Multiple hormonal signalling pathways function cell-nonautonomously to control protein homeostasis in *Caenorhabditis elegans*

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ABSTRACT

Protein homeostasis is crucial for viability of all organisms and the development of many different human pathologies are caused by mutations that enhance protein aggregation. For example, several forms of muscular dystrophy are caused by mutations that enhance protein aggregation in muscle cells. Here, we report that *unc-1*/Stomatin like protein 3 functions in the nervous system to maintain appropriate protein homeostasis in muscle cells. We show that UNC-1 regulates protein aggregation in muscle cells by antagonizing neurohormonal signalling from the cytosolic sulfotransferase SSU-1, which functions in the ASJ sensory neurons, to the nuclear hormone receptor NHR-1 in muscle cells. In addition, we demonstrate that this signalling pathway functions antagonistically to a second hormonal signalling pathway that signals via the DAF-12/vitamin D receptor to regulate protein homeostasis. Our results indicate that protein aggregation in muscle cells is controlled by multiple hormonal signalling pathways that function cell-nonautonomously. In addition, our data suggest that abnormal hormonal signalling might contribute to some of the pathologies caused by protein aggregation.

KEYWORDS

Caenorhabditis elegans, protein homeostasis, polyglutamine aggregation, protein aggregation, endoplasmic reticulum stress, steroid hormone signalling, nuclear hormone receptor, stomatin-like protein, sulfotransferase, sulfatase.

BACKGROUND

Protein homeostasis is essential to maintain appropriate cell function. This consists of both, an adequate expression of specific sets of proteins and proper folding and localisation of these molecules. When proteins do not fold correctly they tend to expose hydrophobic domains, which normally lay in the interior of the protein, to the cytoplasm and therefore become prone to aggregation between themselves and other proteins. These aggregates are very toxic and can substantially alter the function of cells and cause disease[1]. To prevent the formation of aggregates there are natural mechanisms to control proper folding of proteins and to remove proteins that are already misfolded[2]. Once a considerable amount of proteins have collapsed, due to improper folding, they induce stress in the cytoplasm, by poisoning the machinery of the proteasome for example, which in turn activates unfolded protein response (UPRs) pathways[3]. UPR signals activate nuclear transcription factors that induce expression of genes associated with folding and degradation of proteins, autophagy, among other protective processes[3]. While many UPR signals are thought to function cell-autonomously, recent studies of *C. elegans* have demonstrated that cell-nonautonomous pathways also regulate protein aggregation. For example, chemical synaptic function in neurons was shown to regulate polyglutamine (polyQ) misfolding in muscle cells[4, 5]. Specifically, Silva and co-workers showed that a small increase in physiological cholinergic signalling, at the motor synapses, induced a calcium-dependent activation of muscular HSF-1, which in turns activates the expression of chaperons that protect against protein aggregation[5]. Despite these and similar observations, the mechanisms by which neuronal signalling can control protein aggregation in distant tissues remains unknown.

Nuclear hormone receptors (NHRs) comprise a group of transcription factors that regulate the expression of genes in a ligand binding-dependent manner. These ligands are usually lipid molecules, such as steroid hormones[6]. After ligand binding, NHRs dimerise (homo- and heterodimers) and bind DNA domains in the vicinity of promoters. The wide range of metabolic processes regulated by NHRs has made them outstanding druggable targets against inflammation, metabolic and neurodegenerative diseases (reviewed in refs. 7-11). Interestingly, it has been described that stress responses in the ER can be modulated by the function of NHRs. For example, the nuclear hormone receptor liver homolog-1 (LRH-1) is required to reduce stress in the ER, in type 2

diabetes mellitus mice models[7, 8]. Moreover, the thyroid hormone receptor α, which is essential to maintain postnatal pancreatic β-cells, is required to reduce oxidative stress in the ER in these cells, to avoid apoptotic death[9].

In this manuscript, we report the isolation of a mutant allele in *unc-1*, a gene encoding one of the stomatin-like proteins in *C. elegans*, that enhances polyQ aggregation in muscle cells. We show that UNC-1 functions in neurons, likely by controlling electrical synapse function, to regulate protein homeostasis in distant tissues. Disruption of UNC-1 function causes a malfunction of ASJ sensory neurons which excretes a sulfated signal and activates NHR-1 in muscle cells. Activation of NHR-1 down-regulates components of the ER stress response and this compromises proteostasis in muscle cells. In addition, we show that signalling through DAF-12, a NHR related to the mammalian vitamin D receptor, functions antagonistically to signalling via NHR-1 to control protein aggregation, indicating that multiple hormonal signalling pathways control protein aggregation in *C. elegans*. Finally, our experiments present evidence of the first example of a NHR where activation induces disruption of protein homeostasis.

RESULTS

EMS screening and isolation of *unc-1* as a modulator of polyQ aggregation

García and co-workers have shown that signalling from chemical synapses modulates protein homeostasis[4]. However, little is known about upstream and downstream processes involved in this regulation. To expand our knowledge on these signalling pathways controlling protein homeostasis we did an EMS screening to find regulatory genes (Fig. 1a and Supplementary Material and Methods), in a worm model of polyQ aggregation[10]. We used animals that express 40 glutamines in frame with a yellow fluorescent protein (40Q::YFP) in muscle cells. In these animals, 40Q::YFP aggregates in an age-dependent manner and forms inclusion bodies that can be observed and counted under a dissecting microscope (Fig. 1a, b). Among several mutants, we obtained a mutant strain, RVM10 that carries the allele *vlt10*, which substantially enhanced the aggregation pattern (Fig. 1b, c). In addition to the enhanced polyQ aggregation pattern, the *vlt10* animals showed a kinky phenotype (or Unc phenotype, from uncoordinated), suggesting that this mutation may affect the function of the nervous system and/or muscle cells.

To determine the molecular identity of this allele we sequenced the genome of this strain after six outcrossing steps and also the genome of the original strain (RVM10). EMS causes random lesions through the chromosomes and after outcrossing RVM10 most of these mutations would be lost except the DNA changes that lay around the allele responsible for the aggregation phenotype. We took advantage of this, and when we compared both genomes we observed a region with dense amount of mutations that "peaked" around the left arm of chromosome X (Fig. 1d). Detailed analysis of this region showed that the *unc-1* gene of RVM10 had a nonsense mutation that gives rise to a premature stop codon (Fig. 1e). *unc-1* encodes a homologue of the Stomatin-like protein (SLP) family from mammals[11], most closely related to STOML3 (Fig. 1e and Supplementary Fig. S1). The product of this gene has been shown to be involved in modulation of the electrical synapse[12] and sensitivity to anaesthetics[13]. Null alleles of this gene are well-known to cause strong uncoordination in *C. elegans*[12], similar to *vlt10* mutants from our screen.

Depletion of *unc-1*/Stomatin-like protein enhances polyglutamine aggregation

To test if the loss of *unc-1* is responsible for the enhanced aggregation phenotype of *vlt10* mutants, we introduced known mutant alleles of *unc-1* (*e179* and *e1598*) in 40Q::YFP expressing animals. Analysis of the inclusion bodies produced by the aggregation of polyQs showed that indeed mutations in *unc-1* phenocopy the aggregation pattern of *vlt10* mutants (Fig. 1f). However, the *vlt10* allele seems to produce a weaker phenotype than *e179* or *e1598*, suggesting that although it is a nonsense mutation *unc-1,* it may somehow produce a protein with partial function. We also cannot rule out that an unknown *unc-1*-linked mutation may be reducing the protein aggregation phenotype of *vlt10* mutants.

We investigated whether *unc-1(vlt10)* would modify the aggregation phenotype of a strain expressing 35Q::YFP in body wall muscles. These animals are believed to have a weaker protein aggregation phenotype than animals expressing 40Q::YFP, and they only accumulate inclusion bodies at very late adult stages[10]. Analysis of 35Q::YFP in *unc-1(vlt10)* mutants showed that the *vlt10* allele is able to enhance the phenotype of these animals to a similar extent as other *unc-1* alleles, *e179* and *e1598* (Fig. 1g). Altogether, these data strongly suggest that *unc-1* is required to prevent aggregation of polyQ proteins.

unc-1 is required in the nervous system to maintain protein homeostasis

UNC-1, the product of the *unc-1* gene, is expressed both thorough the nervous system and in muscle cells[12-14]. To investigate whether the function of UNC-1 is required in a specific tissue, we reintroduced the cDNA of *unc-1*, under the control of the promoter of *myo-3* (*myo-3p*), a gene that is exclusively expressed in muscle cells[15] in 40Q::YFP; *unc-1(vlt10)* animals. Analysis of the aggregation patterns showed that this construct did not rescue the aggregation pattern in larvae, although it produced a small reduction of inclusion bodies in young adult worms (Supplementary Fig. S2a and data not shown). By contrast, when we reintroduced *unc-1* in the nervous system, using a pan-neuronal promoter, from the gene *rab-3*[16], they showed a substantial reduction of the aggregation phenotype in all stages (Fig. 2a and 2b). In addition, introduction of a dominant negative form of *unc-1* (i.e. a cDNA of *unc-1* bearing the *n494* allele[17]) (*unc-1(nd)*), in the nervous system increases the number of inclusion bodies (Figure 2c). All these data suggests that *unc-1* is required in the nervous system to regulate protein homeostasis in body wall muscles.

The results of the tissue-specific expression of *unc-1*, *unc-1(RNAi)* and expression of *unc-1(nd)* further support the hypotheses that *unc-1(vlt10)* is the mutation responsible of the enhanced aggregation of the RVM10 worms.

Selective depletion of the electrical synapse increases polyQ aggregation

The *C. elegans* genome encodes three other neuronally expressed SLP genes[23]. Analysis of the other two neuronally expressed SLPs genes[18], *mec-2* (mechanosensory neurons) and *unc-24* (wide neuronal expression) showed that only *unc-24* mutants have a phenotype comparable to *unc-1* mutants, suggesting that not all neuronal SLP genes are involved in the regulation of polyQ aggregation (Table 1).

Since *unc-1* mutants show a defect of motor coordination due, potentially, to a disrupted electrical synapse, we sought to investigate whether polyQ aggregation in muscle cells is a general result of disrupted electrical synapse function. To test this we introduced mutant alleles of genes encoding innexins, which are known components of the electrical synapse, to investigate the effects of these mutations on polyQ aggregation in muscle cells. Innexins are transmembrane proteins that form gap junctions between cells, including electrical synapses between neurons and neuromuscular synapses (please see Altun *et al*., for a review[19]). Specifically, we introduced mutant alleles of all innexins widely expressed in the nervous system (*unc-7*, *unc-9*, and *inx-7*), and two expressed in a restricted set of neurons (*inx-2* and *inx-6*), into a polyQ background (Table 1). From this analysis, we observed that mutants of two of the innexins expressed widely in the nervous system (*unc-7* and *unc-9*), reproduce the phenotype of *unc-1* (Table 1). Of the innexins expressed in a particular set of neurons, only *inx-2* increased polyQ aggregation, suggesting that disrupting electrical synapse function, in specific neurons, promotes protein aggregation in muscle cells.

Ablation of the sulfotransferase gene *ssu-1* rescues *unc-1*-associated protein homeostasis unbalance

Carroll and co-workers showed that mutant alleles of *ssu-1*, a gene that encodes the only known cytosolic sulfotransferase in the *C. elegans* genome, partially rescues uncoordinated locomotion of *unc-1* defective worms, among other phenotypes[18]. Cytosolic sulfotransferases are promiscuous enzymes that are able to add a sulfate

moiety to a variety of molecules such as steroid hormones and xenobiotics targeted for elimination (reviewed by Gamage *et al.*,[20]).

To investigate the potential role of *ssu-1* as an interactor of *unc-1*, in regard to protein homeostasis, we introduced the insertion expressing 40Q::YFP in body wall muscle cells into a strain containing the loss-of-function alleles *ssu-1(fc73)* and *unc-1(e580)*, *ssu-1* single mutant animals did not exhibit a modified aggregation phenotype (Fig. 3b). However, consistent with previous observations, we found that mutations in *ssu-1* suppressed the effects caused by mutations in *unc-1* on protein aggregation in muscle cells (Fig. 3b).

SSU-1 is only expressed in a single pair of sensory neurons, the ASJ neurons[23]. To test whether SSU-1 functions in the ASJ sensory neurons to regulate protein aggregation in muscle cells we induced cell-specific RNAi against this gene[21, 22]. Briefly, we injected into a 40Q::YFP; *unc-1(vlt10)* background sense and antisense DNA fragments of *ssu-1* fused to the promoter of *trx-1*, which is known to be expressed exclusively in ASJ neurons[23]. Expression from *trx-1p* of these inverted *ssu-1* fragments produces sense and antisense RNA of this gene, which hybridizes to become dsRNA and induces RNAi against *ssu-1*. As expected, reducing the expression of *ssu-1* in ASJ partially rescued the aggregation phenotype of *unc-1* (Fig. 3c). These data indicate that SSU-1 activity in the ASJ sensory neurons is required for the disrupted protein homeostasis in muscle cells exhibited by *unc-1* mutants.

Arylsulfatase activity modulates the aggregation dynamics of polyQs

Our results suggest that SSU-1 produces a sulfonated signalling molecule in *unc-1* mutants and that this sulfonated molecule promotes the aggregation of polyQs in muscle cells. Thus, we hypothesised that removal of sulfate from the signal may alter the aggregation phenotype. To test this hypothesis we sought to disrupt sulfatases, which are enzymes that remove sulfate moieties from small molecules. There are only three genes encoding potential enzymes with predicted sulfatase activity in *C. elegans*: *sul-1*, *sul-2* and *sul-3* [24]. *sul-1* encodes a predicted protein with similarity to mammalian 6-O-endosulfatases (www.wormbase.org; and Fig. 3a) and *sul-2* and *sul-3* predicted enzymes are closely related to arylsulfatases (www.wormbase.org; and Fig. 3a). We sought to investigate whether disruption of any of these enzymes alters the aggregation pattern of *unc-1* animals using RNAi to inhibit gene expression. Analysis of animals fed

bacteria expressing dsRNA targeting each of the three genes showed that reduction of function of both *sul-2* and *sul-3* decreased the aggregation pattern observed in *unc-1* mutants (Fig. 3d). In contrast, *sul-1(RNAi)* animals did not show a reduction of inclusion bodies (Fig. 3d), which suggests that only arylsulfatase function is able to modulate aggregation of polyQs in *unc-1* mutants. Then we analysed the aggregation phenotype of the only arylsulfatase mutant available, *sul-2(gk187)*, and observed that ablation of *sul-2* substantially reduces the aggregation phenotype of *unc-1* mutants (Fig. 3e). These results indicate that arylsulfatase activity regulates protein homeostasis, possibly by antagonizing the sulfonation of the target of SSU-1.

The Nuclear Hormone Receptor NHR-1 modulates protein homeostasis

The activity of both sulfotransferases and sulfatases regulates signalling via nuclear hormone receptors (NHR), by adding or removing a sulfate moiety to hormones, or their precursors (reviewed by He et al., [25]). Inversely, NHRs are able to modulate the expression of genes encoding these enzymes, through negative feedback loops operated by their enzymatic products [26]. Interestingly, Burton and co-workers showed that the *C. elegans* sulfotransferase SSU-1 controls hormonal signalling through NHR-1 to regulate insulin sensitivity and specificity [27]. These data provided us with a hint about a potential downstream effector of SSU-1 in the context of the phenotype shown by *unc-1* mutants. Therefore, we decided to test whether the same signalling pathway was involved in the modulation of the polyQ aggregation phenotype observed in *unc-1* mutants. To do so, we introduced mutant alleles of *nhr-1* in a 40Q::YFP*; unc-1* background. We used gene editing by CRISPR to generate a lesion in *nhr-1*, *vlt16*, reproducing the allele described by Burton *et al.*, *n6242*[27], in the 40Q::YFP*; unc-1* background. Additionally, we isolated and characterized a new allele of *nhr-1*, *vlt15*, which was produced by an abnormal homologous recombination and is a potential null (Supplemental Fig. S3b, c). Analysis of both mutant alleles showed that disruption of NHR-1 in the *unc-1* background produces a substantial reduction of inclusion bodies (Fig. 5a), indicating that NHR-1 acts downstream of SSU-1 signalling to regulate protein homeostasis.

NHR-1 seems to be the target of a signal produced by SSU-1 in ASJ, but in which tissue the function of this nuclear receptor is required? To answer this question, we sought to reintroduce NHR-1 in body wall muscles, using the promoter of the *unc-54* gene[27]. We found that expressing NHR-1 specifically in muscle cells partially restored the

aggregation phenotype shown by *unc-1* worms (Fig. 5b), which demonstrates that NHR-1 function is required cell autonomously to regulate protein homeostasis.

Similar to our observations of protein homeostasis, we found that disruption of *nhr-1* also restored motility in *unc-1* mutants and that this effect on motility was reversed when a wild-type copy of NHR-1 was expressed specifically in muscle cells (Fig. 5c). These results suggest that the immobility observed in *unc-1* mutants might be caused by protein aggregation in muscle cells.

NHR-1 regulates transcription of molecules involved in protein homeostasis

To further understand how UNC-1 and NHR-1 antagonistically regulate protein homeostasis we performed RNA sequencing (RNA-seq) on *unc-1(vlt10)* mutants and wild-type animals. Analysis of the transcriptome of these animals showed that a substantial number of genes, in a range of pathways, were dysregulated in the *unc-1(vlt10)* animals. Among them many genes related to synaptic signalling were up- or down-regulated. For example, Bonferroni analysis shows that genes involved in G-protein coupled receptor signalling (GO:0007186) are upregulated in *unc-1* animals (50% upregulated, p-value= 0 and p-value adjusted=0.028) (Supplementary Table S1). neuropeptides, G proteins, etc. (Supplementary Table S1).

Interestingly, this analysis showed that genes involved in the response to unfolded protein stress and endoplasmic reticulum (ER) stress were upregulated in *unc-1* mutants (Supplementary Fig. S4). This upregulation of genes involved in unfolded protein stress and ER stress might be due to increased protein aggregation, similar to our observations of enhanced polyQ aggregation in *unc-1* mutants.

Since our RNA-seq data showed that the expression of many genes involved in protein homeostasis maintenance are upregulated in *unc-1* mutants, we sought to evaluate the level of expression of some of these genes in *nhr-1* mutants. We chose to analyse genes from the unfolded protein response (UPR) of mitochondria (*dve-1*, *atfs-1* and *hsp-60*), UPR of the endoplasmic reticulum (*pek-1*, *rtcb-1*, *ire-1* and *atf-6*) and the heat shock response (*hsp-16.41*) in *unc-1; 40Q* and *unc-1; nhr-1; 40Q* worms, to investigate if the expression of these genes correlated with our phenotypic data. Our experiments showed worms lacking *nhr-1* have reduced expression of some of the components of these pathways when compared to *unc-1* mutants (Supplementary Fig. S5). These results are

consistent with our phenotypic data and suggest that NHR-1 functions by controlling the expression of genes involved in protein homeostasis.

Activation of the nuclear hormone receptor DAF-12 reduces polyQ aggregation

Although there is no evidence, to our knowledge, that dafachronic acids (DAs) are substrates and/or products of sulfotransferases or sulfatases, we sought to test whether the signal from ASJ, produced by SSU-1 and responsible for the phenotype of *unc-1* animals, could be DAs. These steroids induce opposing effects on longevity, depending of the genetic and metabolic context of the worms[28]. The target of these hormones is DAF-12, a steroid hormone receptor homologous to mammalian vitamin D receptor. Hence, we decided to investigate whether DAF-12 modulates the phenotype of 40Q::YFP; *unc-1* animals. We generated a lesion in *daf-12*, *vlt17*, reproducing the allele *daf-12(rh257)* using gene editing through CRISPR. Animals carrying this allele produce a DAF-12 receptor unable to bind DAs[29]. However, homozygous 40Q::YFP; *unc-1(vlt10)*; *daf-12(vlt17)* double mutants were synthetic lethal, so we could not analyse them. In consonance with this, 40Q::YFP; *daf-12(vlt17)* animals showed a substantial increase in the number of polyQ aggregates (Fig. 4a).

To further confirm these data, we investigated the effect of disrupting DA biosynthesis, using dafadine A, a compound that inhibits enzymatic activity of DAF-9, a P450 cytochrome essential for DA synthesis[30, 31]. Culturing worms on this compound phenocopies loss-of-function alleles of *daf-9*[32]. We treated L1 40Q::YFP animals with different concentrations of dafadine A (1, 5, 12.5 µM), and observed that this substance, similar to loss of DAF-12 activity, induced dose-dependent increases in polyQ-aggregates (Fig. 4b). Then, we analysed the behaviour of the 40Q::YFP worms cultured on 1 µM Δ^4-DA, and we observed a reduced amount of polyQ aggregates (Fig. 4c). To confirm these results, we evaluated the effect of both dafadine A and Δ^4-DA in worms expressing polyQs in mechanosensory neurons (112Q), which are defective in touch response (under review Sanchis *et al.*). The Δ^4-DA treatment produced improvement of the touch response in 112Q animals, while dafadine A treatment decreased touch response (Fig. 4d).Collectively, these data suggest that steroid hormone signalling through DAF-12 is required to maintain protein homeostasis and functions antagonistically to hormone signalling via NHR-1. Furthermore, these data demonstrate that multiple hormonal signalling pathways likely function cell-nonautonomously to

regulate protein homeostasis in *C. elegans.*

Metabolomics analysis of *unc-1* mutants shows altered lipid metabolic signatures

Since our genetic analysis strongly suggests that a steroid hormone is involved in the protein homeostasis dysfunction observed in *unc-1* mutants, we sought to perform an untargeted metabolomics analysis, to explore whether any steroid species were different between *unc-1* and wild type animals.

In the OPLS-DA model, the separation between *unc-1* and wild type animals was clearly presented (Supplementary Fig. S6) with high R^2Y (0.792) and Q^2Y (0.527) parameters, indicating potential biochemical changes between the two worm strains. The model was satisfactory validated with a 7-fold cross validation method (*p* CV-anova <0.001) and 1000 permutation tests (R^2Y- intercept=0.661, Q^2Y-intercept=-0.318).

We made a Variance Importance in Projection plot (VIP), which summarises the contribution of each variable to the discriminant model. Discriminant variables with a score VIP> 1.0 and a jack-knife confidence interval that did not include 0 were considered relevant and submitted to identification process. A univariance statistical analysis (Mann-Whitney test), was also performed in order to investigate which discriminant metabolites were statistically different between worm strains.

34 metabolites were selected from the VIP plot, for their potential annotation as true metabolites, and many of them were tentatively annotated as lipids. Among those metabolites, only 7 were confirmed by at least one of the fragmentation strategies (Table 2). The feature *mz*= 311.2019 was of major interest as it was annotated as a potential steroid or steroid derivative. Although the results show that the abundance of this metabolite was reduced in *unc-1* animals, the differences were not statically significant. Even though this metabolite may be of great interest, it has not been possible to fully confirm its identity with chemical standard, so the question about which steroid hormone is signalling through NHR-1 remains open and more in-depth metabolomics studies might need to be performed to identify the hormones that regulate protein homeostasis in *C. elegans.*

DICUSSION

Progressive malfunction of protein homeostasis is a natural side effect of the process of ageing in all organisms. Many pathways and molecules have been shown to influence this process, and they function by regulating several diverse processes including maintaining appropriate protein folding and localization. Deleterious mutations in genes encoding components of some of these modulatory pathways accelerate the decline of protein homeostasis, and contribute to the progression of age-related disorders[33].

In this work we show that disruption of the electrical synapse, by ablation of unc-1/Stomatin like protein 3, causes overproduction of neurohormonal signalling from ASJ neurons, which in turns impairs protein homeostasis in a cell-nonautonomously fashion. Disruption of proteostasis in these mutants requires the function of SSU-1, the only know cytosolic sulfotransferase in the *C. elegans* genome[18], which suggest that these hormones need to be sulfated to induce protein aggregation. When these signals reach the target tissue the sulfate moiety must be removed to bind their target, the nuclear hormone receptor NHR-1. In contrast, we show that activation of the DAF12/Vitamin D receptor, another steroid hormone signalling pathway, functions antagonistically to NHR-1 to maintain protein homeostasis.

Several studies have suggested that neurosteroids may induce protection against neurodegenerative diseases that involves dysregulation of protein homeostasis, such as Alzheimer, Parkinson or Huntington diseases (see Borowicz *et al.*, for a review[34]). To our knowledge, this is the first report showing that different steroid hormone signals have opposing modulator effects in protein homeostasis in animals, and it will be of interest to investigate if this is evolutionary conserved between invertebrates and mammals. If similar effects of hormonal signalling on protein homeostasis are conserved, then it is likely that such pathways are amenable to be modified using drugs. For example, there exist inhibitors against sulfotransferases[35], the cytochrome *daf-9*/ CYP2J2[36], sulfatases[37] and modulators of NHRs[38-42], which are being investigated to treat different kinds of cancer and metabolic diseases. Therefore, we predict that such signalling molecules are potential targets to treat pathological conditions produced by toxic protein aggregates such as neurodegeneration. Finally, our work provides an *in vivo* genetically tractable model, *C. elegans*, to study these opposing hormonal signals and to search for new therapies.

MATERIAL AND METHODS

Maintenance and culture of *C. elegans*

All strains were maintained under standard conditions[43]. Some strains used in this work were obtained from Caenorhabditis Genetics Center (CGC; Minneapolis, MN, USA). The CGC is funded by the National Institutes of Health's National Center for Research Resources (NGRR). *ssu-1(fc73)V; unc-1(e580)X* was kindly provided by Phillip G. Morgan. Supplementary Table S3 provides a detailed list of strains used in this study. All strains were outcrossed at least three times.

Plasmid construction, genetic manipulation of worms and transgenesis

Each primer used in this work is described in Supplementary Table S4. To produce the constructs for tissue-specific rescue and overexpression experiments, we amplified the promoter of the *rab-3* gene using the APG315-APG316 primers from wild type gDNA. We cloned the PCR product into pDONRP1P5r using the Gateway system (Invitrogen, Waltham, Massachusetts, USA) to generate pDONRP1P5r-*rab-3p* (pAPG1). We amplified the cDNA of *unc-1* from wild type gDNA using RVM321-RVM239 to obtain pDONRP5P2-*unc-1* (pAPG2). We used pDONRP1P5r-*myo-3p* kindly provided by H. Baylis. Plasmids containing each promoter, were fused with the cDNA of *unc-1*, using multisite Gateway system, within a destination vector that contains the transcription terminator from the gene *unc-54*, pHP2[44], to generate the following destination constructs: pDESTHP2-*rab-3p::unc-1::unc-54t* (pAPG3) and pDESTHP2-*myo-3p::unc-1::unc-54t* (pAPG4). To induce *nhr-1* expression in muscle cells we used pVD105[27].

To produce a dominant negative form of *unc-1*, the *n494* allele[17], we used RVM600-RVM601 (plasmid pAPG5). We used pAPG1 and pAPG5 as donor vectors and pHP2 as destination vector, to generate pAPG6.

DNA mixtures for injection consisted of 50 ng/µl of the interest DNA [except for *myo-3p* expression analysis (1-5 ng/µl)], a DNA marker (*myo-2p*::GFP) at 2.5-5 ng/µl, together with the empty yeast plasmid pYES (Thermofisher, Waltham, Massachusetts, USA) as carrier DNA, at a total final concentration of 120 ng/µl. We injected each mix as described elsewhere[45].

Construction of knock-in worms using CRISPR

We used CRISPR/Cas9 system to introduce point mutations into both *daf12* and *nhr-1*. We based on our design and strategy using a modified protocol from the Ceron's Lab (IDIBELL Institute, Barcelona, Spain)[46]. We employed Alt-R™ S.p. Cas9 Nuclease 3NLS, and two RNAs to produce the gRNA: Alt-R™ CRISPR-Cas9 tracrRNA and Alt-R™ CRISPR-Cas9 crRNA from IDTDNA (Coralville, IA, USA). The guide RNA sequences used to target the genes were: 5′-ATATTATGGATGTTACCATGAGG-3′ for *daf-12* (*rh257* allele) and 5′ -_CACCACTCCACACCGTCTTCAGG-3′ for *nhr-1* (*n6242* allele).

The ssODN sequences to induce knock-ins to generate the allele of interest were: 5′-GAGTCTTCTCAACAGGTAATCAATCAACGGATAAAGAAGATATTACAAAAC TGGTTTTCAAATACTTTGATTCACTGTTCGGTGAACAAGCCCGCCGTATCTTT GAACGGATTCCAAAAGCACTGGGATTACTTAATGTAAGATATCAT- 3′ to generate *rh257* in *daf-12*; and 5′-CCAACGAAGAAAATCAAGATGAGCAGCGGATCTGACGACGAGCAAGCCAC CACTCCACACAGACTCTAAGACCAGGTCAGTGGGCGAAACACATTTACCCC AATTTGGATGCATCTTGAATTTCAACAA- 3′ to generate *n6242* in *nhr-1*. To produce the mixture for injection, the crRNAs were suspended in 20 µl of IDTE nuclease free buffer for 100 µM stock. ssODNs were suspended in the same buffer at 1 µg/µl. CRISPR components were added at the following final concentrations: Cas9: 4,5 µM; ALT-R trackRNA: 32 µM; target gene crRNA: 35 µM; ssODN target gene: 175 ng/µl. We used disruption of *dyp-10* gene as a selection marker as described [47].

RNA interference

To produce constructs for tissue-specific RNAi we used fusion PCR[48], to produce constructs as described elsewhere[21, 22]. We used the promoters of the genes *rab-3* and *trx-1* to silence *unc-1* and *ssu-1* genes in the nervous system and ASJ sensory neurons, respectively. We used a DNA fragment from the bacterial β-Lactamases gene (which we named AMP^r) as negative control in the RNAi experiments. We injected 2,5 ng/µl of each construct together with pCFJ90 (encodes a pharynx-expressed mCherry as a transgenic marker [49]), as a reporter, and pYES as carrier DNA.

To induce constitutive RNAi against sulfatases, we feed the animals with modified HT115 *E. coli* strain expressing dsRNA of each gene. We generated RNAi vectors for these genes cloning a coding fragment of each, into an EcoRV site of pL4440. The PCR

product for each gene was amplified using Phusion polymerase (Thermofisher, Waltham, Massachusetts, USA) and specific primers (Supplementary Table S4).

HT115 strains containing empty pL4440, as a control, and RNAi vectors (pAPG7, pAPG8, pAPG9) were grown in liquid Luria-Bertani medium containing 50 µg/ml carbenicillin over night at 37°C. Feeding RNAi plates contained 50 µg/ml carbenicillin and 1 mM IPTG. Feeding RNAi plates were incubated for 2 h at 37°C to induce dsRNA before adding worms. L3 animals were feed with feeding bacteria over night at 20°C and L4 animals were scored.

Quantification of polyQ inclusion bodies in *C. elegans*

The inclusion bodies (IB) produced by aggregation of polyQs were counted *in vivo* in L2, L4 and young adult stages at 20°C. Counting was done, at least, in three independent experiments. Thirty animals or more were analysed for each genotype and each independent experiment. The number of IBs was scored using an M165FC Leica dissecting microscope (Leica, Wetzlar, Germany).

Analysis of touch response in *C. elegans*

Touch response assays were performed scoring for 10 touches on the tail of the animal for approximately 50 animals. An eyelash, mounted on a toothpick, was gently passed on the posterior part of the animals, as described elsewhere[50]. Each assay was repeated three times.

Liquid thrashing assay

We evaluated the motility of young adults in M9 buffer for 1 minute. Animals were acclimated for 30 seconds before scoring at 20°C. At least 15-20 animals were analysed for each genotype and transgenic line in replicate three times.

Analysis of the effect of (25S)-Δ^4-dafachronic acid (DA) and dafadine A on worms

(25S)-Δ^4-dafachronic acid (DA) was synthesised as described elsewhere[51, 52]. DA treatment was done as described[30]. Briefly, we grown synchronized L1 animals on DA 1 µM in M9 buffer containing 5 µg/ml cholesterol, 12.5 µg/ml nystatine, 50 µg/ml streptomycin and OP50 *E. coli* at 0.5 Optic density. Dafadine A was added at three concentrations (1, 5 and 12.5 µM)[33] using the same culture conditions. We evaluated the

effect of both compounds at least three times in independent experiments. Dafadine A was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Quantitative PCR

The relative expression of genes was measured by RT-qPCR using the ViiA7 thermal cycler from Applied Biosystems (Waltham, Massachusetts, USA) using Taqman probes. To verify the expression of genes included within extracromosomal arrays, we selected by hand worms expressing reporters for RNA extraction (OMEGA Bio-Tek, Norcross, GA, USA) using a dissecting the M165FC dissecting microscope (Leica). To evaluate expression of genes in mutants, we collected a mix population of worms. RNA samples were treated by DNAase treatment (Qiagen, Hilden, Germany). cDNA synthesis was done using 0.1-1 µg approximately of RNA following the manufacturer protocol (Takara Bio, Kusatsu, Japan). We used the 2xPrimeTime® Gene Expression Master Mix (Takara Bio), and the PCR program was as follows: 1 cycle of 10 min at 50°C; 1 cycle of denaturalization at 95°C 15 s; 40 cycles of polymerization at 60°C 1 min. To normalize relative expression we used the housekeeping control, *pmp-3* (Applied biosystems, Ce02485188_m1). *unc-1* expression was measured using a probe from Applied Biosystems (Ce02491558_m1). All other genes (*40Q::YFP*, *ssu-1*, *rtcb-1*, *ire-1*, *hsp-3*, *pek-1*, *atf-6*, *hsp-60, hsp-70, hsp-16.41*, *dve-1*, *atfs-1*) were analysed using customized PCR primers and probes from IDTDNA (Coralville, Iowa, USA). We considered all isoforms for each gene in the customized design.

Imaging worms by fluorescent microscopy

Fluorescence microscopy was performed using a Leica SP5 confocal microscope (Wetzlar, Germany). Live worms were mounted onto 2% agar pads and were anaesthetized with a drop of 0.025 M sodium azide.

Statistics

We plotted mean values for number of inclusion bodies, thrashing and touch response and we analysed the data using ANOVA tests, with Tukey's multiple comparison test. When we had to compare two groups we used Mann-Whitney tests.

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AUTHOR CONTRIBUTION

APG-E and RPV-M designed the study, performed most experiments, analysed the data and wrote the manuscript. JB, JMM, RS and JK contributed with essential reagents and ideas, and wrote the manuscript. MR and AL performed and analysed metabolomics data and wrote the manuscript. JP analysed the data from RNA sequencing and wrote the manuscript. JB-Y performed some experiments. JAB and JC analysed high-throughput to isolate the *unc-1(vlt10)* mutation. NB provided essential reagents and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflicts of interest to disclose.

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FIGURE LEGENDS

Fig. 1. A random chemical mutagenesis experiment led to isolation of *unc-1* as a modifier of polyQ aggregation. a) The strategy to isolate mutants involved both in synaptic signalling and protein homeostasis, consisted in selecting worms that had different aggregation patterns than wild types, and that were uncoordinated. b) Confocal microscopy images of L4 larva and young adult *vtlt10* mutants and wild type animals. c) *vlt10* mutants shows substantially enhanced polyQ aggregation than controls. d) High-throughput sequencing showed that the mutation of *vlt10* lays in the left arm of the LGX. e) One possible candidate was *unc-1*, which had a non-sense mutation in homozygosis, and encoded a potentially truncated UNC-1 protein. f) Analysis of different mutant alleles of *unc-1* shows that this gene is indeed a potent enhancer of aggregation of polyQs. g) *unc-1* mutants are also able to enhance polyQ aggregation in worms expressing a track of polyQs that is below of the pathologic threshold (i.e. 35Q). ***: p-value < 0.001. ANOVA Test with post-hoc Tukey.

Fig. 2. UNC-1 is required in the nervous system to modulate protein homeostasis. a) Expression of the cDNA of *unc-1* in the nervous system (NS) completely rescues the aggregation pattern *unc-1* mutants. *unc-1* depletion do not affect polyQ expression. b) Tissue-specific RNAi against *unc-1* in the NS increases the number of inclusion bodies in 40Q::YFP worms, compared to controls (AMP^r). c) Overexpression of *unc-1* in NS does not modify aggregation of polyQs, while expression of a dominant negative mutation of *unc-1*, *n494*, enhances the aggregation phenotype. ***: p-value < 0.001. ANOVA Test with post-hoc Tukey.

Fig. 3. Depletion of sulfotransferase SSU-1 and arylsulfatases SUL-2 and SUL-3 rescues protein homeostasis unbalance due to *unc-1* absence. a) Diagram showing the phylogenetic analysis of all sulfatases in the *C. elegans* genome (*sul-1*, *sul-2* and *sul-3*). b) *unc-1(e580)*; *ssu-1(fc73)* animals show reduction of inclusion bodies compared to *unc-1* animals. c) RNAi against *ssu-1* in ASJ neurons decreases significantly the number of inclusion bodies in *unc-1(vlt10)* worms. RNAi against *E. coli*'s β-lactamase (AMP^r) was used as a negative control. d) Knocking-down *sul-2* and *sul-3* reduces inclusion bodies in *unc-1* mutants. e) The loss-of-function allele of *sul-2*, *gk187*, shows a statistically significant reduced amount of inclusion in *unc-1* animals. **: p-value < 0.01; ***: p-value < 0.001. ANOVA Test with post-hoc Tukey.

Fig. 4. Steroid hormones that signal through *daf-12* reduce polyQ aggregation and are neuroprotective. a) *daf-12(vlt17)* mutants have enhanced polyQ aggregation. *vlt17* was created by CRISPR-induced knock-in, emulating the *daf-12(rh257)* lesion. b) Incubation 40Q::YFP worms in dafadine A, an inhibitor of *daf-9*, increases in an age-dependent manner the average number of inclusion bodies. c) Growing 40Q::YFP worms on (25S)-Δ^4-dafachronic acid (DA) reduces the average number of inclusion bodies. d) DA restores neuronal function in worms expressing polyQs in mechanosensory neurons. In contrast, incubation of worms with dafadine A enhances neuronal impairment produced by polyQs in mechanosensory neurons. **: p-value < 0.01; ***: p-value < 0.001. ANOVA with post-hoc Tukey.

Fig. 5. NHR-1 signalling modulates polyQ protein homeostasis in muscle cells. a) The *vlt15* and *vlt16* alleles of *nhr-1* rescue the aggregation phenotype in *unc-1(vlt10)* mutants. b) Tissue-specific expression of *nhr-1* in muscle cells exacerbates aggregation pattern in double mutants *unc-1(vlt10); nhr-1(vlt15).* c) Disruption of *nhr-1* improves motility of *unc-1* mutants, while restoration of NHR-1 in body wall muscles reduce the motility capacity of both transgenic lines compared with *unc-1(vlt10); nhr-1(vlt15)* worms. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. ANOVA Test with post-hoc Tukey.

Fig. 6. Diagram showing a working model compatible with the results presented in this work. a) Animals with functional UNC-1 have normal electric synaptic signaling, and therefore ASJ neurons produce the right amount of sulfated steroid hormones. When *unc-1* is mutated, ASJ function may be somehow disrupted, which in turns induces higher production of sulfated steroid hormone by SSU-1. This steroids species are desulfated by sulfatases, either in the target tissues or in intermediary cells. NHR-1 is the target of these hormones, and when it is targeted by higher concentration of the signal inhibits genes related to unfolded protein response, among other targets. b) The nuclear hormone receptors NHR-1 and DAF-12 have opposite effects on protein homeostasis by modulating the expression of genes.

Table 1. Stomatin-like proteins and innexins are required to maintain protein homeostasis

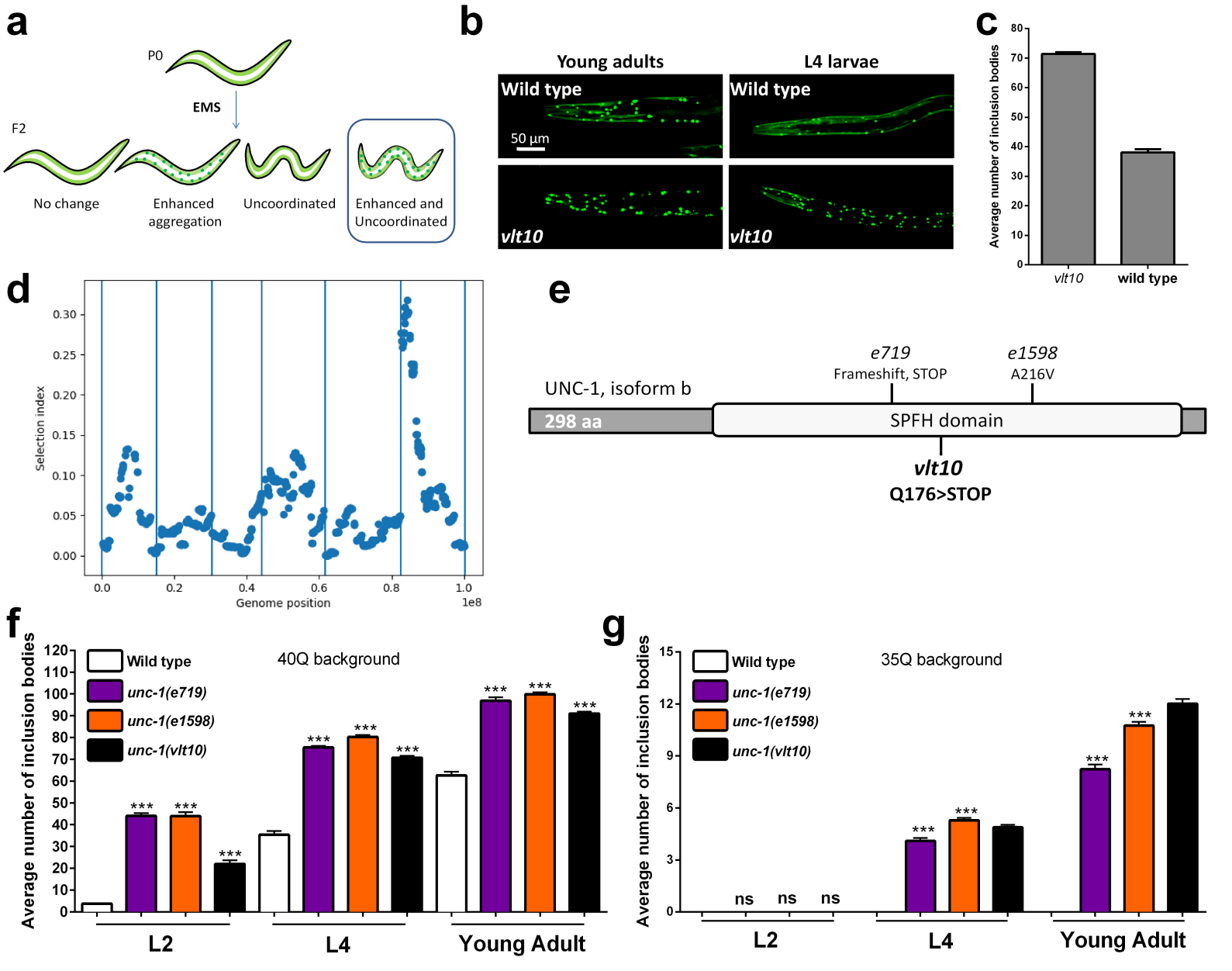
Genotype	Focis (%)[1]	SS[2]	Protein	Function[3]	Expression[3]
unc-1(vlt10)	171.7 ± 3.1	***	Stomatin-like protein	Locomotion Sensitivity to volatile anaesthetics	NS-WE[4]
unc-24(e138)	149.1 ± 3.8	***	Stomatin-like protein	Locomotion Sensitivity to volatile anaesthetics	NS-WE
mec-2(e75)	114.5 ± 5.2	ns	Stomatin-like protein	Mechanosensation	Mechanosensory neurons
unc-7(e5)	178.7 ± 4.7	***	Innexin	Locomotion Sensitivity to volatile anaesthetics Sensitivity to ivermectin	NS-WE Glial cells Mesoderm
unc-9(ec27)	185.4 ± 2.7	***	Innexin	Locomotion Sensitivity to volatile anaesthetics Sensitivity to ivermectin Electrical coupling of muscle cells	NS-WE Enteric muscles RS Hypodermis Glial cells Embryos
inx-2(ok376)	159.2 ± 5.4	***	Innexin	Role initial cellular proliferation stage	NS-AVK neuron Pharynx Intestine Embryos
inx-7(ok2319)	98.7 ± 3.8	ns	Innexin	Unknown	NS-WE Pharynx RS Mesoderm Embryos
inx-6(rr5)	98.8 ± 4.1	ns	Innexin	Synchronized pharyngeal muscle contractions	NS Pharynx Mesoderm Embryos

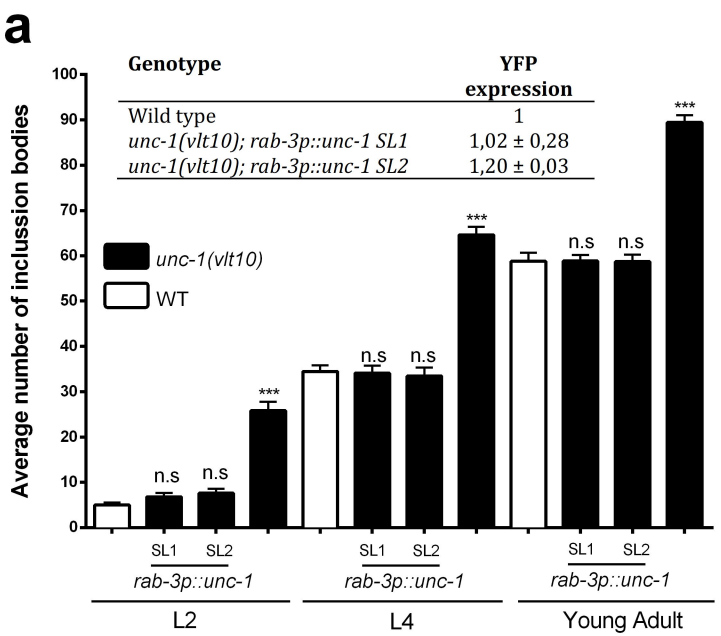
[1]Average of inclusion bodies normalised with the background of wildtype siblings ±Standard Error of the Mean.
[2]SS: statistically significance; ANOVA with post-hoc analysis of Tukey: ***$p<0.001$ referred to wild type; ns: no statistically significant.
[3]Function and expression described in Altun *et al*.,[19].
[4]NS-WE: nervous system-wide expression; NS: some neurons; RS: reproductive system.

Table 2. Identification of metabolites and change trend variation in *unc-1* animals

(m/z)	rt (min)	Adduct	Formula	Metabolite annotation	Compound class	Change trend
658.4446	8.788	M-H	C35H66NO8P	PE(14:1(9Z)/16:1(9Z))[1]	Glycerophospholipids	down*
387.1142	0.653	M-H	C12H22O11	More than 35 carbohydrates[1]	Carbohydrates and carbohydrate conjugates	down*
		M+FA-H	C12H25N2O10P	Fructoselysine-6-phosphate[1]	Organic acids and derivatives	
311.2019	7.899	M-H	C21H28O2	Δ(1)-progesterone[1], tetrahydrogestrinone[1], pregna-4,9(11)-diene-3,20-dione[1], pregna-4,11-diene-3,20-dione[1], norgestrel[1], levonorgestrel[1], 16,17-didehydroprogesterone[1], dydrogesterone[1], 3-Methoxy-19-norpregna-1,3,5(10)-trien-20-one[1], 3-Methoxy-19-nor-17alpha-pregna-1,3,5(10),20-tetraen-17-ol[1], 17-Allylestra-1,3,5(10)-triene-3,17beta-diol[1], ethisterone[1]	**Steroids and steroid derivatives**	down
79.0216	0.716	M+H	C2H6OS	Dimethyl sulfoxide[1]	Sulfoxides	up*
180.0882	0.959	M+NH4	C7H14S2	(-)-1-Methylpropy[1] 1-propenyl disulfide[1]	Organic disulfides	down*
166.0864	0.973	M+H	C9H11NO2	L-Phenylalanine[1,2]	Carboxylic acids and derivatives	down
		M+NH4	C9H8O2	trans-Cinnamic acid[1,2]	Cinnamic acids and derivatives	
		M-H2O+H	C9H13NO3	Epinephrine[1,2], methylnoradrenaline[1,2], levonordefrin[1,2]	Phenols and benzenes and substituted derivatives	
800.5220	8.904	M+NH4	C42 H70 O13	Ginsenosid La[1], momordicoside D[1]		up

FA: formic acid; up: compared with wild type, the compound is up-regulated in *unc-1*; down: compared with wild type, the compound is down-regulated in *unc-1*; * $p < 0.05$; [1]: all ion spectra confirmation; [2]:MS/MS spectra confirmation


a
P0
EMS
F2
No change
Enhanced aggregation
Uncoordinated
Enhanced and Uncoordinated
b
Young adults
L4 larvae
Wild type
Wild type
50 µm
vlt10
vlt10
c
Average number of inclusion bodies
vlt10
wild type
d
Selection index
Genome position
1e8
e
UNC-1, isoform b
e719
Frameshift, STOP
e1598
A216V
298 aa
SPFH domain
vlt10
Q176>STOP
f
Average number of inclusion bodies
Wild type
unc-1(e719)
unc-1(e1598)
unc-1(vlt10)
40Q background
L2
L4
Young Adult
g
Average number of inclusion bodies
Wild type
unc-1(e719)
unc-1(e1598)
unc-1(vlt10)
35Q background
ns
L2
L4
Young Adult


a
Genotype
YFP expression
Wild type 1
unc-1(vlt10); rab-3p::unc-1 SL1 1,02 ± 0,28
unc-1(vlt10); rab-3p::unc-1 SL2 1,20 ± 0,03
Average number of inclusion bodies
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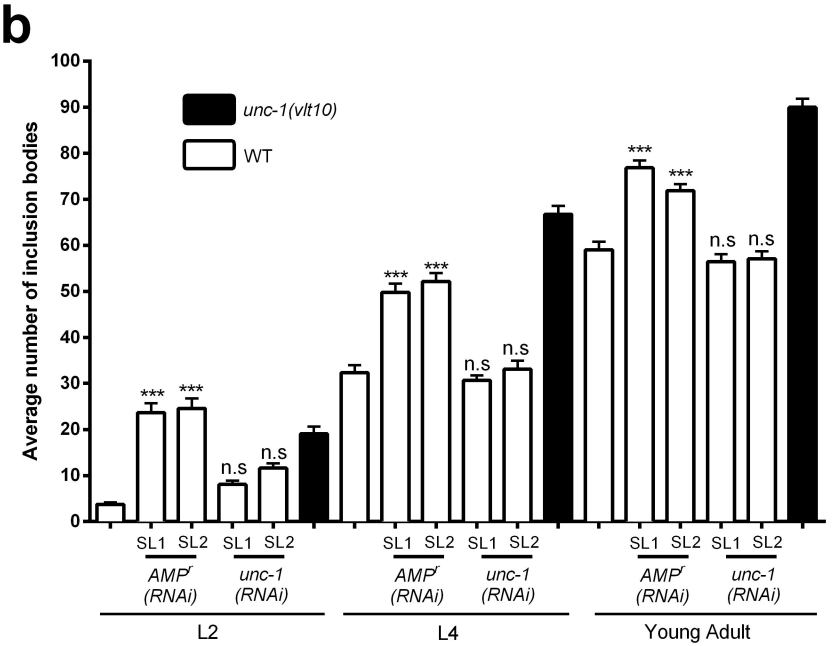
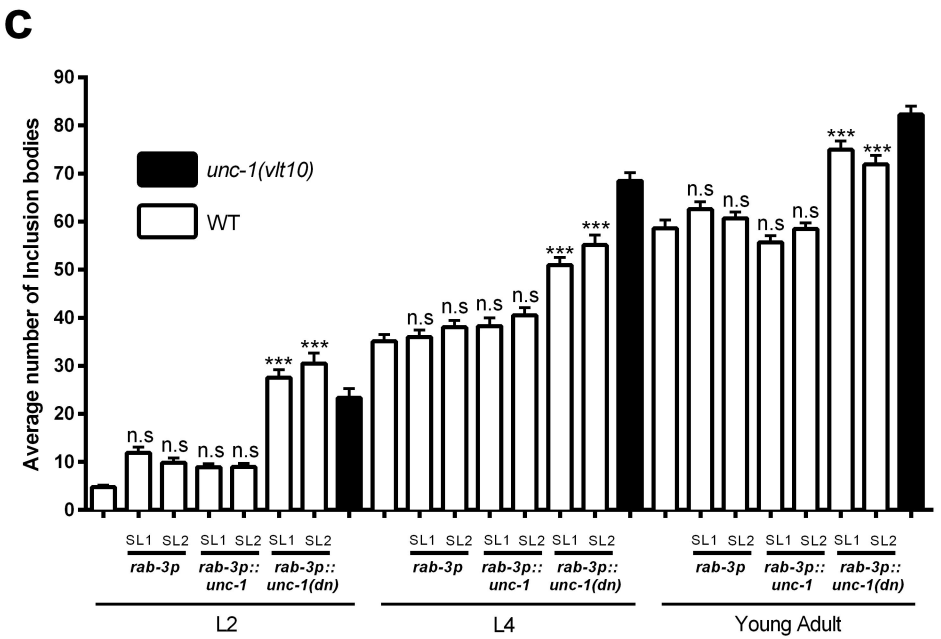
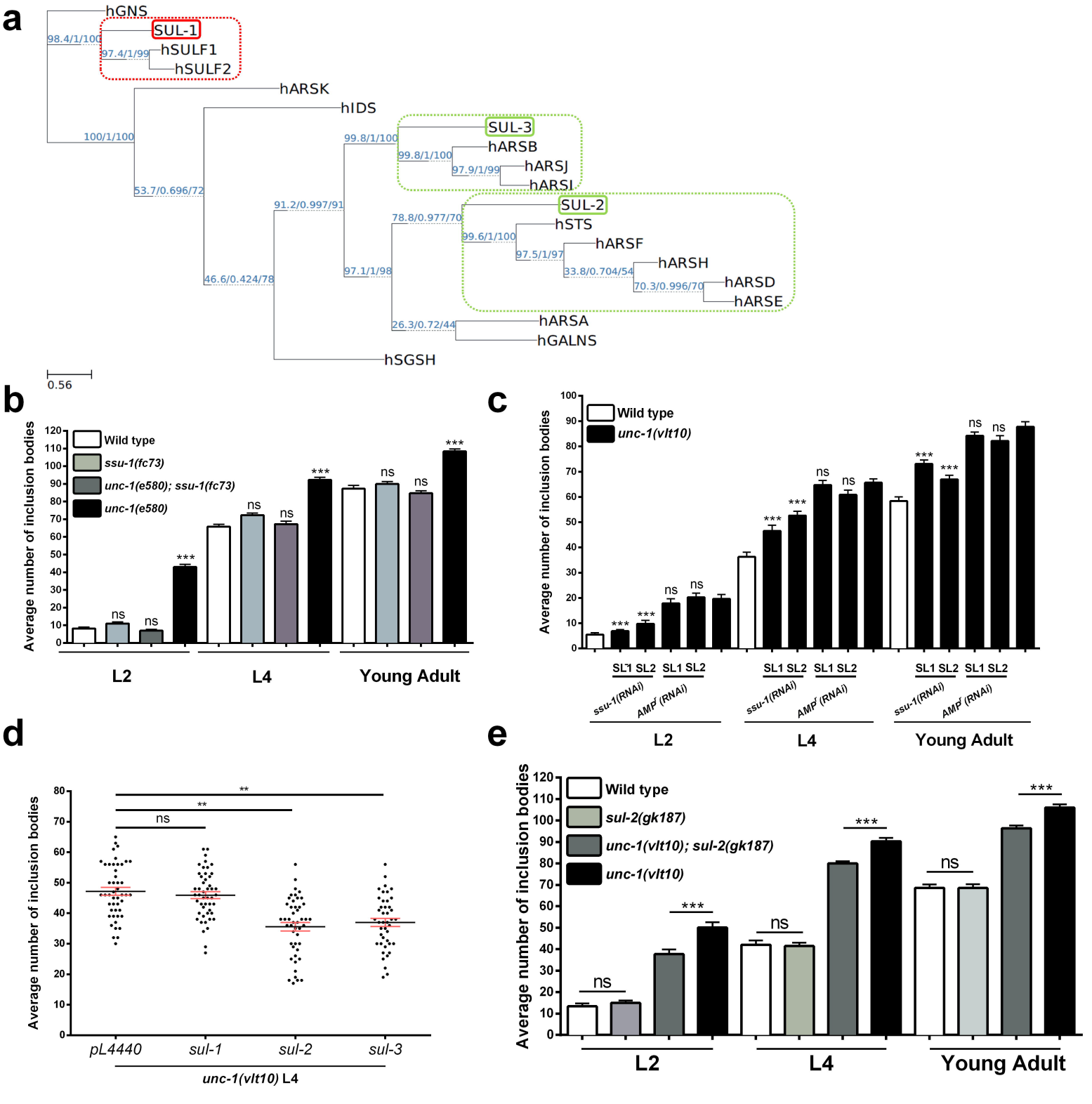
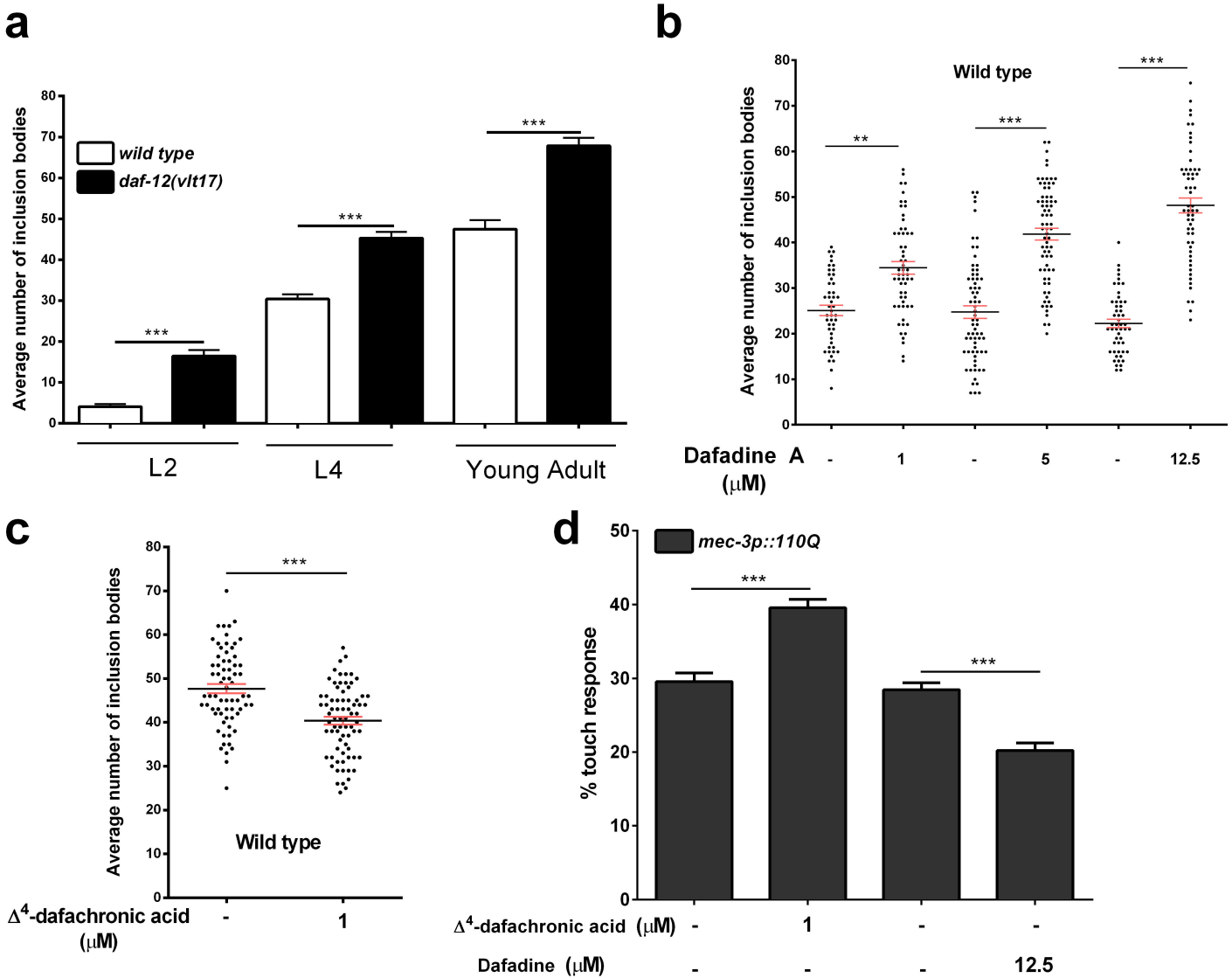
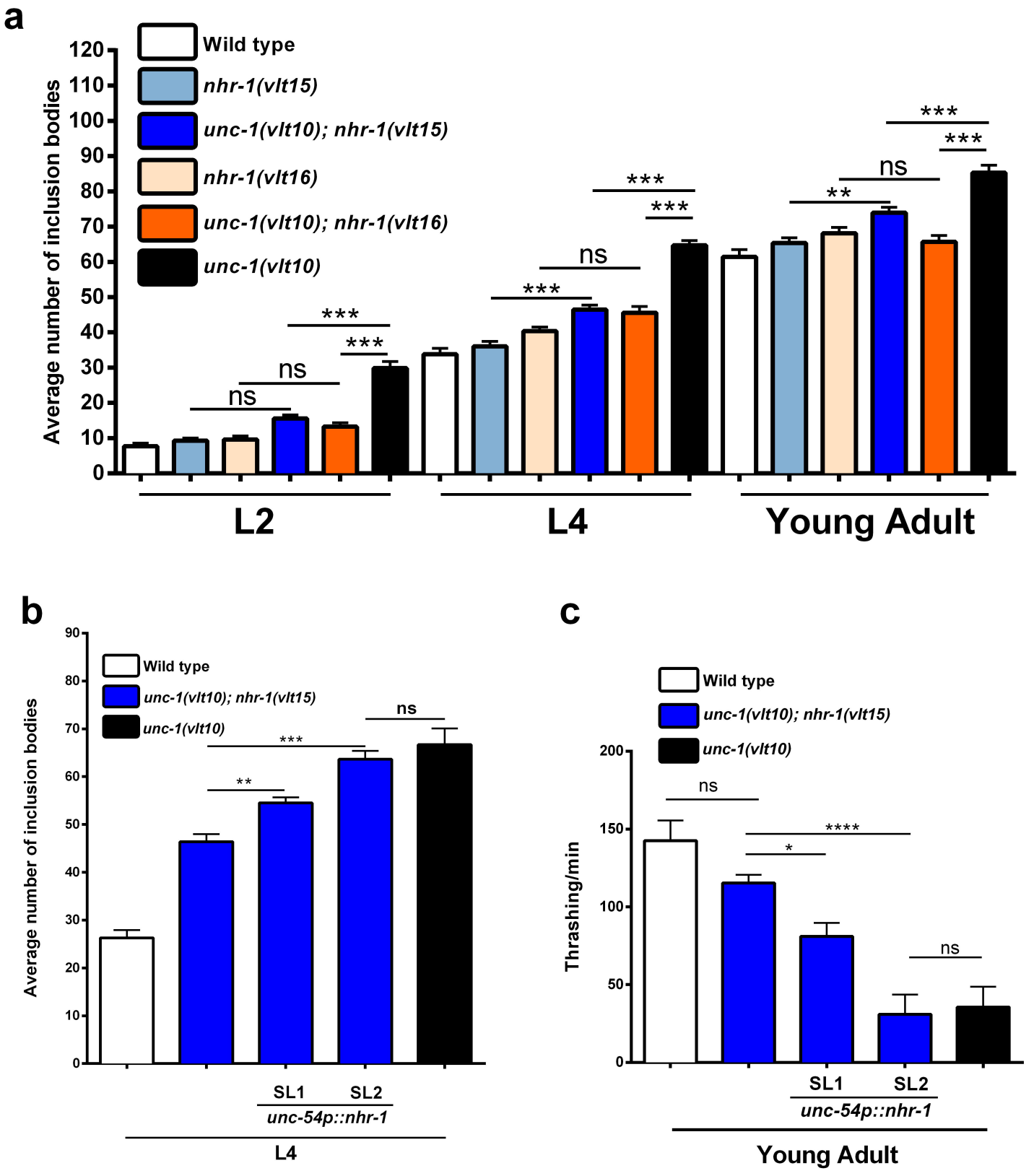
WT
n.s
***
SL1 SL2
rab-3p::unc-1
L2
L4
Young Adult

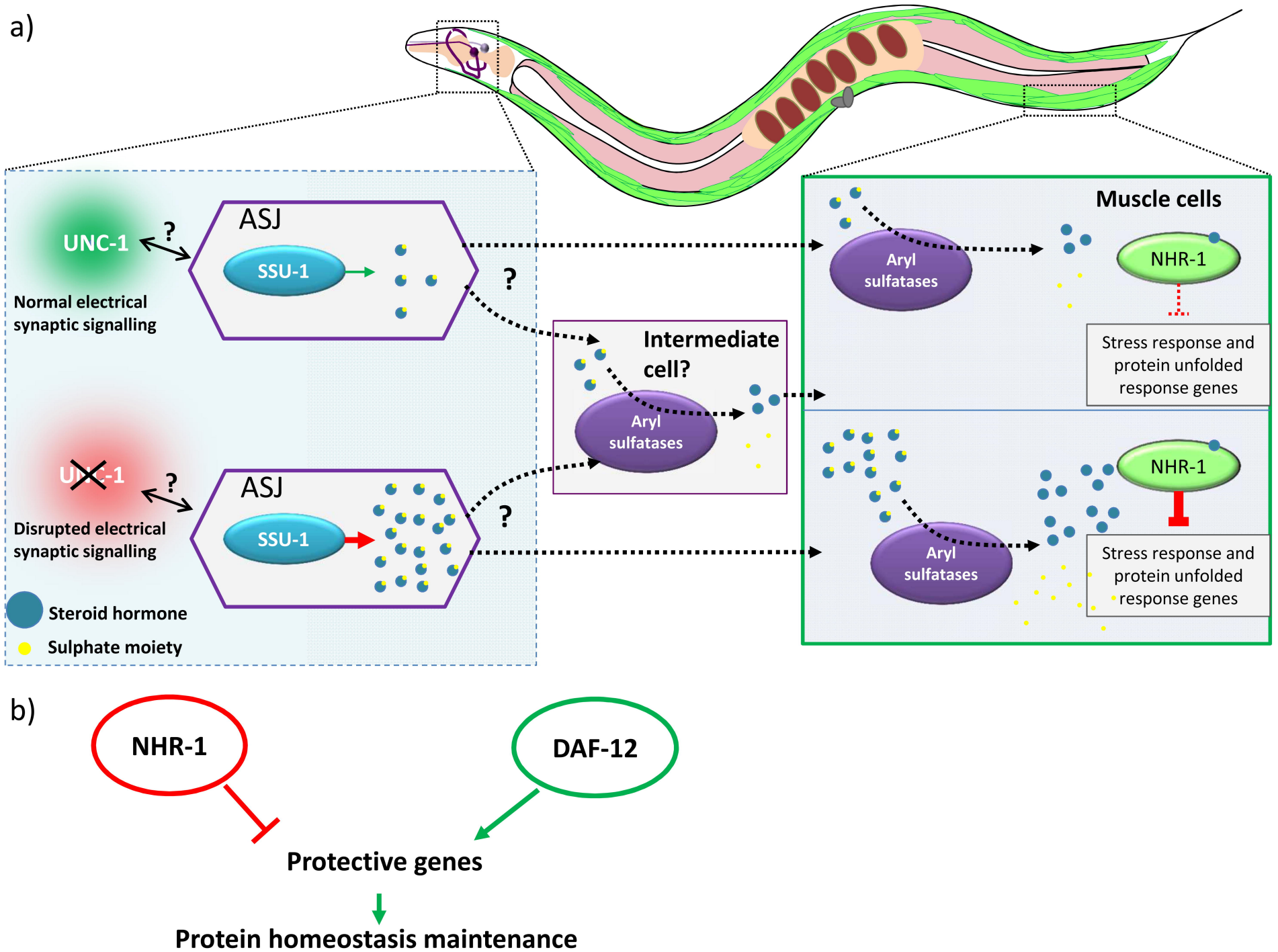

b
Average number of inclusion bodies
unc-1(vlt10)
WT
***
n.s
SL1 SL2
AMP^r (RNAi)
unc-1 (RNAi)
L2
L4
Young Adult


c
Average number of Inclusion bodies
unc-1(vlt10)
WT
n.s
***
SL1 SL2
rab-3p
rab-3p:: unc-1
rab-3p:: unc-1(dn)
L2
L4
Young Adult


a
hGNS
SUL-1
hSULF1
hSULF2
hARSK
hIDS
SUL-3
hARSB
hARSJ
hARSI
SUL-2
hSTS
hARSF
hARSH
hARSD
hARSE
hARSA
hGALNS
hSGSH
98.4/1/100
97.4/1/99
100/1/100
53.7/0.696/72
99.8/1/100
99.8/1/100
97.9/1/99
91.2/0.997/91
78.8/0.977/70
99.6/1/100
97.5/1/97
33.8/0.704/54
70.3/0.996/70
97.1/1/98
46.6/0.424/78
26.3/0.72/44
0.56
b
Average number of inclusion bodies
Wild type
ssu-1(fc73)
unc-1(e580); ssu-1(fc73)
unc-1(e580)
L2
L4
Young Adult
c
Average number of inclusion bodies
Wild type
unc-1(vlt10)
SL1 SL2
ssu-1(RNAi)
AMP^r (RNAi)
L2
L4
Young Adult
d
Average number of inclusion bodies
pL4440
sul-1
sul-2
sul-3
unc-1(vlt10) L4
e
Average number of inclusion bodies
Wild type
sul-2(gk187)
unc-1(vlt10); sul-2(gk187)
unc-1(vlt10)
L2
L4
Young Adult


a
Average number of inclusion bodies
wild type
daf-12(vlt17)
***
***
***
L2
L4
Young Adult
b
Wild type
Average number of inclusion bodies
**
***
***
Dafadine A (μM)
-
1
-
5
-
12.5
c
Average number of inclusion bodies
***
Wild type
Δ4-dafachronic acid (μM)
-
1
d
mec-3p::110Q
% touch response
***
***
Δ4-dafachronic acid (μM)
-
1
-
-
Dafadine (μM)
-
-
-
12.5


a
Wild type
nhr-1(vlt15)
unc-1(vlt10); nhr-1(vlt15)
nhr-1(vlt16)
unc-1(vlt10); nhr-1(vlt16)
unc-1(vlt10)
Average number of inclusion bodies
0
10
20
30
40
50
60
70
80
90
100
110
120
ns
ns
***
***
***
ns
***
***
**
ns
***
***
L2
L4
Young Adult
b
Wild type
unc-1(vlt10); nhr-1(vlt15)
unc-1(vlt10)
Average number of inclusion bodies
0
10
20
30
40
50
60
70
80
90
**
***
ns
SL1
SL2
unc-54p::nhr-1
L4
c
Wild type
unc-1(vlt10); nhr-1(vlt15)
unc-1(vlt10)
Thrashing/min
0
50
100
150
200
ns
*
****
ns
SL1
SL2
unc-54p::nhr-1
Young Adult


a)
UNC-1
?
Normal electrical synaptic signalling
ASJ
SSU-1
?
Intermediate cell?
Aryl sulfatases
Muscle cells
Aryl sulfatases
NHR-1
Stress response and protein unfolded response genes
UNC-1
?
Disrupted electrical synaptic signalling
ASJ
SSU-1
?
Aryl sulfatases
NHR-1
Stress response and protein unfolded response genes
Steroid hormone
Sulphate moiety
b)
NHR-1
DAF-12
Protective genes
Protein homeostasis maintenance