the posterior. At each time point, larvae were removed from food and scored for dye uptake: those positive for ingestion had dark blue staining in the first section of the midgut, those negative had no or very light blue staining. To assay excretion, larvae were fed blue yeast for 4 h, then those with blue dye in the hindgut were transferred to undyed yeast. Larvae scored positive for excretion if the dye had been completely eliminated from their systems. To assay defecation specifically, larvae fed blue yeast as above were placed on a thin layer of undyed yeast for 30 min, and the number of blue spots, representing individual defecation events, was determined. Dorsal vessel (DV) contraction was detected by crossing *GAL4^{mef2.PR}* (14) or *Ryr¹⁶/CyO*; *GAL4^{mef2.PR}* females and *GFP^{UAS}* or *Ryr¹⁶/CyO*; *GFP^{UAS}* males. Newly hatched progeny were fed blue yeast for 4 h, to eliminate the yolk autofluorescence, enhance contrast between the DV and the underlying gut, and detect mutants. The *Ryr¹⁶* larvae were identified by their rounded appearance, slow movement, and blue yeast in the pharynx. Contractions were counted by observing the dorsal vessel through the cuticle after rinsing the larvae in water to remove yeast paste.

Electrophysiological Recordings. Large (>90% of the eye) *Ryr¹⁶* clones were generated in *eyFlp1.2*; *P{ry⁺ hs.neo FRT}42D*, *P{w⁺}*, *M53/P{ry⁺ hs.neo FRT}42D*, *Ryr¹⁶* animals (15). *M53* is a minute mutation, and *eyFlp1.2* is the *flp recombinase* expressed under control of the *eyeless* enhancer (M. Brodsky and G.M.R., unpublished results). Retinas were removed from newly eclosed animals, the white mutant tissue was dissected out and dissociated, and then whole-cell patch-clamp recordings were performed as described (16). The bath solution contained 124 mM NaCl, 4 mM KCl, 10 mM HEPES, 5 mM proline, 25 mM sucrose, 1.5 mM CaCl₂, pH 7.15. The pipette solution contained 95 mM potassium gluconate, 40 mM KCl, 10 mM HEPES, 2 mM MgCl₂, 0.2 mM EGTA, pH 7.15. Photoreceptors were clamped at a holding potential of –70 mV. Whole-cell capacitances were >35 pF, and seal resistances were >1 GW. Junction potentials were nulled just before seal formation and most (80%) series resistance errors were compensated during recording. In all experiments, light was filtered through a bandpass filter (1 = 580 ± 10 nm), neutral density filters, and focused onto the photoreceptor cells by a 0.5 numerical aperture, 40× objective. Stimulation was by means of a 75-W Xenon source; unattenuated output at the stage was 10 mW for white light. Alternatively, heads of *Ryr¹⁶* mosaic animals were cut coronally, and photoreceptor cells were

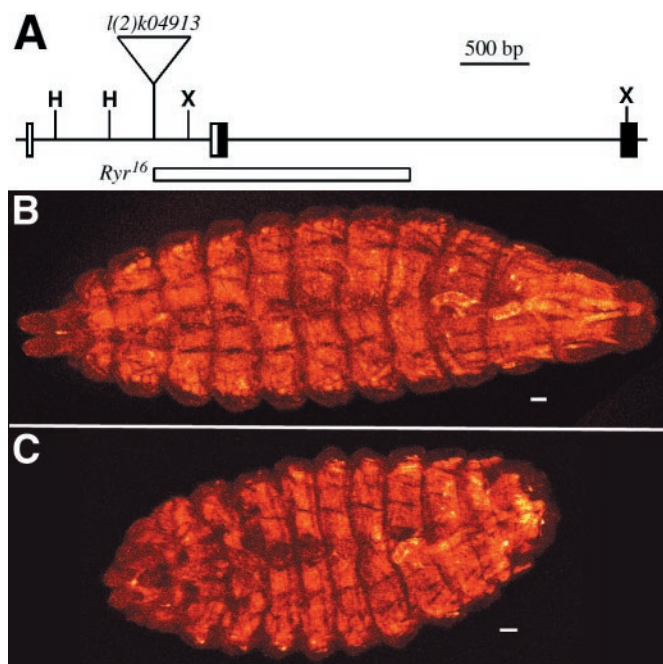


Fig. 1. *Ryr¹⁶* is a deletion in *Ryanodine receptor 44F*. (A) The first three exons of *Ryr* (of 27) are represented as rectangles, the filled portions indicating coding and the open portions 5′ untranslated region sequences. The inverted triangle marks the position of *l(2)k04913*, and the region deleted in *Ryr¹⁶* is indicated by an open rectangle. (H, *HindIII*; X, *XhoI*). (B and C) The muscles of newly hatched *Ryr¹⁶* and *Ryr¹⁶/CyO* larvae are visualized by expressing GFP under control of the *Mef2* promoter with the *UAS/GAL4* system. GFP fluorescence is shown in pseudocolor. (Scale bars, 10 μm.) (B) *Ryr¹⁶* heterozygotes phenotypically resemble wild type (data not shown). (C) The muscles of *Ryr¹⁶* larvae appear to have developed normally, but the mutant animals appear smaller and rounder than wild type, and the head is not properly extended.

penetrated with sharp microelectrodes (70–150 MW) filled with 2 M KCl. Bath solution and light stimulation were exactly as described above, except a 530-nm longpass filter was used. Responses obtained from five *Ryr¹⁶* patches and five wild-type patches were not notably different, in agreement with the patch-clamp results.

Results and Discussion

The *Drosophila* genome contains a single RyR gene at cytological position 44F (ref. 9, and Berkeley *Drosophila* Genome Project, unpublished observations). Although an RyR cDNA had previously been partially sequenced and mapped to 76C (10), this localization could not be confirmed (9), and the cDNA’s sequence was >99% identical to the corresponding exons at 44F. In this paper, we have subsumed both sets of published results under a single gene, renamed the *Ryanodine receptor 44F* (*Ryr*).

We obtained genomic sequence flanking each *P* element in the 44F region and determined that *P{lacW}l(2)k04913* was inserted in an intron of *Ryr*, 255 bp upstream of the first coding exon (Fig. 1A). We excised *l(2)k04913* and found that 80% of the independent excision lines were viable, indicating that the lethal lesion of *l(2)k04913* corresponded to the *P* element insertion site and that there were no other lethal mutations on the chromosome. All but one of the lethal excisions failed to complement *l(2)k04913* and *Df(2R)Np3*, a deficiency that uncovers *Ryr^{k04913}*, confirming that these mutations were allelic to the *P* element. Using PCR, we identified four lethal excision lines that had deletions specifically in *Ryr*. The largest, *Ryr¹⁶*, removed the first coding exon and extended into the first and second introns (Fig. 1A).

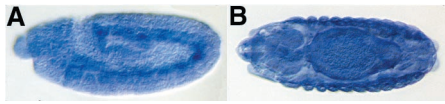


Fig. 2. *Ryr* mRNA is detected in developing visceral and hypodermal muscles. *In situ* hybridization was performed on wild-type embryos with DIG-labeled probes made from a partial *Ryr* cDNA. (A) A stage-11 embryo is shown in a lateral view, with dorsal up and anterior to the left. Expression of *Ryr* mRNA is strongest in the mesoderm. (B) A stage-14 embryo is viewed from the dorsal perspective, anterior to the left. *Ryr* expression appears widespread with high levels in hypodermal and visceral muscles.

To identify mutants, the *Ryr¹⁶* chromosome was placed over the balancer *CyO-GFP^{arm}*, which specifically marked heterozygous animals with GFP. The development of *Ryr¹⁶* embryos is normal as determined by direct observation and with myosin heavy chain (muscle) and fasciclin II antibodies, which stain muscles and a subset of embryonic central nervous system axons (data not shown). However, in contrast to *Ryr¹⁶/CyO-GFP^{arm}* larvae, which were indistinguishable from wild type, *Ryr¹⁶* larvae appeared short and rounded (Fig. 1 *B* and *C*); furthermore, they did not grow and died over 4–7 days as first instar larvae. To control for developmental changes and for defects caused indirectly by malnutrition, all experiments were performed on newly hatched larvae, unless otherwise stated. It should be noted that *Ryr¹⁶* larvae appeared unchanged for the first 12 h after hatching, as assayed by BWC, and not until 12–24 h was an increase in phenotypic severity observed (data not shown).

Lethal stage analysis revealed that approximately 60% of *Ryr¹⁶/Df(2)Np3* embryos failed to hatch, whereas newly hatched *Ryr^{k04913}* larvae appeared normal and did not cease growth and die until the late first/second instar stages (data not shown). The relative allelic strength was tentatively assigned as *Ryr¹⁶/Df(2R)Np3* > *Ryr¹⁶* > *Ryr^{k04913}*. These results indicate that *Ryr¹⁶* is not a null allele, despite deletion of the first coding exon. Reverse transcription–PCR experiments confirmed the presence of *Ryr* mRNA in *Ryr¹⁶* larvae (data not shown); thus, a functional, truncated ryanodine receptor may be synthesized. *Ryr* encodes a protein >5,000 amino acids in length that contains many methionine residues. The transmembrane, channel-forming domain is found at the extreme C terminus, and it has been shown for RyR1 that the C-terminal third of the protein is sufficient to make a functional channel (17).

Although *Ryr* transcript was reported to be exclusively localized to hypodermal and head muscles during late embryogenesis (10), we found *Ryr* to be more widely expressed. RNA *in situ* were performed on embryos using both genomic- and cDNA-derived probes. *Ryr* expression was first detected in the mesoderm around stage 9 and then increased starting at stage 13 (Fig. 2 *A* and *B*). The highest levels were seen in hypodermal muscles and in the visceral muscles surrounding the gut. The transcript was also detected at lower levels in other tissues, notably the central nervous system.

Based on the *in situ* results, we first examined the role of *Ryr* in hypodermal or body wall muscles, which are analogous in structure and function to vertebrate skeletal muscle (18). Forward movement is initiated by contraction of the posterior body wall muscles, moving the tail up, forward, and down. The contraction propagates as a constriction anteriorly, narrowing and lengthening the larval body, and finally terminates on extension of the mouth hooks up and forward. Initiation of a new BWC typically does not occur until termination of the previous one; however, initiation and propagation appear to be independent processes. For example, in *calmodulin* null mutants, initiation of BWCs was greatly decreased, whereas propagation occurred at normal speeds once initiated (19).

BWC was quantified by counting the number of end-to-end contractions a single larva underwent per minute. In *Ryr¹⁶* larvae, the timing of BWC initiation appeared normal, but the contractions propagated more slowly than in wild-type and *Ryr¹⁶/CyO-GFP^{arm}* controls, such that the rate of BWC was reduced by 50% (Fig. 3*A*). Furthermore, contraction in *Ryr¹⁶* animals was attenuated and often had associated tremors; this muscle weakness is probably responsible for the altered larval appearance. As expected, *Ryr¹⁶/Df(2R)Np3* larvae had a more severe defect in BWC propagation, which was reduced by over 90% (Fig. 3*A*). The weakest allele, *Ryr^{k04913}*, had a small but significant decrease in the rate of BWC propagation compared with wild-type and *Ryr^{k04913}/CyO-GFP^{arm}* controls. By the second instar, *Ryr^{k04913}* larvae had visible contraction defects in hypodermal muscle, which included tilting and dragging of the mouth hooks (data not shown).

Recent reports suggest that larval growth defects do not necessarily impair feeding or locomotion (20, 21). To confirm this, BWC was examined in *Itp-r¹* larvae. *Itp-r¹* is a deletion in the *InsP₃* receptor gene, and these mutants do not progress beyond the second instar (22). Despite the larval growth defect, BWC rates in newly hatched *Itp-r¹* were completely normal (Fig. 3*A*).

The drug ryanodine is a highly specific modulator of the RyR channel. *In vitro* studies have demonstrated that low doses of ryanodine (<10 μ M) activate the channel, medium doses (>10 μ M) lock the channel into a subconductance state, and high concentrations (\geq 100 μ M) completely inactivate it (23, 24). A range of ryanodine concentrations was fed to newly hatched larvae in yeast paste, and the effect on BWC rates was determined (Fig. 3*B*). Low concentrations of ryanodine (<5 μ M) had no significant effect on BWC rates when compared to larvae fed yeast paste doped with solvent alone (data not shown). Higher ryanodine levels (5–100 μ M) decreased the rate of BWC propagation, but not initiation, and at the highest doses (\geq 100 μ M), BWC was completely inhibited. Ryanodine similarly inhibited BWC in second and third instar larvae (data not shown). Ryanodine concentrations \geq 10 μ M also caused larvae to round up, altering their appearance in a manner similar to that of *Ryr¹⁶*. These results demonstrate that *Ryr¹⁶* is phenocopied by inhibitory concentrations of ryanodine, which is expected if the drug and the mutation both target *Ryr*. The complete inhibition of BWC by ryanodine provides further evidence that *Ryr¹⁶* is not a null allele and suggests that the ryanodine receptor is essential for ECC in hypodermal muscles. In contrast, *C. elegans* can undergo muscle contraction even in the absence of RyR activity (8).

Visceral muscle function was analyzed in wild-type and *Ryr¹⁶* larvae by assaying the ingestion and excretion of food. The dye bromophenol blue is nontoxic, readily ingested, and completely excreted by larvae, as well as easy to detect through the cuticle. In a typical ingestion assay (Fig. 3*C*), 96–100% of wild-type (not shown), *Itp-r¹*, and *Ryr¹⁶/CyO-GFP^{arm}* larvae scored positive for ingestion within 30 min. A very mild, but reproducible, ingestion defect was seen in *Ryr^{k04913}* larvae. In sharp contrast, *Ryr¹⁶* larvae ingested food much more slowly, and the percentage that scored positive failed to reach 100 even after 2 days (data not shown). Additionally, *Ryr¹⁶* larvae accumulated food in the pharynx, which was never seen in controls, and those that failed to ingest food were indistinguishable in growth and movement from those that did (data not shown). *Ryr¹⁶* mutants may have additional defects in nutrient absorption or metabolism; however, it may simply be that none of the *Ryr¹⁶* larvae ingest sufficient food for growth. Our results demonstrate that *Ryr¹⁶* mutants have a severe defect in the ingestion and passage of food into the gut, suggesting that the head and visceral muscles are impaired.

Pulse–chase and defecation assays were used to measure visceral muscle function specifically. Newly hatched larvae were fed blue yeast for 4 h, then transferred to undyed yeast and scored for complete loss of the dye. In a typical excretion time

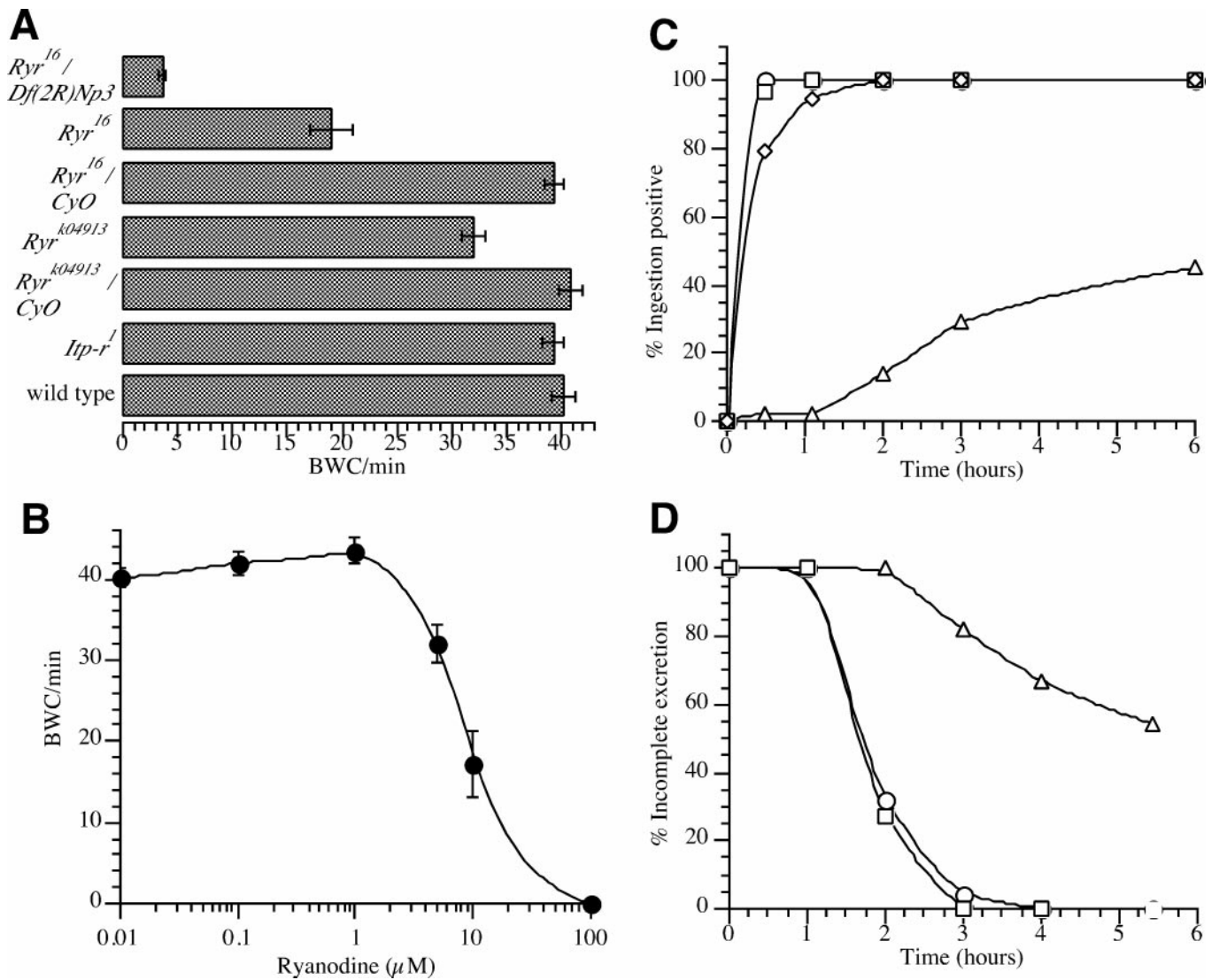


Fig. 3. Muscle function is impaired in ryanodine-treated and *Ryr¹⁶* larvae. (A) The average BWC/min was determined for newly hatched larvae of the specified genotype. The wild type, *Itp-r¹*, *Ryr^{k04913}/CyO-GFP^{arm}*, and *Ryr¹⁶/CyO-GFP^{arm}* larvae performed identically in this assay. *Ryr^{k04913}* larvae had a slight, reproducible reduction, *Ryr¹⁶* a \approx 50% reduction, and *Ryr¹⁶/Df(2R)Np3* hemizygotes a 90% decrease in BWC/min. The BWC/min for each genotype was measured at least three times on separate collections of larvae ($n = 30$), and the error is the SEM. (B) Yeast paste freshly doped with 0.01–100 μ M ryanodine was fed to newly hatched *Ryr¹⁶/CyO-GFP^{arm}*, and the effect on BWC rates was determined after 30 min. Concentrations of ryanodine 1 μ M or less and the ethanol control (not shown) had no significant effect on BWC rates, and concentrations \geq 100 μ M completely inhibited BWC. For each concentration of ryanodine, the BWC measurement was performed on at least two separate larval samples ($n = 30$), and the error is the SEM. (C) Larvae scored positive for ingestion if they had a concentrated level of dye in the first section of the midgut. Representative curves are shown for *Ryr¹⁶/CyO-GFP^{arm}* (\circ) and *Itp-r¹* (\square). *Ryr^{k04913}* larvae (\diamond) showed only a slight lag in reaching 100% positive compared with controls, but the delay was reproducible in multiple trials. In contrast, the percentage of positive *Ryr¹⁶* larvae (\triangle) typically ranged from 40% to 60% at the end of a 6-h time course. (D) To assay excretion, larvae positive for ingestion after feeding blue yeast for 4 h were transferred to unadulterated yeast paste and scored for the complete loss of dye. *Ryr¹⁶/CyO-GFP^{arm}* (\circ), *Itp-r¹* (\square), and wild-type larvae (not shown) had nearly identical excretion time courses. The rate of excretion was significantly decreased in *Ryr¹⁶* larvae (\triangle). For C and D, each larval genotype was assayed in at least three independent trials ($n \geq 30$).

course, 100% of *Ryr¹⁶/CyO-GFP^{arm}*, *Itp-r¹*, and wild-type larvae (Fig. 3D, and data not shown) had completely excreted the dye within 3 h. In contrast, *Ryr¹⁶* larvae excreted the dye extremely slowly, such that roughly half still retained dye in the gut when assayed for up to 12 h. As a second test for visceral muscle function, we measured the rate of defecation. Wild-type and heterozygous larvae on average defecated once every 4 min, *Ryr^{k04913}* once every 7 min, and *Ryr¹⁶* less than once per 60 min. Both assays demonstrate that *Ryr¹⁶* larvae have a severe defect in excretion, consistent with impaired visceral muscle function.

Drosophila visceral muscles, although considered striated muscles, are distinct from those of the hypodermus: they are much

smaller (1 μ m in diameter) and mononucleate; the contractile elements are reduced in density and more disorganized; and the sarcoplasmic reticulum (SR) is less developed (25, 26). It has been proposed that the extent of SR in a given muscle type correlates with the requirement for intracellular Ca^{2+} , and thus the RyR, in contraction (10). However, this hypothesis is inconsistent with the severe visceral defect seen in *Ryr¹⁶* larvae. One possibility is that visceral muscle ECC requires Ca^{2+} release from internal stores, but not the rapid, widespread release enabled by extensively developed stores. Alternatively, intracellular Ca^{2+} stores may be essential for some other process equally vital for the passage of food through the gut, such as Ca^{2+} homeostasis or muscle relaxation.

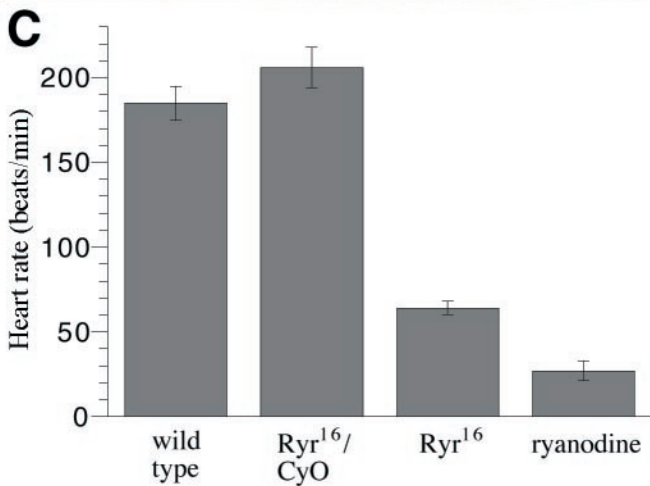
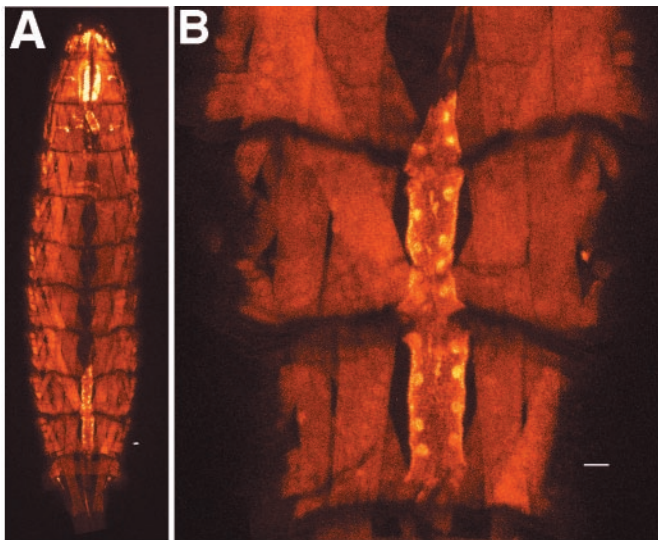


Fig. 4. Dorsal vessel contraction is reduced in *Ryr¹⁶* and ryanodine-treated larvae. (A) GFP is expressed in all muscles of a wild type, second instar larvae (see Fig. 2). (B) The cardiac cells of the heart are shown at higher magnification with the dorsal vessel in the closed position. (Scale bars in A and B, 10 μ m.) (C) Heart rate was measured by counting the number of beats per minute for a population of each larval genotype. There was no significant difference between the heart rate of wild-type (second instar) and *Ryr¹⁶/CyO* (first instar) larvae. Both *Ryr¹⁶* larvae and second instar larvae treated with 100 μ M ryanodine, however, showed a marked reduction in the rate of contraction. Heart rates were measured for each genotype in at least three independent experiments ($n \geq 15$), and the error is the SEM.

The DV is the major pulsatile organ of the *Drosophila* circulatory system (27). The contractile region or heart is a tube-shaped chamber of cardiac cells in the posterior segments that circulates the hemolymph by lateral constriction (Fig. 4 A and B). The aorta extends anteriorly and connects to the ring gland and lymph nodes. Little is known about the structure or physiology of the circulatory muscle, although L-type channels have been implicated in DV contraction (28), as is the case in vertebrate cardiac cells. DV function and development have been compared with that of the vertebrate heart, although it has recently been argued that visceral muscle development is the more analogous (14).

By expressing GFP in muscles, we were able to examine the larval heartbeat *in vivo* for all three larval stages and found that it remained relatively consistent (data not shown). The heartbeat frequency ranged from slow to fast to fibrillating, with the heart

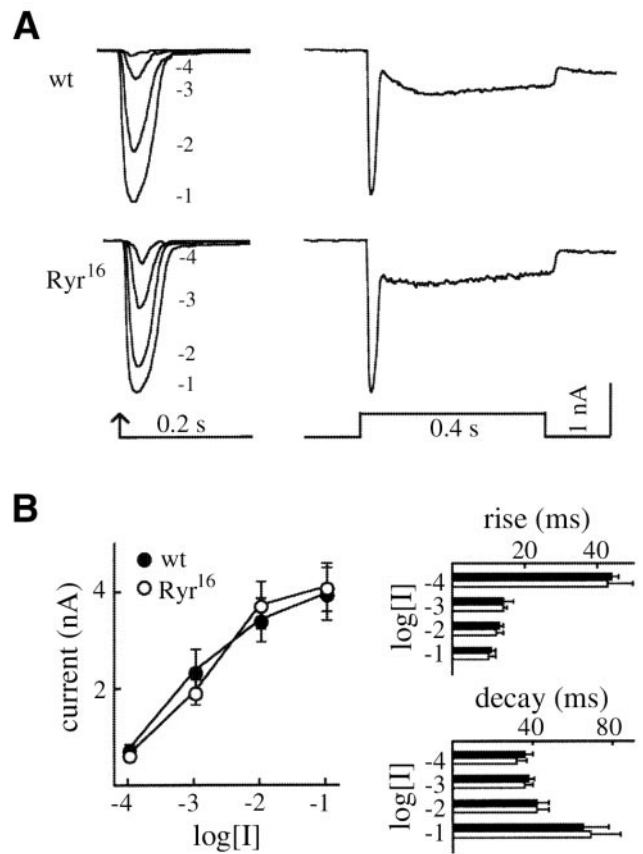


Fig. 5. Phototransduction is normal in *Ryr¹⁶* eye clones. (A) Representative whole-cell voltage-clamp recordings of light-induced currents from a wild type (wt) and a *Ryr¹⁶* photoreceptor. (Left) Cells were stimulated with 10-ms flashes of increasing light intensity given at the arrow. Numbers refer to the log order of light intensity associated with that light response (e.g., -4 is 10 times less light than -3). (Right) Cells were stimulated with a 400-ms pulse of light ($\log [I] = -1$). In each case, *Ryr¹⁶* responses are indistinguishable from wild type. (B) Quantitative analysis of responses to 10-ms flashes of light. The plot shows peak response amplitude vs. light intensity for wild type and *Ryr¹⁶*, and the bar graphs compare rise time (measured as the time between 10–90% rise of the peak current) and decay time (measured as the time between 90–10% decline of the current) vs. light intensity. Black bars correspond to wild type, white to *Ryr¹⁶*. Each data point represents the mean response \pm SE for seven wt cells and 15 *Ryr¹⁶* mutant cells sampled from 12 *Ryr¹⁶* mutant patches.

occasionally pausing for up to several seconds; more rarely, localized contractions or twitches occurred. There was no obvious correlation between larval behavior or movement and heart rate. *Ryr¹⁶/CyO* and wild-type heart rates and behavior were indistinguishable (Fig. 4C). In *Ryr¹⁶* larvae, the heart rate was reduced by $\approx 75\%$ relative to the heterozygotes. The decrease in heart rate was caused by loss of the fast and fibrillating contractions but not an increase in pausing.

The effect of ryanodine on the dorsal vessel was examined in wild-type first (data not shown) and second (Fig. 4C) instar larvae. Feeding larvae $\geq 25 \mu$ M ryanodine decreased heart rates (data not shown), and $\geq 100 \mu$ M ryanodine reduced it by $\approx 85\%$ relative to wild type. It was not possible to completely inhibit circulatory muscle contraction with ryanodine as it was in the case of hypodermal muscles. This may reflect experimental limitations, because the larvae must be washed to observe the GFP signal. As detected by increasing BWC rates, larvae rapidly recovered when washed out of ryanodine. Alternatively, the ryanodine receptor may not be essential for ECC in circulatory muscles.

In previous studies (11), it was observed that depletion of Ca^{2+} stores in dissected photoreceptor cells by ryanodine inhibited subsequent light responses. This effect could be rescued by the addition of Ca^{2+} -calmodulin, which inhibits Ca^{2+} release by the ryanodine receptor. Based on these results, it was proposed that Ca^{2+} release through the RyR is required for phototransduction. However, these experiments could not distinguish a pleiotropic effect of the pharmacological depletion of internal stores from an actual role for the RyR in light transduction. To test this, *Ryr¹⁶* mutant eye clones were generated in heterozygous adults by using the *Flp/FRT* system (15). We generated clones representing >90% of the eye using *eyFlp* and a marker chromosome carrying the minute (*M53*) mutation, which slows cellular growth. The *eyeless* enhancer drives *Flp* expression specifically in the eye disc, and the resulting *Ryr¹⁶* mutant cells outgrow both *M53/Ryr¹⁶* and *M53/M53* cells in mosaic tissue. *Ryr¹⁶* clones appeared morphologically normal (data not shown); furthermore, whole-cell, voltage-clamp recordings on *Ryr¹⁶* ommatidia showed responses indistinguishable from wild type (Fig. 5 *A* and *B*). Taken together, these results are inconsistent with the postulate that *Ryr* is required for phototransduction.

To examine the role of *Ryr* in embryonic development, we generated females with germ-line clones homozygous for *Ryr¹⁶* using the dominant female sterile technique (29). However, the

progeny of these females, when crossed to *Ryr¹⁶* males, were phenotypically identical to either wild type or *Ryr¹⁶* (data not shown). Consistent with this, RNA interference (30) using double-stranded RNA transcribed from *Ryr* cDNA had no apparent effect on embryogenesis, although a majority of the injected embryos that hatched did have muscle contraction defects (data not shown). The overall behavior of *Ryr¹⁶* larvae was indistinguishable from wild type (data not shown): they detected and migrated to food sources; had an enhanced rate of food ingestion after starvation; had normal salt avoidance and response to mechanical stimulation; and showed no spontaneous avoidance behavior. BrdUrd labeling of *Ryr¹⁶* larval brains showed that cell cycle progression is normal in these mutants. Thus, at present our results provide no evidence that *Ryr* has any nonmuscle roles in *Drosophila* development.

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