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Malignant hyperthermia, environmental heat stress and intracellular calcium dysregulation in a mouse model expressing the p.G2435R variant of the RYR1 gene

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Abstract.-

Background. We report the phenotype of a knock-in mouse that expresses the RYR1 mutation p.G2435R, which is isogenetic with the human RYR1 variant most frequently associated with malignant hyperthermia (MH) in the UK.

Methods: We observed the general phenotype; determined the sensitivity of myotubes from these mice to caffeine-, KCl- and halothane-induced Ca^{2+}-release; the in vivo repsonse to halothane or increased ambient temperature; the in vivo myoplasmic intracellular Ca^{2+} concentration in skeletal muscle of the mice, both before and during exposure to volatile anaesthetics.

Results: $RYR1_{G2435R/MHN}$ (MHS-Het) or $RYR1_{G2435R/G2435R}$ (MHS-Hom) mice are fully viable under typical rearing conditions, although some male MHS-Hom mice died spontaneously. The EC_{50} (95% CI) for intracellular Ca^{2+} release in myotubes in response to KCl (MH normal, MHN, 20.6 (20-21.2) mM; MHS-Het 15.7 (15.3-16.1) mM; MHS-Hom 10.4 (10.1-10.8) mM) and caffeine (MHN, 5.03 (4.8-5.3) mM; MHS-Het 3.67 (3.3-4.0) mM; MHS-Hom 1.44 (1.3-1.6) mM) exhibit a gene dose-dependent decrease and there was a gene dose-dependent increase in halothane sensitivity. Intact animals show a gene dose-dependent susceptibility to MH with volatile anaesthetics or to heat stroke. $RYR1_{G2435R}$ mice have elevated skeletal muscle intracellular resting $[Ca^{2+}]_i$ (MHN 123±3 nM; MHS-Het 156±16 nM; MHS-Hom 265±32 nM, $P<0.001$) and $[Na^{+}]_i$ (MHN 8±0.1 mM; MHS-Het 10±1 mM; MHS-Hom 14±0.7 mM, $P<0.001$) that is further increased by exposure to volatile anaesthetics.

Conclusions: $RYR1_{G2435R}$ mice demonstrate gene dose dependent in vitro and in vivo responses to pharmacological and environmental stressors that parallel those in patients

with the RYR1 variant pG2434R.

Malignant hyperthermia (MH) is a hypermetabolic condition triggered in genetically predisposed individuals by any of the volatile anaesthetics and succinylcholine[1]. The MH crisis is characterized by hypermetabolism, hypercapnia, tachycardia, hypoxaemia, muscle rigidity, respiratory and metabolic acidosis and hyperthermia. The great majority of malignant hyperthermia (MH)-susceptible patients remain subclinical until challenged with pharmacological triggering agents[1-4]. If left untreated, the mortality of a fulminant MH episode is >70%, but improved understanding, better monitoring, and availability of dantrolene has reduced the mortality to <8% [1, 5]. The prevalence of MH susceptibility based on clinical incidence is estimated to range from as low as 1 in 250,000 to as high as 1 in 200, depending on the age and geographic location of the patient population, though accurate measures of MH susceptibility prevalence as a genetic predisposition remains difficult due to variable penetrance and the poor epidemiological data [6-8]. MH susceptibility may be diagnosed in patients with high a priori risk using an in vitro contracture test (IVCT) that measures contractile responses to halothane or caffeine of vastus lateralis or vastus medialis muscle biopsies[1, 9].

Molecular genetic studies have established the type 1 ryanodine receptor gene (RYR1) encoding the skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+} release channel (RyR1 protein) as the primary locus for both MH susceptibility and central core disease (CCD) [4, 6, 7, 10]. More than 200 RYR1 variants have been associated with MH and/or CCD [7]. Although MH related RYR1 variants can be found in all regions of the gene, most have been described in three clusters corresponding to the: N-terminal (Cys35-Arg614, MH region 1), central (Asp2129-Arg2458, MH region 2), and C-terminal (Ile3916-Ala4942, MH

region 3) regions of the RyR1 protein. To date, a porcine and 3 knock-in murine models that express human MHS mutations have been studied. Two murine, p.R163C [11], and p.Y522S [12], and the porcine p.R615C models have mutations in MH region 1 of the N-terminal domain of RyR1 [13-16]. The third currently available murine mutant RyR1 MH model, p.T4826I involves MH region 3 in the putative cytoplasmic linker between transmembrane segments S4 and S5[17]. All four models reported to date exhibit anaesthetic-triggered fulminant MH episodes and environmental heat stress, with varying gene dose relationships. The molecular mechanisms by which RYR1 variants confer MH susceptibility are unknown. A common characteristic of all animal models with MH-RyR1 mutations to date is an increased resting skeletal muscle intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)[14, 18-21] compared to non-susceptible muscle. In experimental murine models we have shown that exposure to halothane or isoflurane at clinically relevant concentrations causes $[Ca^{2+}]_i$ to rise several fold in MH muscle, whereas exposure to the same concentrations of halothane or isoflurane has no effect in non-susceptible muscle [15, 20, 22].

Although interesting observations have been made using these models involving MH regions 1 and 3, the most prevalent RYR1 variant associated with human MH is p.G2434R, involving MH region 2 of the RyR1 protein[23]. Because of its prevalence, the p.G2434R variant has been used as the comparator variant for describing differences in human phenotypes associated with different RYR1 genotypes. For example, MH susceptible patients with the p.G2434R variant have a relatively weak IVCT phenotype and are less likely to have an elevated serum creatine kinase concentration compared with humans harbouring either the p.T4826I or p.R163C RYR1 variants. Furthermore, pG2434R is not associated with CCD and is the most frequent variant associated with

MH in the UK to be implicated in familial genotype-phenotype discordance.

If we are to identify fundamental molecular mechanisms that are generically implicated in MH then detailed study of the p.G2434R variant is crucial. Furthermore, if we are to have confidence that human RYR1 genotype-phenotype relationships are recapitulated in the isogenetic murine models, a model of p.G2434R is necessary. The aim of the present study, therefore, was to develop a new knock-in murine model of MH with a mutation in RyR1 MH region 2 (p.G2435R), and to characterize whole animal and cellular phenotypes of the heterozygous and homozygous states.

Materials and Methods.-

Creation of p.G2435R Knock-In MH mice

Site-directed mutagenesis (QuickChange Multi-Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA) was used to mutate the glycine at codon 2435 in the murine RyR1 to Arginine (p.G2435R). This mutation is equivalent to position 2434 in the human RyR1. A 5.862 Kb NotI fragment harbouring RYR1 exons 40–47 (Fig. 1A) was isolated from a 129Sv/J mouse genomic library and used to construct the targeting vector. A bacterial LoxP (locus of crossover in P1) recombination site flanked neomycin (G418) cassette was inserted between the 3.9 Kb upstream arm and the 1.9 Kb downstream arm. The vector was linearized and electroporated into 129Sv embryonic stem cells and subjected to positive selection with G418 using standard techniques, as described previously[11, 22]. Polymerase chain reaction (PCR) analysis was used to identify homologous recombination at this location (Fig 1B and 1C). A clone identified as carrying the p.G2435R mutation was injected into C57BL/6 murine blastocysts and implanted into pseudopregnant mice. Male chimeric mice were mated with female C57BL/6 mice, and germ line transmission was confirmed by the presence of agouti coat colour and PCR screening. These mice were then bred to Tnap-Cre (tissue-nonspecific alkaline phosphatase promoter-driven Cre recombinase) transgenic mice[11,22], to excise the LoxP-flanked neo cassette. The resulting progeny with the neomycin cassette excised, which were used in the present study, were backcrossed for 2 rounds with C57BL/6 wild type (MH-N) mice and then crossbred to produce mice carrying either 1 (HET) or 2 (HOM) alleles with the mutation and maintained as either Hom (male) x Hom (female) or Het (male) x Hom (female) crosses.

Experimental Preparations.-

Experiments were conducted:

(i) In vitro using myoblasts from C57BL/6 (MHN), heterozygous MHS-p.G2435R (MHS-Het) and homozygous MHS-p.G2435R (MHS-Hom) mice. Myoblasts were obtained by enzymatic digestion of excised rear and foreleg skeletal muscles and isolated as a pure myoblast culture[24]. For measurements of Ca^{2+} transients, myoblasts were expanded to >60% confluence in growth media and differentiated into myotubes by culturing them for 4-6 days in DMEM containing 2% heat inactivated horse serum and no added growth-factors in Greiner µCLEAR® 96 well plates[18],[19] (Greiner Bio One, Stonehouse, UK).

(ii) In vivo, a) in intact awake mice exposed to increased environmental temperature or halothane and b) in ion-selective microelectrode studies of vastus lateralis fibres of anaesthetized (ketamine 100mg/kg and xylazine 2 mg/kg) MH-N, MHS-Het and MHS-Hom mice that were exposed and cleaned of fascia and adipose tissue, as described previously [25].

All experiments on animals from the creation of knock-in mice to establishment of their physiological and biochemical phenotypes were conducted using protocols approved by the institutional animal care and use committees at Harvard Medical School, the University of California at Davis, MRC Harwell and University of Leeds, the latter two both through project licenses approved by the UK Home office.

Measurements of Ca^{2+} transients in MHN, MHS-Het and MHS-Hom skeletal myotubes during exposure to caffeine, KCl and halothane

Differentiated myotubes were loaded with 5 µM Fluo-4 AM (Molecular Probes Inc., Eugene, OR) at 37℃, for 20 min in imaging buffer. The cells were then washed three times with imaging buffer and transferred to a fluorescence microscope. During the study Fluo-4 was excited at 494 nm and its emission was measured at 516 nm using a 40x 1.3 NA oil objective. Images were collected with an intensified 12-bit digital intensified charge-coupled device (ORCA-ER, Hamamatsu Photonics, Japan), and analysed from regions of interest in 1–15 individual cells using Image J software (NHLBI) and PRISM 7 software (GraphPad, California, USA). Using a multivalve perfusion system (Automate Scientific Inc., Oakland, CA) a dose-response curve for each agent was performed in myotubes in 4-5 wells to compare the response to any given agent among primary myotubes from MH-N, MHS-Het or MHS-Hom mice. The average fluorescence of the calcium transient (defined by the area under the transient curve) was measured and compared among genotypes. Individual areas under the curve were calculated in the following way: the average fluorescence during the challenge minus the average baseline fluorescence for 1 s immediately before the challenge. Because of variability in responses from cell to cell, to compare different experiments, individual response amplitudes for caffeine and KCl were normalized to the maximum response amplitude obtained by that cell to the highest concentration of the reagent that was being tested (20 mM caffeine, 60 mM KCl) and halothane responses were normalized to the maximum response of that cell to 60mM KCl. Data were obtained from 1-11 differentiated myotubes per well in 2-4 different wells (in 96-well plates) in at least 3-6 plates cultured on different days. Halothane concentrations were confirmed with gas chromatography[26].

Basal core temperature and responses to heat stress in vivo:

Basal rectal (core) temperature was measured in equal numbers of male and female awake mice with all three genotypes, using a thermistor probe when they were first removed from their home cage (environmental range 24-26℃) and recorded digitally (TC-324B Automatic Temperature Controller extension; Warner Instruments, Hamden, CT, USA) after the reading stabilized (30 s). To investigate heat stress responses, mice were gently restrained in a mouse restrainer and transferred to a 38℃ test chamber (MHN, n=9, 8–18 months; MH-Het, n=14, 10–16 months; MH-Hom, n=14, 4–13 months). Rectal temperature was measured continuously during the heat stress challenge (up to 80 min) or until the time of fulminant heat stroke, whichever came first. All MHN animals survived the exposure to increased ambient temperature and were euthanized at this time, however all MHS animals regardless of zygosity expired as a result of heat stroke with limb and tail rigidity, within the experimental time.

Response to halothane exposure in vivo:

Mice were weighed, their baseline temperature recorded, after which they were placed into an anaesthetic chamber, and induced with 2.0% halothane in oxygen using a precision vaporizer (Ohmeda Tec-4 halothane vaporizer system; GE Healthcare, Piscataway, NJ, USA) with flows at 1.5 L/min until there was no detectable response to toe pinches (30–60 s). Next, the mice were rapidly placed atop a bed warmed by recirculating warm water to achieve a bed surface temperature of 37.0℃. Anaesthesia was maintained with halothane, 1.5% in oxygen via a nose-cone attachment and a rectal thermistor sited. Animals were then continuously monitored for fluctuations in body temperature and signs of fulminant MH (limb and tail rigidity). Data were recorded every 2 min for the first 10 min and then every 5 min for up to 70 min or until the animal died of

an MH crisis. The onset of fulminant MH was determined by close surveillance for increased body temperature, hyperacute rigor in extremities (limb flex response) and the cessation of respiratory function. All MHN animals survived the halothane challenge and were then euthanized at this time under anaesthesia. All MHS animals regardless of zygosity expired as a result of an MH episode within the experimental time.

Ca^{2+} and Na^{+} selective microelectrodes

Double-barrelled Ca^{2+} and Na^{+} selective microelectrodes were prepared as described previously [18, 25]. Each ion-selective microelectrode was individually calibrated before and after the in vivo determinations of its selective ion concentration as described previously [25]. Only those Ca^{2+} selective microelectrodes with a linear relationship between pCa 3 and 7 (Nernstian response, 29.5 and 30.5 mV/pCa unit at 23℃ and 37℃, respectively) were used experimentally. The Na^{+} selective microelectrodes gave virtually Nernstian responses at free $[Na^{+}]_e$ between 100 and 10 mM. However, at concentrations between 10 and 1 mM $[Na^{+}]_e$, the electrodes had a sub-Nernstian response (40–45 mV), but their overall response was of a sufficient amplitude to be able to measure $[Na^{+}]_i$ in all genotypes. The sensitivity of the Ca^{2+} and Na^{+} selective microelectrodes was not affected by any of the reagents used in the present study.

In Vivo intracellular [Ca^{2+}] and [Na^{+}] determinations in muscle fibres

Microelectrode recordings were performed as described previously [25]. MHN and MHS mice were anaesthetized with ketamine and xylazine and both vastus lateralis muscles were exposed and trimmed of fascia and fat. The exposed myofibers on the right side were impaled with a double-barrelled Ca^{2+}-selective microelectrode and on the left side with a double-barrelled Na^{+}-selective microelectrode, and their potentials were

recorded via a high-impedance amplifier (WPI Duo 773 electrometer; WPI, Sarasota, FL, USA). The potentials from the 3 M KCl microelectrode (V_m) were subtracted electronically from either the potential of the ion selective electrode (V_{CaE} or V_{NaE}) to produce a differential Ca^{2+}- specific potential (V_{Ca}) or Na^+ -specific potential (V_{Na}) that represents the free $[Ca^{2+}]_i$ or $[Na^+]_i$, respectively. V_m, V_{Ca}, V_{Na} were filtered (30–50 kHz) to improve the signal-to-noise ratio and stored in a computer for further analysis.

In animals exposed to halothane and isoflurane, an initial set of measurements prior to exposure to the volatile anaesthetic was made to get the baseline and then the animal exposed to either 2% v/v halothane or 2% isoflurane delivered by facemask from a calibrated metered vaporizer. Measurements of $[Ca^{2+}]_i$ and $[Na^+]_i$ were made 2-4 min after commencing exposure to the inhalation agent.

Solutions

For in vitro studies stock concentrations of caffeine, KCl and halothane solutions were prepared in imaging buffer with the following composition: 133 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM MgSO4, 5.5 mM glucose, and 10 mM HEPES, pH 7.4. In KCl containing solutions, the concentration of NaCl was adjusted as necessary to maintain a total ionic strength (K^+ + Na^+) at 138 mM.

For in vivo studies the mammalian Ringer solution used to keep the exposed muscle hydrated had the following composition (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, pH 7.4.

Statistics

Values for myotube experiments are expressed as mean ± 95% confidence intervals (CI) with n for the number of myotubes for each condition. Values are expressed

as mean ± SD, with n_{mice} representing the number of mice and n_{cell} representing the number of myofibres used for the in vivo experiments. Statistical analysis was performed using 1-way ANOVA and Tukey's t test for multiple measurements to determine significance which was accepted at the $P<0.05$ level.

Results.-

Basic RyR1$_{G2435R}$ mouse phenotype and survival

MHS-Het mice and MHS-Hom mice had no apparent abnormality and appeared healthy at birth. Although initially Hom mice had no issues and could be maintained and the colony expanded as Hom x Hom mating trios, in the ~F6 generation it became evident that the MHS-Hom males did not make good breeding partners and were frequently found dead in their cage at ages <6 months. At the same time, it was noticed that MHS-Hom males housed with littermate MHS-Het or MHN males frequently died spontaneously at 3-5 months and if housed only with MHS-Hom littermates they survived to 7-8 months before spontaneous death, which was of no apparent outside cause. Only 4 MHS-Hom females died spontaneously with 18 months being the longest time for any animal to be kept prior to being used as an experimental animal. Although a definitive survival study has not yet been undertaken, a comparison of the death rates of the 3 genotypes is seen in supplemental figure 2.

In vitro myotube Ca^{2+} responses to caffeine, KCl and halothane

There were significant differences in both the threshold and the EC_{50} of the intracellular Ca^{2+} response to increasing concentrations of caffeine and KCl, and increased sensitivity to halothane among MHN, MHS-Het and MHS-Hom myotubes (see Fig 1 A-C). The normalized EC_{50}'s for caffeine were 1.44, 3.67 and 5.03 mM (fig 1A) and for KCl were 10.4, 15.7 and 20.6 mM (Fig 1B) respectively. Both Het<Hom had a significant response to 0.04 mM Halothane which was magnified at 0.1 mM, whereas MHN myotubes had no significant response to halothane at either of these concentrations (Fig 1C).

Response to exposure to 38^{o}C ambient temperature

Exposure to increased environmental temperature triggered fulminant heat stroke resulting in death in all MHS-Het and MHS-Hom animals. The mean time to death for MHS-Homs was 17.9±3.5 min and the max temperature reached was 39.8^{o}C whereas the mean time to death for MHS-Het animals was significantly longer (68.6 min) and they died with a significantly higher mean max temperature of 41.8^{o}C. All MH-N animals survived the 80 min challenge with a mean ending temperature of 37.4^{o}C. Both sets of MHS data were significantly different than the MH-N set $p<0.05$. (See Fig 2)

Response to exposure to halothane anaesthesia

The mean time to death for MHS-Homs was 18±2.6 min and their mean max temperature was 41 ^{o}C. The mean time to death for MHS-Het animals was significantly longer, 65.2 min, but as with exposure to high environmental temperature they died with a significantly higher max temperature of 42.5 ^{o}C. All MHN animals survived with a mean ending temperature at 70 min of 37 ^{o}C. Both sets of MHS data were significantly different than the MH-N set. (See Fig 3)

Resting $[Ca^{2+}]_i$ and $[Na^{+}]_i$

Malignant hyperthermia is characterized by intracellular Ca^{2+} and Na^{+} dyshomeostasis. Consequently, $[Ca^{2+}]_i$ or $[Na^{+}]_i$ was measured in-vivo in quiescent fibres of the vastus lateralis from MHN and MHS mice. In MHN $[Ca^{2+}]_i$ was 123±3 nM (n_{cel}=32, n_{mice}=8) compared to 156±16 nM (n_{cell}=30, n_{mice}=10) and 265±32 nM (n_{cell}=60, n_{mice}=20) in MHS-Het and MHS-Hom fibres respectively (Fig 4A). $[Na^{+}]_i$ was 8±0.1 mM (n_{cell} =16, n_{mice}=4) in MHN compared to 10±1 mM (n_{cell} =25, n_{mice}=5) and 14±0.7 mM (n_{cell}=25, n_{mice}=5) in MHS-Het and MHS-Hom fibres respectively (Fig 4B). Taken together, the

results demonstrate that there is an intracellular Ca^{2+} and Na^{+} overload which has a gene dose effect in MHS muscle fibres compared to MH-N.

$[Ca^{2+}]_i$ and $[Na^{+}]_i$ during an MH episode.

Malignant hyperthermia is triggered by halogenated volatile anaesthetics. Therefore, we studied the effects of halothane and isoflurane on $[Ca^{2+}]_i$ and $[Na^{+}]_i$ in vivo in MHN and MHS mice. $[Ca^{2+}]_i$ and $[Na^{+}]_i$ were measured simultaneously as described before. In short, $[Ca^{2+}]_i$ was measured in the right and $[Na^{+}]_i$ in the left vastus lateralis muscles in MHN, MHS-Het and MHS-Hom mice before and after exposure to 2% halothane or 2% isoflurane vapour in their inspired gas. Halothane exposure elicited an elevation of $[Ca^{2+}]_i$ by 1.8-fold in MHS-Het and by 3.6-fold in MHS-Hom compared to their values prior to exposure (Fig 5A). Also, there was an increase of $[Na^{+}]_i$ by 1.4-fold in MHS-Het and by 1.6-fold in MHS-Hom, compared to their values prior to exposure to halothane (Fig 5B). On the other hand, isoflurane inhalation produced an elevation of $[Ca^{2+}]_i$ by 2-fold in MHS-Het and by 4.6-fold in MHS-Hom compared to their values prior to exposure (Fig 5C). Isoflurane inhalation raised $[Na^{+}]_i$ by 1.7-fold and 1.9-fold in MHS-Het and MHS-Hom respectively compared to their values prior to exposure to isoflurane (Fig 5D). Although isoflurane appeared to be slightly more potent at raising $[Ca^{2+}]_i$ and $[Na^{+}]_i$ this difference was not statistically different. Inhalation of either volatile anaesthetic in MHN mice had no effect on muscle $[Ca^{2+}]_i$ and $[Na^{+}]_i$ (Fig's 5A,B,C and D).

Discussion.-

The major findings of the present study are as follows:

(1) Mice with the RyR1 p.G2435R MH mutation appear grossly normal and can be bred to homozygosity, allowing the study of gene dose effects on their physiology and pharmacology

(2) There were unexpected deaths from no apparent cause in MHS-Hom animals, with the frequency being much higher in males than in females, suggesting an association with a factor on the Y chromosome. This will be pursued in future studies.

(3) MHS-Het and MHS-Hom myotubes have a lower threshold and EC_{50} for caffeine- and KCl-evoked Ca^{2+} release and a lower threshold for halothane evoked Ca^{2+} release.

(4) All MHS-Het and MHS-Hom animals died from heat stroke when exposed to increased ambient temperature (38^{o}C) or in an MH crisis during halothane anaesthesia. MHS-Hets survived longer than MHS-Homs in both instances but the increased time to death allowed MHS-Hets to develop higher body temperatures before succumbing. There was no effect of either increased ambient temperature or halothane anaesthesia in MHN animals.

(5) MHS-Het and MHS-Hom adult muscle fibres in vivo have chronically elevated intracellular resting $[Ca^{2+}]_i$ and $[Na^{+}]_i$ (Hom>Het) compared to MHN muscle.

(6) MHS-Het and MHS-Hom adult muscle fibres in vivo increase their intracellular resting $[Ca^{2+}]_i$ and $[Na^{+}]_i$ (Hom>Het) after the animal is exposed to 2% halothane or isoflurane anaesthesia, and die in an MH crisis

MH is a life-threatening clinical syndrome of hypermetabolism involving the skeletal muscle [1, 9]. It is triggered in susceptible individuals primarily by the volatile inhalational anaesthetic agents and also possibly the muscle relaxant succinylcholine [27]. The clinical picture is characterized by intense tachycardia, overproduction of CO_2, variable degrees of muscular rigidity, increase in core temperature, respiratory and metabolic acidosis, hyperkalaemia and terminal haemodynamic collapse [28]. Molecular studies have established the type 1 ryanodine receptor (RYR1) gene encoding the skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+} release channel as the primary locus for MH susceptibility [3, 7, 29]. An additional locus implicated for susceptibility is CACNA1S, encoding the pore-forming subunit of the skeletal muscle L-type voltage-dependent Ca^{2+} channel ($Ca_{V1.1}$) [30, 31]. The molecular mechanisms by which both RyR1 and $Ca_{V1.1}$ mutations confer MH susceptibility remain unknown. To date we have shown a common characteristic of muscle expressing MH-RyR1 and MH-$Ca_{V1.1}$ mutations is an inherited membrane defect that disrupts both resting intracellular Ca^{2+} and Na^+ homeostasis in skeletal muscle [11, 14, 15, 18, 19].

Calcium has a significant role in the regulation of numerous muscle processes including contraction, transcription factor regulation, metabolism, muscle plasticity, and survival [32]. Muscle intracellular [Ca^{2+}] is maintained at a low level (100–120 nM) in resting cells against a large concentration gradient (~1 mM) in both the extracellular space and in the Ca^{2+} store in the SR. Muscle $[Ca^{2+}]_i$ is tightly regulated by complex regulatory mechanisms which balance Ca^{2+} influx and release from intracellular stores with intracellular sequestration and extracellular extrusion. Disturbance of the normal regulation of intracellular [Ca^{2+}] (increased plasma membrane influx and/or increased

release from the intracellular store, and reduced uptake into the SR) leads to a sustained rise in $[Ca^{2+}]_i$. The association between MH-RyR1 and MH-$Ca_{V1.1}$ mutations and intracellular Ca^{2+} dyshomeostasis has been validated in MHS patients [33], as well as in porcine and rodent experimental models [11, 14, 19, 34]. Furthermore, we have previously shown the role of sarcolemmal cation entry channels as a significant contributor to chronically elevated $[Ca^{2+}]_i$ in quiescent MHS muscle fibres and during a fulminant MH episode [25].

In humans, RYR1 p.G2434R is the most common globally reported variant associated with MH, although the data are biased because more than 50% of MH families that have been subject to detailed genetic studies are from the UK. In the UK MH cohort, p.G2434R is present in approximately 16% of families [23]. Of the probands subsequently found to harbour p.G2434R 64% were male and 36% female, a distribution which is representative of all MH probands[35]. There have been 9 deaths associated with p.G2434R, 5 in males and 4 in females: the incidence of death of 8.5% is lower than the overall death rate from MH in the UK of 16% (unpublished data). The p.G2434R variant is not associated with muscle weakness but some patients report episodes of muscle pain and recurrent rhabdomyolysis, although p.G2434R has not been associated with exertional heat illness to date.

Similar to what has been observed in the human IVCT data, the caffeine sensitivity of myotubes from p.G2434R MHS-Het mice is higher and nearer to that of MHN myotubes, than we have previously observed in studies of myotubes from animals harbouring other RyR1 MH mutations[16]. However, their responses to KCl and halothane showed an increased sensitivity and lower EC_{50} than the response in MHN myotubes.

$RYR1_{G2435R}$ MH muscle fibres in vivo have a chronically elevated resting $[Ca^{2+}]_i$ and $[Na^+]_i$ compared to MHN (see Figures 5A, B, C, and D). $[Ca^{2+}]_i$ was increased by 20% and by 55% in MHS-Het and MHS-Hom muscle fibres respectively in vivo compared to MHN muscle fibres. Similarly resting $[Na^+]_i$ was also increased 20% and by 41% in MHS-Het and MHS-Hom muscle fibres respectively compared to MHN muscle fibres. Although that these intracellular ions are elevated compared to MHN, the p.G2435R MHS-Het elevations are not as high as we have previously reported in both p.R163C and p.T4826I MHS-Het fibers and the p.G2435R MHS-Hom elevations are not as high as were seen in pT4826I MHS-Hom fibers. One explanation for the combined elevation in $[Ca^{2+}]_i$ and $[Na^+]_i$ is increased activity in sarcolemmal non-specific cation channels (e.g., TRPC1, 3, and 6) in p.G2435R animals as we have previously reported in p.R163C and p.T4826I MHS animals[11,22]. The exposure of MHS mice to halothane or isoflurane produced an increase of $[Ca^{2+}]_i$ and $[Na^+]_i$ in MH (Hom>Het), with no detectable ionic change in MHN muscle cells.

The present study demonstrated that $RYR1_{G2435R}$ mice exhibit impairments in intracellular Ca^{2+} handling consistent with a high resting $[Ca^{2+}]_i$ levels at physiological temperature (37℃) and an abnormal elevation of intracellular Ca^{2+} upon exposure to anaesthetics or increased environmental temperature. This study, also provides experimental evidence showing that a mutation in RyR1 in region 2 is closely associated with enhanced sarcolemmal Ca^{2+} and Na^+ influx.

In summary, these results demonstrate that knock-in mice expressing the isogenetic RYR1 mutation p.G2435R have an elevated resting Ca^{2+} and are capable of developing a MH crisis upon exposure to halothane or isoflurane. In addition, these

results support the working hypothesis that nonselective sarcolemmal cation permeability plays a critical role in producing the intracellular Ca^{2+} and Na^{+} overload both at rest and during the MH crisis.

References.-

1 Nelson TE. Malignant hyperthermia: a pharmacogenetic disease of Ca++ regulating proteins. Curr Mol Med 2002; **2**: 347-69

2 Riazi S, Kraeva N, Hopkins PM. Malignant Hyperthermia in the Post-Genomics Era: New Perspectives on an Old Concept. Anesthesiology 2018; **128**: 168-80

3 Jurkat-Rott K, McCarthy T, Lehmann-Horn F. Genetics and pathogenesis of malignant hyperthermia. Muscle Nerve 2000; **23**: 4-17

4 Groom L, Muldoon SM, Tang ZZ, et al. Identical de novo mutation in the type 1 ryanodine receptor gene associated with fatal, stress-induced malignant hyperthermia in two unrelated families. Anesthesiology 2011; **115**: 938-45

5 Jurkat-Rott K, Lerche H, Lehmann-Horn F. Skeletal muscle channelopathies. J Neurol 2002; **249**: 1493-502

6 Litman RS, Rosenberg H. Malignant hyperthermia: update on susceptibility testing. JAMA 2005; **293**: 2918-24

7 Robinson R, Carpenter D, Shaw MA, Halsall J, Hopkins P. Mutations in RYR1 in malignant hyperthermia and central core disease. Hum Mutat 2006; **27**: 977-89

8 Brady JE, Sun LS, Rosenberg H, Li G. Prevalence of malignant hyperthermia due to anesthesia in New York State, 2001-2005. Anesth Analg 2009; **109**: 1162-6

9 Denborough M. Malignant hyperthermia. Lancet 1998; **352**: 1131-6

10 Parness J, Bandschapp O, Girard T. The myotonias and susceptibility to malignant hyperthermia. Anesth Analg 2009; **109**: 1054-64

11 Yang T, Riehl J, Esteve E, et al. Pharmacologic and functional characterization of malignant hyperthermia in the R163C RyR1 knock-in mouse. Anesthesiology 2006; **105**: 1164-75

12 Chelu MG, Goonasekera SA, Durham WJ, et al. Heat- and anesthesia-induced malignant hyperthermia in an RyR1 knock-in mouse. FASEB J 2006; **20**: 329-30

13 Jiang D, Chen W, Xiao J, et al. Reduced threshold for luminal Ca2+ activation of RyR1 underlies a causal mechanism of porcine malignant hyperthermia. J Biol Chem 2008; **283**: 20813-20

14 Lopez JR, Alamo LA, Jones DE, et al. [Ca2+]i in muscles of malignant hyperthermia susceptible pigs determined in vivo with Ca2+ selective microelectrodes. Muscle Nerve 1986; **9**: 85-6

15 Lopez JR, Allen PD, Alamo L, Jones D, Sreter FA. Myoplasmic free [Ca2+] during a malignant hyperthermia episode in swine. Muscle Nerve 1988; **11**: 82-8

16 Yang T, Allen PD, Pessah IN, Lopez JR. Enhanced excitation-coupled calcium entry in myotubes is associated with expression of RyR1 malignant hyperthermia mutations. J Biol Chem 2007; **282**: 37471-8

17 Du GG, Sandhu B, Khanna VK, Guo XH, MacLennan DH. Topology of the Ca2+ release channel of skeletal muscle sarcoplasmic reticulum (RyR1). Proc Natl Acad Sci U S A 2002; **99**: 16725-30

18 Lopez JR, Alamo L, Caputo C, DiPolo R, Vergara S. Determination of ionic calcium in frog skeletal muscle fibers. Biophys J 1983; **43**: 1-4

19 Yang T, Esteve E, Pessah IN, Molinski TF, Allen PD, Lopez JR. Elevated resting [Ca(2+)](i) in myotubes expressing malignant hyperthermia RyR1 cDNAs is partially restored by modulation of passive calcium leak from the SR. Am J Physiol Cell Physiol 2007; **292**: C1591-8

20 Eltit JM, Bannister RA, Moua O, et al. Malignant hyperthermia susceptibility arising from altered resting coupling between the skeletal muscle L-type Ca2+ channel and the type 1 ryanodine receptor. Proc Natl Acad Sci U S A 2012; **109**: 7923-8

21 Feng W, Barrientos GC, Cherednichenko G, et al. Functional and biochemical properties of ryanodine receptor type 1 channels from heterozygous R163C malignant hyperthermia-susceptible mice. Mol Pharmacol 2011; **79**: 420-31

22 Yuen B, Boncompagni S, Feng W, et al. Mice expressing T4826I-RYR1 are viable but exhibit sex- and genotype-dependent susceptibility to malignant hyperthermia and muscle damage. FASEB J 2012; **26**: 1311-22

23 Robinson RL, Brooks C, Brown SL, et al. RYR1 mutations causing central core disease are associated with more severe malignant hyperthermia in vitro contracture test phenotypes. Hum Mutat 2002; **20**: 88-97

24 Altamirano F, Eltit JM, Robin G, et al. Ca2+ influx via the Na+/Ca2+ exchanger is enhanced in malignant hyperthermia skeletal muscle. J Biol Chem 2014; **289**: 19180-90

25 Eltit JM, Ding X, Pessah IN, Allen PD, Lopez JR. Nonspecific sarcolemmal cation channels are critical for the pathogenesis of malignant hyperthermia. FASEB J 2013; **27**: 991-1000

26 Stoelting RK. Halothane and methoxyflurane concentrations in end-tidal gas, arterial blood, and lumbar cerebrospinal fluid. Anesthesiology 1973; **38**: 384-8

27 Hopkins PM. Malignant hyperthermia: pharmacology of triggering. Br J Anaesth 2011; **107**: 48-56

28 Rosenberg H, Pollock N, Schiemann A, Bulger T, Stowell K. Malignant hyperthermia: a review. Orphanet J Rare Dis 2015; **10**: 93

29 MacLennan DH, Duff C, Zorzato F, et al. Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia. Nature 1990; **343**: 559-61

30 Weiss RG, O'Connell KM, Flucher BE, Allen PD, Grabner M, Dirksen RT. Functional analysis of the R1086H malignant hyperthermia mutation in the DHPR reveals an unexpected influence of the III-IV loop on skeletal muscle EC coupling. Am J Physiol Cell Physiol 2004; **287**: C1094-102

31 Carpenter D, Ringrose C, Leo V, et al. The role of CACNA1S in predisposition to malignant hyperthermia. BMC Med Genet 2009; **10**: 104

32 Tu MK, Levin JB, Hamilton AM, Borodinsky LN. Calcium signaling in skeletal muscle development, maintenance and regeneration. Cell Calcium 2016; **59**: 91-7

33 Lopez JR, Alamo L, Caputo C, Wikinski J, Ledezma D. Intracellular ionized calcium concentration in muscles from humans with malignant hyperthermia. Muscle Nerve 1985; **8**: 355-8

34 Perez CF, Eltit JM, Lopez JR, et al. Functional and Structural Characterization of a Novel Malignant Hyperthermia-susceptible variant of DHPR-beta1a subunit (CACNB1). Am J Physiol Cell Physiol 2017: ajpcell 00187 2017

35 Ellis FR, Halsall PJ, Harriman DG. The work of the Leeds Malignant Hyperpyrexia Unit, 1971-84. Anaesthesia 1986; **41**: 809-15

Figures:

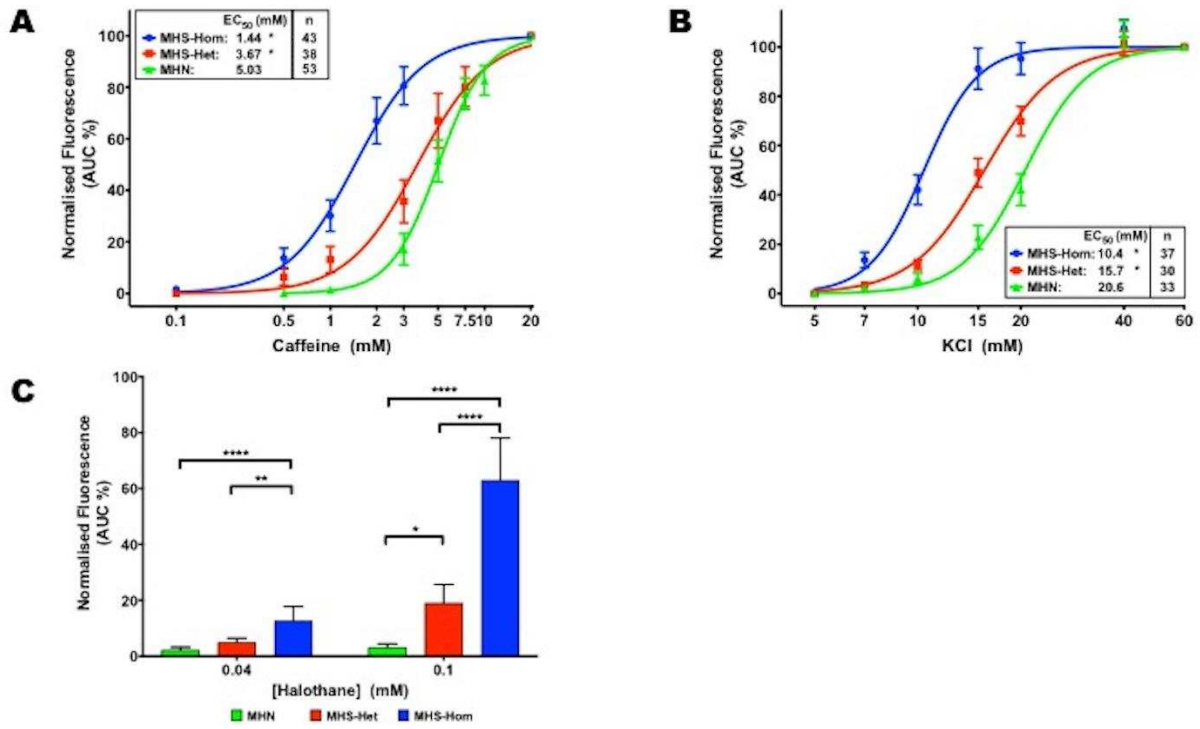


Figure 1: Sigmoidal dose-response analysis of Ca^{2+} imaging in myotubes generated from MHN, MHS-Het, and MHN-Hom p.G2435R mice in response to caffeine (**A**) and KCl (**B**). EC_{50}s are shown inset together with the number of myotubes. *EC_{50}s are significantly different between each genotype (P<0.0001, one-way ANOVA of log(EC_{50})s with Tukey's multiple comparison test). The threshold response defined as ³10% of the maximal response was observed at 3.0, 1.0 and 0.5 mM Caffeine, and 15.0, 10.0 and 7.0 mM KCl for MHN, MHS-Het and MHS-Hom myotubes respectively. (**C**) The effect of 0.04 mM and 0.1 mM halothane on myotubes from MHN, MHS-het and MHS-Hom p.G2435R mice. The halothane response has been normalised to the maximal response observed with 60 mM KCl (*P<0.05, ** P<0.005, *** P<0.0001, One-way ANOVA with Tukey's multiple comparisons test, n = 33-46 myotubes per genotype and response). All data presented as the mean±95% CI.

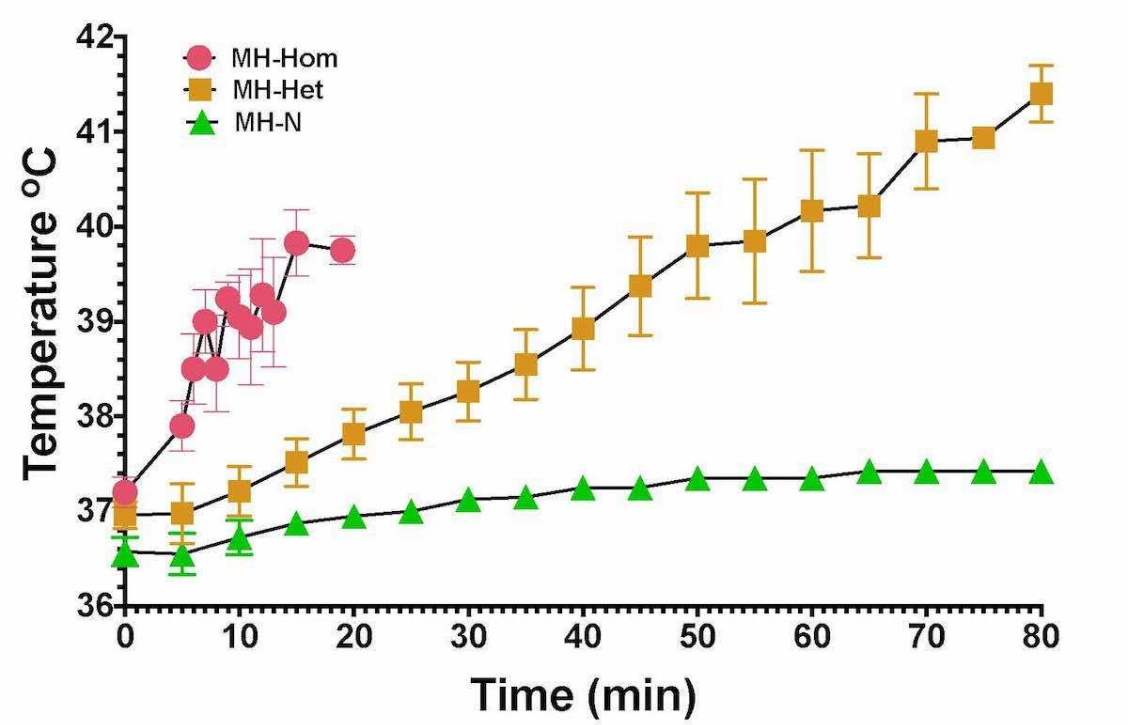


Figure 2: Graph of time vs rectal temperature of MHS-Hom, MHS-Het and MHN animals when exposed to an increased environmental temperature (38ºC). Both MHS-Hom and MHS-Het animals did not survive the exposure but there was a significant difference between the time of exposure and time to death between MHS-Hom and MHS-Hets, while none of the MHN animals showed a significant increase in body temperature during the 80 min exposure time and completed the experiment unharmed.

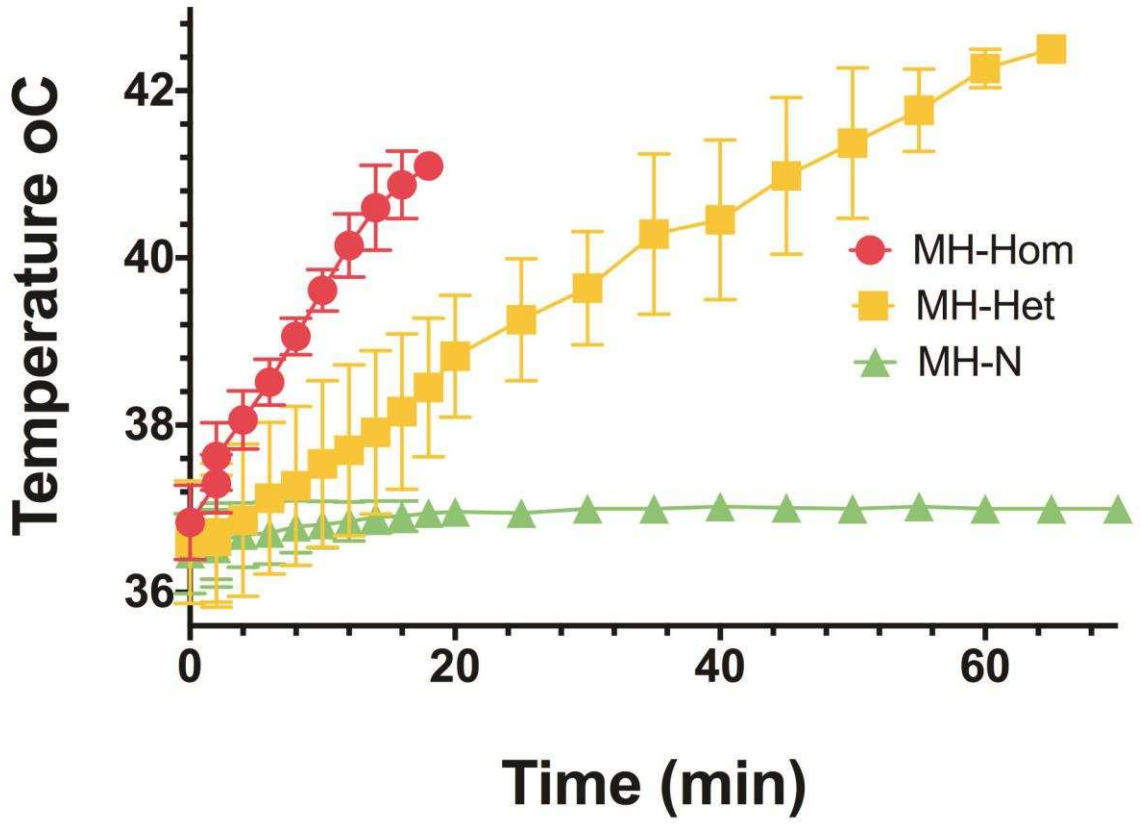


Figure:3 Graph of time vs rectal temperature of MHS-Hom, MHS-Het and MHN animals when exposed 2% halothane (vol/vol). Both MHS-Hom and MHS-Het animals did not survive the exposure but there was a significant difference between time to death and the time of exposure between MHS-Hom and MHS-Hets. None of the MHN animals showed a significant increase in body temperature during the 80 min exposure time and completed the experiment unharmed.

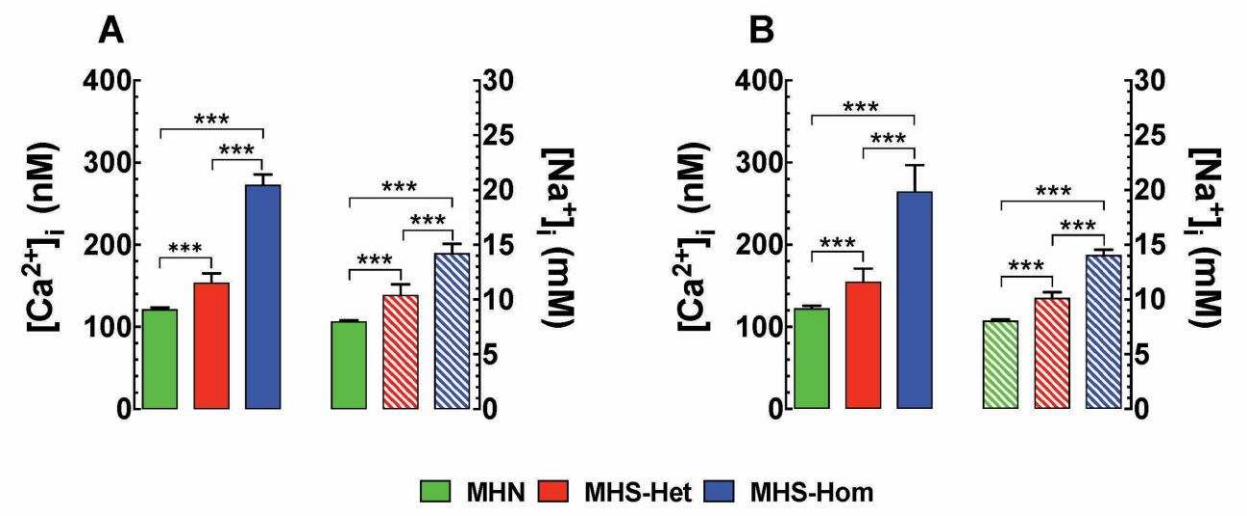


Figure 4: **A**. Measurements of in vivo resting $[Ca^{2+}]_i$ and $[Na^+]_i$ in skeletal muscle and **B**. similar measurement in vitro in isolated FDB muscle fibers. In both preparations both resting $[Ca^{2+}]_i$ and $[Na^+]_i$ were significantly different between each other and both were significantly different than control. $P<0.001$

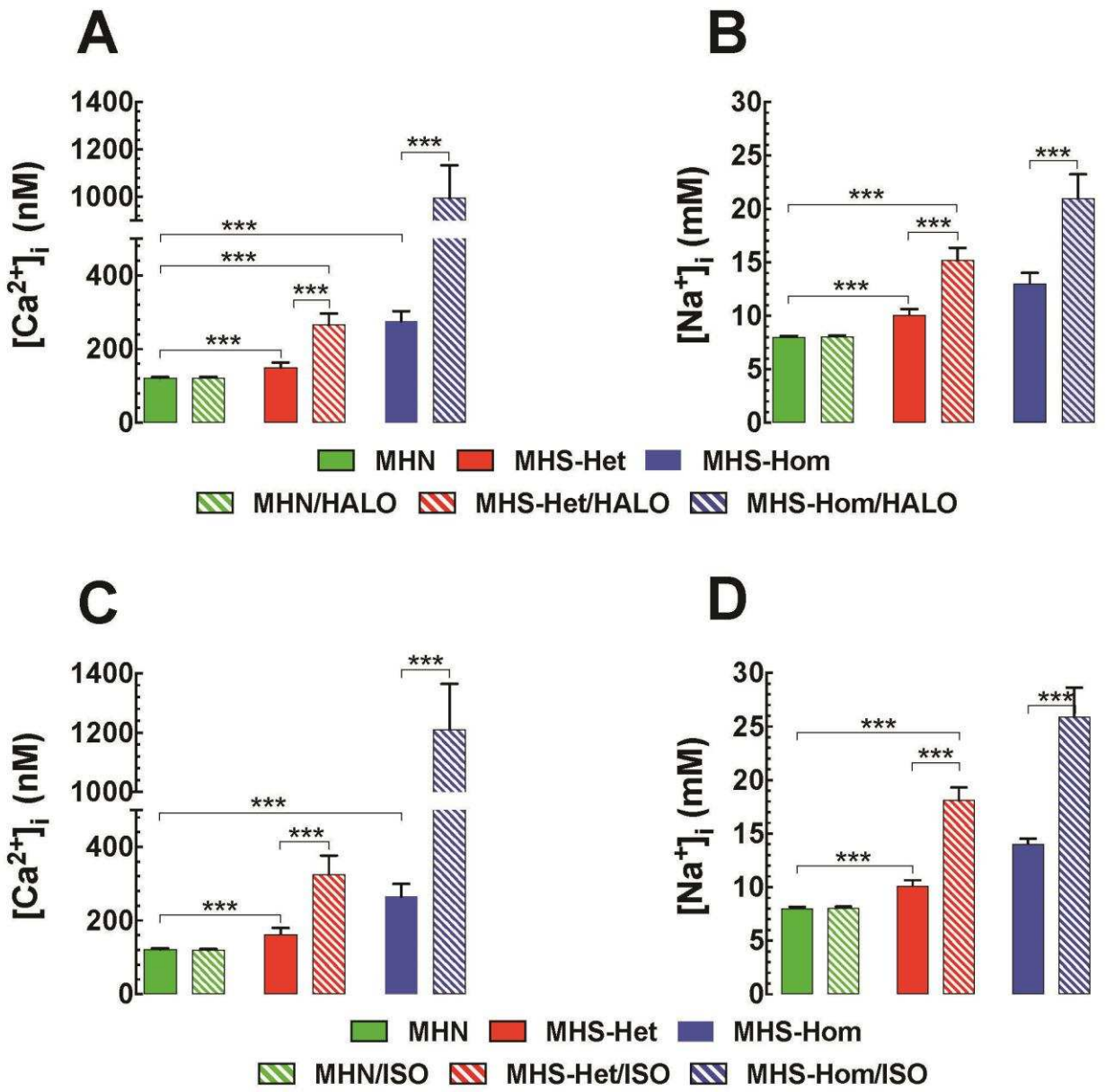
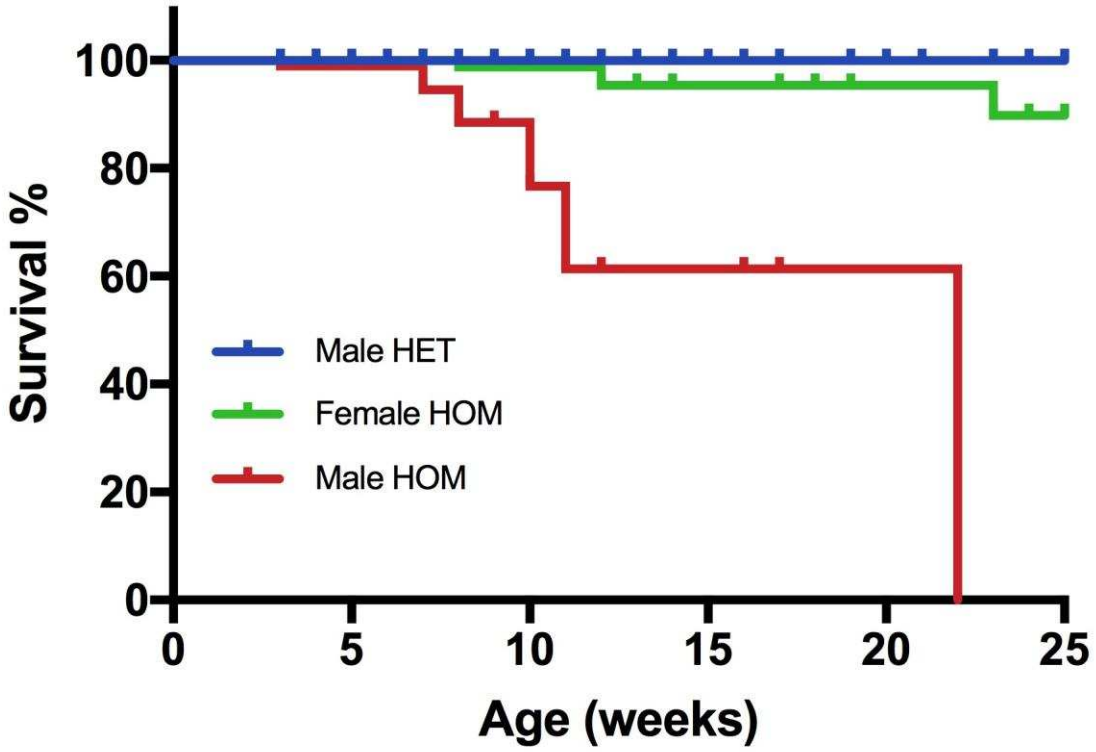


Figure 5: **A**. In vivo measurements of resting $[Ca^{2+}]_i$ and **B.** $_{resting}$ $[Na^+]_i$ before and after being exposed to 2% halothane. **C.** and **D.** In vivo measurements of resting $[Ca^{2+}]_i$ and $[Na^+]_i$ before and after being exposed to 1.5% isoflurane. In all cases as before resting values of $[Ca^{2+}]_i$ and $[Na^+]_i$ were significantly increased at rest ($p<0.001$) and both rose after the animals or fibers were expoed to halothane or isoflurane ($p<0.001$)

Supplemental Figure 1: A. line figure showing the ES cell targeting sequence with floxed G418 selection cassette. The mutation was in exon 45 in the left arm of the targeting cassette. B: Numeral identification of primers used to confirm targeting of the mutant cassette. C Primer pairs used to confirm homologous gene targeting by the targeting cassette. D: Primer pairs used for genotyping.



Supplemental Figure 2: Kaplan Meyer curve for G2434R MHS-Hom male and female, and MHS-Het mouse survival. It can be seen from the graph that there is a male vs female difference in the survival rates of MHS-Hom animals, but no difference from WT in MHS-Het animals of both sexes.