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SYMPOSIUM REVIEW

Calsequestrin-1: a new candidate gene for malignant hyperthermia and exertional/environmental heat stroke

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Malignant hyperthermia (MH) and exertional/environmental heat stroke (EHS) in humans present as similar life threatening crises triggered by volatile anaesthetics and strenuous exercise and/or high temperature, respectively. Many families (70–80%) diagnosed with MH susceptibility (MHS), and a few with EHS, are linked to mutations in the gene for the ryanodine receptor type-1 (RyR1), Ca^{2+} release channel of the sarcoplasmic reticulum (SR) of skeletal muscle and a key protein in excitation–contraction (EC) coupling. However, mutations in the *RyR1* gene are not found in all MH families, suggesting that alternative genes remain to be identified. In our laboratory we have recently characterized a novel knockout model lacking skeletal muscle calsequestrin (CASQ1), a SR Ca^{2+}-binding protein that modulates RyR1 function, and investigated whether these mice present a MH/EHS-like phenotype. Ablation of CASQ1 results in remodelling of the EC coupling apparatus and functional changes, which in male mice causes a striking increase in the rate of spontaneous mortality and susceptibility to trigger MH-like lethal episodes in response to halothane and heat stress. The demonstration that ablation of CASQ1 results in MH- and EHS-like lethal episodes validates *CASQ1* as a viable candidate gene for linkage analysis in MH and EHS families where mutations in *RyR1* are excluded.

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Abbreviations Ca^{2+}, calcium ions; CASQ, calsequestrin; CVPT, catecholamine-induced polymorphic ventricular tachycardia; CRUs, calcium release units; EDL, extensor digitorum longus; EM, electron microscopy; EHS, environmental heat stroke; IVCT, in vitro contracture test; MH, malignant hyperthermia; MHS, MH susceptibility; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TT, transverse tubule.

Several skeletal muscle disorders result from abnormalities in proteins involved in excitation–contraction (EC) coupling, a highly coordinated process that controls myoplasmic Ca^{2+} dynamics during muscle activation (MacLennan, 2000). Malignant hyperthermia (MH), a pharmacogenetic disorder characterized by a hypermetabolic response to volatile anaesthetics, such as halothane and isofluorane (Denborough, 1998; Rosenberg *et al.* 2007), is life-threatening if not corrected immediately by suspension of triggering agent and treatment with dantrolene (Wedel *et al.* 1995). Mutations in the ryanodine receptor type 1 gene (*RyR1*), encoding the sarcoplasmic reticulum (SR) Ca^{2+} release channel of skeletal muscle account for MH susceptibility (MHS) in most MH families (70–80%) (Robinson *et al.* 2006). Interestingly, MH-like hyperthermic episodes, but unrelated to anaesthetic administration, have also been reported in humans during strenuous exercise conducted under conditions of excessive elevations in temperature, a condition referred to as exertional/environmental heat-stroke (EHS) (Pamukcoglu, 1988; Bouchama & Knochel, 2002). Interestingly, two of these cases have been reported in young, otherwise fit male athletes with documented family histories of MHS (Ryan & Tedeschi, 1997; Tobin *et al.* 2001), further validating a possible association between these two disorders (Hopkins *et al.* 1991; Muldoon *et al.* 2004).

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While most MHS cases are linked to mutations in the *RyR1* gene, about 20–30% of all MHS individuals do not possess an *RyR1* gene mutation. In these cases, MH (and potentially EHS) could arise from mutations to genes for other EC coupling proteins, possibly those modulating directly RyR1 activity (Robinson *et al.* 1997). A mutation in the α_1-subunit of the dihydropyridine receptor (DHPR), which controls directly RyR1 during EC coupling, has been reported (Monnier *et al.* 1997). One other possible candidate would be calsequestrin (CASQ)-1, a Ca^{2+} binding protein located in the SR terminal cisternae of striated muscle (MacLennan & Wong, 1971), which possibly acts as a luminal regulator of RyR1-mediated Ca^{2+} release (Beard *et al.* 2002). To date, no human disease has yet been associated with mutations in the *CASQ1* gene. However, catecholaminergic polymorphic ventricular tachycardia (CPVT), a rare arrhythmogenic disorder characterized by physical/emotional stress-induced syncopal events and sudden cardiac death, is caused by both gain-of-function mutations in the cardiac *RyR2* gene (Priori *et al.* 2001) and loss-of-function mutations in the cardiac *CASQ2* gene (Lahat *et al.* 2001). By analogy to cardiac muscle, we hypothesized that MH and EHS may result from increased SR Ca^{2+} leak caused by either gain-of-function mutations in *RyR1* (Chelu *et al.* 2006; Yang *et al.* 2006) or loss-of-function mutations in *CASQ1*. We have tested this hypothesis in *CASQ1*-knockout (null) mice (Paolini *et al.* 2007).

The phenotype of *CASQ1*-null mice

In 2007 we published in *The Journal of Physiology* the structural–functional characterization of the first

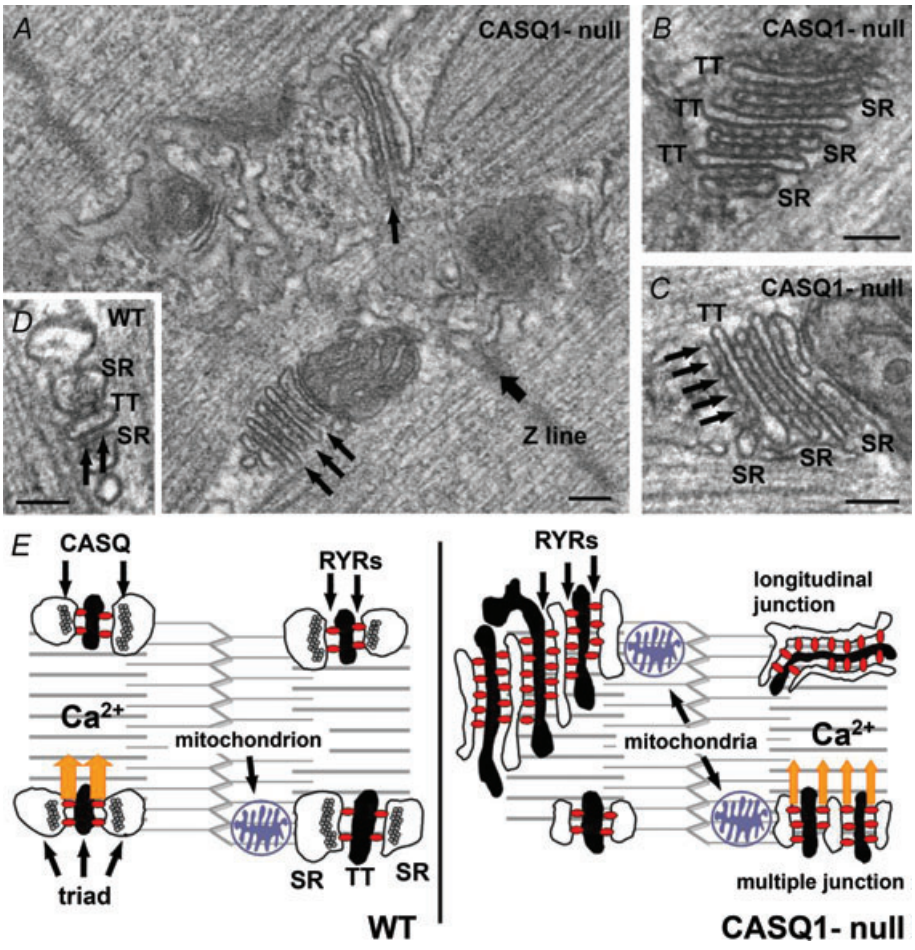

Figure 1. Remodelling of CRUs in EDL muscle following depletion of CASQ1
A, in *CASQ1*-null fibres CRUs are often formed by multiple elements and more variably oriented (arrows). *B* and *C*, multilayered CRUs usually contain more than two rows of RyRs, or feet, between SR and TT (arrows). The lumen of the SR terminal cisternae is significantly narrower than in controls (compare with *D*). *D*, in WT fibres, CRUs are almost exclusively in the form of triads, SR has a wide profile, and feet usually form two rows between SR and TT (arrows). *E*, cartoon summarising the structural modifications in *CASQ1*-null CRUs: junctions are often multilayered; RyR-feet and mitochondria are increased in number; junctional SR width is smaller. See Paolini *et al.* 2007 for more detail. Bars: 0.1 μm.

knockout mouse lacking CASQ1 (Paolini *et al.* 2007). A year earlier Knollmann *et al.* (2006) had characterized the first *CASQ2* knockout mouse. Lack of CASQ1, as the *CASQ2* knockout, resulted in a non-lethal phenotype and, apparently, *CASQ1*-null mice did not present noticeable differences under standard housing conditions. The biochemical, structural and functional characterization of calcium release units (CRUs) – intracellular junctions between SR and transverse tubule (TT) – revealed significant remodelling possibly caused by compensatory postnatal adaptation. Figure 1 summarizes some of the findings of Paolini *et al.* 2007: (a) reduction in size of SR terminal cisternae, which in wild-type (WT) muscle contains CASQ; (b) proliferation of junctional SR–TT; (c) increased amount of RyRs. These results demonstrated the importance of CASQ1 for the correct assembling of CRUs and for the structural shaping of mature SR in fast twitch fibres. Also interesting was the increased frequency of mitochondria (Paolini *et al.* 2007), which have been shown to be functionally and structurally connected to the SR compartment (Rudolf *et al.* 2004; Boncompagni *et al.* 2009).

One unexpected finding of our initial studies (Paolini *et al.* 2007) was the relatively small reduction in the size of electrically evoked Ca^{2+} transients and in the total amount of releasable Ca^{2+} from the SR (caffeine-induced responses). If, as commonly thought, CASQ1 is the main buffer inside the SR of skeletal muscle, one would have expected a far more dramatic effect following complete ablation of CASQ1 from fast twitch fibres. Results of others are also against the expectations (Wang *et al.* 2006; Knollmann *et al.* 2006) and in line with our results (Paolini *et al.* 2007), suggesting that means of Ca^{2+} storage unrelated to CASQ must play a key role in muscle, at least in the *CASQ1*-null animals.

After the first study (Paolini *et al.* 2007), one puzzling observation prompted us to investigate the phenotype of *CASQ1*-null mice under conditions of increased stress:

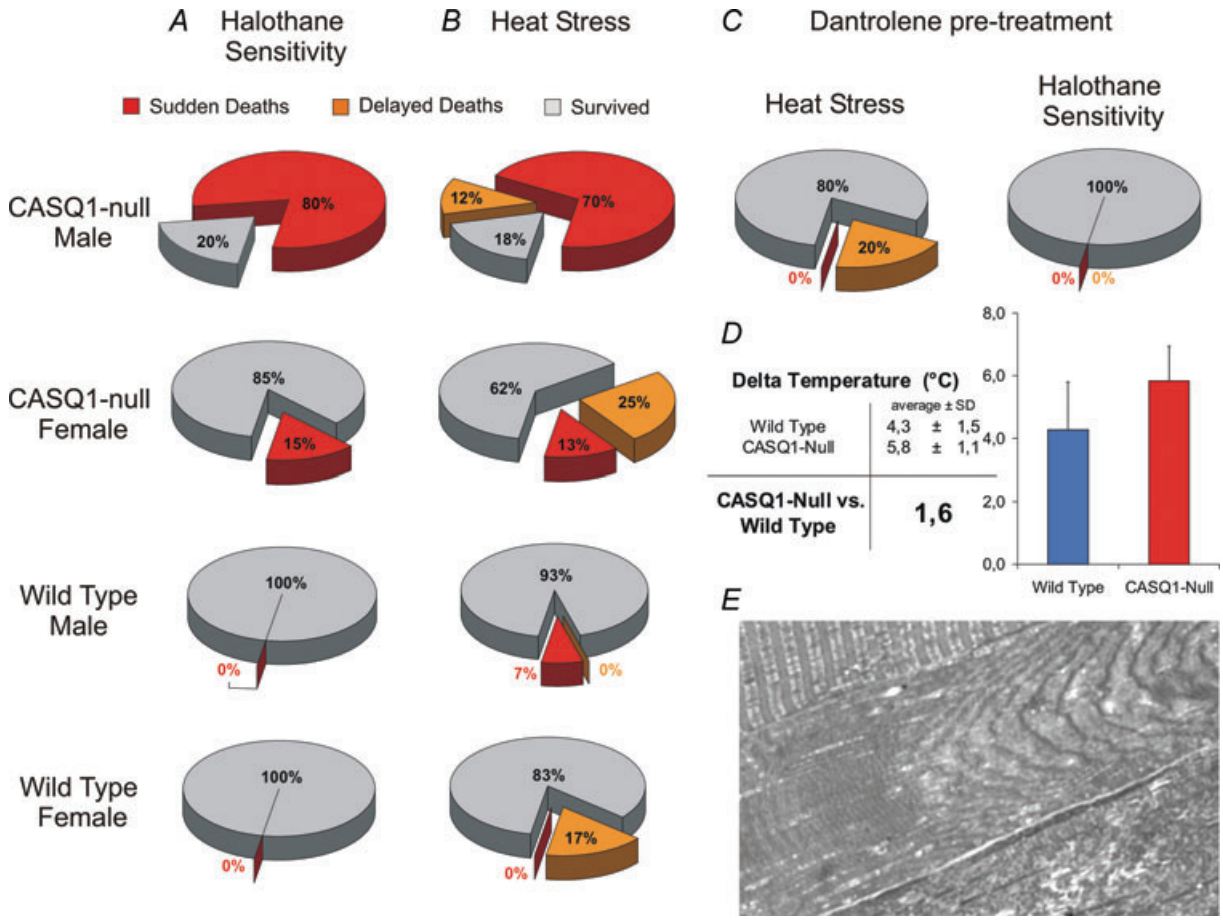

Figure 2. *CASQ1*-null male mice exhibit enhanced sensitivity to halothane and heat exposure (prevented by dantrolene pre-treatment), hyperthermia and rhabdomyolysis
A and *B*, incidence of sudden death in male/female WT and *CASQ1*-null mice as a result of halothane and heat exposure. *C*, sudden death may be prevented by prior administration of dantrolene. *D*, core temperature increases significantly ($P < 0.05$) more in *CASQ1*-null mice compared with WT animals. *E*, about 40% of EDL fibres appear severely damaged immediately after heat-induced sudden death. See Dainese *et al.* 2009 for more detail.

compared to their WT counterparts, we observed a significantly increased rate of spontaneous mortality in male *CASQ1*-null mice, particularly after 3 months of age (Dainese *et al.* 2009). Based on this initial observation, we hypothesized the presence of a pathological phenotype and decided to determine if *CASQ1*-null mice were susceptible to trigger MH-like episodes in response to halothane and heat. Protocols similar to those recently used to test the phenotype of *RyR1*-knockin mice expressing human MH mutations were employed (Chelu *et al.* 2006; Yang *et al.* 2006; Durham *et al.* 2008). Male *CASQ1*-null animals exhibited a very high mortality rate during exposure to either halothane (2%, 1 h) or heat (41°C, 30 min): lethal episodes were characterized by impaired movement, difficulty in breathing, whole-body contractions, arched back, followed by death (Dainese *et al.* 2009). Specifically, about 80% of male *CASQ1*-null mice died during both stress protocols (halothane: 16 out of 20; heat: 17 + 3 delayed out of 24). Female *CASQ1*-null mice displayed a higher survival rate following both procedures compared to male *CASQ1*-null mice, but still lower than WT mice (Fig. 2*A* and *B*, and Dainese *et al.* 2009 for more detail). Importantly, both halothane- and heat-induced crises could be completely prevented by prior intraperitoneal injection of dantrolene (Fig. 2*C* and Dainese *et al.* 2009 for more detail), the only pharmacological intervention for MH in humans (Wedel *et al.* 1995) and also been considered in the treatment of EHS (Hausfater, 2005).

The parallel between MH and the phenotype of *CASQ1*-null mice was strengthened by assessing the presence of hyperthermia and rhabdomyolisis during heat stress (Fig. 2*D* and *E*) and by experiments that demonstrated the enhanced sensitivity of *CASQ1*-null *vs.* WT specimens (whole extensor digitorum longus (EDL) fibres, single flexor digitorum brevis (FDB) fibres, and myotubes) to increasing temperature and to caffeine (not shown, see Dainese *et al.* 2009 for detail).

Summary and conclusions

Mutations in the gene that encodes the skeletal muscle ryanodine receptor (RyR1) result in life-threatening MH

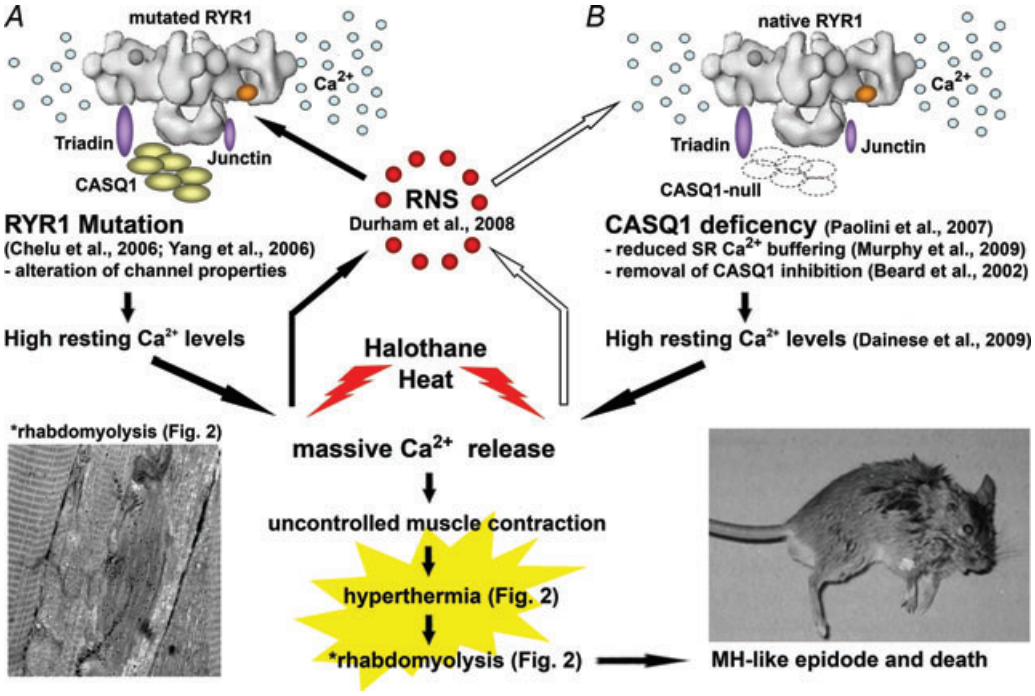

Figure 3. MH/EHS susceptibility may result from either gain-of-function mutations in *RyR1* or loss-of-function mutations in *CASQ1*

A, gain of function mutations in *RyR1* changes the properties of the SR Ca^{2+} release channels. *RyR1* channels carrying human MH mutations have been expressed in knock-in mice causing high resting Ca^{2+} levels and MH susceptibility (Chelu *et al.* 2006; Yang *et al.* 2006). *B*, CASQ1 deficiency in knockout mice also results in high resting Ca^{2+} levels (Dainese *et al.* 2009). Lack of CASQ1 causes a reduction in the SR Ca^{2+} buffering (Murphy *et al.* 2009) and may remove the inhibitory influence on RyR1-mediated Ca^{2+} release (Beard *et al.* 2002). The precise mechanism(s) underlying the abnormal intracellular Ca^{2+} levels in *CASQ1*-null fibres and myotubes has not yet been elucidated (see Discussion). High resting Ca^{2+} leak resulting from either *RyR1* mutations or *CASQ1*-deficiency results in massive anaesthetic- and heat-induced Ca^{2+} release, uncontrolled muscle contraction, hyperthermia, and rhabdomyolysis followed by sudden death. Durham *et al.* (2008) have recently proved that overproduction of reactive nitrogen species (RNS) due to abnormally high resting Ca^{2+} levels generates a feed-forward mechanism by nitrosilating RyR1. This mechanism remains to be tested in *CASQ1*-null mice (open arrows).

and EHS in humans (Ryan & Tedeschi, 1997; Denborough, 1998; Tobin *et al.* 2001; Robinson *et al.* 2006; Rosenberg *et al.* 2007). However, since not all MH/EHS families are linked to *RyR1* mutations, it is likely that other MH gene loci and different pathophysiological mechanisms remain to be identified. Our recent demonstration that loss-of-function mutations in *CASQ1* strongly enhances MH and EHS susceptibility in mice (Dainese *et al.* 2009) validates *CASQ1* as a powerful alternative candidate gene for linkage analysis in MH and EHS families where mutations in *RyR1* are excluded.

The phenotype of *CASQ1*-knockout mice (Dainese *et al.* 2009) is strikingly similar to that resulting from gain-of-function mutations in *RyR1* (Chelu *et al.* 2006; Yang *et al.* 2006; Durham *et al.* 2008) (Fig. 3). These findings create an important parallel with cardiac muscle, where loss-of-function mutations in cardiac *CASQ2* and gain-of-function mutations in *RyR2* result in analogous arrhythmogenic disorder, i.e. CPVT (Lahat *et al.* 2001; Priori *et al.* 2001). Thus, our study provides crucial evidences that disruption in RyR regulation by CASQ may represent an important novel pathogenic mechanism that underlies both cardiac (CPVT) and skeletal muscle (MH and EHS) diseases. We believe that these findings could be of clinical relevance to the fields of muscle disease, Ca^{2+} signalling, and medical genetics.

In addition, our studies reinforce a parallel between MH and EHS (Hopkins *et al.* 1991; Muldoon *et al.* 2004), and support the notion that the two syndromes may in some cases arise from a common pathogenic mechanism, i.e. lack of proper regulation of SR Ca^{2+} release. Indeed, MH and EHS are becoming more and more relevant, since global warming is affecting many world regions potentially rendering a greater number of susceptible humans exposed to environmental temperatures sufficient to trigger EHS with higher frequency (Semenza *et al.* 1996; Bouchama & Knochel, 2002).

Our study leaves, though, some important questions that will need to be addressed in future work to fully understand some of the observations of Dainese *et al.* 2009.

(1) What is the molecular mechanism leading to high resting Ca^{2+} concentrations and to MH/EHS episodes in *CASQ1*-knockout mice? The mechanism(s) underlying abnormal Ca^{2+} levels in *CASQ1*-null fibres and myotubes (Dainese *et al.* 2009) has not yet been elucidated. Lack of CASQ1 causes a reduction in the SR Ca^{2+} buffering (Murphy *et al.* 2009) and may remove the inhibitory influence on RyR1-mediated Ca^{2+} release (Beard *et al.* 2002) (see Fig. 3). However, since the final steady value of free intracellular $[Ca^{2+}]$ cannot be directly determined by intracellular organelles, one possible explanation is that these changes are the result of a net Ca^{2+} entry through the plasmalemma. This would demands some kind of link between the primary effect (lack of CASQ1 in the SR) and the fibre's surface membrane. As is well known, the mechanism that allows such a communication has been identified as store-operated Ca^{2+} entry (SOCE). Recent data presented at the Biophysical Society Meeting 2009 support this hypothesis: in skeletal fibres with knockdown of *CASQ1* SOCE is significantly increased with respect to control fibres (Kee Min *et al.* 2009).

(2) What is the contribution of adaptative changes (i.e. increase in RyR and mitochondria, see Fig. 1) and of oxidative stress (Durham *et al.* 2008) in the MH/EHS-like phenotype of knockout mice? Increased mitochondrial frequency could indeed be considered an expected consequence of increased intracellular Ca^{2+} level, given that Murphy *et al*. (2009) have pointed out that this would inevitably mean higher resting consumption of ATP, which might readily predict an adaptive increase in mitochondrial density in fast-twitch muscle fibres.

(3) What are the reasons for the striking difference in spontaneous and stress-induced mortality between male and female *CASQ1*-null mice (Fig. 2)? Possible explanations may be found in hormonal differences between the two sexes, but also other possibilities (possible differences in muscle mass, different capacity to handle oxidative stress) need to be taken into consideration. To date, the precise mechanism underlying this sex difference remains unclear and will require further investigation.

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