Quantitative reduction of RyR1 protein caused by a single-allele frameshift mutation in *RYR1* ex36 impairs the strength of adult skeletal muscle fibers.

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Abstract

Here we validated a mouse model knocked-in for a frameshift mutation in *RYR1* exon 36 (p.Gln1970fsX16) that is isogenic to that identified in one parent of a severely affected patient with recessively inherited Multiminicore disease. This individual carrying the *RYR1* frameshifting mutation complained of mild muscle weakness and fatigability. Analysis of the RyR1 protein content in a muscle biopsy from this individual showed a content of only 20% of that present in a control individual. The biochemical and physiological characteristics of skeletal muscles from RyR1Q1970fsx16 heterozygous mice recapitulates that of the heterozygous parent. RyR1 protein content in the muscles of mutant mice reached 38% and 58% of that present in total muscle homogenates of fast and slow muscles from WT littermates. The decrease of RyR1 protein content in total homogenates is not accompanied by a decrease of $Ca_v1.1$ content, whereby the $Ca_v1.1$/RyR1 stoichiometry ratio in skeletal muscles from RyR1Q1970fsx16 heterozygous mice is lower compared to that from WT mice. High-resolution electron microscopy revealed a 40% reduction in the number of calcium release units and a reduction of the double strip arrays of RyR1 in the junctional sarcoplasmic reticulum, as indicated by a 2.5 fold increase of the number of diads. Compared to WT, muscle strength and depolarization-induced calcium transients in RyR1Q1970fsx16 heterozygous mice muscles were decrease by 20% and 15%, respectively. Altogether our results demonstrate that an average 50% decrease of the RyR content does not correlate with similar decay of muscle functional properties.

Introduction

The propagation of the action potential deep into the muscle fibre by means of the transverse tubular (TT) system leads to a massive release of Ca^{2+} from the sarcoplasmic reticulum (SR) throughout the entire length of the muscle fibre via a process called excitation-contraction coupling (ECC)(1, 2) The voltage dependent release of calcium from the SR initiates muscle contraction, whereas muscle relaxation is brought about by calcium re-uptake into the SR by the sarco(endo)plasmic CaATPAse (SERCA). ECC occurs at the contact region between the TT and the SR membrane, a domain encompassing a macromolecular complex formed by the dihydropyridine receptor (DHPR), the ryanodine receptor 1 (RyR1) and the calcium buffering protein calsequestrin (3-5). Under resting conditions, DHPRs which are apposed to RyR1, inhibit RyR1 mediated calcium release; however, upon depolarization of the TT, DHPRs generate an orthograde signal to RyR1s causing their opening and leading to calcium release from the SR (2). Subsequently RyR1s send retrograde signals important to support the DHPR channel activity, back to DHPRs. (6, 7).

In the past decades, experimental evidence has demonstrated that alterations of RyR1 function are the underlying cause of a number of neuromuscular disorders, bleeding abnormalities (8) and are potentially involved in critical care illnesses including sepsis and intensive care polyneuropathy (9, 10). Dominant mutations in *CACNA1S*, the gene encoding the alpha 1 subunit of the DHPR, and in *RYR1*, the gene encoding the RyR1 calcium channel, are the underlying genetic causes of neuromuscular disorders including the Malignant Hyperthermia Susceptibility/ exertional rhabdomyolysis and Central Core Disease phenotypes (OMIM #145600, OMIM #601887, OMIM #117000), and their functional effects have been extensively investigated (11-15). Recessive *RYR1* mutations on the other hand, have been associated with Multiminicore Disease, Centronuclear myopathy and Congenital Fiber Type Disproportion (OMIM # 255230) (16-18). Most affected patients harboring recessive *RYR1* mutations are compound heterozygotes between a non-sense or frame-shift mutation in one allele and a missense mutation in the other allele. This leads to the hemyzygous expression of the allele carrying the missense mutation and is associated with an overall decrease of RyR1 protein content in skeletal muscle (17-20). Although the extent of RyR1 depletion varies from patient to patient, common clinical signs are proximal, axial and respiratory muscle weakness, in some

patients accompanied by loss of ambulation and respiratory failure leading to a significant impact on physical performance and quality of life of the affected individuals. To date, few studies have focused on the elucidation of the specific pathophysiological mechanisms of disease and in general, to disease treatment. Analysis of *ryr1*$^{+/-}$ heterozygous mice showed that ablation of one *RYR1* allele leads to a 15% decrease of RyR1 protein content (21) Such a modest decrease of RyR1 does not impact muscle function as determined by the hanging wire test, and does not affect peak calcium transients in cultured satellite-derived myotubes. Although the results on satellite-derived myotubes may not recapitulate the situation occurring in vivo in human adult skeletal muscle fibres, they raise the important question of whether a quantitative reduction RyR1 expression in skeletal muscle may account for the muscle weakness exhibited by patients carrying recessive *RYR1* mutations. In this context it should be pointed out that the reduction of protein content in muscle biopsies from patients carrying recessive *RYR1* mutations (17) is 2 to 3 fold lower compared to that observed in the *ryr1*$^{+/-}$ mice. In addition, the mutation of the *ryr1*$^{+/-}$ mice investigated by Cacheux et al. (21) is not isogenic with mutations identified so far in patients.

In the present study we investigated the effect of reduced RyR1 expression in mature muscle, by studying the physiological and biochemical changes occurring in the muscles of a mouse model we generated to mimic a clinically relevant *RYR1* mutation. Specifically, the mouse carries a knocked-in inducing a frameshift mutation in *RYR1* exon 36 that is isogenic to that identified in one of the alleles of a severely affected MmD patient (18, 22). The *RYR1* frameshift mutation RYR1 p.Gln1970fsX16 (abbreviated RyR1Q1970fsX16), leads to a premature stop codon causing a decrease in RYR1 transcript levels and RyR1 protein content but did not affect the expression of other sarcoplasmic reticulum proteins. Motor activity assessed with a voluntary running wheel revealed that heterozygous RyR1Q1970fsX16 mice run less compared to age-matched WT littermates, a results consistent with atrophy of fast EDL fibers. Analysis of the mechanical properties of isolated muscles showed lower twitch and tetanic specific force in both EDL and soleus muscles in RyR1Q1970fsX16 mice compared to age-matched WT littermates. Additionally, electrically evoked Ca^{2+} transients were lower in EDL fibres from RyR1Q1970fsX16 compared to WT mice. Our results show that quantitative reduction of the RyR1 complex is associated with

mild reduction of muscle performance and decreased muscle strength, these results indicate that in adult muscle fibers there is a functional reserve of RyR1

RESULTS

A premature stop codon in ex36 of the human *RYR1* is associated with mild muscle symptoms and a decrease of RyR1 protein:

Klein et al., (22) identified a severely affected MmD patient carrying the compound heterozygous *RYR1* mutations c.5908C>T in exon 36 (p.Q1970X, nonsense mutation) and c.12986C>A (p.A4329D, missense mutation) in exon 91. The two mutations were inherited from the 2 parents and lead to a dramatic decrease of the RyR1 content in the skeletal muscle of the affected child (17). Though the parent carrying the frameshifting mutation did not have overt muscle weakness, she complained of generalized muscle weakness and fatigability. Histological evaluation of the muscle biopsy from this parent revealed a variability in fiber size due to hypotrophic and hypertrophic fibres (range 33-96 µm), the presence of a few internal nuclei, peripheral aggregation of oxidative stains in some fibers and a predominance of slow, type 1 fibers. Fast fibers tended to be smaller in size. A lower leg muscle MRI did not show any selective muscle involvement (not shown). A small sample of the original muscle biopsy was studied for western blot analysis. Immunostaining of the proteins present in the skeletal muscle total homogenate with anti-RyR1 Ab revealed a remarkable decrease of the RyR1 protein content (Fig. 1). A similar decrease of the RyR1 protein content in total skeletal muscle homogenate was also observed in an MmD patient (19) carrying a paternally inherited frameshift mutation in *RYR1* ex36 (c.5938delC, p.L1980SerfsX1) and a maternally inherited missense mutation in exon 79 (c.11315G>A, p.R3772Q). In depth evaluation of the impact of the ex36 *RYR1* premature stop codon on muscle function could not be done on the muscle biopsy of the patient. Thus, to elucidate the pathophysiological consequences of the mutation, we created of a mouse model carrying a frameshift mutation in ex36, namely the RyR1Q1970fsX16 mouse.

The presence of the RyR1Q1970fsX16 mutation functionally impacts *in vivo* muscle function in mice:

The presence of the heterozygous frame shift mutation in ex36 of the *RYR1* gene in the mouse line was confirmed by PvuII digestion of PCR amplified genomic DNA (Figure 2A). Homozygous RyR1Q1970fsX16 mice exhibited a lethal neonatal

phenotype while heterozygous mice were undistinguishable from their WT littermates and did not exhibit defects in embryonic or postnatal development. Furthermore, the body weight growth curves of WT and RyR1Q1970fsX16 mice were similar (Fig. 2B). We investigated skeletal muscle function by analyzing the running capacity of the mice using with voluntary running wheel set-up. Mice had free access to the wheel at any time of day; this experimental approach avoids potential problems linked to the effect of circadian rhythm on animal activity and/or animal compliance in performing non-voluntary motor activity. We examined spontaneous motor activity by measuring the total dark phase running distance of three-month-old RyR1Q1970fsX16 mice and their WT littermates (Fig. 2C). In the first week, the total running distance of WT mice, was approximately double that of RyR1Q1970fsX16 mice. Two weeks of training improved skeletal muscle performance in both groups. After three weeks of training, the total running distance increased and WT mice ran approximately 34% more compared to RyR1Q1970fsX16mice (Mann-Whitney two-tailed P test, calculated over the 21 days running period $p<0.05$, $n=13$). The shorter running distance is also associated with a lower median cruise speed of RyR1Q1970fsX16 mice compared to WT littermates (Fig. 2D; $P <0.01$ Mann-Whitney test, two-tailed test).

Isolated muscles from RyR1Q1970fsX16 mice exhibit a decrease of isometric force development:

The reduced speed and total running distance of the RyR1Q1970fsX16 mice may results from reduced muscle strength caused by alterations of the macromolecular complexes involved in ECC. We examined such a possibility by investigating the mechanical properties of intact *extensor digitorum longus* (EDL) and soleus muscles from WT and RyR1Q1970fsX16 mice upon delivery of a single action potential or by a train of pulses of 0.5 ms duration delivered at 150 Hz for 400 ms to obtain maximal tetanic contracture (Fig. 3). The averaged specific twitch peak force induced by a single action potential in EDL from RyR1Q1970fsX16 mice was reduced by approx 20% compared to that of EDL from WT mice (86.26±14.83* n=7, vs 106±20.80, n=7, respectively; values are expressed as mean±S.D., *$p<0.05$). Soleus muscles from RyR1Q1970fsX16 mice also exhibited a 30% decrease of twitch specific peak force compared to WT littermates (56.73±17.59*, n=7 vs 78.92±19.50, n=7, respectively; values are expressed as mean± S.D., *$p<0.01$). (Table1). The specific tetanic force of EDL and soleus muscles from RyR1Q1970fsX16 mouse

muscles was also significantly impaired (Fig. 3 C-F). The maximal specific tetanic force developed by EDL muscles from RyR1Q1970fsX16 mice was approximately 17% lower than developed by EDL from WT mice (mean±S.D., 356.36±50.68 mN mm^{-2}, n=7 vs 465.72±55.94 mN mm^{-2}, n=7, respectively; ; values are expressed as mean± S.D., $p<0.001$). A similar decrease in maximal specific tetanic force generation was observed in soleus muscles from RyR1Q1970fsX16 mice (310.10±46.44, n=7 compared to 273.21±59.10 in WT n=7; values are expressed as mean± S.D., $p<0.05$). The decrease in force output is not due to fast-to-slow fiber transition since we observed no changes in the expression of myosin heavy chain (MyHC) isoforms in EDL and soleus muscles (Fig. 4A and B). The reduced absolute force observed in their skeletal muscles cannot be attributed to a major loss of contractile proteins, as the wet weight of the EDL and soleus muscles from RyR1Q1970fsX16 and WT mice were similar. On the other hand, there was a shift of minimal Feret's fiber diameter distribution to lower values in EDL muscles, indicating an atrophy of the fast fibers (Fig. 4 A and C). The fast fibers present in soleus muscles from RyR1Q1970fsX16 did not show atrophy and were similar to those of WT littermates (Fig. 4, Soleus), a result likely reflecting differences in the fiber type composition between EDL and soleus muscles. The latter muscles are composed of: 60% slow type I fibers and approximately 40% of fast oxidative type 2A/X fibers while EDLs contain approximately 90% type 2B/2X fiber and only a small fraction of type I fibers (23-25).

The RYR1Q1970fsX16 mutation decreases RyR1 content in sarcotubular membranes:

The decreased muscle strength of RyR1Q1970fsX16 mice is apparently not due to fast to slow fiber type transition, but rather may be linked to the atrophy of fast fibers, to alterations of the expression of ECC components or to a combination of both. Thus, we investigated the protein content of the major protein components involved in skeletal muscle ECC coupling, namely $Ca_v1.1$, RyR1, SERCA and calsequestrin. $Ca_v1.1$ is the voltage sensor, generating the charge movement that is transmitted to the RyR1 to initiate the calcium release necessary to activate muscle contraction. Muscle relaxation relies on the activity of SERCA pumps and once calcium is pumped back into the SR lumen, it is bound by the high capacity calcium binding protein calsequestrin. The content of the main protein components of the

sarcotubular membranes of total homogenate from EDL and soleus muscles from WT and RyR1Q1970fsX16 heterozygous mice was determined by quantitative western blot analysis. Supplementary Figure 1 shows the specific immunoreactivity of the antibodies that were used for western blot analysis. No significant changes in the content of $Ca_v1.1$, SERCA1, SERCA2, sarcalumenin, calsequestrin, calreticulin, JP45, parvalbumin and β1a were observed (Fig. 5A). However, the RyR1 content was significantly reduced in homogenate from EDL and soleus muscles from RyR1Q1970fsX16 mice compared to WT littermates. Relative to levels observed in WT, the RyR1 content was 37.6±19.8% in EDL (n=9) and 58.7±19.6% in soleus muscles (n=10), (mean+/- S.D., $P<0.01$ for EDL and $P<0.05$ for Soleus Mann-Whitney test). The decrease of the RyR1 protein content is paralleled by a 50% decrease of the transcript encoding RyR1, with no effect on the levels of expression of CACNA1S transcripts (Fig. 5B), indicating that the mRNA transcribed from the mutant allele is most likely not expressed in the heterozygous RyR1Q1970fsX16 mice. RyR1 and Cav1.1 form a supramolecular complex referred to as calcium release units (CRU), which are localized at the T tubules-sarcoplasmic reticulum junctions. To verify whether the quantitative decrease of RyR1 induces alterations of the CRU arrays along the muscle fiber we analyzed the intracellular distribution of RyR1s and $Ca_v1.1$ by confocal immunohistochemistry. No apparent change in the distribution pattern of either protein along the muscle fibers was apparent (Supplementary Fig. 2). This could be due to the low optical resolution of the confocal microscope. Thus we investigated the distribution of the CRU in muscle fibers by electron microscopy.

Ultrastructure of EDLs from WT and RyR1Q1970fsX16 heterozygous mice:

In adult WT EDL fibers CRUs are quite frequent, uniformly distributed in proximity of the sarcomere I-A interface (Fig. 6A)(26). CRUs are made up of two junctional SR (jSR) cisternae closely opposed to a central T-tubule oriented transversally with respect to the longitudinal axis of the myofibrils (Fig.6A, inset). These membrane structures are also known as triads. Small electron dense structures referred to as ryanodine receptor calcium release channels bridge the gap between SR and T tubules within the triads (Fig.6A, inset; arrowheads). In the triads there are two RyR1 on either side of the T tubule, which form parallel rows whose length depends upon fibre type and species (27).

In EDL muscles from RyR1Q1970fsX16 mice CRUs show an uneven distribution and altered morphology (Fig. 6B; inset): CRUs formed by only two elements (one T-tubule and one jSR) named dyads (Fig.6B, inset). Quantitative analysis confirmed the visual observations. Indeed, in muscle fibers from RyR1Q1970fsX16 mice there was a significant reduction of CRU frequency (triads plus dyads) and a significant structural modification of the CRUs (Supplementary Table 1). Specifically, quantitative analysis of CRUs revealed: (i) a significant decrease in the number/100 μm2 of CRUs in RyR1Q1970fsX16 EDL fibers compared to those from WT littermates (39.7 ± 2.0 vs. 62.7 ± 2.5, respectively) and (ii) a significant increase in CRUs formed by only two elements (diads). These date taken together suggest a reduction in the overall number of calcium release sites (i.e. RyRs) available in the muscle (28).

Calcium transients in isolated EDL and soleus fibers from WT and RyR1Q1970fsX16 heterozygous mice:

If the full arrays of CRUs are activated during calcium release induced by an action potential, then one would expect that a quantitative reduction of the RyR1 content (ranging from 38 to 58%) should result in changes of the calcium transients. In the next set of experiments, we investigated calcium homeostasis in single EDL and soleus fibers from WT and RyR1Q1970fsX16 heterozygous mice. The resting calcium concentration ($[Ca^{2+}]_i$) was measured with the ratiometric Ca^{2+} indicator Fura-2, and did not reveal differences between muscle fibers from RyR1Q1970fsX16 and WT mice. The resting $[Ca^{2+}]_i$ of soleus fibers from WT and RyR1Q1970fsX16 heterozygous mice was 82.1±4.4 nM (n=29) and 82.7±4.7 nM (n=25), respectively (mean±S.E.M.), and that of EDL was 67.5±2.9 nM (n=37) and 64.3±3.2 nM (n=24) in WT and RyR1Q1970fsX16, respectively. In line with previous results (29), we found that the resting $[Ca^{2+}]_i$ of soleus fibers was 30% higher than that of EDL fibers.

In the presence of 1.8 mM Ca^{2+} in the extracellular solution, the averaged peak Ca^{2+} transient induced by a single action potential in EDL fibers from RyR1Q1970fsX16 mice was decreased by 17% (Fig. 7A) compared to that from WT EDL fibers ($\Delta F/F_o$ was 1.00±0.28*, n=66 vs 1.18±0.26, n=62, respectively; values are mean±S.D; Mann-Whitney test *p<0.01). The peak Ca^{2+} transients elicited in soleus fibers from RyR1Q1970fsX16 mice were reduced by approximately 10% (Fig. 7B) compared to WT ($\Delta F/F_o$ was 0.61±0.13*, n=25 vs 0.66±0.17, n=17, respectively;

values are mean± S.D; Mann-Whitney test *p<0.05). The kinetics of the Ca^{2+} transients were similar in fibers from WT and RyR1Q1970fsX16 heterozygous mice (Supplementary Table 2). The summation of Ca^{2+} transient peaks evoked by a train of pulses delivered at 100 Hz in the presence of 1.8 mM Ca^{2+} in the extracellular solution in EDL fibers from RyR1Q1970fsX16 mice was approx. 20% lower compared to that of WT (Fig. 7C)($\Delta F/F_0$ was 1.47±0.43*, n=17 vs 1.83±0.35, n=14, respectively; values are mean± S.D., unpaired t test *p> 0.05). In soleus fibres the peak calcium transients induced by tetanic stimulation (Fig.7D) RyR1Q1970fsX16 mice was approx. 20% lower compared to that of WT ($\Delta F/Fo$ was 1.02±0.35*, n=23 vs 1.32±0.38, n=20, respectively; values are mean ±S.D., unpaired t test *p<0.05)

Discussion

Here we investigated the phenotype of a mouse model carrying a frameshift mutation in ex36 of the *RYR1* gene, which is isogenic with a mutation present in the *RYR1* gene of the parent of a severely affected child suffering from a recessive form of RyR1-related of MmD (19, 22). The presence of the frameshift mutation causes an average 50% reduction of the RyR1 protein content not only in the total homogenate from (i) fast and slow muscles from RyR1Q1970fsX16 heterozygous mice, but also (ii) in the skeletal muscle biopsy of the parent carrying the frameshifting mutation. The remarkable quantitative decrease of RyR1 protein content in the muscle biopsy of this individual is associated with a relatively mild clinical phenotype mostly characterized by reduced stamina, sufficient to justify a diagnostic muscle biopsy in this individual. We exploited the RyR1Q1970fsX16 heterozygous mouse model to obtain a deeper quantitative correlation between the muscle phenotype and the reduction of RyR1 protein content, which occurs in heterozygous and compound heterozygous *RYR1* mutation-bearing patients. Our data shows that the phenotype of RyR1Q1970fsX16 heterozygous mice is consistent with that of the heterozygous parent, i.e., the reduction of RyR1 protein content is accompanied by a relatively mild decrease of muscle performance and reduced muscle strength. Nevertheless, the histopathology of the skeletal muscle of RyR1Q1970fsX16 mice differs from that obtained from the muscle biopsy of the patient. In particular, skeletal muscle from RyR1Q1970fsX16 mice did not reveal fiber type 1 predominance, that was reported in the human muscle biopsy; however both murine model and the patients displays an atrophy of the fast fibers. Although we do not have an exact explanation for these results, we cannot exclude the possibility that such discrepancies might be due to (i) fundamental differences between human and murine species; (ii) the fact that the mice live in cages and have few opportunities to exercise their muscles and potentially release important myokines; (iii) a different effect of the RyR1Q1970fsX16 mutation on the signaling pathways involved in the mechanisms responsible for the maintenance of trophic status of the muscle fibers.

Skeletal muscle phenotype and calcium release unit content.

An unexpected result of our investigation is the partial correlation between the quantitative reduction of the RyR1 protein content and reduced muscle function *in vivo* and *in vitro*. Indeed the RyR1 protein content of the RyR1Q1970fsX16 mice

reaches 38% and 58% of that present in total muscle homogenate of fast and slow muscles from WT muscles, and yet we found an approx 20% reduction of muscle strength and a 15% decrease of peak calcium transients induced by single action potentials. This may represent an adaptive event to the chronic quantitative reduction of the RyR1 protein content. In the heterozygous mice the mutant allele carrying the *RYR1* Q1970fsX16 mutation in not translated, most likely because of non-sense mediated RNA decay. The consequence of such an event is the exclusive expression of the WT *RYR1* allele alone, leading to the production of fewer RyR1 channels. The quantitative decrease of RyR1 was not paralleled by a decrease of $Ca_v1.1$ either in EDL or in soleus muscles. These results are consistent with changes of the stoichiometry of $Ca_v1.1$ and RyR1, namely the $Ca_v1.1$/RyR1 ratio is lower in skeletal muscles from RyR1Q1970fsX16 mice compared to WT.

High resolution electron microscopy studies have demonstrated that RyRs calcium release channels form a double strip of arrays on the junctional SR membrane (29). The length of the double strip arrays of RyR1 is variable and depends on the type of muscle and species being analysed. The RyRs arrays localised in the junctional SR are opposed to the T tubule membranes containing $Ca_v1.1$. The latter are organised in groups made up of four units regularly oriented in the T-tubular membrane to form structures referred to as tetrads, which correspond to the position of every other RyR present in the arrays localised in the opposite junctional SR membrane (29,30). If the reduction of the RyR1 membrane density in muscle is uniformly distributed over the junctional SR membrane compartment of the entire muscle fibre of RyR1Q1970fsX16 mice, one should expect a reduction of the array of RyR1s constituting the double strip of RyR1 of the calcium release units (CRU). Our electron microscopy data indeed revealed a reduction of the number of the calcium release units (CRU) and a reduction of the double strip arrays of RyR1 as indicated by the increase of the CRU formed by diads. If we assume that all the $Ca_v1.1$ present in the T-tubules are coupled by default to the available RyR1s of the junctional SR membrane, our quantitative analysis is consistent with the following concepts: (i) in sarcotubular membranes from RyR1Q1970fsX16 mice, the fraction of RyR1 which are coupled to the voltage sensor $Ca_v1.1$ (V channels) is larger compared to the uncoupled RyR1, which are controlled by calcium via interaction with activating and inhibiting sites on the RyR1s (C channels) (4, 31); (ii) the quantity of RyR1s coupled to $Ca_v1.1$ (V channels) in WT mice is similar to that present in

RyR1Q1970fsX16 mice. However, we cannot exclude another possibility, i.e. that not all the $Ca_v1.1$ present in the T- tubule membranes are by default coupled to the RyR1s making up the arrays in junctional SR. In the latter situation, then the T tubule-SR membrane junctions of RyR1Q1970fsX16 mice would also contain a fraction of uncoupled $Ca_v1.1$ in addition to uncoupled RyR1s (C channels). The function of the uncoupled $Ca_v1.1$ fraction, if any, in the skeletal muscles of RyR1Q1970fsX16 mice is difficult to foresee. Current models of the control mechanisms underlying ECC coupling predict that at large depolarization pulses the calcium release flux is mostly supported by the activation of the voltage-sensor coupled RyR1s, (V channels), while at lower depolarization pulses a larger fraction of the calcium release flux relies on the opening of uncoupled RyR1s (C channels) which are recruited by calcium released via RyR1 coupled to $Ca_v1.1$ (V channels) (29, 30). We activated calcium release in skeletal muscle fibers isolated from WT and RyR1Q1970fsX16 mice by supramaximal field stimulation, a condition deemed to causes large T tubule membrane depolarization, whereby peak calcium release mostly occurs via activation of voltage sensor coupled RyR1s (V channels). If this is the case, the lack of major differences in twitch peak calcium release between WT and RyR1Q1970fsX16 mice, despite a remarkable quantitative reduction of the RyR1 protein, likely reflect similar $Ca_v1.1$ content, which in turn dictates a similar content of voltage-sensor coupled RyR1s (V channels) in both WT and RyR1Q1970fsX16 mice. Nevertheless, the twitch peak calcium transients were 15% higher in WT fibers compared to those from heterozygous RyR1Q1970fsX16 mice. The additional peak calcium fraction observed in EDL fibers from WT mice may results from the recruitment of C channels present in the RyR1 arrays expressed in muscle fibers from WT mice, whose fraction is larger compared to that present in muscle fibers from RyR1Q1970fsX16 mice.

In conclusion, the results of the present investigation demonstrate that the mild skeletal muscle phenotype of RyR1Q1970fsX16 mice is caused by haploinsufficiency that does not fully correlate with an average 50% reduction of the *RYR1* dosage. In addition, the results of this study indicate that a V channel fraction of the RyR1 arrays localised in the membrane compartments involved in ECC is recruited to support muscle activation and that the C channel fraction may contribute, at least in part, to optimise the calcium fluxes leading to complete force output of the skeletal muscles during repetitive T tubule membrane depolarisation.

Materials and Methods:

Compliance with Ethical standards*:* The procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethikkommission beider Basel (permit N° EK64/12); all subjects gave written informed consent to carry out this work. Experiments involving animals were carried out on 8-12 weeks old male mice unless otherwise stated. All experimental procedures were approved by the Cantonal Veterinary Authority of Basel Stadt (BS Kantonales Veterinäramt Permit numbers 1728, 2115). All experiments were performed in accordance with relevant guidelines and regulations. The muscle biopsy of the patient mentioned in this paper was stored with the appropriate ethics in the Biobank for Neuromuscular Disorders (approved by The Hammersmith and Queen Charlotte's and Chelsea Research Ethics Committee - 06/Q0406/33).

Creation of the *RYR1* p.Gln1970fsX16 mouse line: Knock-in mice engineered for the RyRQ1970fsX16 mutation in exon 36 were generated by the Center for Transgenic Models, University of Basel, Switzerland using CRISPR-Cas9 gene-editing technology in a C57BL/6J mouse background (Supplementary Figure 3). The ex36 gRNA was designed to target the following sequence in the mouse genome: aagatgcagggcaaccagcgggg (the last 3nt being the PAM sequence). Homologous recombination was achieved by using a targeting oligo encompassing the following mouse genomic sequence: CAGATGTGCCACCTCCTGGAGTATTTCTGTGACCAAGAGCTGCAGCACCGGGT GGAGTCCTTGGCGGCCTTTGCAGAGTGTTATGTGGACAAGATGACAGCTGGGC AACCAGCGGGGTCGCTACGGCCTCCTCATGAAAGCCTTCACCATGAGCGCAGC CGAGACCGCAAGGCGCACCCGAGAGTTCCGTTCTCCACCCC. To identify mutant mice a *PvuII* restriction site flanking the mutation site was introduced (Supplementary Fig. 3A). Modification of the exon36 sequence was carried out using Cas9/CRISP directly in fertilized mouse oocytes. C57BL/6J female mice underwent ovulation induction by i.p. injection of 5 IU equine chorionic gonadotrophin (PMSG; Folligon–InterVet), followed by i.p. injection of 5 IU human chorionic gonadotropin (Pregnyl–Essex Chemie) 48 h later. For the recovery of zygotes, C57BL/6J females were mated with males of the same strain immediately after the administration of human chorionic gonadotropin. All zygotes were collected from oviducts 24 h after the

human chorionic gonadotropin injection, and were then freed from any remaining cumulus cells by a 1–2 min treatment of 0.1% hyaluronidase (Sigma-Aldrich) dissolved in M2 medium (Sigma-Aldrich). Mouse embryos were cultured in M16 (Sigma-Aldrich) medium at 37°C and 5% CO_2. For micromanipulation, embryos were transferred into M2 medium. All microinjections were performed using a microinjection system comprised of an inverted microscope equipped with Nomarski optics (Nikon), a set of micromanipulators (Narashige), and a FemtoJet microinjection unit (Eppendorf). Injection solution containing sgRNA (300ng/ul), Cas9 mRNA (100ng/ul) Cas9 protein (50ng/µl) and ssDNA homologous recombination template (10ng/ul) was microinjected into the male pronuclei of fertilized mouse oocytes until 20-30% distension of the organelle was observed.

Embryos that survived the microinjection were transferred on the same day into the oviducts of 8–16-wk-old pseudopregnant Crl:CD1 (ICR) females (0.5 d used after coitus) that had been mated with sterile genetically vasectomized males (32) the day before embryo transfer. Pregnant females were allowed to deliver and raise their pups until weaning age.

Mutation analysis and RyR1Q1970fsX16 mouse genotyping: PCR amplification of *RYR1* exon 36 was performed on genomic DNA of WT and RyR1Q1970fsX16 mice using specifically designed primers (Supplementary Table 2). For sequencing, amplified DNA was first purified using the QIAquick PCR-purification kit 250 (QIAGEN), and subsequently subjected to Sanger Sequencing in the forward and reverse directions (Microsynth) (Supplementary Figure 3B). For mouse genotyping, after extraction of genomic DNA from mouse pup biopsies, DNA was amplified using GoTaq® DNA Polymerase (Promega) using the primers listed in Supplementary Table 1 and the following amplification conditions: initiation, 5 min. at 95°C, 30 amplification cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, and final extension step at 72°C for 7 min. The amplified DNA products were digested with *PvuII* restriction enzyme (R0151L BioLabs) for 1 hour at 37ºC, separated on a 7.5% polyacrylamide gel and stained with ethidium bromide to visualize DNA bands.

Analysis of mutant allele expression: cDNA was prepared from total RNA extracted from frozen hind limb muscles of WT (n=6) and RyR1Q1970fsX16 (n=5) mice using TRIzol® (ThermoFischer scientific; 15596026) according to the manufacturer's instructions. DNA was removed using DNase I (Invitrogen; 18068-015) and 1000 ng RNA were reverse-transcribed into cDNA using the High capacity

cDNA Reverse Transcription Kit (Applied Biosystems; 4368814). Genomic DNA was isolated by phenol:chloroform extraction as previously described (20). Primers (Supplementary Table 2) were designed to PCR amplify the area surrounding the *RYR1* mutation inserted in ex36. Genomic DNA and skeletal muscle cDNA were amplified using the following conditions: initiation 5 min. at 95°C, followed by 37 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 40 s, and a final extension step at 72°C for 3 min. The final products were separated on a 7.5% acrylamide gel to monitor proper amplification and then subjected to Sanger sequencing. Subsequently qPCR was performed to quantify the expression of the mutant allele (Supplementary Figure 3C). The cDNA was amplified using Power SYBR® Green reagent Master Mix (Applied Biosystems; 4367659), using the Applied Biosystems 7500 Fast Real-time PCR System running 7500 software version 2.3. Transcript quantification was based on the comparative ΔΔCt method. Each reaction was performed in duplicate and results are expressed as relative gene expression normalized to desmin (DES).

***In Vivo* Muscle Strength Assessment:** Mice (WT n=13 and RyR1Q1970fsX16 (n=12) were individually housed in cages equipped with a running wheel carrying a magnet. Wheel revolutions were registered by a reed sensor connected to an I-7053D Digital-Input module (Spectra), and the revolution counters were read by a standard laptop computer via an I-7520 RS-485-to-RS-232 interface converter (Spectra). Digitized signals were processed by the ‘‘mouse running’’ software developed at Santhera Pharmaceuticals. Total running distance (in meters) and speed (Km/hr) were evaluated.

***In Vitro* Muscle Strength Assessment**: For *in vitro* force measurements, EDL and soleus muscles were dissected (WT n=7 and RyR1Q1970fsX16 (n=7) and mounted on a MyoStation muscle force transducer (Myotronic, Heidelberg Scientific Instruments). Muscle force was digitized at 4 kHz by using an AD Instruments converter and stimulated with 15-V pulses for 1.0 ms. EDL tetanus was recorded in response to 400-ms pulses at 10/20/50/100 and 150 Hz, whereas, for soleus, 1100-ms pulses at 10/20/50/100 and 120 Hz were applied. Specific force was normalized to the muscle cross-sectional area [CSA_wet weight (mg)/length (mm)_1.06 (density mg/mm^3)] (33).

Biochemical Analysis and qPCR: Total muscle homogenates were prepared from EDL and Soleus muscle from WT and RyR1Q1970fsX16 mice. SDS-polyacrylamide electrophoresis and Western blots were carried out as previously

described (20, 23). Western blots were incubated with the following primary Abs diluted in Tris Buffer Saline containing 0.01% Tween (TBST): anti-RyR1 (Cell signaling, catalogue number D4E1), anti-SERCA1 (Santa Cruz, catalogue number sc-8093), anti-SERCA2 (Santa Cruz, catalogue number sc-8095), anti-$Ca_v1.1$ (Santa Cruz, catalogue number sc-8160), anti- DHPR β1a (Santa Cruz, catalogue number sc-32079), anti-JP45 (Anderson, A. A., 2006), anti-calsequestrin 1 (Sigma, catalogue number C0743), anti-calreticulin (Abcam, catalogue number ab-92516), anti-sarcalumenin (ThermoFischer scientific, catalogue number MA3-932), anti-parvalbumin (Swant, catalogue number PV25), anti-myosin heavy chain (MyHC, Millipore 05-716) followed by peroxidase-conjugated Protein G (Sigma P817) or peroxidase-conjugated anti-mouse IgG ((Sigma A2304). The immunopositive bands were visualized by chemiluminescence using the WesternBright ECL- HRP Substrate (Witec AG). Densitometry of the immunopositive bands was carried out using the Fusion Solo S (Witec AG).

Gene expression was monitored by qPCR using the Power SYBR® Green reagent Master Mix (Applied Biosystems; 4367659) on skeletal muscle cDNA obtained as described in the previous section. Amplification was performed using the Applied Biosystems 7500 Fast Real-time PCR System running 7500 software version 2.3. Transcript quantification was based on the comparative ΔΔCt method. Each reaction was performed in duplicate and results are expressed as relative gene expression normalized to desmin (DES). The primer sequences are listed in Supplementary Table 2.

Human muscle biopsies and total homogenates: Quadriceps muscle biopsies from the subject with the genetically confirmed *RYR1* c.5908C>T (p.Q1970X) mutation and from a healthy non-affected individual undergoing the *in vitro* contracture test were used. Total muscle homogenates were prepared as previously described (20), proteins were separated on a 6% SDS PAG, transferred onto nitrocellulose and probed with anti-RyR1 and anti-MyHC antibodies as described in the previous section.

Isolation of single muscle fibers: 6-8 weeks old male mice were killed by pentobarbital overdose as approved by the Cantonal animal care authorities. The heart was exposed and washed by injecting Tyrode's normal mammalian Ringer buffer (Tyrode's buffer, 137mM NaCl, 5.4mM KCl, 0.5mM $MgCl_2$, 1.8mM $CaCl_2$, 0.1% glucose, 11.8 mM HEPES, pH 7.4 NaOH), followed by an injection of the following

enzymatic mix; 0.1% Collagenase type I (Clostridium hystoliticum Type I, Sigma-Aldrich), 0.08% Collagenase type II (Clostridium hystoliticum Worthington biochemical corporation) and 0.05% Elastase (porcine pancreas Worthington biochemical corporation) diluted in Tyrode's buffer. After washing, EDL and Soleus muscles were isolated and further digested with 0.2% of Collagenase type I (Clostridium hystoliticum Type I, Sigma-Aldrich) in Tyrode's buffer for 45-55 minutes at 37 °C 5% CO_2. Muscles were subsequently washed with Tyrode's buffer and gently separated from the tendons using a fire-polished Pasteur pipette. EDL and Soleus muscle fibers isolated by this procedure remained viable and contracted when assayed during the experiments. For the final step, fibers were placed either on 35 mm glass bottom dishes (MatTek corporation, P35G-0-14-C) or on ibiTreat 15µ-Slide 4 well (ibidi cell in focus), previously coated with 5 µl of 1 mg/ml mouse laminin (Sigma, L2020). The isolated muscle fibers were then used either for Ca^{2+} measurement or for immunofluorescence.

Calcium Measurements: Resting $[Ca^{2+}]_i$ measurements were carried out as previously described (35) on single EDL and soleus muscle fibers loaded (20 min at 20°C) with 5 µM of the fluorescent indicator Fura-2 AM (Invitrogen, F1201). For measurements of electrically evoked Ca^{2+} transients, EDL and Soleus fibers were plated on a chamber with a glass coverslip bottom previously coated with 2 µl of laminin 1 mg/ml (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 10 min at 20°C in Tyrode's buffer containing 10 µM of the low affinity calcium indicator Mag-Fluo-4 AM (Invitrogen, M14206). To avoid movement artifacts the calcium measurements were carried out in Tyrode's buffer containing 50 µM N-benzyl-p-toluene sulfonamide (BTS, Tocris). Fiber stimulation was obtained using two platinum plate electrodes, connected to a Grass S88 stimulator (Grass medical instruments, Quincy Mass. U.S.A). Single twitches were delivered by stimulating the muscles with supra-threshold rectangular current pulses of 1 ms duration. For tetanic stimulation, the stimulator was triggered by a TTL pulse with a length of tetanic stimulation of 300-ms, and 50-V pulses of 1 ms duration delivered at 100 Hz. Calcium measurements on whole fibers were carried out using a Nikon Eclipse TE2000-E fluorescent microscope (Nikon Instruments Inc.) with a 20× Plan Apo VC Nikon objective (numerical aperture, 1.4) acquisition frequency was 4000 lps. Analysis of calcium transients was performed using a homemade software as described (36). Results are presented as peak $\Delta F/F$ $((F_{max}- F_{rest})/(F_{rest}))$ and kinetic parameters

(Time-To-Peak, Half Time-To-Peak and Half Relaxation Time) for a single twitch; tetanic stimulations are presented as ΔF/F.

Immunostaining: Single EDL and Soleus fibers were fixed in 4% paraformaldehyde in Phosphate Buffer Saline (PBS) for 30 minute at room temperature, followed by a 10 minute incubation in 0.1M glycine to quench free aldehyde groups. Fibers were permeabilized with 2% Triton X-100 in PBS for 30 minutes rinsed with PBS and incubated with 1% blocking buffer (Roche 11500694001) in TBS for 1 hour at room temperature. Fibers were in incubated with the primary antibodies anti-RyR1 (Cell Signaling) and anti-$Ca_V1.1$ (Developmental Studies Hybridoma Bank) diluted in TBS overnight at 4º C. After extensive washing they were then incubated for 60 min at room temperature with the appropriate conjugated secondary antibody (chicken anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 568 (ThermoFischer scientific). Actin filaments were stained for 30 min using Phalloidin-iFluor 647 (Abcam) and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (ThermoFischer scientific). Fibers were visualized using a Nikon A1R laser-scanning confocal microscope (Nikon Instruments Inc.) equipped with Nikon Plan Apo TIRF 60x oil objective (NA 1.4) and with Coherent® Sapphire lasers (405, 488, 561 nm) and an MPBC®CW Visible Fiber laser (647 nm) controlled by Nikon® NIS-Elements Confocal software.

Histological examination: EDL and soleus muscles from WT and RyR1Q1970fsX16 mice were isolated and examined for muscle fiber type composition and minimal Ferret diameter as previously described (37). Images were obtained using an Olympus IX series microscope and analyzed using the CellP Software. The fiber type composition was determined as described (37).

Preparation and analysis of samples for electron microscopy (EM*)*: EDL muscles were dissected from sacrificed animals, pinned on a Sylgard dish, fixed at room temperature with 3.5% glutaraldehyde in 0.1 M NaCaCO buffer (pH 7.4), and stored in the fixative at 4ºC. Fixed muscle were then post-fixed in a mixture of 2% OsO_4 and 0.8% $K_3Fe(CN)_6$ for 1-2 h, rinsed with 0.1M sodium cacodylate buffer with 75 mM $CaCl_2$, en-block stained with saturated uranyl acetate, and embedded for EM in epoxy resin (Epon 812) as in Pietrangelo et al. 2015. Ultrathin sections (~40 nm) were cut in a Leica Ultracut R microtome (Leica Microsystem, Austria) using a Diatome diamond knife (DiatomeLtd. CH-2501 Biel, Switzerland) and examined at 60 kV after double-staining with uranyl acetate and lead citrate, with a FP 505 Morgagni

Series 268D electron microscope (FEI Company, Brno, Czech Republic), equipped with Megaview III digital camera (Munster, Germany) and Soft Imaging System (Germany).

Quantitative analyses by EM: Data contained in Table 1 were collected from three months-old Wild Type (WT, n=2 mice) and RyR1Q1970fsX16 (n=2 mice) EDL muscles. In each sample, 20 fibres were analysed. In each fibre 2-3 micrographs (all at the same magnification, 14K, and of non-overlapping regions) were randomly collected from longitudinal sections. CRUs were marked and counted in each micrograph. In Table 1, column A, number of CRUs/area was reported as average number /100 μm^2. In each EM image, we also determined the number of dyads i.e. incomplete triads (Table 1, column B) expressed as percentages over the total number of CRUs. Note: Since in RyR1Q1970fsX16 mouse muscles there is a small but visible percentage of fibers presenting large areas of severely altered morphology (10%, not shown), EM quantification was performed only on the apparently normal fibers.

Statistical Analysis: Statistical analysis was performed using the Student's unpaired *t* test and the Mann–Whitney U test when values were not normally distributed. Means were considered statistically significant when the P value was <0.05. Data was processed, analyzed and plotted using the software OriginPro 2018 (OriginLab Corporation, Northampton, MA., U.S.A). Images were assembled using Adobe Photoshop CS (version 8.0).

Acknowledgements:

This work was supported by a grant from the Swiss National Science Foundation (SNF N° 31003A-169316), a grant from the OPO-Stiftung, from the Swiss muscle foundation (FSRMM) and a grant from NeRAB. The MRC Centre for Neuromuscular Diseases Biobank and the UMCG Pathology department are greatly appreciated for providing patients' sample. The support of the Department of Anesthesia Basel University Hospital is gratefully acknowledged.

We would like to thank Heide Oller, Anne-Sylvie Monnet and Christoph Bachmann for expert technical assistance and support.

All research at Great Ormond Street Hospital NHS Foundation Trust and UCL Great Ormond Street Institute of Child Health is made possible by the NIHR Great Ormond Street Hospital Biomedical Research Centre, which in the context of this study supported the human pathology studies and not the animal work. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Conflict of interest statement: The Authors declare that they have no conflicts of interest

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LEGENDS TO FIGURES

Figure 1: Western blot shows that the presence of the ex 36 *RYR1* c.5908C>T (p.Q1970X) nonsense mutation causes a substantial decrease in RyR1 protein content. Fifty micrograms of proteins from total skeletal muscle homogenates from control or patient quadriceps muscle biopsy were separated on a 6% SDS PAG, transferred onto nitrocellulose and probed with rabbit anti-RyR1 monoclonal antibody (D4E1) followed by protein-G peroxidase. The blot was then stripped and re-probed with anti-MyHC mouse monoclonal antibody (Millipore catalog number 05-716) for protein loading quantification.

Figure 2: RyRQ1970fsX16heteozygot mice show normal body weight but exhibit reduced running capacity. A. Genotyping of WT and heterozygous RYRQ1970fsX16 mice; in the WT mice the 450nt PCR fragment from the WT allele lacks the engineered *Pvu*II site and therefore is resistant to *Pvu*II digestion. In the Het mice the mutant allele yields fragments of 140 and 240 nucleotides after digestion with *Pvu*II resulting in a stoichiometric mix of 450nt, 240nt and 140nt fragments. **B.** The body weight of RYR1Q1970fsX16 mice (empty squares) was similar to that of WT littermates (black squares) during the period of 18 weeks. Each symbol represents the mean (±S.E.M.) body weight of 11-13 male mice. **C.** Spontaneous dark phase activity and **D**. Total running speed measured for 21 days in 12-weeks old RYR1Q1970fsX16 mice (empty squares) and WT littermates (black squares). Each mouse was individually housed in cages equipped with a running wheel. The total running distance and median speed were significantly reduced in RYR1Q1970fsX16 mice. Data points are expressed as mean ± SEM (n = 12-13 mice). $p \leq 0.05$ (Mann–Whitney two-tailed test calculated for the 21 day period).

Figure 3: Mechanical properties of skeletal muscles from WT and heterozygous RYRQ1970fsX16 mice. **A.** and **B.** Representative traces of twitch force in EDL and soleus muscles from WT (continuous line) and heterozygous RYR1Q1970fsX16 (dotted line). **C.** and **D.** Representative traces of maximal tetanic force in EDL and soleus muscles from WT (continuous line) and heterozygous RYR1Q1970fsX16 (dotted line) mice. **E.** Statistical analysis of force generated after tetanic stimulation of isolated EDL muscles from WT mice (continuous black boxes) and RYRQ1970fsX16$_{X16}$ mice (dotted grey boxes) at 50Hz, 100Hz and 150Hz. **F.**

Statistical analysis of force generated after tetanic stimulation of isolated Soleus muscles from WT mice (continuous black boxes) and RYR1Q1970fsX16 mice (dotted grey boxes) at 50Hz, 100Hz and 120Hz. Data points are expressed as Whisker plots (n = 7 mice). p ≤0.05 (Mann–Whitney two-tailed test).

Figure 4: EDL muscles from EX36 RYR1Q1970fsX16 heterozygous mice are atrophic. **A.** EDL and Soleus were sectioned with a cryostat, stained with anti laminin and anti-MHCI Ab, imaged with an inverted fluorescent microscope and analyzed as described in the Methods section. Bar indicates 100 μm. **B.** Quantification of the fiber type composition of Soleus (left panel) and EDL (right panel) muscle from WT (black bars) and RYR1Q1970fsX16 heterozygous mice (white bars). Values are presented as mean (± S.E.M.) % fibers. **C.** Minimal Feret fiber distribution. Measurements were carried out on cross sections of Soleus (left) and EDL (right) muscles from WT (black squares, continuous line) and RyR1Q1970fsX16 (white squares, dotted line). WT soleus= 1958 fibers, RyR1Q1970fsX16 soleus =2940 fibers, WT EDL= 2033 fibers and RyR1Q1970fsX16 EDL= 2212 fibers, (n=2 mice per genotype). Data points are expressed as mean ± SEM. p ≤ 0.05 (Mann–Whitney two-tailed test)

Figure 5: RYR1 transcript and protein levels are decreased in RYRQ1970fsX16$_{X16}$ mouse muscles. A. Biochemical characterization of total homogenate of EDL and Soleus muscles from WT and RYRQ1970fsX16 mice. Left panels show representative Immunoblots with the indicated antibodies. Right panels show relative protein content in EDL (top) and soleus (bottom) muscles. Each bar represents the mean relative protein content of n=9-10 mice; a minimum of 3 repeats were done for each data point, and data are expressed as mean (± SEM). Values were normalized to the intensity values obtained from WT mice, which were considered 100%. **B.** Quantitative real-time PCR (RTqPCR) of RYR1 and CACNA1S transcripts in skeletal muscle from WT (filled circles) and RYRQ1970fsX16$_{X16}$ (empty circles) mice. Transcript levels were normalized using the ΔΔCt method and DES as a muscle specific gene. *p<0.05; **p<0.001;***p<0.005 (Mann–Whitney two-tailed test).

Figure 6 Ultrastructure of EDL from WT and RYR1Q1970fsX16 mice. A-F) representative EM images of CRUs distribution and morphology in adult EDL fiber from WT (A) and RYR1Q1970fsX16 mice (B-F). A) In adult WT EDL fibers CRUs (pointed by black arrows) are usually placed at the sarcomere I-A junctions and are mostly in the form of triads: two SR vesicles closely opposed to a central T-tubule (inset). B) In EDL fibers from RYR1Q1970fsX16 mice CRUs are less frequent and often found in the form of dyad (inset). RyRs-feet are visible as small electrondensity (arrowheads, insets). C-D) Representative EM pictures of triads undergoing a process of disarrangement where T tubules (labeled in light-blue) are barely but still recognizable. Scale bars: A-B, 0.5 μm (inset, 0.1 μm); C-F: 0.2 μm

Figure 7: Electrically evoked peak Ca^{2+} transients are reduced in EDL from RYR1Q1970fsX16 mice. Enzymatically dissociated EDL (panels A and C) and soleus (panels B and D) fibers were loaded with Mag-Fluo-4 and electrically stimulated by field stimulation **A and B.** Representative calcium transient elicited by a single twitch of 0.5 msec and 50 volts. **C and D.** Representative calcium transient elicited by a train of pulses delivered at 100 Hz for 300 msec. **C.** Data points are expressed as traces of experiments on fibers (n=……) isolated from three mice from each genotype.

TABLE 1

	A	B
	CRUs/100 μm^2	**Dyads (%)**
WT	62.7 ± 2.5	1.4 ± 0.2 (2%)
RYR1$_{Q1970fsx16}$	39.7 ± 2.0*	3.5 ± 0.6 (8%)*

Quantitative analysis of CRUs in EDL muscles from WT and RYR1Q1970fsx16 mice. In fibers from RYR1Q1970fs16 mice there is a decrease in the N°/area of CRUs compared to fibers from WT mice (column A). The average frequency of dyads (column B), given as a percentage over the total number of CRUs, is higher in samples from RYR1Q1970fs16 than in WT. Data are shown as mean ± SEM (*p < 0.01). Sample size: 2 mice each, 20 fibers, 2-3 micrographs/fiber.

Abbreviations: $Ca_V1.1$**, alfa 1 subunit of the DHPR;** CRU, calcium release units; DHPR, dihydropyridine receptor; ECC, excitation contraction coupling; EDL, extensor digitorum longus; MmD, multiminicore disease; RyR, ryanodine receptor; SR, sarcoplasmic reticulum;