Zebrafish relatively relaxed mutants have a ryanodine receptor defect, show slow swimming and provide a model of multi-minicore disease

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Wild-type zebrafish embryos swim away in response to tactile stimulation. By contrast, relatively relaxed mutants swim slowly due to weak contractions of trunk muscles. Electrophysiological recordings from muscle showed that output from the CNS was normal in mutants, suggesting a defect in the muscle. Calcium imaging revealed that Ca2+ transients were reduced in mutant fast muscle. Immunostaining demonstrated that ryanodine and dihydropyridine receptors, which are responsible for Ca2+ release following membrane depolarization, were severely reduced at transverse-tubule/sarcoplasmic reticulum junctions in mutant fast muscle. Thus, slow swimming is caused by weak muscle contractions due to impaired excitation-contraction coupling. Indeed, most of the ryanodine receptor 1b (ryr1b) mRNA in mutants carried a nonsense mutation that was generated by aberrant splicing due to a DNA insertion in an intron of the ryr1b gene, leading to a hypomorphic condition in relatively relaxed mutants. RYR1 mutations in humans lead to a congenital myopathy, multi-minicore disease (MmD), which is defined by amorphous cores in muscle. Electron micrographs showed minicore structures in mutant fast muscles. Furthermore, following the introduction of antisense morpholino oligonucleotides that restored the normal splicing of ryr1b, swimming was recovered in mutants. These findings suggest that zebrafish relatively relaxed mutants may be useful for understanding the development and physiology of MmD.

KEY WORDS: Zebrafish, Ryanodine receptor, Muscle, Calcium, Multi-minicore disease

INTRODUCTION
Zebrafish are useful for the study of motor development and disorders. First, forward genetics can be applied to this organism to identify genes that are essential for proper behaviors (Granato et al., 1996). Second, embryos are transparent, facilitating the visualization of dynamic events, such as cell migration, axonal outgrowth and calcium transients, in live fish (Fetcho and Bhatt, 2004; Wilson et al., 2002). Third, electrophysiological techniques can be used to analyze the physiology of embryonic neurons and muscles (Drapeau et al., 2002; Fetcho, 2006). Fourth, zebrafish embryos exhibit readily assayable and well-characterized behaviors (Eaton and Farley, 1973; Saint-Amant and Drapeau, 1998). Fifth, zebrafish mutants can potentially serve as animal models of human motor disorders (Bassett and Currie, 2003; Kunkel et al., 2006; Lieschke and Currie, 2007).

Zebrafish embryos display three stereotyped behaviors by 36 hours post-fertilization (hpf) (Saint-Amant and Drapeau, 1998). The earliest behavior consists of repetitive, slow and alternating coiling of the trunk and tail. This coiling is independent of sensory stimulation and observed from 17 to 26 hpf. After 21 hpf, embryos respond to mechanosensory stimulation with two or three rapid C-bends of the trunk and tail. By 26 hpf, embryos swim in response to tactile stimulation. The frequency of muscle contractions during swimming increases from 7 Hz at 26 hpf to 30 Hz at 36 hpf, the latter being similar to the frequency of swimming by adult zebrafish (Buss and Drapeau, 2001).

The process of touch-induced swimming involves a number of steps, starting with the sensing of tactile stimuli and ending with the contraction of muscles. Touch is sensed by Rohon-Beard neurons in the trunk and tail or trigeminal sensory neurons in the head (Drapeau et al., 2002). Once triggered by sensory inputs, interneuronal networks located in the hindbrain and spinal cord create the appropriate motor pattern that alternately activates motor neurons in each side of the spinal cord (Fetcho, 1992; Gahtan et al., 2002). Motor terminals release acetylcholine at the neuromuscular junction (NMJ) to depolarize the muscle membrane (Buss and Drapeau, 2001; Wen and Brehm, 2005) and the change of membrane potential is converted to muscle movement by excitation-contraction (E-C) coupling (Franzini-Armstrong and Protasi, 1997). Depolarizations of the plasma membrane spread down the transverse-tubules (t-tubules), which are invaginations of the plasma membrane, and cause conformational changes of the dihydropyridine receptor (DHPR), a voltage sensor located in the t-tubule membrane. DHPRs then trigger the opening of ryanodine receptor 1 (RYR1) in the adjacent sarcoplasmic reticulum (SR) to allow Ca2+ release from the SR to the cytosol (Meissner, 1994). Elevated cytoplasmic Ca2+, in turn, activates the sliding of actin/myosin to produce muscle contraction.

The membranes of t-tubules and SR are juxtaposed and permit direct physical interactions between DHPR and RyR1 in skeletal muscle (Block et al., 1988). The skeletal muscle DHPR is composed of the voltage-sensing and pore-forming α1S subunit, intracellular modulatory β1 subunit, and auxiliary α2δ1 and γ1 subunits (Catterall, 2000; Flucher et al., 2005). A tetrad, which is a cluster of four DHPRs, associates with a Ca2+-releasing RyR1 channel, which is formed with four RyR1 monomers. The RyR1 protein, the largest known ion channel protein, weighs 560 kDa.
(Takehshima et al., 1989) and, thus, RyR1 channels can be observed in electron micrographs as dots of high electronic density (Franzini-Armstrong and Protasi, 1997). Although a hydrophobic C-terminus domain might contain several transmembrane domains as well as the channel pore, the exact number and position of membrane domains is not known. Three RyR isoforms (RyR1, RyR2 and RyR3) are encoded by different genes in mammals (Fill and Copello, 2002). RyR1 is the most abundant isoform in skeletal muscle. RyR2 predominantly functions in cardiac muscle and RyR3 is expressed by many tissues, but at relatively low levels. RyR1-deficient mice do not move because of the absence of E-C coupling and die from dysfunction of the diaphragm muscles shortly after birth (Takehshima et al., 1994). The formation of DHPR tetrads is also impaired in RyR1-deficient myotubes (Takekura et al., 1995).

Mutations in RyR1, encoded by the RYR1 gene in humans, are involved in a pharmacogenetic muscle disorder, malignant hyperthermia (MH), and two congenital myopathies, central-core disease (CCD) and multi-minicore disease (MmD). Inherited as a dominant trait, MH appears as a hypermetabolic crisis when a susceptible individual is exposed to certain anesthetics, such as d-tubocurarine (Sigma) without tricaine. Patch electrodes were pulled from borosilicate glass (Narishige) to yield electrodes with resistances of 2-10 MΩ.

The protocols for Ca²⁺ imaging have been described previously (Hirata et al., 2004). Briefly, we injected Calcium Green-1 dextran (10,000 M₀, Molecular Probes) into one blastomere of 8- to 16-cell-stage progeny of zebrafish carrier in-

Animals

Zebrafish were bred and raised according to established procedures (Nüsslein-Volhard and Dahm, 2002; Westerfield, 2000), which meet the guidelines set forth by the University of Michigan and Nagoya University. ryr⁺/⁻ was identified as a spontaneous mutation in our breeding stock of zebrafish.

Video-recording of zebrafish behavior

Embryonic behaviors were observed and video-records were taken with a dissection microscope. Mechanosensory stimuli were delivered to the tail with forceps. Videos were captured with a CCD camera (WVBP330, Panasonic) and a frame grabber (LG-3, Scion Corporation), and were analyzed with Scion Image on a G4 Macintosh (Apple).

Muscle recording

The muscle recording protocols for in vivo patch recordings have been described elsewhere (Buss and Drapeau, 2000). Briefly, 48 hpf zebrafish embryos were anesthetized with 0.02% tricaine (ethyl 3-aminoenobenzoate methanesulfonate, Sigma), pinned on a Sylgard dish and immersed in Evans solution (134 mM NaCl, 2.9 mM KCl, 2.1 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose and 10 mM Heps at 290 mOsm and pH 7.8). The skin was peeled off to allow access to the underlying muscles. For electrophysiological recordings, embryos were partially curarized in Evans solution containing 3 mM d-tubocurarine (Sigma) without tricaine. Patch electrodes were pulled from borosilicate glass (Narishige) to yield electrodes with resistances of 2-10 MΩ.

The electrode was visually guided to patch muscle cells using Hoffman modulation optics (40× water immersion objective). The electrode solution consisted of 105 mM potassium gluconate, 16 mM KCl, 2 mM MgCl₂, 10 mM Heps, 10 mM EGTA and 4 mM Na₃ATP at 273 mOsm and pH 7.2. Recordings were performed with an Axopatch 200B amplifier (Axon Instruments), low-pass filtered at 5 kHz and sampled at 10 kHz. Data were collected with Clampex 8.2 and analyzed with Clampfit 9.0 (both Axon Instruments). Mechanosensory stimulation was delivered by injecting bath solution (20 psi, 20 milliseconds pulse) into a pipette with a 20 μm tip to the tail of the pinned embryo using a Picospritzer III (Parker Hannifin Corporation) to induce fictive swimming.

Ca²⁺ imaging in muscle

The protocols for Ca²⁺ imaging have been described previously (Hira et al., 2004). Briefly, we injected Calcium Green-1 dextran (10,000 M₀, Molecular Probes) into one blastomere of 8- to 16-cell-stage progeny of zebrafish carrier in-

RyR1b mRNA, encoding RyR1, carried a nonsense mutation in relatively relaxed (ryr) mutant, which was identified as a spontaneous mutation in our breeding stock of zebrafish. Mutants displayed slow swimming due to weak muscle contractions despite normal output from the CNS. Ca²⁺ transients in the muscle cytosol and RyR1 at the t-tubules were dramatically decreased in mutant fast muscles, suggesting a defect in E-C coupling. In fact, most of the ryr₁b mutants displayed small amorphous cores in muscle fibers, as observed in electron micrographs as dots of high electronic density (Takeshima et al., 1989) and, thus, RyR₁ channels can be observed in electron micrographs as dots of high electronic density (Franzini-Armstrong and Protasi, 1997). Although a hydrophobic C-terminus domain might contain several transmembrane domains as well as the channel pore, the exact number and position of membrane domains is not known. Three RyR isoforms (RyR1, RyR2 and RyR3) are encoded by different genes in mammals (Fill and Copello, 2002). RyR1 is the most abundant isoform in skeletal muscle. RyR2 predominantly functions in cardiac muscle and RyR3 is expressed by many tissues, but at relatively low levels. RyR1-deficient mice do not move because of the absence of E-C coupling and die from dysfunction of the diaphragm muscles shortly after birth (Takehshima et al., 1994). The formation of DHPR tetrads is also impaired in RyR1-deficient myotubes (Takekura et al., 1995).

In this paper, we characterized the relatively relaxed (ryr) mutant, which was identified as a spontaneous mutation in our breeding stock of zebrafish. Mutants displayed slow swimming due to weak muscle contractions despite normal output from the CNS. Ca²⁺ transients in the muscle cytosol and RyR1 at the t-tubules were dramatically decreased in mutant fast muscles, suggesting a defect in E-C coupling. In fact, most of the ryr₁b mutants displayed small amorphous cores in muscle fibers. Interestingly, analysis of antisense morpholino oligonucleotides against ryr₁b that blocked the aberrant splicing in mutants restored normal swimming. These findings suggest that analysis of the relatively relaxed mutant may be useful for understanding the development of amorphous cores and the physiology of MmD.

E-C coupling

E-C coupling was assessed using a calcium secretion assay (Takekura et al., 1995). Amorphous cores, which run along the long axis of the muscle fibers, can be observed in histological sections of CCD muscle. MmD is characterized by muscle weakness, sclerosis and respiratory insufficiency, but is inherited through a recessive RYR1 mutation (Engel, 1967; Junghbluth et al., 2004). MmD is defined by the presence of multiple small cores in histological sections. Although little is known about the development of cores, it has been proposed that cores are formed as a secondary cellular response to isolate regions of defective Ca²⁺ regulation from regions of normal Ca²⁺ homeostasis (Lyfenko et al., 2004).

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The following primers were used for RT-PCR and genomic PCR: Primer #1, 5'-GTGGTTTTCCGCCATGGAGCTTCA-3' and 5'-AACAGTGGGGGCACTTTAGTGAGTCA-3'; Primer #2, 5'-AACAGTGGGGGACATTTAGTGAGCAGAGG-3'.

Knockdown by morpholino

To knockdown RyR1b protein synthesis (Nasevicius and Ekker, 2000), antisense morpholino (MO1) was designed against acceptor and donor sites of the aberrant exon: 5'-TATCTTACACTTACCTTTAAATAAG-3'; reverse primer 11, 5'-CTTAAAGTACTGGTTTATGAGCGGGAGCAG-3'; reverse primer 12, 5'-AGGAGAGTGAATGCTACAGTGCAGTCA-3'.

In situ hybridization

In situ hybridizations to wholeembryo zebrafish were performed as described previously (Hirata et al., 2004). For sectioning after color development, embryos were equilibrated in 15% sucrose/7.5% gelatin in PBS at 37°C and then embedded in it at –80°C. Sections (10 μm) were cut with a cryostat (CM3050S, Leica). An ryr1 probe covering 1089 bp of the C-terminus amino acids and 148 bp of the 3'-UTR was used for in situ hybridization. An ryr1a probe covering 1157 bp of the C-terminus was cloned with the following primers: ryr1a forward primer, 5'-ATGATTGAGTTTACCGTATCCAGAG-3'; ryr1a reverse primer, 5'-CGTCATACATGTCGTCAGAGAGG-3'.

Transmission electron microscopy

The protocols for transmission electron microscopy have been described elsewhere (Hatakeyama et al., 2004; Schredelseker et al., 2005). Briefly, embryos were fixed with 6% glutaraldehyde-2% paraformaldehyde in 0.1 M sodium cacodylate buffer, the embryos were post-fixed with 1% OsO4 for 60 minutes, and then dehydrated and embedded in Epon 812. Ultrathin sections (80 nm) were cut and examined using an electron microscope (H-7000, Hitachi) operated at 75 kV.

RESULTS

ryr mutants show slow swimming because of weak muscle contractions

An autosomal, recessive mutation, relatively relaxed (ryrmi340), was identified from our breeding stock of zebrafish. Mutant embryos exhibited significantly slower swimming after 36 hpf, but spontaneous coiling at 22 hpf (wild-type siblings: 0.21±0.11 Hz, n=34; ryr mutants: 0.19±0.09 Hz, n=14) and touch-induced fast C-bends at 24 hpf were unperturbed (data not shown). The swimming phenotype was examined by video-microscopy, with wild-type embryos swimming away rapidly upon tactile stimulation and ryr embryos swimming away much less efficiently (wild-type at 36 hpf: 0.71±0.16 cm/s, n=12; ryr at 36 hpf: 0.17±0.16 cm/s, n=12; Student’s t-test, P<0.001; Fig. 1A-O, and see Movies 1, 2 in the supplementary material). To examine the frequency and strength of contractions during swimming, 48 hpf embryos were video-recorded with their heads restrained on a Sylgard dish, leaving the
trunk and tail free to move. The frequency of muscle contractions was comparable between wild-type siblings (38.7±5.2 Hz, n=10) and ryr embryos (35.9±4.5 Hz, n=10), but the amplitude of trunk and tail movements was significantly smaller in ryr mutants compared with wild-type siblings (Fig. 1P,Q). There were no obvious anatomical defects in ryr embryos and larvae, but mutants died at around 7-15 days post-fertilization (dpf), possibly from their inability to feed effectively. These observations suggest that weak contractions by trunk and tail muscles are the cause of slow swimming in ryr.

**Output of the CNS is normal in ryr mutants**

The fact that muscle contractions in ryr mutants were weaker than those in wild type suggested that the output from the CNS onto the muscle was decreased because of a defect in the CNS and/or NMJ, or that contractile responses of muscles were compromised due to a muscle defect. To see whether there was a decrease in signaling from the CNS and/or the NMJ, the voltage responses in muscles evoked by tactile stimulation were recorded (Fig. 2A). Voltage recordings from both wild-type-sibling and ryr mutant fast and slow muscles showed rhythmic depolarizations indicative of normal fictive swimming in response to touch (Fig. 2B-E). The amplitudes of the rhythmic depolarizations from wild-type-sibling and mutant fast muscles were comparable (wild type: 1.16±0.07 mV, n=4; ryr: 1.21±0.14 mV, n=4), as were the depolarizations in slow muscles (wild type: 1.02±0.25 mV, n=5; ryr: 0.90±0.17 mV, n=5). These results indicate that the CNS and the NMJ function normally in ryr mutants and suggest that they harbor a defect in muscles, downstream of the NMJ.

**Ca\(^{2+}\) transient is smaller in ryr mutant fast muscle**

Depolarization of the muscle membrane causes a transient increase in cytoplasmic Ca\(^{2+}\) mediated by E-C coupling that results in actin/myosin sliding and the contraction of muscle (Franzini-Armstrong and Protasi, 1997). Because muscles were defective in ryr mutants, we examined whether the increase in cytosolic Ca\(^{2+}\) was perturbed in mutant muscles by injecting live embryos with Ca\(^{2+}\) indicator dye, Calcium Green-1 dextran (Fig. 3A). The amplitude of Ca\(^{2+}\) transients in fast muscle was 3.3-times smaller in ryr mutants compared with wild-type siblings at 48 hpf [wild-type relative level of Calcium Green-1 fluorescence (\(\Delta F/F\)): 0.43±0.13, n=7; ryr \(\Delta F/F\): 0.13±0.05, n=7; Student’s t-test, P<0.001, Fig. 3B]. By contrast, Ca\(^{2+}\) transients in slow muscle were not perturbed in ryr mutants (wild-type \(\Delta F/F\): 0.35±0.08, n=5; ryr \(\Delta F/F\): 0.35±0.07, n=5; Fig. 3C). Furthermore, Ca\(^{2+}\) transients in mutants were comparable with wild-type siblings at 24 hpf (data not shown), when all mutants responded normally to tactile stimulation. Thus, a defect in E-C coupling in fast muscles appears to be the basis for weak contractions in ryr mutant muscles.

**Proteins for E-C coupling are not clustered at t-tubule–SR junctions in ryr mutants**

E-C coupling is mediated by direct interaction between DHPRs and RyRs, both of which are clustered at the juxtaposed membranes of t-tubule–SR junctions. To see how E-C coupling might be defective in mutant fast muscles, the distribution of RyRs and DHPRs were examined in ryr mutant muscles. Labeling with anti-RyR showed that RyRs were distributed in a striated pattern in wild-type fast muscles (Fig. 4C,E,G), whereas double labeling with anti-RyR and anti-DHPR\(_{\alpha 1}\) confirmed that RyRs and DHPRs were colocalized at presumptive t-tubule–SR junctions in wild-type fast muscles (Fig. 4C,E,G). These results indicate that the CNS and the NMJ function normally in ryr mutants and suggest that they harbor a defect in muscles, downstream of the NMJ.
presumptive RyR/DHPR particles in slow muscles was comparable between wild type and \( \text{ryr} \) mutants (Fig. 4W,X). Thus, \( \text{ryr} \) mutants are deficient in E-C coupling in their fast muscles because of a lack of clustering by RyRs and DHPRs at the t-tubule–SR junctions.

The mutated gene in \( \text{ryr} \) mutants encodes RyR1

To identify the gene responsible for the \( \text{ryr} \) phenotype, the mutation was meiotically mapped to a region of chromosome 18 defined by two microsatellites, z737 (2.1 cM, 23 recombinants in 1110 meioses) and z8343 (6.9 cM, 77 recombinants in 1110 meioses) (Fig. 5A). A gene encoding for muscle ryanodine receptor 1 (\( \text{ryr1b} \)) was found in between these two markers in the Zv6 Ensemble assembly of the zebrafish genome. Furthermore, there was no recombination with a polymorphic marker in \( \text{ryr1b} \) (<0.09 cM, 0 recombinants in 1110 meioses). Thus, both genetic mapping and the RyR1 phenotype in mutants suggested that \( \text{ryr1b} \) was a good candidate for the \( \text{ryr} \) mutation. To see whether \( \text{ryr1b} \) was the \( \text{ryr} \) gene, \( \text{ryr1b} \) cDNA was cloned and sequenced from wild-type and mutant embryos. Wild-type \( \text{ryr1b} \) encodes 5076 amino acids (GenBank #AB247454, Fig. 5B). The \( \text{ryr1b} \) cDNA from \( \text{ryr} \) mutants contained a 32-bp insertion. This insertion generated a premature stop codon in the middle of the full-length protein that was 5’ to the sequences for the predicted transmembrane domains located at the C-terminus (Meissner, 1994). Reverse transcriptase (RT)-PCR with primers flanking the insertion was performed to confirm that the insertion was specifically found in \( \text{ryr} \) mutants (Fig. 5C). Both longer and shorter PCR fragments were amplified using the cDNA from a group of wild-type siblings as template (Fig. 5C, lane 1), whereas the longer fragment was predominant in mutants (Fig. 5C, lane 2). Only the shorter fragment was amplified from the cDNA of wild-type embryos of another strain, the AB strain, of zebrafish (Fig. 5C, lane 3), confirming that the shorter band corresponded to wild-type \( \text{ryr1b} \). Sequencing of the longer and shorter fragments verified that the 32-bp insertion was found only in the longer product. Thus, the \( \text{ryr} \) phenotype is very likely to be due to mutation in \( \text{ryr1b} \).

Genomic sequencing revealed that \( \text{ryr} \) mutants carry a 4046-bp DNA insertion, including the 32-bp cDNA insertion, in the intron between exon 48 and 49 of the \( \text{ryr1b} \) gene (Fig. 5D). Sequences flanking the 32 bp in the genomic insert contained splicing acceptor and donor sites, confirming that the 32-bp sequence acts as an additional exon in the mutant \( \text{ryr1b} \) gene. This genomic insertion might represent a transposable element, because it contained a repeated motif at both ends that are characteristic of Tc1/mariner family transposons (data not shown) (Ivics et al., 2004; Kawakami, 2005). Because the aberrant splicing results in a premature stop codon that predicts a truncated RyR1b lacking the channel domains, the great majority of fast muscle RyR1b would probably be non-functional.

To confirm whether a loss of RyR1b is responsible for the \( \text{ryr} \) phenotype, we attempted to slow swimming by antisense knockdown of RyR1b and application of a specific inhibitor of RyR. We injected antisense morpholino oligonucleotides (MO1), which were complementary to the splice donor site of exon 48, into wild-type embryos and assayed touch responses at 36 hpf. MO1-injected wild-type embryos swam more slowly than control MO-injected wild-type embryos (MO-1 injected: 0.81±0.20 cm/s, \( n = 12 \); control MO-injected: 1.78±0.35 cm/s, \( n = 12 \); Student’s \( t \)-test, \( P < 0.001 \)), much like mutant embryos. Interestingly, most of the MO1-injected embryos also exhibited weak coils of the trunk and tail following tactile stimulation at 24 hpf (82.0±7.8%, \( n = 43-76 \), five trials) rather than the normal fast, vigorous coils, suggesting that RyR1b is required for touch-induced coiling at earlier stages as well as for swimming at later stages. Correlated with the behavioral defects, the amount of \( \text{ryr1b} \) mRNA with normal splicing at 24, 36 and 48 hpf in MO1-injected embryos was reduced compared with control MO-injected embryos (Fig. 5E), confirming the efficacy of knockdown by MO1. Treatment of wild-type embryos with Ruthenium red, an inhibitor of RyR (Pessah et al., 1985), also phenocopied touch-induced slow swimming at 36 hpf (\( n = 20 \)). Thus, RyR1 is essential for normal muscle function in zebrafish, as it is in mammals.

The fact that weak muscle contractions in mutants were obvious after 36 hpf but not earlier than 30 hpf, whereas wild-type embryos in which \( \text{ryr1b} \) was knocked down exhibited weak contractions at 24 hpf, suggests that defective splicing was stage-dependent. To test this possibility, the head and trunk of individual embryos from a cross of two \( \text{ryr} \) carriers were subjected to genomic PCR and RT-PCR, respectively, to assay the genotype and splicing at 24 and 48 hpf (Fig. 5F). Wild-type embryos of the wt/wt genotype showed only a wild-type (short) RT-PCR fragment at both stages (Fig. 5F, lanes 1, 4), whereas wt/\( \text{ryr} \) embryos exhibited both wild-type (short)
and mutant (long) fragments at both stages (Fig. 5F, lanes 2, 5). Mutant embryos (rry/rry) gave both wild-type and mutant fragments with comparable intensity at 24 hpf (Fig. 5F, lane 3), whereas the mutant product became predominant at 48 hpf (Fig. 5F, lane 6). These results suggest that the mutant behavioral phenotype was due to stage-dependent aberrant splicing in mutants.

The zebrafish genome contains potentially duplicated ryr genes

To identify other zebrafish genes encoding RyRs, we blasted the Zv6 Ensemble assembly of the zebrafish genome with protein sequences for human RYR1, RYR2 and RYR3, and found 14 genomic contigs that included sequences encoding for RyRs (Table 1). Each of the zebrafish RyR sequences were physically mapped with the LN54 radiation hybrid panel (Hukriede et al., 1999) and identified as RyR1, RyR2 or RyR3 based on homology with the human sequences. This classification suggested that there were at least five different ryanodine receptor genes in zebrafish: two for RyR1 [rry1a in linkage group (LG)10 and rry1b in LG18], two for RyR2 (rry2a in LG12 and rry2b in LG17) and one for RyR3 (rry3 in LG20).

rry1b is expressed by fast muscle

Because rry mutants were defective in E-C coupling in fast muscle, rry1b should be expressed by fast muscles. RT-PCR showed that both rry1b and rry1a were expressed from 1 to 5 dpf as well as by the adult trunk (Fig. 6A). RT-PCR using cDNA from dissected adult fast muscles, however, showed that rry1b, but not rry1a, was expressed in fast muscle. In situ hybridization revealed that rry1b was expressed by deep axial muscles probably representing fast muscles at 24 and 48 hpf (Fig. 6B-E). By contrast, expression of rry1a was observed in superficial muscles under the skin, representing slow muscles (Devoto et al., 1996) (Fig. 6F-I). These results indicate that the potential duplicates of RyR1 were both expressed by muscles but that only RyR1b is expressed by fast muscles.
The *ryr* mutant is a disease model of MmD

MmD, a recessive myopathy, is caused by RYR1 mutations in human and is pathologically defined by multiple amorphous cores in muscle fibers (Engel, 1967; Jungbluth et al., 2004). To examine whether *ryr* mutants displayed morphological defects in muscle, transverse sections of larval axial muscles were analyzed by transmission electron microscopy. Superficial slow muscles appeared comparable between wild type and mutant (data not shown). Well-formed actin/myosin bundles and SR were observed in wild-type fast muscle at 2, 7 and 14 dpf (Fig. 7A-C). Mutant fast muscle, however, displayed small amorphous cores (50-100 nm in diameter) at 2 dpf (Fig. 7D). The diameter of the cores in mutant fast muscles increased with development (50-500 nm at 7 dpf; 100-800 nm at 14 dpf; Fig. 7E,F). Disorganization of the SR was also evident at 7 dpf and, in some cases, the SR was missing at 14 dpf. Thus, *ryr* mutant fast muscles displayed ultrastructural defects similar to those seen in MmD muscles.

Because the *ryr* mutation was due to defective splicing of *ryr1b* mRNA, we wondered whether normal swimming could be restored by preventing aberrant splicing. This treatment was examined by injection of antisense morpholino oligonucleotides (MO2) against the splice acceptor site of the 32-bp insert into recently fertilized progeny of *ryr* carriers (Fig. 7G). After testing their response to tactile stimulation at 36 hpf, the head and trunk of embryos were subjected to genomic PCR and RT-PCR, respectively, to assay genotype and splicing (Fig. 7H). Injection of control MO had no effect either on swimming or on splicing (Fig. 7H, lanes 1-3). MO2-injected *wt/wt* embryos exhibited normal swimming and expressed only the normal (short) fragment (Fig. 7H, lane 5). However, 49% (23/47) of MO2-injected *wt/ryr* embryos exhibited recovery (>1.0 cm/s) in swimming (MO2 injected: 1.34±0.32 cm/s; control MO-injected: 0.74±0.22 cm/s; Student’s *t*-test, *P*<0.001).
They expressed an increase in the proportion of wild-type, short mRNA to mutant, long mRNA (Fig. 7H, lanes 6, 7) compared with that in control MO-injected ryr/ryr embryos (Fig. 7H, lane 3). By contrast, MO2-injected mutant embryos that exhibited slow swimming (<1.0 cm/s, 51%, 24/47) predominantly expressed the mutant, long fragment, with little normal product (Fig. 7H, lanes 8, 9) much like control MO-injected mutants (Fig. 7H, lane 3). However, rescue of swimming was transient, because the 23 embryos that exhibited recovery at 36 hpf exhibited slow swimming by 60 hpf, presumably due to breakdown of the MO. These MO2-injected mutants died at around 7-15 dpf, much like uninjected ryr mutants. Furthermore, electron micrographs of the fast muscles of MO2-injected ryr mutants displayed amorphous cores in fast muscle at 7 dpf (n=4, data not shown). Similar results were obtained with another antisense morpholino oligonucleotides (MO3) that was complementary to the donor splice site of the 32-bp insert (data not shown). Thus, prevention of aberrant splicing with antisense MO treatment can transiently restore normal swimming, confirming that the aberrant splicing is responsible for the ryr phenotype. It also indicates that ryr mutants might be useful for examining antisense-mediated treatment in vivo.

DISCUSSION

rry mutants have an E-C-coupling defect in fast muscles

Several findings demonstrate that ryr mutants exhibit weak muscle contractions due to an E-C-coupling defect in fast muscles. First, depolarization of the muscle membrane evoked by output from the CNS was normal in ryr fast muscles. Second, Ca\textsuperscript{2+} transients following the depolarization of muscles were decreased in ryr fast muscles. Third, there was a dramatic decrease in E-C-coupling components, such as RyRs and DHPRs, at the t-tubules–SR junctions in ryr fast muscles. Indeed, most of the ryr1b transcripts were aberrantly spliced in the mutants, leading to a hypomorphic condition due to reduced synthesis of normal mRNA. Therefore, a reduction of RyR1b in fast muscles leads to a decrease in DHPR and RyR at the t-tubule–SR junctions and to a decrease in Ca\textsuperscript{2+} released from the SR into the cytosol, leading to weak muscle contractions in ryr mutants.

The zebrafish immotile mutant, relaxed (also known as cacnb1 – Zebrafish Information Network), is deficient in E-C coupling because of a null mutation in DHPR\textsubscript{B1} (Schredelseker et al., 2005; Zhou et al., 2006). In relaxed mutant muscles, DHPR\textsubscript{A1} was significantly reduced, but RyR1 was correctly targeted to the t-tubules–SR junctions. In ryr mutants, by contrast, there was a dramatic decrease in both DHPR and RyR1 even when the t-tubules and the SR were not damaged. Taken together, the phenotypes of the two zebrafish mutations corroborate the finding that the formation...
of DHPR tetrads requires the presence of RyR1 (Takekura et al., 1995). Indeed, a cytoplasmic domain of RyR1 is essential for the physical interaction with an intracellular loop of DHPRα1 in cultured mammalian myotubes (Kugler et al., 2004; Proenza et al., 2002). The failure of DHPR to localize to the t-tubule–SR junctions is probably a direct consequence of the failure of RyR1b to be targeted to the SR in *ryr* mutants.

**ryr1b is the *ryr* gene**

In *ryr* mutants, we found a 32-bp insertion in most of the *ryr1b* transcripts, which resulted in the generation of a nonsense codon. The 32-bp exon was included in a 4046-bp DNA insertion in the mutant *ryr1b* gene. This large insertion might be derived from a transposable element in zebrafish. Indeed, sequencing of the insertion revealed inverted repeats characteristic of the Tcl/mariner family of transposons (Ivics et al., 2004; Kawakami, 2005). Although *ryr1b* was expressed by fast muscles at 24 hpf and was essential for E-C coupling at later stages, *ryr* mutants responded to touch with normal C-bends at 24 hpf and exhibited slow swimming after 36 hpf. This lack of a mutant phenotype at 24 hpf can be explained by the possibility that splicing of *ryr1b* might be developmentally regulated and that the aberrant splicing may only occur during later stages. In fact, half of the *ryr1b* transcripts were normally processed at 24 hpf, whereas most *ryr1b* mRNA transcripts were aberrantly spliced at 48 hpf. A splicing factor, which determines exon preference, might be differentially regulated between 1 and 2 dpf. The finding that knockdown of RyR1b translation in wild-type embryos leads to weak muscle contractions at 24 hpf is consistent with this hypothesis.

We found that zebrafish have two genes encoding RyR1; *ryr1a* expressed by slow muscles and *ryr1b* by fast muscles. A similar division of labor between duplicated RyR1s in slow and fast muscles appears in other fish, such as blue marlin (*Makaira nigricans*) and yellowfin tuna (*Thunnus albacares*) (Franck et al., 1998; Morrissette et al., 2000; Morrissette et al., 2003). Although single-channel analysis indicates that channel activity of RyR1 in fast muscle is higher than that in slow muscle (Morrissette et al., 2000), any functional difference in E-C coupling has not been examined. It would be interesting to examine potential differences in E-C coupling between slow and fast muscles to see how the requirements of the two muscle types dictate divergent RyR1s and how these differences may have evolved.

**The *ryr* mutant is an animal model for MmD**

The zebrafish *ryr* mutant phenotype shares several crucial features with human MmD. First, mutations in genes encoding RyR1 are responsible for both the *ryr* phenotype and MmD. Second, both the *ryr* phenotype and MmD are inherited as autosomal recessives. Third, both *ryr* mutants and individuals with MmD display myopathy characterized by minicores in histological sections. The amorphous cores in MmD (2-25 μm in diameter) are...
associated with labeling for reductase activity in mitochondria with NADH-TR (tetrazolium reductase) (Engel, 1967; Martin et al., 1986; Swash and Schwartz, 1981). Unfortunately, NADH-TR staining appears to not be useful in fish (Johnston et al., 1975; Matsuoka and Iwai, 1984). However, electron microscopy clearly demonstrated amorphous cores in zebrafish ryr mutant muscles and showed that they are evident in embryonic muscles. The fact that RYR1-deficient mice die on the day of birth limits their usefulness as an animal model of MmD (Takeshima et al., 1994). By contrast, zebrafish ryr mutants die 7-15 dpf, but their fast development and accessibility might be useful for detailed physiological and pathological analysis of the consequences of MmD.

We succeeded in treating muscle weakness in ryr mutants by the application of an antisense morpholino that increased the normal splicing of ryr1b and restored normal swimming. Germaine to our finding, antisense-mediated exon skipping restored normal similarity to the human ryr1 gene (Bell, 1995). In this case, an aberrant splice donor site was generated by a point mutation application of an antisense morpholino that increased the normal splicing of ryr1 in the human genetic disease and showed that they are evident in embryonic muscles. The fact that RYR1-deficient mice die on the day of birth limits their usefulness as an animal model of MmD (Takeshima et al., 1994). By contrast, zebrafish ryr mutants die 7-15 dpf, but their fast development and accessibility might be useful for detailed physiological and pathological analysis of the consequences of MmD.

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