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Aging Effects of Caenorhabditis elegans Ryanodine Receptor Variants Corresponding to Human Myopathic Mutations

Katie Nicoll Baines*†‡, Célia Ferreira†, Philip M Hopkins*, Marie-Anne Shaw*‡ and Ian A Hope†‡

*Leeds Institute of Biomedical and Clinical Sciences, St James's University Hospital, Leeds, LS9 7TF, UK.

†School of Biology, Faculty of Biological Sciences, University of Leeds, LS2 9JT, UK.

‡Corresponding authors

Running title

C. elegans RyR Variants and Aging

Key words

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Corresponding author

Ian A. Hope, School of Biology, Faculty of Biological Sciences, University of Leeds, Woodhouse Lane, Leeds, LS2 9JT, UK.

Tel: (44) 113 343 2889

e-mail: i.a.hope@leeds.ac.uk

Abstract

Delaying the decline in skeletal muscle function will be critical to better maintenance of an active life-style in old age. The skeletal muscle ryanodine receptor, the major intracellular membrane channel through which calcium ions pass to elicit muscle contraction, is central to calcium ion balance, and is hypothesized to be a significant factor for age-related decline in muscle function. The nematode Caenorhabditis elegans is a key model system for the study of human aging and strains with modified C. elegans ryanodine receptors corresponding to human myopathic variants linked with malignant hyperthermia and related conditions were generated. The altered response of these strains to pharmacological agents reflected results of human diagnostic tests for individuals with these pathogenic variants. Involvement of nerve cells in the C. elegans responses may relate to rare medical symptoms concerning the central nervous system that have been associated with ryanodine receptor variants. These single amino acid modifications in C. elegans also conferred a reduction in lifespan and an accelerated decline in muscle integrity with age, supporting the significance of ryanodine receptor function for human aging.

Article Summary

Mutations in the human ryanodine receptor gene RYR1 lead to muscle disorders such as malignant hyperthermia. Equivalent changes in the corresponding Caenorhabditis elegans gene led to alterations in movement after challenge with the pharmacological agents caffeine and halothane, reflecting observations seen for humans. The single amino acid changes generated by

these mutations also led to reduced lifespan and accelerated muscle aging, supporting the hypothesis that failure to maintain calcium ion balance in muscle cells appropriately contributes to reduced mobility in human old age. The change in response to caffeine also depended upon a neural component.

Introduction

To improve the health of the world's aging population we need a better understanding of the aging processes, and age-related decline of skeletal muscle function is of key importance. Defective excitation-contraction coupling (Payne and Delbono 2004) and reduced capacity of Ca^{2+} homeostasis (Weisleder et al. 2006; Zhao et al. 2008) have been suggested to contribute to the human muscle contractile dysfunction that occurs with age. The ryanodine receptor isoform 1 (RyR1) is the channel through which Ca^{2+} is released from the skeletal muscle sarcoplasmic reticulum to elicit contraction. In the mouse there is an age-related increase in the ryanodine receptor 'leakiness' (Anderson et al. 2011) and age-related decrease in both the number of RyR1s and their degree of coupling to regulatory proteins (Ryan et al. 2000). Single-point variants in human RYR1 have been associated with the impairment of calcium handling in malignant hyperthermia (MH) (Robinson et al. 2006; Bouchama and Knochel 2002; McCarthy et al. 2000; Robinson et al. 2002; Tong et al. 1997; Jungbluth et al. 2009; Loseth et al. 2013; Nishio et al. 2009). The clinical incidence of MH is age-dependent and there is evidence of premature aging in MH mouse models (Boncompagni et al. 2009; Boncompagni et al. 2006). During an MH episode the sensitised RyR1 is activated by inhalational anesthetics and remains open

without neural stimulation resulting in sustained muscle contraction across the body (Larach et al. 1994), with death in the absence of a prompt and aggressive treatment regimen. The primary method of diagnosing susceptibility to this condition is through an in vitro contracture test (IVCT) (Ording et al. 1997) which measures the response of patients' muscle biopsies to the inhalation anesthetic halothane and to caffeine.

Mammalian RyR1 is a very large tetrameric membrane protein (>5000 amino acid residues per monomer) (Robinson et al. 2006) making it difficult to study. Similarly, the human RYR1 gene, with its many introns across such a large coding region, is awkward to manipulate. The nematode Caenorhabditis elegans, however, has a compact genome and the RYR1 orthologue, unc-68, is only 30 kb (WormBase). Nevertheless, UNC-68 has approximately 40% amino acid identity with the human RyR1, distributed along the entire length of the proteins, suggesting that the mammalian and nematode ryanodine receptor operate and are controlled in similar fashion (Sakube et al. 1997). The short life span and many other attributes of this species for experimental study make C. elegans the ideal subject for investigating the contribution of human RyR1 variants to aging.

Materials and Methods

Recombineering

Amino acid sequence alignment identified residues of RyR1 variants in human genetic conditions but conserved in C. elegans UNC-68 (Table 1). Modification of the target gene, unc-68, was achieved by a two-step counter-selection recombineering technique (Feng et al. 2012). A PCR amplified variant-specific counter selection cassette was inserted into the target fosmid (WRM069cA02) by bacterial transformation using positive selection for the cassette. The

cassette was then replaced, with incorporation of the desired point mutation in a second bacterial transformation with a second PCR product bearing the desired sequence alteration, using negative selection against the cassette. A dicistronic cassette was used containing the positive marker tetA(C) conferring tetracycline resistance (Tc^R), and the negative marker $rpsL^+$ conferring streptomycin sensitivity (Str^S) in the $rpsL^-$ (thus Str^R) EL350 host, a recombineering competent Escherichia coli strain (Feng et al. 2012). Confirmation of insertion and replacement of the cassette was carried out using colony PCR. The final recombineered fosmid for each variant was sequenced across the manipulated region and subjected to EcoRI restriction enzyme digestion to confirm that the correct variant had been introduced into the fosmid, with at least no substantial rearrangements

Strains

Manipulated and wild type unc-68 fosmids were introduced into unc-68(e540) worms by microinjection (Mello et al. 1991). Unc-68(e540) carries a point mutation towards the centre of the gene and behaves genetically as a null (Maryon 1996). Those worms bearing the fosmid in an extrachromosomal array encoding a functional unc-68 display a wild type phenotype of movement through which they could be selected and transgenic strains established. One fosmid was also co-injected in a mixture with pRF4, a plasmid bearing rol-6(su1006), which causes an obvious dominant roller phenotype (Mello et al. 1991).

GFP-myosin strains were developed by mating N2 males with unc-68(e540) hermaphrodites to generate male progeny heterozygous for unc-68. These males were mated with RW1596 (stEx30 (myo-3::gfp, rol-6(su1006))). The resulting hermaphrodite progeny were then allowed to self-fertilize to generate uncoordinated rollers, homozygous for unc-68(e540) and bearing the extrachromosomal array containing myo-3::gfp, rol-6(su1006). These worms were subjected to

UV mutagenesis and screened for uncoordinated rollers with progeny that are all rollers due to the extrachromosomal array having been stably integrated into a chromosome (Mariol et al. 2013). Hermaphrodites from this new strain were mated with males from each of the unc-68 fosmid transgenic strains screening for worms with well-coordinated roller movement due to the unc-68 bearing extrachromosomal array and the chromosomally integrated myo-3::gfp, rol-6(su1006) transgenes. Self-fertilization and selection for well-coordinated rollers yielded myo-3::gfp, rol-6(su1006) homozygous strains bearing the unc-68 transgenes.

Strain maintenance

All C. elegans strains were routinely maintained in culture at 20 °C on 50 mm plates of Nematode Growth Medium (NGM) (51 mM NaCl, 1.7 % agar, 0.25 % Bacto-peptone in 1 L H_2O, autoclaved, before addition of 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M KPO_4 pH 6.0, 1 ml cholesterol 5 mg/ml in ethanol) (Stiernagle 2006). NGM plates were seeded with E. coli strain OP50. Transgenic strains were maintained by serial transfer of transgenic worms selected for their altered phenotype.

Age Synchronising

Eggs were prepared by bleaching to synchronise worm populations for assay. Mixed stage population of nematodes were washed from the NGM plates in 500 µl M9 buffer (20 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$) (Stiernagle 2006). 150 µl of Sainsbury's thin bleach and 100 µl 4 M NaOH were added and the solution left at room temperature for 5 minutes. After microcentrifugation at 13500 rpm for 30 seconds the supernatant was removed and the pellet re-suspended in 1 ml fresh M9 buffer. Centrifugation was repeated and the pellet re-suspended in approximately 50 µl of residual supernatant for transfer to a freshly seeded NGM plate. This protocol kills all post-embryonic stages leaving the

eggs, which subsequently hatch across the 14 hours of embryogenesis, and then develop together into adults, effectively ensuring that the worms assayed will be of approximately the same age in days.

Phenotyping assays

The transgenic strains were assayed to determine their sensitivity to caffeine and halothane. Individual adult worms 4 days after synchronisation, were selected from NGM plates using a sterile worm pick and placed in 1 ml of 0, 1, 5, 10, 20, 40 or 80 mM caffeine dissolved in S-medium (1 litre S Basal (5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml in ethanol), H_2O to 1 L and autoclaved), 10 ml 1 M potassium citrate pH 6, 10 ml trace metals solution (1 L stock: 1.86 g Na_2 EDTA, 0.69 g $FeSO_4$ •7 H_2O, 0.2 g $MnCl_2$•4 H_2O, 0.29 g $ZnSO_4$ •7 H_2O, 0.025 g $CuSO_4$ •5 H_2O, H_2O to 1 litre, autoclaved and stored in the dark), 3 ml 1 M $CaCl_2$, 3 ml 1 M $MgSO_4$) (Stiernagle 2006). After 5 minutes the effect of the chemical was quantified by assessing the number of body bends in 30 seconds. Halothane assays were carried out in a similar manner but using 1 ml of 0, 0.5, 1, 1.5, 2 and 2.5 mM halothane solution (prepared from a 25 mM stock in DMSO and mixed into S-medium) and assaying after 1 minute exposure. Fifty worms were assayed for each strain at each concentration for each reagent.

RNAi assays

Synchronized L1s were transferred to new NGM plates, including 1 mM IPTG and 50 μg/ml Ampicillin, and seeded with E. coli (HT115) producing dsRNA for cbd-1, osm-3 or che-3. Cbd-1 RNAi was used in the longevity assays to exclude progeny from the assay (Johnston et al. 2010). Cbd-1 is only required for eggshell production and cbd-1 RNAi knockdown appears to have no effect on longevity of hatched animals. Lifespan assays were initiated at the young adult stage

and populations were scored every day. Animals that were lost from the plates or died from vulval extrusion were excluded from the analysis. Osm-3 and che-3 RNAi treated animals were used in caffeine assays as described above, with controls treated identically except using HT115 containing L4440 RNAi plasmid lacking an insert so that no dsRNA was present.

Muscle aging assays

The transgenic strains expressing the myo-3::gfp were assayed on days 0, 2, 4, 6, 8, 10, 12 and 14 of adulthood. Day 0 was considered to be 3 days post-hatching. Only live worms were selected for analysis. 20 worms were assayed for each strain at each time point. The extent of muscle aging was quantified by direct observation using an aging scale of 1-5 (Figure 4). A score of 1 indicates perfectly ordered myofilament structure, through to a score of 5 indicating total disorder, with half scores for worms that lay between the defining states. Visualisation and image capture was carried out using a Leica DMR fluorescence microscope and Improvision Openlab software. In preparation for microscopy animals were immobilised using 5 mM levamisole and placed in individual wells of an 8 well microscope slide. Each individual was scored for extent of muscle aging at the head, vulva and tail regions of the body at 20x magnification. These scores were combined to provide a whole-body score.

Statistical Analysis

Results of phenotyping assays were analysed to establish any potential differences in the rate of body bends when the worms are subjected to halothane and caffeine. Each strain containing an altered fosmid was compared to the strain containing the unaltered fosmid at each discrete concentration of the reagent in question. A linear model was established describing body bends being dependent upon presence of the variant and statistical significance was measured by carrying out one-way analysis of variance on the linear model. Body bends data on RNAi treated

worms were also compared by one-way analysis of variance at each discrete concentration to determine whether there was a significant difference in movement from a mock RNAi treatment with the L4440 empty plasmid RNAi control.

Categorical whole-body muscle score data were analysed using ordered logistic regression with p values calculated by comparing the T-statistic to the standard normal distribution. This enabled examination of any differences between the strains with modified unc-68 and the strains rescued with the wild-type unc-68, evaluation of the effect of increasing age of the worm in days, and interactions between these two variables. Differences between the scores for the regions of the worm down the anterior-posterior axis were similarly carried out. All statistical analyses were completed using RStudio version 3.0.2.

Comparison of life span data was subjected to survival analysis with curve comparison using the log-rank test to determine significant differences between variant strains and the UL4140 wild type control as well as testing for any difference between the UL4140 wild type control and N2.

Reagent and Data Availability

Strains and recombineered fosmids described are available upon request. File S1 contains the raw data for the experiments presented.

Results and Discussion

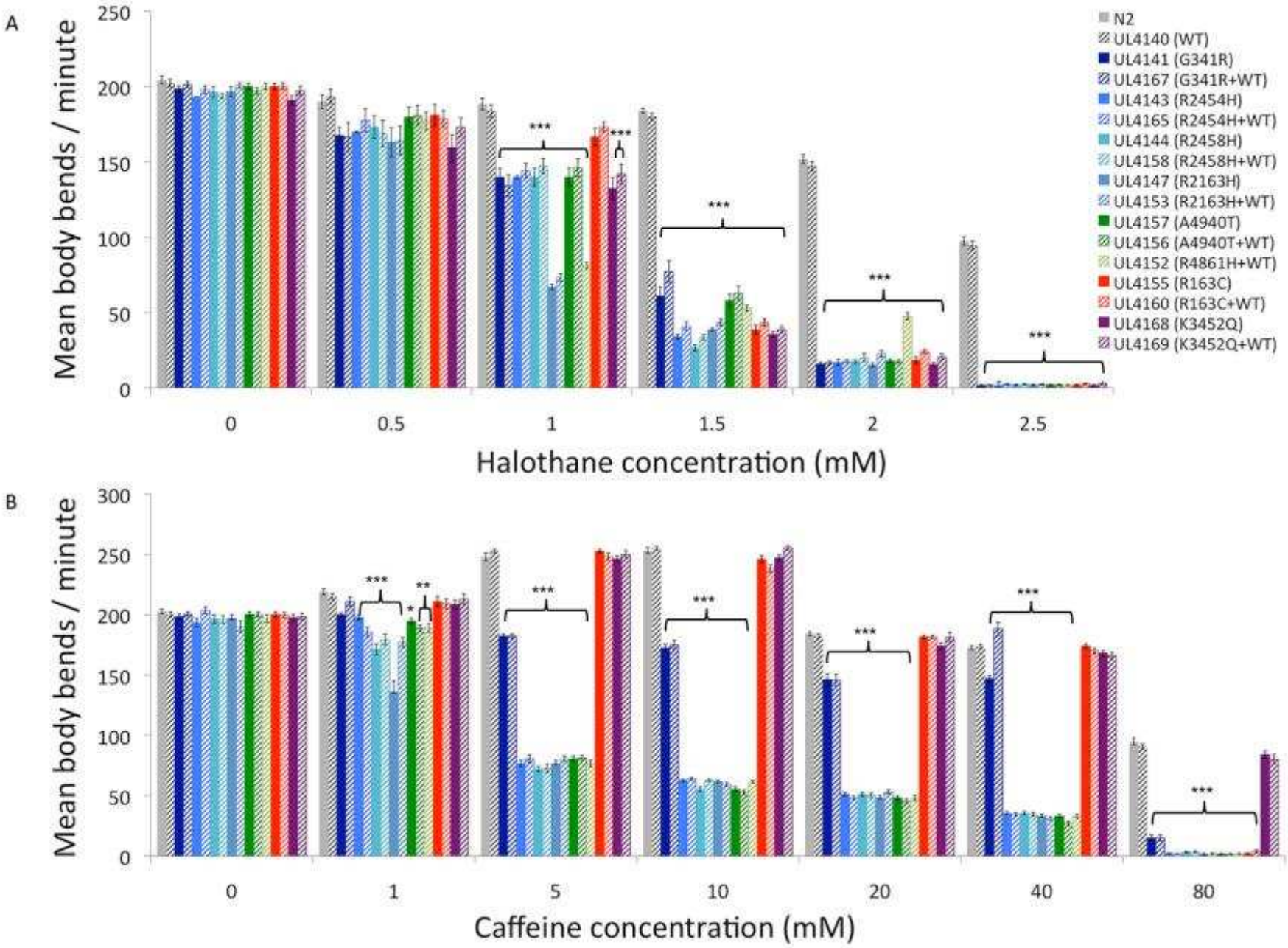
A single fosmid DNA clone contains the whole of unc-68

UNC-68, and release of Ca^{2+} from the sarcoplasmic reticulum, is needed for wild type locomotion. C. elegans locomotion is achieved through the coordinated contraction and relaxation of opposing dorsal and ventral muscle cells resulting in a sinusoidal wave passing

along the body propelling the worm forwards or backwards (Nicholas 1984). While mouse mutants that lack RyR1 are not viable, unc-68 null mutants survive (Sakube et al. 1997). The relatively small size of the nematode's muscle cells means influx of calcium through the cell membrane alone is sufficient for muscle contraction. However, muscle contractions of the unc-68 mutant are not as strong or rapid as in the wild type and consequently the overall appearance of locomotion is affected (Sakube et al. 1997). Microinjection transformation of unc-68 mutant hermaphrodites with genomic DNA fosmid clone WRM069cA02 (WormBase) yielded progeny with apparently wild type locomotion suggesting WRM069cA02 contains the entire unc-68 gene and all that is required for its expression. Each rescued line moved with around 200 body bends per minute in liquid medium, considerably faster than the 60 mean body bends per minute of the unc-68 null mutant. Transmission of this rescued phenotype to subsequent generations established that this fosmid, in an extrachromosomal transgenic array, provides appropriate levels of expression of the ryanodine receptor and yielded the reference strain, UL4140, used in subsequent comparisons, below, examining ryanodine receptor variants.

Not only did the wild type unc-68 transgene in UL4140 fully rescue for locomotion but the strain responded to increasing concentrations of halothane and caffeine, the drugs used in the MH IVCT, in the same manner as the standard wild type strain, N2 (Figure 1). C. elegans responds to inhalation anesthetics such as halothane in a similar manner to humans; initial excitation leads on to lack of co-ordination, before complete cessation of movement, first with and then without response to mechanical stimulation, with longer term exposure being fatal (Morgan and Carscobi 1985). Halothane inhibited UL4140 and N2 locomotion progressively and to the same degree with increasing concentrations of the anesthetic, across concentrations tested (0.5 to 2.5 mM)

(Figure 1A). Previous work showed unc-68 mutants to have altered responses to caffeine (Adachi and Kagawa 2003), yet locomotion of both UL4140 and N2 individuals was stimulated and then inhibited to the same degree by increasing concentrations of caffeine (from 1 to 80 mM) (Figure 1B).


A
Mean body bends / minute
250
200
150
100
50
0
0
0.5
1
1.5
2
2.5
Halothane concentration (mM)
N2
UL4140 (WT)
UL4141 (G341R)
UL4167 (G341R+WT)
UL4143 (R2454H)
UL4165 (R2454H+WT)
UL4144 (R2458H)
UL4158 (R2458H+WT)
UL4147 (R2163H)
UL4153 (R2163H+WT)
UL4157 (A4940T)
UL4156 (A4940T+WT)
UL4152 (R4861H+WT)
UL4155 (R163C)
UL4160 (R163C+WT)
UL4168 (K3452Q)
UL4169 (K3452Q+WT)
***
B
Mean body bends / minute
300
250
200
150
100
50
0
0
1
5
10
20
40
80
Caffeine concentration (mM)
***
**
*

234

Figure 1. Comparison of the rate of locomotion of unc-68 variant strains and wild type C. elegans in increasing concentrations of halothane or caffeine. Mean body bends per minute for 50 individuals in the presence of various concentrations of halothane (A) or caffeine (B) are presented for each strain. C. elegans strains corresponding to Malignant Hyperthermia (MH) associated variants are in shades of blue, to Central Core Disease (CCD) associated variants are in shades of green, to the Exertional Heat Illness (EHI) associated variant is in red and to the Late Onset Axial Myopathy (LOAM) associated variant is in purple. In the key, strain names are provided, with the nature of the RyR1 variant and whether the wild type fosmid was also present (WT) indicated in brackets. Transgenic strains generated using only a variant fosmid are represented by solid bars, while strains generated using a variant fosmid and the wild type fosmid are represented by striped bars, in adjacent corresponding pairs. The solid grey bars are for the wild type N2 while the dashed grey bars are for the control transgenic strain, UL4140, generated with just the wild type unc-68. Error bars are standard error of the mean. Significant differences between variant strains and UL4140 are indicated: *$p<0.05$, **$p<0.01$, *** $p<0.001$ (ANOVA).

UNC-68 variants equivalent to RyR1 myopathic variants retained ryanodine receptor function

Eight human RYR1 variants were selected for study in C. elegans (Table 1). Selection of variants took into account that some RYR1 variants are implicated in other myopathic conditions beyond MH; central core disease (CCD) (Robinson et al. 2002), exertional heat illness (EHI) (Davis et

al. 2002; Nishio et al. 2009) and late-onset axial myopathy (LOAM) (Jungbluth et al. 2009; Loseth et al. 2013). CCD is a congenital myopathy that presents with progressive proximal muscle weakness; type 1 skeletal muscle fibers exhibit cores with unstructured myofibrils lacking mitochondria (Zhou et al. 2007). In EHI, individuals suffer potentially lethal hyperthermic responses to exercise. Patients with LOAM exhibit lumbar hyperlordosis, scapular winging and camptocornia, due to skeletal muscle defects, with onset between the ages of 30 and 70 years (Jungbluth et al. 2009; Loseth et al. 2013). Strikingly, the amino acid residues variant in the different conditions are not segregated to distinct domains of the ryanodine receptor and are distributed throughout the protein (Robinson et al. 2006).

The RyR1 variants selected for study were: G341R, R2163H R2454H and R2458H for MH (Robinson et al. 2006); R4861H implicated in CCD only (Monnier et al. 2001); A4940T implicated in CCD and MH (Kraeva et al. 2013); R163C implicated in EHI, CCD and MH (Estève et al. 2010); and K3452Q implicated in LOAM (Jungbluth et al. 2009; Loseth et al. 2013). Single base pair changes were generated by recombineering of the unc-68 fosmid WRM069cA02 such that the change in the encoded UNC-68 would be precisely equivalent to the single amino acid changes in these RyR1 variants.

Table 1. Amino acid alignment in region of all eight variants studied and the corresponding transgenic C. elegans strains generated.

HUMAN CONDITION	*RYR1* VARIANT	PROTEIN	ALIGNMENT	TRANSGENIC STRAIN WITH VARIANT FOSMID ONLY	TRANSGENIC STRAIN ALSO INCLUDING WILD TYPE FOSMID
Malignant hyperthermia	p.G341R c.1021G>A	RyR1	KRDVEGMGPPEIKYGESLCFVQHVASGLW	UL4141 (UL4193)	UL4167 (UL4200)
		UNC-68	EKEEEGMGNATIRYGETNAFIQHVKTQLW		
	p.R2163H c.6488G>A	RyR1	DTMSLLECLGQIRSLLIVQMGPQEENLMI	UL4147 (UL4194)	UL4153 (UL4198)
		UNC-68	DVTDFLVYLIQIRELLTVQFEHTEEAILK		
	p.R2454H c.7361G>A	RyR1	CAPEMHLIQAGKGEALRIRAILRSLVPLE	UL4143 (UL4195)	UL4165 (UL4206)
		UNC-68	CAPDPMAIQAGKGDSLRARAILRSLISLD		
	p.R2458H c.7373G>A	RyR1	IQAGKGEALRIRAILRSLVPLEDLVGIIS	UL4144 (UL4201)	UL4158 (UL4197)
		UNC-68	IQAGKGDSLRARAILRSLISLDDLGQILA		
Central Core Disease	p.R4861H 14582G>A	RyR1	VVVYLYTVVAFNFFRKFY-NKSEDEDEPD	–	UL4152 (UL4205)
		UNC-68	VVVYLYTVIAFNFFRKFYVQEGEEGEEPD		
	p.A4940T c.14820G>A	RyR1	FFFFVIVILLAIIQGLIIDAFGELRDQQE	UL4157 (UL4203)	UL4156 (UL4202)
		UNC-68	FFFFVIIILLAIMQGLIIDAFGELRDQQE		
Exertional Heat Illness	p.R163C c.487C>T	RyR1	CWWTMHPASKQRSEGEKVRVGDDIILVSV	UL4155 (UL4191)	UL4160 (UL4192)
		UNC-68	CWWTIHPASKQRSEGEKVRVGDDVILVSV		
Late-onset axial myopathy	p.K3452Q c.10354A>C	RyR1	IFIYWSKSHNFKREEQNFVVQNEINNMSF	UL4168 (UL4196)	UL4169 (UL4199)
		UNC-68	IFRIWSQSQHFKREELNYVAQFEEDAAAT		

Human variant residues and corresponding C. elegans residues are in red. Other amino acids identical in RyR1 and UNC-68 are in blue. Strains in brackets also have the chromosomally-integrated myo-3::gfp, rol-6(su1006) transgenes. UL4140 is transgenic for the wild type unc-68 fosmid and UL4190 is the corresponding strain with the chromosomally-integrated myo-3::gfp, rol-6(su1006) transgenes.

Multiple strains were generated by microinjection transformation of the unc-68 mutant with each modified fosmid. All the variants constructed, apart from that for R4861H, rescued the mutant phenotype. Therefore, the identity of these particular amino acid residues, with the one exception, is not critical for basic ryanodine receptor function, under normal conditions, despite their evolutionary conservation from humans to nematodes. The progeny of unc-68 null mutants microinjected with the CCD associated R4861H variant all appeared to retain the unc-68 phenotype and transgenic progeny could not be distinguished from their non-transgenic siblings. Co-injection of the fosmid for the R4861H variant, along with a distinct transformation marker, yielded transgenic worms, recognizable from the roller phenotype, but still with a slow moving unc-68 mutant phenotype, suggesting that the modification equivalent to R4861H had indeed inactivated, or at least severely compromised, UNC-68 function. The rolling phenotype means a numeric comparison of body bends would not be meaningful. No human individuals homozygous for R4861H are known and this amino acid substitution may inactivate the human ryanodine receptor too.

Increased halothane sensitivity was conferred by the UNC-68 variants

All the rescued strains established with the modified unc-68 fosmids exhibited an increased sensitivity to halothane (Figure 1A, solid bars) revealing that these single amino acid changes did modify the function of the ryanodine receptor and conferred an altered phenotype. An approximately 10% decrease in the rate of body bends in liquid, in comparison to the control strains with wild type unc-68, was apparent even at 0.5 mM halothane. At 1.5 mM halothane, a

concentration that has little effect on the wild type, the rate of movement in the variant strains was reduced to 15% of that in the absence of halothane. The variant strains were completely paralyzed by 2.5 mM halothane, while the wild type strains retained a reduced but still substantial mobility. Previous extensive investigations identified genes such as unc-79, unc-80 and gas-1 from mutations conferring halothane hypersensitivity in C. elegans (Kayser et al. 1999; Sedensky and Meneely 1987). Mutations in unc-68 may not have been isolated in these studies due to the need for specific point mutations that sensitize but do not eliminate ryanodine receptor function.

Increased caffeine sensitivity was conferred by some of the UNC-68 variants

Young adults of the variant strains generated specific to MH or CCD also demonstrated a modified response to caffeine across the range of concentrations assayed, while those for EHI and LOAM did not (Figure 1B). The strains generated for the human RYR1 variants G341R, R2454H, R2458H, R2163H, A4940T and R4861H all failed to show the stimulation of locomotion of the wild type as caffeine was increased from 1 to 10 mM. These same strains, with the exception of that for variant G341R, actually displayed a substantial inhibition of locomotion from 1 mM to 5 mM caffeine with a small progressive further inhibition from 10 to 40 mM, and almost complete paralysis at 80 mM, a concentration at which the wild type still remains motile. The G341R variant strain only shows a substantial inhibition of locomotion upon raising the caffeine concentration to 80 mM, but this is still not quite to the same degree as the other variant strains. The LOAM associated K3452Q variant strains showed a wild type response to all concentrations of caffeine assayed, as did the EHI associated R163C variant strain with the

exception of almost total inhibition of locomotion specifically at the highest concentration of 80 mM.

The modified unc-68s showed genetic dominance

Genetic dominance, a striking property of many pathogenic human RYR1 variants and all those selected for study, was also demonstrated by the equivalent versions of unc-68. This was revealed when transgenic strains of C. elegans were generated by microinjection transformation using the modified unc-68 fosmids but mixed with the unmodified fosmid, containing wild type unc-68 (Figure 1, striped bars). These strains all behaved in essentially the same manner as the strains transformed with only the corresponding modified unc-68 across the range of caffeine and halothane concentrations assayed: i.e. variant UNC-68s that conferred a modified sensitivity to caffeine or halothane did so even when wild type UNC-68 was also present. Remarkably, when a wild type UNC-68 was also present, the unc-68 variant for R4861H, the one variant unable to rescue the unc-68 mutant and apparently non-functional, nevertheless induced the same modified response to caffeine and halothane (and the same aging effects – see below) as other MH and CCD associated versions of UNC-68, like the corresponding human condition. This could result from the non-functional variant being stably expressed and, in combination with the wild type protein, generating a malfunctioning heteromeric ryanodine receptor.

The modified unc-68s conferred age-related phenotypes

Given the potential links between RyR1 variants and age related phenotypes in mammals, the unc-68 variant strains were examined for age-related phenotypes in C. elegans. First, a dramatic

age-related change in response to caffeine was found, for the LOAM (K3452Q) variant strain (Figure 2). Young adults of the LOAM variant strain showed the same response to caffeine as the wild type, with stimulation of locomotion at low concentrations and inhibition at higher concentrations. All the other strains, i.e. those with other unc-68 variants, showed simply a progressive dampening of the rate of locomotion with age but the same general response profile, with increasing concentrations of caffeine, characteristic of each strain (Figure 2). In contrast, rather than an inhibition of locomotion, older LOAM variant strain adults showed a considerable stimulation of locomotion, increasing with increasing caffeine concentrations, a response attained progressively as the animals aged. It is tempting to link this striking effect in C. elegans directly to the specific age-related symptoms characteristic of LOAM.

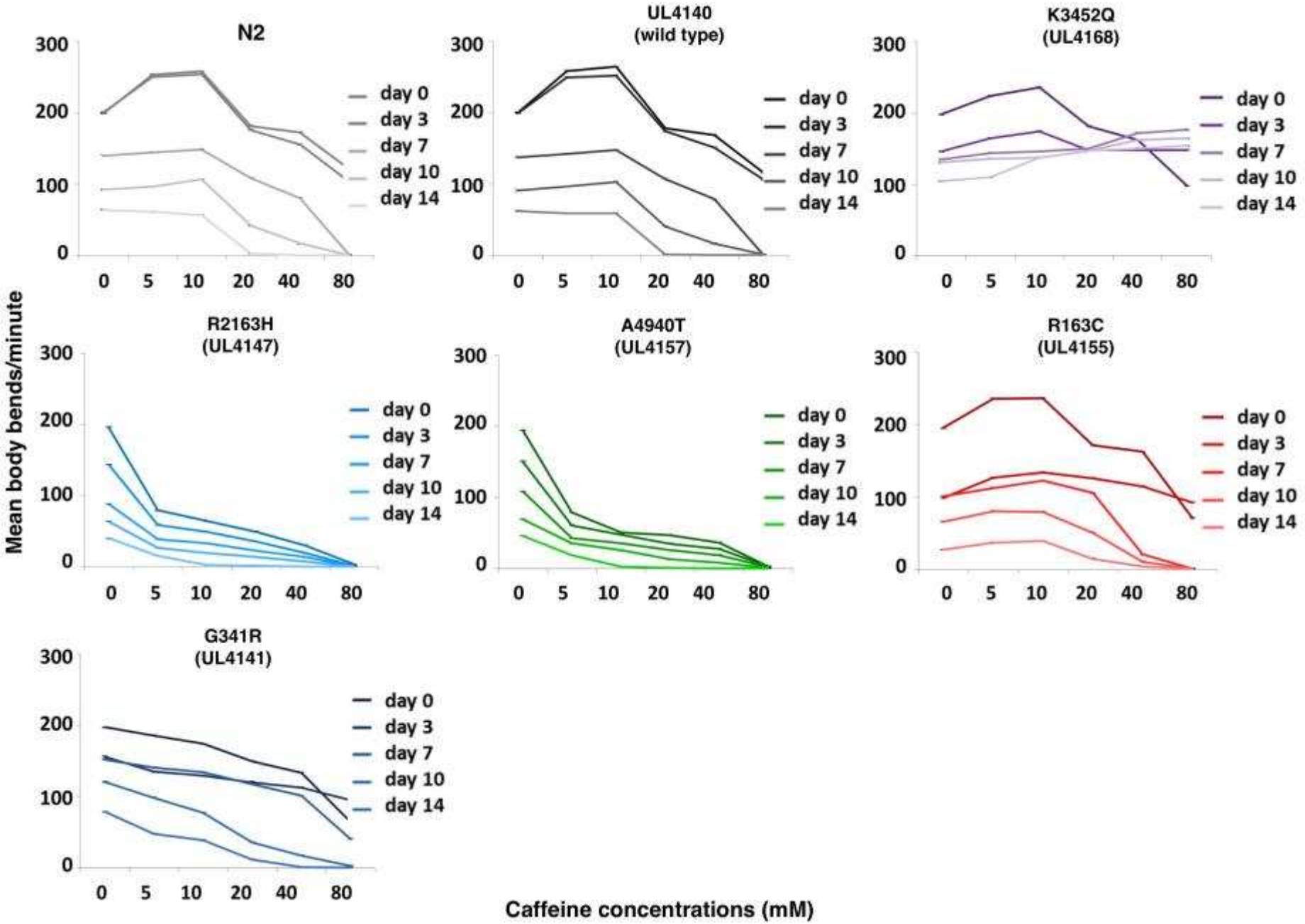

N2
UL4140
(wild type)
K3452Q
(UL4168)
R2163H
(UL4147)
A4940T
(UL4157)
R163C
(UL4155)
G341R
(UL4141)
day 0
day 3
day 7
day 10
day 14
300
200
100
0
0 5 10 20 40 80
Mean body bends/minute
Caffeine concentrations (mM)

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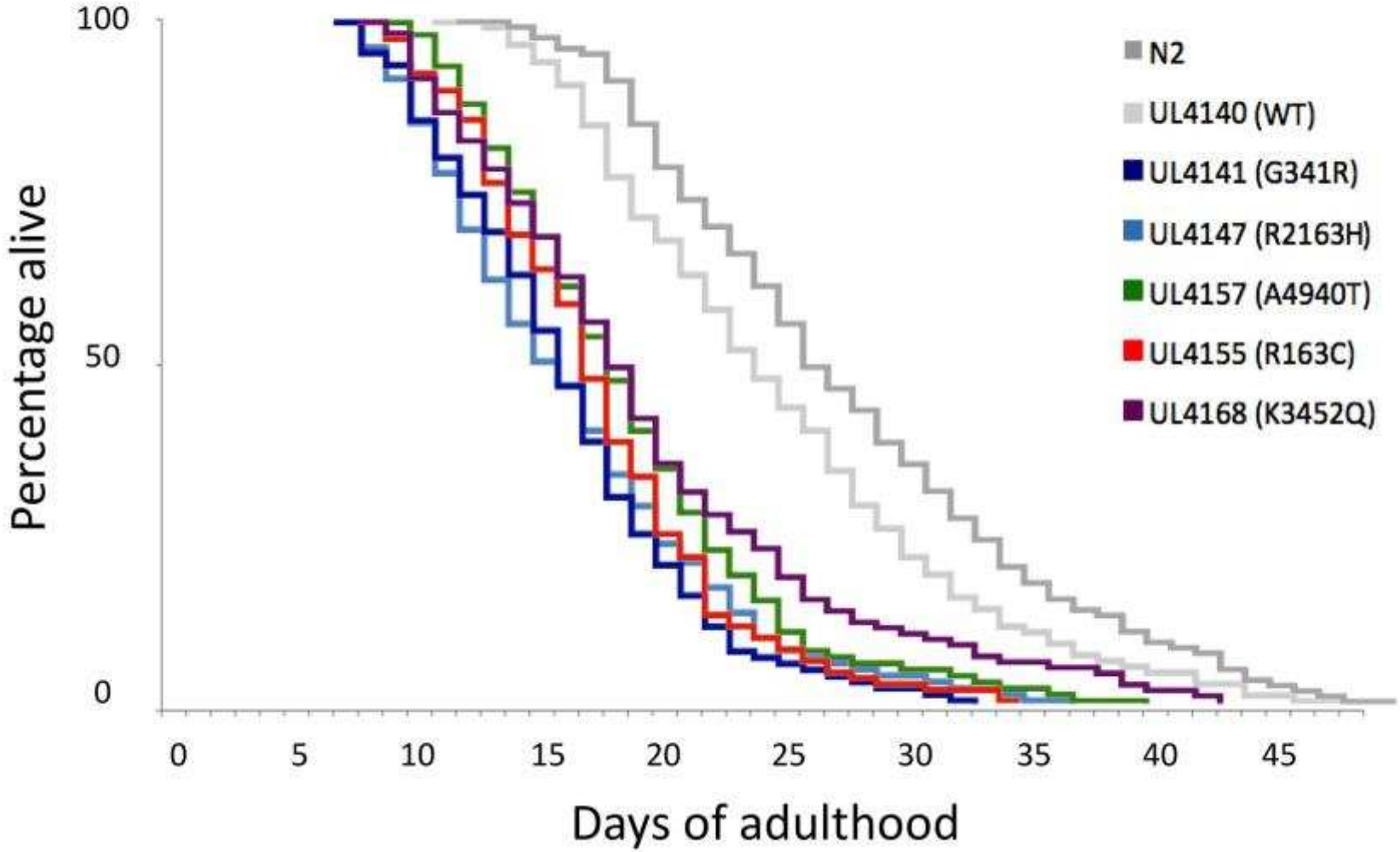
Figure 2. Locomotion of C. elegans expressing the LOAM associated variant of unc-68 is specifically increasingly-stimulated by caffeine with age. The locomotion of the strain for the RyR1 variants G341R (UL4141), R2163H (UL4147), A4940T ((UL4157) R163C (UL4155) and K3452Q (UL4168) are compared with the strain transgenic for only the wild type unc-68 (UL4140) and the standard wild type strain (N2). Mean body bends per minute in the presence of increasing concentrations of caffeine are presented for 50 individuals at 0, 3, 7, 10, and 14 days of adulthood. The colour coding from Figure 1 is retained with, broadly, blue for MH, green for CCD, red for EHI and purple for LOAM. Error bars are standard error of the mean.

Second, the unc-68 variants caused a decrease of median lifespan (Figure 3). All the unc-68 variant transformed strains which were assayed had median life-spans of 14 to 17 days into adulthood, compared to the 22 or 24 days for the wild type control strains. A log-rank test on the survival curves revealed that lifespans of strains transgenic for variant UNC-68s were statistically shorter than the lifespan of UL4140, the control strain transgenic for the wild type UNC-68 ($p<0.0001$ in each case, File S1). Hence although the ryanodine receptor appeared to remain functional, these single amino acid changes did shorten C. elegans lifespan.

It is also noted that the lifespan of UL4140 is significantly shorter than the lifespan of the non-transgenic wild type control, N2, although the difference is not as marked ($p<0.05$, File S1). Presumably this small reduction in life span results from the difference in expression of unc-68 when present in multiple copies, as an extrachromosomal transgene, rather than in the endogenous location, in single copy, within a chromosome.


100
50
0
Percentage alive
0
5
10
15
20
25
30
35
40
45
Days of adulthood
N2
UL4140 (WT)
UL4141 (G341R)
UL4147 (R2163H)
UL4157 (A4940T)
UL4155 (R163C)
UL4168 (K3452Q)

394

Figure 3. **Single amino acid changes in UNC-68 shorten C. elegans lifespan.** The percentage of animals surviving on successive days of adulthood is presented for each strain: strains transgenic for different unc-68 variants; UL4140, transgenic for just the wild type unc-68 (light grey); and the non-transgenic wild type N2 (dark grey). In the key, strain names are provided, with the nature of the RyR1 variant indicated in brackets. The colour coding from Figure 1 is retained with, broadly, blue for MH, green for CCD, red for EHI and purple for LOAM.

Third, we found a specific aging effect of unc-68 variants on C. elegans body-wall muscle. The progressive disorganization of the sarcomeric structure with age can be followed in live C. elegans by fluorescence microscopy using GFP tagged myosin (Herndon et al. 2002). An extra-chromosomal myo-3::gfp transgene was chromosomally integrated, before introduction into the strains bearing the various extra-chromosomal unc-68 transgenes through mating. The extent of muscle aging was quantified by comparison to a set of standard states on a scale of 1-5 (Figure 4), from 1 indicating fully-organized thick filament alignments through to 5 being totally disorganized. As only live worms were scored and worms that die early are more likely to have more disordered myofilaments, the degree of age-related structural decline will be underreported. A whole-body score was derived from assessment of three regions along the anterior-posterior axis in each individual. Differences in muscle aging rates between the strains with different unc-68 variants were small, but apparent upon statistical analysis by ordered logistic regression. No significant difference in muscle aging was found between strain pairs, i.e. with versus without wild type unc-68 present, for specific unc-68 variants. Therefore, these data were combined when testing for differences in muscle aging that were specifically due to the change in UNC-68 sequence (Figure 5 and Table 2). Overall, the presence of an UNC-68 variant was found to

significantly affect the whole-body score ($p<0.001$). No significant difference in whole-body score on day 0 or day 2 of adulthood was found for any of the variant strains when compared to the UL4140 based control, transformed with just wild-type unc-68. The whole-body score was significantly increased compared to the control by day 4 for the R163C, A4940T and K3452Q variant strains, by day 6 for R2454H and R2458H, by day 8 for G341R and by day 10 for R2163H and R4861H, indicating that the single amino acid change in all unc-68 variants examined did induce faster muscle aging, although the age when this became statistically significant varied.

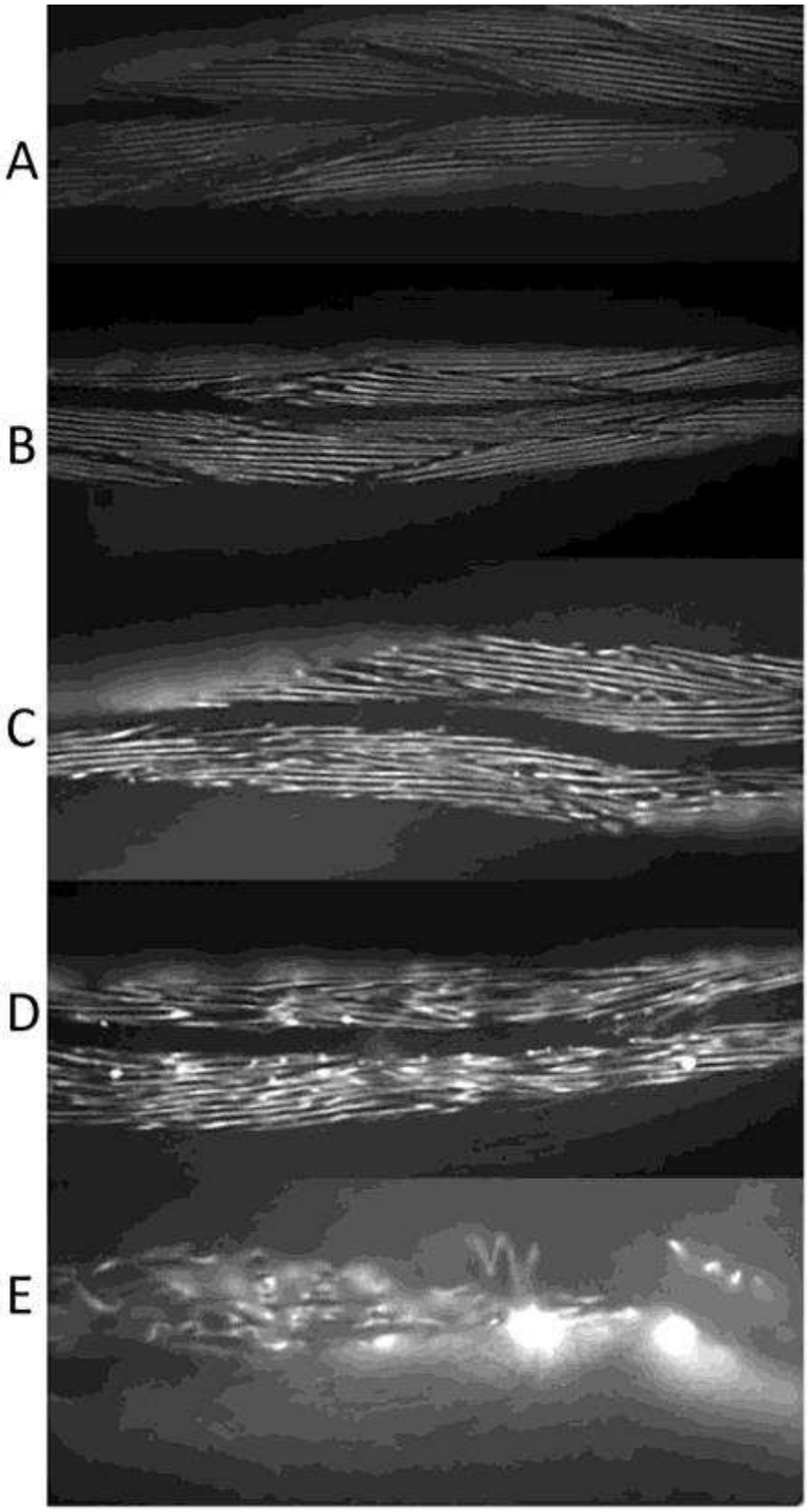


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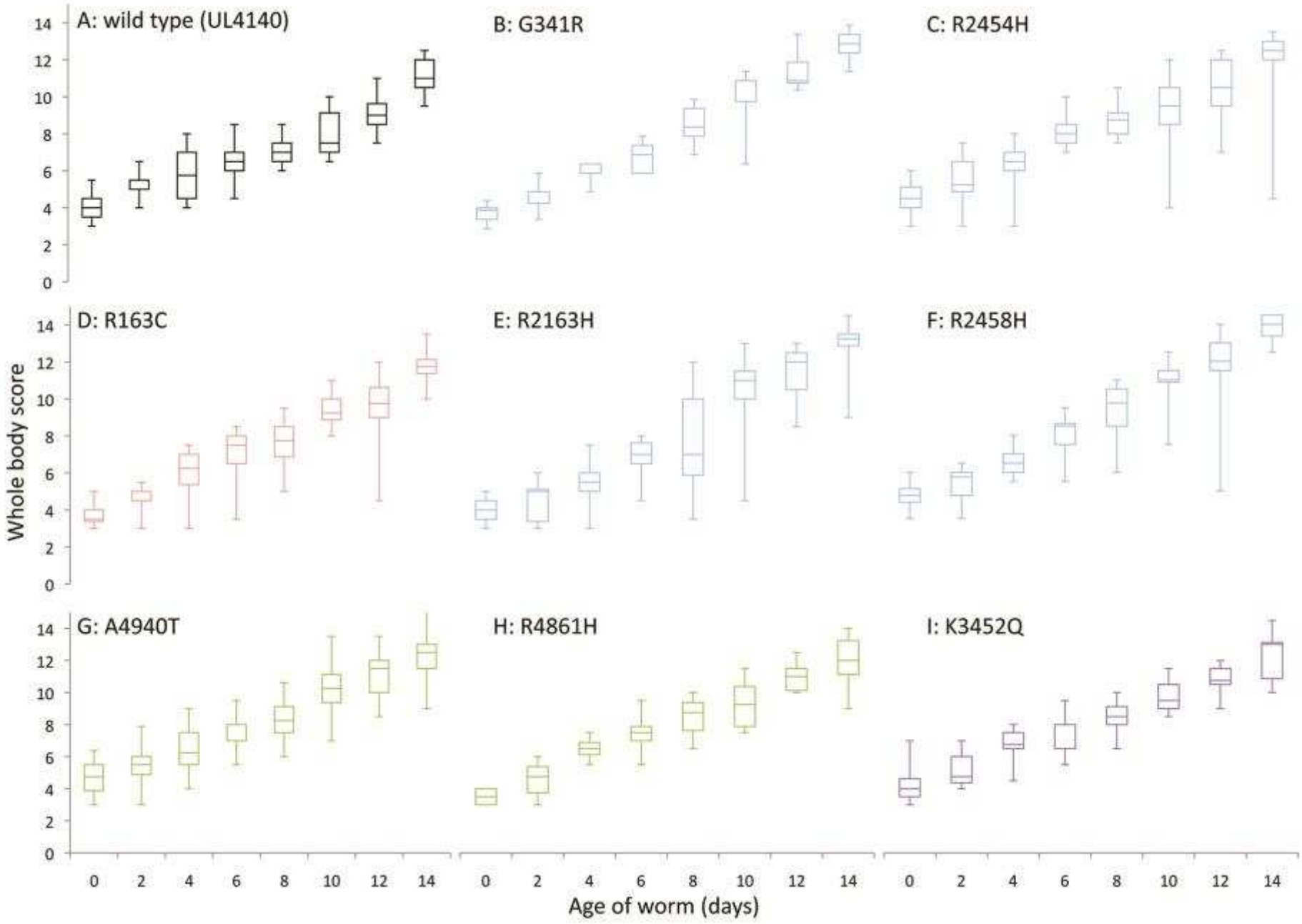
Figure 4. Examples illustrating the 5 grades in the muscle disorganization scoring scale. The myosin::gfp fusion protein is localized to the thick filaments and so distribution of the fluorescence reports on the regularity in the arrangement of the sarcomeres. Images captured by fluorescence microscopy. A: Typical structure of a grade 1 muscle score; myosin filaments are linear and well organised. B: Typical structure of a grade 2 muscle score; myosin filaments are starting to show more bends but the pattern is still well organised. C: Typical structure of a grade 3 muscle score; myosin filaments are more fragmented and there are apparently overlapping filaments. D: Typical structure of a grade 4 muscle score; myosin filaments are further fragmented and the regularity of pattern is no longer clear. E: Typical example of grade 5 muscle score; the pattern of myosin filaments is severely disorganized. Figure compiled by Matt Pipe.


A: wild type (UL4140)
B: G341R
C: R2454H
D: R163C
E: R2163H
F: R2458H
G: A4940T
H: R4861H
I: K3452Q
Whole body score
Age of worm (days)
0
2
4
6
8
10
12
14

440

Figure 5. Comparison of increase in myofilament disorganization with age, for each unc-68 variant. The whole-body scores for extent of disorganization of the sarcomeric structure from 0 to 14 days of adulthood are presented for the strain transgenic for wild type unc-68 (UL4140) (A), and strains transgenic for unc-68 with amino acid changes equivalent to RyR1 variants associated with: MH in blue (G341R (B), R2454H (C), R2163H (E) and R2458H (F)); EHI in red (R163C (D)); CCD in green (A4940T(G) and (R4861H (H)); and LOAM in purple (K3452Q (I)). Each boxplot represents the median, inter-quartile range and minimum and maximum for the whole-body scores.

449 Table 2. Comparison of strains transgenic for variant and wild type unc-68, in terms of the combined effects of variant and worm age

450 on whole-body score of muscle organization.

		DAY of ADULTHOOD						
		2	4	6	8	10	12	14
VARIANT	G341R	-0.71 (0.48)	1.23 (0.22)	1.48 (0.14)	3.72 (0.0001)	5.33 (<0.0001)	4.83 (<0.0001)	4.53 (<0.0001)
	R2454H	-0.66 (0.51)	0.55 (0.58)	2.35 (0.018)	2.45 (0.014)	1.86 (0.063)	1.64 (0.0101)	2.07 (0.038)
	R2458H	-0.96 (0.34)	0.61 (0.54)	2.15 (0.018)	3.39 (<0.0001)	4.77 (<0.0001)	4.47 (<0.0001)	4.79 (<0.0001)
	R2163H	-1.65 (0.099)	-0.65 (0.515)	1.27 (0.204)	0.71 (0.478)	5.05 (<0.0001)	4.97 (<0.0001)	4.72 (<0.0001)
	R163C	-0.24 (0.81)	2.01 (0.04)	2.73 (0.006)	2.31 (0.02)	3.75 (0.0002)	2.75 (0.006)	2.75 (0.006)
	A4940T	-0.77 (0.85)	2.69 (0.007)	3.13 (0.002)	3.65 (<0.0001)	3.36 (<0.0001)	4.38 (<0.0001)	2.91 (0.0036)
	R4861H	0.19 (0.44)	0.27 (0.78)	0.67 (0.501)	1.19 (0.234)	2.61 (0.009)	2.71 (0.007)	1.38 (0.168)
	K3452Q	0.70 (0.48)	1.91 (0.055)	2.09 (0.036)	2.99 (0.003)	2.87 (0.004)	3.23 (0.001)	2.45 (0.01)

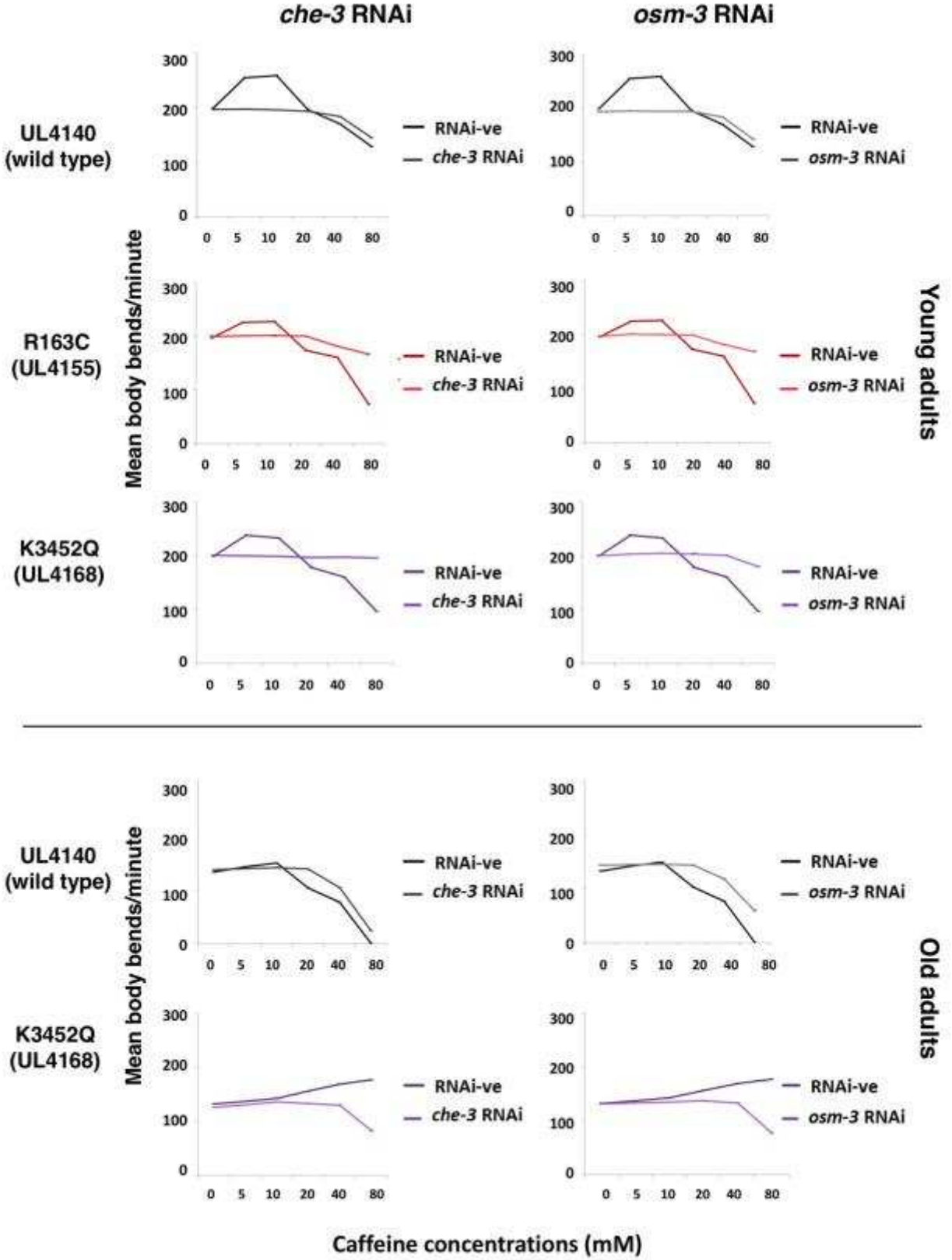
451 T-statistic is displayed correct to 2 decimal places with associated p-value in brackets. Day 2 represents 5 days after hatching.

The modified responses to caffeine depended upon neural function

While RyR1 is considered predominantly a skeletal muscle isoform and other isoforms are expressed in other cell types, there is only a single ryanodine isoform in C. elegans UNC-68 and this is likely to be a key intracellular Ca^{2+} channel in all cells, and critically in excitable cells. Furthermore, caffeine resistant mutations have been localized to two C. elegans genes, osm-3 and che-3, specifically required for chemosensory nerve cell function (WormBase ; Hartman 1987). Therefore, the role of these genes in the differential response to caffeine attributed to the unc-68 variants was tested by RNAi knockdown (Figure 6 and Figure S1). In young adults of the strain transgenic for only wild type unc-68, the stimulation of locomotion at low concentrations of caffeine was completely lost, and the level of inhibition of locomotion at high concentrations was reduced, upon osm-3 and che-3 RNAi knockdown. Exactly the same result was observed for strains transgenic for the EHI associated variant R163C, for the LOAM associated variant K3452Q, and for the standard wild type strain, N2. The inhibition of locomotion by caffeine, across the entire range of concentrations assayed, in the strains transgenic for the MH associated variant R2163H and the CCD associated variant A4940T was reduced by osm-3 and che-3 knockdown, at each caffeine concentration. Furthermore, the dramatic progressive stimulation of locomotion in response to increasing concentrations of caffeine in old adults with the LOAM variant version of unc-68 was also eliminated with knockdown of these genes. In summary, for all strains examined, the locomotory response to each caffeine application was eliminated or markedly reduced by osm-3 and che-3 RNAi. These results could indicate that the focus of influence of the UNC-68 amino acid changes upon caffeine response is actually in these chemosensory nerve cells. UNC-68 does have a function presynaptically at neuromuscular junctions (Liu et al. 2005) and genetic analysis points to a presynaptic focus of anesthetic action

475 in C. elegans (Morgan et al. 2007). However, our results are also consistent with the primary site
476 of action of caffeine being in the chemosensory nerve cells with downstream consequences
477 dependent on UNC-68 in other cells, including or specifically muscle cells.

478


che-3 RNAi
osm-3 RNAi
UL4140 (wild type)
R163C (UL4155)
K3452Q (UL4168)
RNAi-ve
che-3 RNAi
osm-3 RNAi
Mean body bends/minute
Young adults
Old adults
Caffeine concentrations (mM)
0
100
200
300
0 5 10 20 40 80

479

Figure 6. The locomotory response to caffeine that is modified by amino acid changes in UNC-68 is dependent upon the chemosensory neuron specific genes che-3 and osm-3. The locomotion of strains transgenic for the wild type unc-68 (UL4140), for the EHI associated variant R163C (UL4155) and for the LOAM associated variant K3452Q (UL4168) was recorded upon RNAi knockdown of che-3 or osm-3 or in precisely equivalent blank control RNAi experiments. Mean body bends per minute in the presence of increasing concentrations of caffeine are presented for 50 individuals at 0 and 7 days of adulthood, i.e. young and old adults respectively. The colour coding from Figure 1 is retained with, broadly, red for EHI and purple for LOAM. Error bars are standard error of the mean. Equivalent results for other strains are presented in Figure S1.

That compromising chemosensory nerve cells eliminates the unc-68 variant specific changes in the response to caffeine may be pertinent to human biology. Central nervous system damage has been reported occasionally after malignant hyperthermia episodes. The skeletal muscle-specific effects of RYR1 variants may be simply a reflection of the predominant tissue specific distribution of this gene's expression. While RYR2 is expressed predominantly in cardiac muscle and RYR3 is more broadly and weakly expressed, RYR1 is also expressed in other tissues including the central nervous system (De Crescenzo et al. 2012; Giannini et al. 1995). A clinical report has linked RYR1 variants to central nervous system damage in response to triggering events (Forrest et al. 2015).

The response of C. elegans to caffeine and halothane, upon directed modification of unc-68, as described here, emphasizes the conservation of functionality of the ryanodine receptor from humans to nematodes. Sequence changes in UNC-68, equivalent to human disease causing variants, conferred increased sensitivity of the whole organism to pharmacological agents of direct relevance to the medical conditions. The variants even induced this increased sensitivity in C. elegans in the presence of the wild type protein, mirroring the genetic dominance of the human variant alleles. The distinction of increased caffeine sensitivity being seen for the unc-68 variants corresponding solely to MH or CCD, but not those implicated in EHI or LOAM, is based on analysis of a limited number of variants. However, this finding may also be relevant to muscle biopsies of EHI patients and some MH susceptible individuals responding to halothane but not to caffeine in the diagnostic IVCT (Carpenter et al. 2009; Hopkins et al. 1991).

Processes determining animal lifespan appear remarkably conserved and studies on C. elegans longevity have been instrumental in delivery of our current understanding on this subject (Rodriguez et al. 2013). Here, single amino acid changes in the ryanodine receptor have been shown to decrease lifespan and increase muscle aging, in C. elegans, adding further support to the evidence suggesting equivalent effects in mammals. The progressive increase in sensitivity to caffeine stimulation of locomotion with age in the strain with an unc-68 variant equivalent to LOAM may reflect changes that occur with age in humans and contribute to this condition.

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