

GENETIC AND ENVIRONMENTAL MODULATION OF NECROSIS AND HEALTH SPAN
IN *CAENORHABDITIS ELEGANS*

by

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ABSTRACT OF THE DISSERTATION

Genetic and environmental modulation of necrosis and health span in

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Living systems rely on tightly regulated gene expression networks in order to maintain a stable internal milieu, a process known as homeostasis, and to interact with their environment. The study of regulatory networks involved in key processes such as energy metabolism and cellular responses to hostile environmental conditions has immense therapeutic potential and is the focus of a large and growing body of research. This dissertation describes two processes – neuronal necrosis, and the metabolic effects of the phytoecdysteroid 20-hydroxyecdysone in the invertebrate model system *C. elegans*, with a focus on the underlying molecular mechanisms and their potential therapeutic value.

The first part of this dissertation deals with the cellular response to necrosis, a catastrophic event caused by extreme departure from homeostatic conditions due to

exposure to toxic chemicals, extreme temperature or mechanical trauma. Using genetically encoded hyperactive DEG/ENaC ion channels as inducers of necrosis, I describe the role of ER chaperone NRA-2 in the modulation of necrosis caused by calcium excitotoxicity and suggest a broader role for ER chaperones in the regulation of channel expression, with potential therapeutic value in the treatment of disease pathology caused by malformed DEG/ENaC channels. The second part of this dissertation deals with the effects of the plant-derived steroid hormone 20-hydroxyecdysone (20HE) that extends *C. elegans* health span and modulates energy metabolism. I describe the effects of this compound on conserved metabolic pathways and suggest a potential mechanism of action. This study highlights the conservation of gene networks governing nutrient metabolism and the importance of *C. elegans* as a model system in identifying therapeutic targets for the treatment of metabolic syndrome.

In sum, the dissertation describes two unrelated processes in *C. elegans* with the common theme being a conserved genetically encoded regulatory network and its interaction with a specific environmental cue.

DEDICATION

To Aji

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Introduction

Part 1: Neuronal necrosis

Overview

All cellular systems contain multiple intricate and robust pathways to resist external threats to survival and to maintain integrity in the face of insult – mechanical, chemical or biological. These pathways govern cellular responses to pathogens, noxious chemicals, extreme temperature and mechanical trauma. Loss or compromise of these systems thus results in a breakdown in the internal cellular milieu that governs the overall health of the cell – a state known as homeostasis. This breakdown in homeostasis can result in a variety of harmful outcomes, including senescence, malignant transformation, programmed cell death (apoptosis) or necrosis – a process previously described as an uncontrolled response to cellular insult leading to cellular death and lysis, with a pathology that is distinct from the other insults mentioned before. All these pathological conditions are under intense scientific scrutiny in order to better understand their progression and their underlying molecular interactions, and to devise strategies to delay or reverse their outcomes.

Necrosis is characterized by cellular swelling (caused by loss of osmoregulation), loss of organelle integrity, catastrophic dysregulation of cellular signaling

mechanisms and activation of cytotoxic proteins. These processes lead to lysis of the cell and release of noxious chemicals into the surrounding milieu. Thus, necrosis can be described as a progressive condition – a necrosis-inducing insult causes death and rupture of a group of cells, which precipitates further insult and injury to surrounding tissue, a phenomenon known as secondary injury. Secondary injury degrades tissue integrity further, compounds its pathology, and worsens prognosis. Thus, limitation of secondary injury is a key area of clinical research and drug development.

Neuronal necrosis is mainly seen in the context of traumatic brain injury, ischemic stroke or seizure. The insult may be induced by mechanical force, lowered pH due to anaerobic glucose metabolism, or the excessive release of neurotransmitters like glutamate. Necrosis was initially thought of as an uncontrolled response to injury. However, recent efforts have identified molecular events underlying the pathology of necrosis, and conserved molecular pathways that mediate the progression of necrosis. One of the goals of the study of neuronal necrosis is to discover and characterize the cellular networks that respond to the injury. In order to do so, model organisms are studied that have conserved genetic and physiological mechanisms with respect to human neurons. One of the most widely studied model organisms is the microscopic, free-living soil nematode *Caenorhabditis elegans*. This animal is true breeding with a 3-day life cycle that makes genetic manipulation tractable, transparent (which aids the study of internal structures and cells), and straightforward to culture and maintain, making it an ideal model organism for cell

biological study. Furthermore, it was the first organism whose genome was completely sequenced. This information has made it easier to identify genes that are involved in neuronal necrosis, compare the sequences of nematode and mammalian counterparts and look for conservation of structure and function. Past studies have shown that several key processes are conserved from *C. elegans* to humans, and the characterization of neuronal necrosis pathways in *C. elegans* can lead to valuable insights that may aid therapeutic intervention in event of traumatic brain injury, stroke or seizure in humans.

Degenerins and neuronal necrosis

The degenerin family of proteins encodes several transmembrane proteins that act as subunits of multimeric channel complexes. These proteins contain conserved domains that are homologous to mammalian proteins belonging to the family of proteins known as epithelial sodium channels (ENaCs). The DEG/ENaC family of proteins has diverse functions, from mediating mechanosensation to osmoregulation to pain perception, in diverse tissues, both in *C. elegans* and mammals. The degenerin family member MEC-4 is expressed in the six mechanosensory neurons in *C. elegans* that mediate gentle touch. Gain of function alleles *mec-4(u231)* and *mec-4(e1611)* cause degeneration of the mechanosensory neurons, with the process occurring more rapidly in *mec-4(u231)* expressing cells, over a period of ~4 hours after expression of the protein, or about 20 hours after differentiation of the cells into mechanosensory neurons (Hall, Gu et al. 1997). The process of degeneration involves infolding of the plasma membrane, formation of

electron-dense whorls and cellular swelling. This is followed by chromatin aggregation and nuclear invagination. The final stages of the degeneration are characterized by disintegration of organelles and cell lysis (Hall, Gu et al. 1997). Mutations in another degenerin family member *deg-1* lead to similar outcomes (Hall, Gu et al. 1997), with *deg-1(u38)* being the strong necrotic inducer similar to *mec-4(u231)* – henceforth referred to as *mec-4(d)*. Further studies revealed numerous other members of the degenerin family that, when mutated, gave rise to abnormal morphological and functional phenotypes. UNC-8 is a degenerin protein expressed in ventral cord motor neurons and interneurons, along with DEL-1, another degenerin. Gain of function alleles *unc-8(e15)* and *unc-8(n491)* cause uncoordinated movement and swelling of ventral cord motor neurons (Tavernarakis, Shreffler et al. 1997). UNC-105 is a degenerin expressed in muscle that, when mutated causes hypercontraction (Garcia-Anoveros, Garcia et al. 1998). DEG-3 is a degenerin that functions as an acetylcholine receptor α subunit. *de-3(u662)* – hereafter referred to as *deg-3(d)* - is a gain of function allele that causes degeneration. DEG/ENaC homologs in mammals include the family of acid sensing ion channels (ASICs) that are expressed in mammalian brains and activated by H^+ . Studies have shown that knockdown of ASIC1a in rat brains has a neuroprotective effect in models of ischemia (Xiong, Zhu et al. 2004). Thus, DEG/ENaC proteins across species are involved in the induction of neuronal necrosis.

Other models of necrosis

In addition to hyperactivated DEG/ENaC channels, other insults may also contribute to neuronal necrosis. Expression of a constitutively activated GTP binding protein Gas causes necrosis in *C. elegans* (Berger, Hart et al. 1998). This phenotype is aggravated by deletion of the glutamate transporter *glt-3* (Mano, Straud et al. 2007). ACR-2 is a non- α -nAChR subunit expressed in *C. elegans* ventral cord motor neurons. A gain of function *acr-2* mutant causes degeneration in cholinergic motor neurons in the ventral cord (Barbagallo, Prescott et al. 2010). These phenotypes form the basis of the characterization of cellular pathways that mediate the progression of necrosis.

Role of ER Ca^{2+} and ER chaperones in necrosis

Major downstream effectors of necrosis have been characterized by assaying for enhancement or suppression of toxic DEG/ENaC associated neuronal death. The ER chaperone calreticulin (*crt-1*) was identified as a suppressor of *mec-4(d)* – induced necrosis in *C. elegans* (Barbagallo, Prescott et al. 2010). *crt-1* suppresses *mec-4(d)* – induced necrosis in touch neurons and ventral cord neurons as well as activated $\text{G}\alpha\text{s}$ – induced necrosis in PVC neurons (Barbagallo, Prescott et al. 2010). Calreticulin is an ER resident chaperone that binds and sequesters calcium within the ER. One of the major events following *mec-4(d)* induced insult is a catastrophic release of ER calcium that activates downstream effectors of necrosis. As a result, loss of *crt-1* protects against *mec-4(d)* – induced necrosis. However, *crt-1* does not protect against *deg-3(d)* induced insult, as the *deg-3(d)* mutation alters channel

desensitization and allows for greater influx of calcium into the cell than hyperactivated MEC-4(d) channel. Loss of calnexin function also protects against *mec-4(d)* – induced necrosis, indicating a broad role for calcium binding ER chaperones in the progression of necrosis. In support of this theory, mutations in *itr-1* and *unc-68* (IP3 and RyR receptors respectively in *C. elegans*) block ER calcium release and suppress *mec-4(d)* induced necrosis (Xu, Lambert et al. 2001). Pharmacological intervention also shows consistent results --- thapsigargin, which promotes ER calcium release and prevents its reuptake, enhances necrosis in a *crt-1*; *mec-4(d)* background, while dantrolene, which suppresses ER calcium release, protects against *mec-4(d)* - induced necrosis. EGTA, which reduces cellular calcium also protects against *mec-4(d)* – induced necrosis. These pharmacological interventions are neuroprotective without changing MEC-4(d) levels *in vivo* (Xu, Lambert et al. 2001).

Role of calpains and cathepsins in neuronal necrosis

Increase in intracellular calcium activates a number of proteases that bind to and are activated by calcium. Among these, the aspartyl proteases *asp-3* and *asp-4* and the lysosomal cathepsin *cad-1* have been shown to modulate *mec-4(d)* - induced necrosis (Syntichaki, Xu et al. 2002). The calpain inhibitor MDL-28170 (Z-Val-Phe-CHO) markedly reduces necrosis induced by *mec-4(d)*, *deg-1(d)*, *deg-3(d)* and *Gαs* without reducing expression of the necrotic inducers themselves (Syntichaki, Xu et al. 2002). Activation of calpain proteases is required for thapsigargin – induced necrosis. Moreover, it has been shown that aspartyl and calpain proteases work in

the same pathway; there is no synergistic effect of the knockdown of both these sets of proteases in the suppression of *mec-4(d)* – induced necrosis (Syntichaki, Xu et al. 2002).

Effect of lysosomal biogenesis and acidification on necrosis

Lysosomal biogenesis and acidification enhance *mec-4(d)* –, *deg-3(d)* –, and activated Gαs - induced necrosis (Syntichaki, Samara et al. 2005, Artal-Sanz, Samara et al. 2006). Knockdown of *unc-32* and *vha-12*, which encode vacuolar proton translocating ATPases, suppress thapsigargin – induced necrosis and improve survival under hypoxic conditions, but do not enhance *crt-1*, *unc-68* or *itr-1* conferred suppression of necrosis. This suggests that intracellular acidification occurs after ER calcium release (Syntichaki, Samara et al. 2005, Artal-Sanz, Samara et al. 2006). Treatment of *mec-4(d)* mutants with NH₄Cl or Acridine Orange, which accumulate in lysosomes and neutralize lysosomal pH, suppresses touch neuron degeneration without altering lysosomal distribution. *glo-1* loss of function mutants, which show defective lysosomal biogenesis, suppresses *mec-4(d)* - induced necrosis. *cup-5(ar465)* mutants that show an increased number of acidic lysosomes, enhance *mec-4(d)* – induced necrosis (Artal-Sanz, Samara et al. 2006). In support of these results, knockdown of an intracellular serpin (serine protease inhibitor) *srp-6* that binds to and inhibits calpains and lysosomal cysteine proteases, protects *glo-1* worms against hypotonic shock – induced death (Luke, Pak et al. 2007). SRP-6 protects cells even after lysosomal rupture, and *C. elegans* expressing a transgenic SRP-6 are protected from necrosis induced by heat shock, hypoxia (induced by

sodium azide), hyperoxia (induced by paraquat) and hyperactivated ion channels (induced by ectopic expression of MEC-4(d) (Luke, Pak et al. 2007).

These results suggest that lysosomal biogenesis and acidification play a key role as effectors of necrosis downstream of ER calcium release.

Cellular trafficking proteins and necrosis

Proteins that mediate intracellular membrane trafficking are also known to modulate *mec-4(d)* – induced necrosis. These include synaptotagmin (*snt-1*), which mediates both exocytosis and clathrin-mediated endocytosis; endophilin (*unc-57*), which regulates membrane dynamics; the clathrin adaptor protein homologs *dpy-23* and *unc-11* and the PIP2 regulator synaptojanin (*unc-26*). Motor proteins that bind cellular cargo such as the kinesin heavy chain (*unc-116*) and kinesin related protein (*unc-104*) also suppress *mec-4(d)* – induced necrosis. These studies serve to show the interconnectedness of pathways that are responsible for efficient maintenance of cellular homeostasis.

Autophagy and necrosis

Knockdown of proteins involved in autophagy such as *bec-1* and *unc-51* have been shown to suppress *mec-4(d)* – induced necrosis in *C. elegans*, as well as 6-OHDA mediated death in dopaminergic neurons (Aladzsity, Toth et al. 2007). *unc-51* knockdown suppresses activated Gαs – mediated necrosis in *C. elegans*. Knockdown of the calpain protease gene (*clp-1*) does not enhance this suppression, indicating

that these genes may be functioning in the same pathway (Artal-Sanz, Samara et al. 2006, Dinan and Lafont 2006).

These studies have highlighted the roles of diverse cellular proteins in inducing and effecting necrosis. While diverse cellular pathways are involved, the role of ER calcium regulation remains paramount in the progression of necrosis. Thus, the study of calcium binding proteins, in the ER and elsewhere, can advance our knowledge of the mechanisms underlying neuronal necrosis. A detailed study of the role of one such ER resident chaperone protein in the modulation of neuronal necrosis is described in the following chapter.

Part 2: Modulation of *C. elegans* health span by bioactive compounds

Overview

As described previously, homeostasis in a single cell can be defined as a state of optimal equilibrium between the various intricate processes comprising a living cell. Extrapolated to an organism, this becomes a definition of good health --- the ability to exist optimally and resist sub-optimal conditions. Disruption of homeostasis, by contrast, causes disease. Homeostasis is dependent on intrinsic factors such as gene regulatory networks, as well as extrinsic factors such as food and temperature, and on the interplay between the two. One of the most important functions of the cell (and, by extrapolation, the organism) is the regulation of energy. Eukaryotic cells can metabolize carbohydrates, fats and proteins as well as metabolites derived from these three groups of compounds to generate energy in the form of ATP. In order to achieve optimal health, components of these three groups are vital and consumed in far greater quantities than any other nutrient. However, an imbalance in the uptake of either of these dietary compounds can result in a loss of homeostasis and the onset of disease pathology. This is most evident in humans in the current epidemic of our time – obesity and metabolic syndrome. In order to treat obesity and metabolic syndrome it is important to understand the effect of dietary components on gene regulatory networks that are responsible for energy metabolism.

Bioactive compounds and human health

Human beings use several species of plants and animals in their diet in order to promote vigor and longevity. The systematic characterization of bioactive compounds and their impact on health is thus a large and growing body of work. One class of bioactive compounds and the focus of this section is plant-derived analogs of the insect molting hormone ecdysone. These compounds, known as ecdysteroids, are cholesterol derivatives that are acquired through diet and have been shown to improve muscle function, increase protein synthesis and possess anti-diabetic effects (Dinan and Lafont 2006, Kizelsztejn, Govorko et al. 2009, Gorelick-Feldman, Cohick et al. 2010). The mechanisms behind their action remain elusive, however. Characterization of the metabolic effects of ecdysteroid administration has the potential to improve our understanding of the effects of bioactive dietary lipids on human health as well as elucidating the role of different regulatory networks that govern metabolic processes.

Model systems like *C. elegans* can contribute significantly to this process because many core genetic and biochemical pathways are conserved from *C. elegans* to humans. Moreover, the effects of bioactive compounds on the ageing process can be more readily studied in model systems like *C. elegans* that have short life spans than in mammalian systems.

Biomarkers of health and ageing

In order to effectively measure health span in *C. elegans* it is necessary to have quantifiable measures of health. Studies on *C. elegans* ageing has mostly focused on the life span of the organism. Life span, however, is only one biomarker of aging and not an absolute indicator of health. It has been observed that an age-matched population of wild type *C. elegans* can exist on a spectrum of health from being active and having few age-associated morphological and functional impairments to being decrepit and having multiple age-associated phenotypes (Herndon, Schmeissner et al. 2002). One of the phenotypes that are indicative of ageing worms is the presence of advanced glycation end products or AGE pigments in the body of the worm over time. These are byproducts of *C. elegans* metabolism that contain glycosylated lipids and proteins that accumulate over time (Gerstbrein, Stamatas et al. 2005). The relative levels of AGE pigments between individuals in a population of worms correlates strongly with its survival chances. Thus, worms that live longer show lower levels of accumulated AGE pigments compared to worms that do not (Gerstbrein, Stamatas et al. 2005). AGE pigment accumulation can thus inform on the relative viability of an ageing *C. elegans* population. *C. elegans* swimming behavior is also age-dependent. Younger worms swim faster and for longer periods without fatigue as compared to older worms. This behavior can be quantified by computerized tracking software and the resultant measures can be used to correlate swimming performance with viability (Restif et al, submitted). This phenotype moreover can help distinguish between healthy and unhealthy individuals in an age-matched population.

Chapter 3 describes the detailed characterization of the bioactive ecdysteroid 20-hydroxyecdysone, its impact on *C. elegans* metabolism and health and the implications of the study on human health.

NRA-2/Nicalin regulates neuronal death by controlling localization of toxic DEG/ENaCs in *C. elegans*

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Running title: NRA-2/nicalin and toxic DEG/ENaCs localization

Abstract

Hyperactivated, DEG/ENaC channels induce neuronal death through excessive cation influx and disruption of intracellular calcium homeostasis. *C. elegans* DEG/ENaC subunit MEC-4 is hyperactivated by the (d) mutation and induces death of the touch neurons. Homologous mutation in subunit MEC-10 (MEC-10(d)) co-expressed in the same neurons, is only mildly neurotoxic. We exploited the less toxic nature of MEC-10(d) to identify mutations that enhance neuronal death. We report here that knockout of the *C. elegans* nicalin homolog NRA-2 enhances MEC-10(d)-induced neuronal death. Cell biological assays in *C. elegans* neurons show that NRA-2 controls the distribution of MEC-10(d) channels between the ER and the cell surface. Electrophysiological experiments in *Xenopus* oocytes support this notion and suggest that control of channel distribution by NRA-2 is dependent on the subunit composition. We propose that nicalin/NRA-2 functions in a quality control mechanism to retain mutant channels in the ER, ultimately establishing the extent of neuronal death. Nicalin in mammals may have a similar role in DEG/ENaCs biology, therefore influencing pathological conditions such as ischemia.

Key words: *C. elegans*, Nicalin, quality control, trafficking

Introduction

Under conditions of localized oxygen deprivation in the brain, neuronal energy deficits promote an excitotoxic cascade in which ion channels are excessively activated to induce neuronal necrosis (McCall 2010). Although the primary insult is often localized to a small site, successive rounds of secondary neuronal death ensue as dying neurons release channel-hyperactivating glutamate to lethally excite their neighbors, expanding the region of cell death to markedly exacerbate functional losses. Understanding the molecular mechanisms operative in necrotic neuronal death is critical for the design of neuroprotective strategies, although challenges of *in vivo* dissection of the death process in higher organisms are substantial.

Genetic dissection of necrosis mechanisms in model organisms has revealed conserved pathways that contribute to necrotic death {Driscoll, 2003 #6855;McCall, 2010 #16}. In *C. elegans*, specific gain-of-function mutations in DEG/ENaC family channel subunits induce the necrotic death of the neurons in which they are expressed {Huang, 1994 #368;Driscoll, 1991 #72;Shreffler, 1995 #1862}. MEC-4, a pore-forming subunit of the mechanosensory channel complex in *C. elegans* touch receptor neurons (TRNs) that is required for sensing gentle mechanical stimuli (typically delivered to the animal as a brush with an eyelash hair), can be altered to a neurotoxic form (Driscoll and Chalfie 1991). Large sidechain amino acid substitutions for a small AA near the pore domain (MEC-4(A713V), referred to here as MEC-4(d)) create a hyper-activated channel that is permeable to Na⁺ and Ca²⁺ (Chelur, Ernstrom et al. 2002, Goodman, Ernstrom et al. 2002, Bianchi, Gerstbrein et

al. 2004) Calcium dysregulation in *mec-4(d)* neurons is amplified by calreticulin-dependent release of calcium from ER stores (Xu, Lambert et al. 2001, Bianchi, Gerstbrein et al. 2004), which activates cytosolic calpain proteases (Syntichaki, Xu et al. 2002) that ultimately influence deleterious lysosomal activity (Luke, Pak et al. 2007). That the mammalian neuronal DEG/ENaC channel subunit ASIC1 contributes to necrotic death in mouse ischemia models (Xiong, Zhu et al. 2004, Yermolaieva, Leonard et al. 2004, Chu and Xiong 2013), supports that conserved mechanisms of neuronal DEG/ENaC hyper-activation toxicity are operative across phyla.

MEC-10 is a second DEG/ENaC channel subunit co-expressed with MEC-4 in *C. elegans* TRNs (Huang and Chalfie 1994). Introduction of an engineered transgene encoding the *mec-4(d)*-analogous toxic amino acid substitution into *mec-10* – A693V - to create *mec-10(d)*, induces a low level of necrosis associated with neuronal swelling and featuring a similar toxicity mechanism (Zhang, Bianchi et al. 2008). The low level of toxicity associated with the *mec-10(d)* transgene presents an opportunity to screen for genes that can mutate, or be knocked down with RNAi approaches, to enhance necrosis. Such genes would be anticipated to identify negative regulators of channel biosynthesis and activity, or to define factors that normally protect against downstream neurotoxic events.

Here we report that disruption of mammalian nicalin homolog *nra-2*, an ER protein implicated in *C. elegans* muscle AChR channel maturation (Almedom, Liewald et al. 2009), enhances MEC-10(d)-induced necrosis via a mechanism that increases surface expression of the MEC-10(d) channel. Our data further underscore the role

of excessive channel activity in necrosis induction, identify NRA-2/nicalin as a new player in DEG/ENaC channel biogenesis, and reveal an unexpected concentrated surface distribution of MEC-10(d) channel in touch neurons. Since mammalian DEG/ENaC functions modulate cystic fibrosis, renal diseases, hypertension, and ischemia-associated neurotoxicity, insights into regulated surface expression might be exploited in design of interventions that modulate channel activity in human disease.

Results

A targeted RNAi screen for calcium-responsive genes that modulate channel-induced necrosis identifies ER chaperone *nra-2*

To extend molecular understanding of necrosis mechanisms, we sought to identify modulators of genetically-induced touch neuron degeneration. Because calcium is a key factor in neuronal necrosis across species (Bianchi, Gerstbrein et al. 2004, Yamashima 2004, Sendrowski, Rusak et al. 2013), we selected a collection of 191 *C. elegans* genes that encode EF hand calcium-binding proteins for targeted knockdown by feeding RNAi (Kamath, Martinez-Campos et al. 2001). We first constructed a strain with weak necrosis inducer *mec-10(d)* (Huang and Chalfie 1994) that co-expresses GFP in touch receptor neurons (TRNs) and is sensitive to RNAi in neurons (*P_{mec-4}mec-10(d)::GFP; nre-1(hd20); lin-15B(hd126)*; screen strategy summarized in Figure 1A). At 15°C we observed baseline PLM tail touch neuron degeneration of ~2%, as described previously (Zhang, Bianchi et al. 2008). We then

fed this strain either empty vector control (L4440) or dsRNA EF hand gene clones from the L1 to L4 larval stages and evaluated the extent of neuronal death by counting surviving fluorescent PLM tail TRNs. Of the 191 EF hand genes tested, RNAi directed against clone T05F1.1 (*nra-2*) exhibited the greatest enhancement of *mec-10(d)*-induced necrosis: a 3 -fold increase compared to vector control treatment. We confirmed the RNAi phenotype using a second, neuronally sensitized RNAi strain (Calixto, Chelur et al. 2010) (*P_{mec-18}::sid-1*; *P_{sng-1}::YFP* in *P_{mec-4}::mec-10(d)::GFP*; *P_{mec-4}::mCherry*). We grew this strain at 20°C on empty vector or *nra-2* dsRNA-expressing bacteria from the L1 to L4 larval stages and counted all surviving TRNs at the L4 stage. We found that *nra-2* knockdown caused over four-fold enhancement of *mec-10(d)*-induced TRN death (Figure 1B), confirming the enhancer outcome for *nra-2(RNAi)*.

To rule out any extraneous effect of RNAi on death enhancement, we constructed a *nra-2(Δ)*; *mec-10(d)* double mutant strain. The double mutant exhibited over five-fold increase of *mec-10(d)* – induced TRN degeneration as compared to *mec-10(d)* alone (Figure 1C). As further confirmation that *nra-2* was the enhancer locus, we expressed the *nra-2(+)* transgene in TRNs of *nra-2(Δ)*; *mec-10(d)* animals and observed a 3-fold reduction of TRN degeneration compared to control *nra-2(Δ)*; *mec-10(d)* animals, i.e., a restoration of TRN degeneration to *mec-10(d)* levels (Figure 1C). This transgene rescue both confirms that the *nra-2* mutation confers the death-enhancing phenotypes and establishes that *nra-2* acts cell autonomously within the touch receptor neuron to modulate necrosis. Since *nra-2(Δ)* itself does

not induce any TRN necrosis (Figure 1C), our data establish that loss of *nra-2* function results in increased necrosis due to *mec-10(d)*-induced insult.

NRA-2 does not play an essential role in the differential distribution of MEC-10 and MEC-4 subunits in touch receptor neurons

NRA-2 encodes a transmembrane ER-resident protein implicated in regulation of acetylcholine receptor subunit composition (AChRs) in *C. elegans* muscle, possibly by allowing only specific subunit combinations of that receptor to exit the ER (Almedom, Liewald et al. 2009). This role in channel assembly raises the question as to whether NRA-2 might similarly regulate distribution, assembly, or surface expression of the touch-sensing MEC channel. The gentle touch-sensing MEC channel is proposed to be composed of MEC-4 and MEC-10 subunits (Huang and Chalfie 1994, O'Hagan, Chalfie et al. 2005, Gessmann, Kourtis et al. 2010). However, some data suggest that the MEC channel assembled *in vivo* on neuronal processes is primarily made up of MEC-4 subunits (Chatzigeorgiou, Grundy et al. 2010) and the MEC channel at the cell body is heteromeric, containing MEC-4 and MEC-10 subunits. In support of this model, MEC-4::GFP localizes in puncta along the process length (Zhang, Arnadottir et al. 2004, Chatzigeorgiou, Grundy et al. 2010) (Figure 2A), but MEC-10::GFP is localized at the cell body (Chatzigeorgiou, Grundy et al. 2010) (Figure 2B). Consistent with localized functional roles, *mec-4* null mutants are insensitive to touch delivered anywhere along the length of the process, but *mec-10* null mutants are insensitive only to touch administered in the vicinity of the soma, but are otherwise sensitive to touch along the process. Thus, MEC-4

homomeric channels might be the primary transducers of touch along the process, but MEC-4/MEC-10 heteromers might function in sensing touch at the cell body (Chatzigeorgiou, Grundy et al. 2010).

If NRA-2 plays a role in restricting MEC-4 and MEC-10 to different regions within the touch neuron, the distributions of MEC-4::GFP and/or MEC-10::GFP might be altered in the *nra-2* mutant background. We constructed lines that were MEC-4::GFP; *nra-2*(Δ) and MEC-10::GFP; *nra-2*(Δ) and compared basic subunit distribution +/- *nra-2*. (Note that both translational fusion constructs have C-terminal tags and can functionally rescue cognate mutations (Hong, Mano et al. 2000, Chatzigeorgiou, Grundy et al. 2010)). Our comparison of distribution of tagged MEC-4 and MEC-10 proteins did not reveal any striking distribution changes +/- *nra-2* (Figure 2A and 2B). General level of channel expression as measured by fluorescence intensity also appeared similar +/- *nra-2* (Figure 2C and 2D). Thus analysis of tagged reporters does not support a major role for NRA-2 in changing the distinctive distribution of MEC-4::GFP and MEC-10::GFP channel subunits or in regulating overall GFP-tagged subunit stabilities.

To address whether native subunits at physiological expression levels might be differentially affected by *nra-2* deficiency, we scored touch sensitivity over the process length +/- *nra-2* (primarily a score of MEC-4 function). We observed that the *nra-2*(Δ) mutants were only modestly touch insensitive (Figure 2E). Moreover, when we touched in the vicinity of the cell body +/- *nra-2*, we noted only a slight

change from 2 to 10% touch insensitive in *nra-2(Δ)* (n=100, not significant by paired 2-tailed t test; Supplementary Fig. 1). Taken together, our data support that most mechanotransducing complexes are distributed and function even when NRA-2 is lacking. These data suggest that NRA-2 acts on toxic rather than wild type DEG/ENaC subunits.

***nra-2(Δ)* does not enhance *mec-4(d)*-induced neuronal death**

We identified *nra-2(RNAi)* as an enhancer of *mec-10(d)*-induced necrosis, but potential effects on the stronger necrosis inducer *mec-4(d)* were unknown. To test whether *nra-2(Δ)* can enhance *mec-4(d)*-induced neuronal death, we constructed *nra-2(Δ); mec-4(d)* double mutants and measured the extent of TRN death. As previously reported, *mec-4(d)* induces a high level of TRN degeneration (~85% of cells die), in striking contrast to *mec-10(d)* (Driscoll and Chalfie 1991, Huang and Chalfie 1994). We found that the absence of *nra-2* does not enhance neuronal death further than occurs in *mec-4(d); nra-2(+)* (Figure 2F). These data, together with localization and touch sensitivity data, are also consistent with a model in which NRA-2 does not play a major role in MEC-4 biology, although we cannot be certain of a “ceiling effect” for *mec-4(d)*-induced necrosis in which higher levels of neuronal death are not possible. Our findings raise the possibility that NRA-2 might interact specifically with only the MEC-10 or MEC-10(d) subunit *in vivo*.

MEC-10(d) concentration in the ER compartment is diminished when *nra-2* is absent

Toxicity of the MEC-4(d) channel subunit is associated with enhanced channel activity (Chelur, Ernstrom et al. 2002, Goodman and Schwarz 2003, Bianchi, Gerstbrein et al. 2004, Brown, Fernandez-Illescas et al. 2007), and thus enhanced MEC-10(d) channel activity is a plausible mechanism for increased cell death. Given that NRA-2 is an ER protein involved in AChR channel maturation, we wondered whether NRA-2 acts in the ER to limit surface expression of the MEC-10(d) channel. To address this hypothesis, we first monitored subcellular distribution of MEC-10(d)::GFP in various intracellular compartments, +/- *nra-2*.

We elected to work with cultured *C. elegans* touch neurons {Sangaletti, 2013 #6848; Bianchi, 2006 #60}, which allowed us greater freedom to use immunohistochemical and imaging techniques that are impractical *in vivo*. We measured colocalization of MEC-10(d)::GFP with fluorophore-labeled antibodies for endosome (EEA-1) and Golgi body (mAbSQV8) and we dye-labeled lysosomes and ER in cultured TRNs of *nra-2*(Δ);*mec-10(d)::GFP* and control *mec-10(d)::GFP* animals (Figure 3A-3H). Mean MEC-10(d)::GFP colocalization with endosomes, lysosomes or Golgi bodies did not significantly differ between *nra-2*(Δ) and control animals. Mean ER colocalization of the MEC-10(d)::GFP and ER, however, was ~ 25% for *nra-2* mutants as compared to 50% for control *mec-10(d)* animals (Figure 3I). Our data suggest that at the intracellular level, MEC-10(d)::GFP is localized primarily to the ER, and that the absence of *nra-2* reduces ER-associated MEC-10(d)::GFP.

NRA-2 regulates surface expression of MEC-10(d)

Low levels of MEC-10(d)::GFP in the ER might reflect enhanced degradation or enhanced trafficking out of the ER to the cell surface. Given the necrosis-enhancing phenotype of *nra-2* deletion and the overall similar fluorescence levels for MEC-10(d)::GFP +/- *nra-2*, the latter model seemed more plausible. To test the hypothesis that low MEC-10(d)::GFP in the ER might result from altered trafficking from ER to the surface, we quantified surface distribution of MEC-10(d)::GFP in cultured TRNs of *nra-2(Δ);mec-10(d)::GFP* and control *mec-10(d)::GFP* animals by total internal reflection fluorescence microscopy (TIRFM). TIRFM uses an angled incident laser beam to illuminate surface fluorescent probes by inducing total internal reflection of the incident beam at the interface of coverslip and culture medium (Martin-Fernandez, Tynan et al. 2013). The resultant low-intensity evanescent wavelets penetrate ~150 nm into the medium and thereby excite fluorescent proteins at or near the plasma membrane. TIRFM thus allows live imaging of plasma membrane-associated proteins with high X-Y resolution and very low noise.

Our TIRFM imaging revealed a striking concentration of surface MEC-10(d)::GFP at a site near the nucleus, either with or without *nra-2*. The biological reason for this highly restricted distribution of MEC-10(d)::GFP is unclear, but might underlie its role in touch perception near the cell body (see Discussion). Importantly, our TIRFM studies showed that mean fluorescence intensity of surface-localized MEC-10(d)::GFP was significantly increased in the *nra-2(Δ)* background ($p < 0.0001$, ~1200 units control, ~2400 units *nra-2(Δ)*; Figure 4A-B). These data suggest that

loss of *nra-2* function results in an increase of surface-associated MEC-10(d)::GFP. Coupled with data supporting decreased ER-associated MEC-10(d)::GFP in *nra-2(Δ)*, our data suggest a role for NRA-2 in ER retention of MEC-10(d). The increased MEC-10(d) surface expression in the *nra-2(Δ)* background, accompanied by the expected increased mutant channel ion influx, could be the mechanistic basis for the enhanced neurotoxicity.

NRA-2 limits MEC channel currents in an oocyte expression system

Co-expression of MEC-4(d), MEC-2, and MEC-6, in *Xenopus* oocytes generates an amiloride-sensitive channel with a large inward Na^+ current as well as a Ca^{2+} -activated Cl^- current that reveals inward Ca^{2+} conductance (Chelur, Ernststrom et al. 2002, Goodman, Ernststrom et al. 2002, Bianchi, Gerstbrein et al. 2004). MEC-4(d) is essential for the generation of both currents and, interestingly, addition of MEC-10(d) diminishes these currents (Chelur, Ernststrom et al. 2002, Goodman, Ernststrom et al. 2002, Bianchi, Gerstbrein et al. 2004, Brown, Liao et al. 2008). Our *in vivo* and cell culture data suggest that enhanced necrosis in TRNs of *nra-2* mutants may be attributed to increased ion influx due to increased surface expression of MEC-10(d) channels. We tested this model by measuring Na^+ current in oocytes expressing MEC-4, MEC-10(d), MEC-2 and MEC-6 (henceforth described as the MEC-10(d) complex), with or without NRA-2 (Figure 5A-B). We found that MEC-10(d) complex-expressing oocytes showed a mean inward current of 1.5 μA . The addition of NRA-2 to the injected MEC-10(d) subunits reduced mean inward current to 0.15 μA . That co-expression of NRA-2 with the MEC-10(d) complex suppressed Na^+ current by

~90%, is consistent with a negative role for NRA-2 in MEC-10(d) currents (Figure 5C).

We wondered whether the Na⁺ current suppression was due to a MEC-10(d)-specific interaction with NRA-2, as suggested by genetic studies. Surprisingly, however, we found that the addition of NRA-2 could also suppress currents in oocytes expressing only MEC-4 and MEC-4(d) ($p < 0.05$; Figure 5D), indicating that the MEC-10(d) subunit is not essential for NRA-2 impact. Notably, combinations that included MEC-4(d) without native MEC-4(+), were not susceptible to NRA-2-induced current suppression, either with MEC-10(+) or MEC-10(d) co-expressed (Figure 5D). These data support the *in vivo* scoring of neuronal death +/- *nra-2* in *mec-4(d)* animals (Figure 2F) and suggest that a MEC-4(+) subunit might be needed for NRA-2 impact on channel surface expression or that a channel composed of a specific number of mutant versus wild type subunits is impacted by NRA-2. Note that it was not possible to address whether M4/M10 channels or channels composed of M10d only could be suppressed because no current is detectable with these combinations (Goodman, Ernstrom et al. 2002).

In sum, from our electrophysiological studies in the oocyte expression system we make three observations – (i) NRA-2 limits currents for the co-expressed MEC-4, MEC-10(d), MEC-6, MEC-2 channel; (ii) The presence of at least one MEC-4 appears essential to allow NRA-2 current suppression in a channel complex containing either MEC-4(d) or MEC-10(d); and (iii) NRA-2 current suppression does not require the

presence of either MEC-10 or MEC-10(d). These results suggest that the mechanism of NRA-2 action may not be MEC-10(d) – specific, but rather might involve a more complex capacity of NRA-2 to sense the correct conformation of an assembled, or partially assembled, channel complex that involves more than one MEC subunit.

A model for NRA-2 influence on MEC-10(d) neurotoxicity

Our data suggest that NRA-2 acts in the ER to mediate surface expression of properly folded/assembled MEC-4/MEC-10 channels. Correctly folded heteromers traffic to the plasma membrane. When the aberrantly folded MEC-10(d) or MEC-4(d) subunit is present, the specific 3D conformation of the assembling channel (which includes MEC-4) is sensed as being abnormal, and NRA-2 preferentially retains those channels in the ER. In the absence of NRA-2, more channels that contain aberrant subunits escape quality control in the ER, which creates more leaky channels at the cell membrane, leading to enhanced neurotoxicity.

Discussion

Our studies document the role of ER- resident chaperone protein NRA-2 in modulation of MEC-10(d) channel-induced necrosis in *C. elegans*. Combining genetic approaches, live imaging, immunohistochemistry, and electrophysiological assays in *Xenopus* oocytes, we show that loss of *nra-2* function results in an increase in the surface expression of toxic MEC-10(d) channels to enhance TRN necrosis. Conversely, NRA-2 limits channel activity in heterologous expression assays. We

propose that NRA-2 acts in the ER to distinguish specific conformations of assembling channel complexes, restricting exit of immature or mal-formed channels. Our work underscores the theme of enhanced channel activity in necrosis induction, extends understanding of NRA-2 function in channel biogenesis, and suggests a potential *in vivo* molecular strategy for increasing/decreasing surface expression of DEG/ENaC subunits that might be of therapeutic value in some human disease states.

Increased MEC-10(d) channel surface expression is associated with enhanced neurotoxicity.

Previous work has supported that increased activity of mutant DEG/ENaC channels and their associated Na⁺ and Ca²⁺ influx initiates neuronal necrosis (Driscoll and Chalfie 1991, Bianchi, Gerstbrein et al. 2004, Brown, Fernandez-Illescas et al. 2007) {Wang, 2013 #6850}. (Driscoll and Chalfie 1991, Huang and Chalfie 1994) Here we have shown that *nra-2(RNAi)* and *nra-2* deletion enhance *mec-10(d)*-dependent neuronal loss via a cell-autonomous mechanism that increases MEC-10(d) surface expression. In the absence of NRA-2, more MEC-10(d) channels reach the neuronal surface, where they collectively conduct higher cation influx than normal, enhancing neurotoxicity. Thus, our data reinforce the idea that increased channel activity—either induced via subunit abnormality or by increased numbers of plasma membrane channels—is a necrosis-initiating insult.

An unusual subcellular localization of MEC-10(d)::GFP subunits.

Our TIRFM studies highlighted a striking and unexpected concentration of surface-expressed MEC-10(d)::GFP to a highly localized spot at the periphery of the soma.

The MEC-10(d)::GFP is not concentrated near lysosomal compartments, but rather is more prevalently partitioned between the ER and the cell surface domain.

Neurotoxic outcomes support that MEC-10(d) is a component of an active channel.

The concentrated MEC-10::GFP distribution contrasts strongly with the punctate distribution of MEC-4::GFP subunits in the process thought to correspond to gentle-touch transducing touch complexes (Zhang, Arnadottir et al. 2004). The dramatic difference in distribution is consistent with previous studies indicating that MEC-4 is needed for response to touch stimulation all along the neuronal process, whereas MEC-10 is needed for responses to touch stimuli only near the soma

(Chatzigeorgiou, Grundy et al. 2010). Restriction of DEG/NaCs to specific raft domains is known for both invertebrate and mammalian members of this channel class (Sedensky, Siefker et al. 2004, Zhang, Arnadottir et al. 2004, Huber, Schermer et al. 2006, Eshcol, Harding et al. 2008). Interestingly studies of β NaC distribution in mouse C-fiber small diameter neurons suggest a soma rather than process distribution (surface expression not assayed) (Garcia-Anoveros, Samad et al. 2001); and neuronal ASIC1a has been observed to be largely ER concentrated, with regulated surface expression under conditions in which ASIC2a is not similarly distributed (Chai, Li et al. 2010), suggesting differential trafficking and localization as a feature of the channel class shared across phyla. Still, why a distinct MEC-10 channel type might be needed in a focused cellular domain in the soma is not clear.

One possibility is the MEC-10-enriched domain corresponds to a specialized mechanosensory site. Alternatively, this cellular domain might constitute a holding site for end-stage MEC channel surface assembly prior to transport down the process. MEC-10, which can negatively regulate channel activity (Goodman, Ernstrom et al. 2002, Brown, Liao et al. 2008), might be held and added to the MEC complex only under specific physiological conditions, or might even provide a chaperone function. The function of this MEC-10-concentrating cellular domain clearly remains to be elucidated. Because MEC-10 plays a minor role in gentle touch, a novel activity could be anticipated.

Expanding understanding of NRA-2 function

A broad role in channel biogenesis in multiple cell types. NRA-2 was originally identified as a protein that co-immunoprecipitated with a muscle-specific *C. elegans* acetylcholine receptor (Gottschalk, Almedom et al. 2005). Later studies revealed a role for *nra-2* in AChR subunit selection and/or surface expression in muscle, but failed to detect *nra-2*-dependent changes in neuronal GABAergic signaling, raising the possibility of tissue-specific activity (Almedom, Liewald et al. 2009). In demonstrating cell autonomous functions of *nra-2* in touch receptor neurons, our data expand the documented cell types in which *nra-2* has been shown to function. Neuronal *nra-2* function is consistent with studies of fluorescently tagged NRA-2 that showed expression in neurons (Almedom, Liewald et al. 2009). Our work also shows that NRA-2 exerts functions on more than a single channel type (i.e. both AChRs and DEG/ENaCs), suggestive of a broad role in channel biosynthesis.

What might NRA-2 recognize during channel biogenesis? The effect of adding NRA-2 to the channel in *Xenopus* oocytes holds implications for NRA-2 mechanism of action. Our genetic studies revealed that *nra-2* impacts *mec-10(d)*-, but not *mec-4(d)*-induced neuronal death, initially raising the possibility that NRA-2 acts exclusively on MEC-10(d). In heterologous channel assays in *Xenopus* oocytes, activity of the M4 M10d channel is ~90% inhibited by the addition of NRA-2. However, we also noted significant suppression of current (~45%) in channels that were M4 M4d, which lacked any M10 subunit, ruling out a required targeted action on MEC-10. M4d M10 and M4d M10d channels were refractory to NRA-2 addition. Oocyte assays thus suggest: 1) MEC-10(d) is not required for NRA-2 inhibition of MEC channel current, but, 2) wild type MEC-4 subunits might be required since they were present in all the combinations that were inhibited by NRA-2 addition. Although technical aspects limited testing all possible combinations of interest (M4, M10, and M10(d) do not form assayable channels on their own (Goodman, Ernstrom et al. 2002)), we hypothesize that NRA-2 susceptible channel complexes need to include a wild type MEC-4 subunit and a d-type substitution in MEC-4(d) or MEC-10(d) to be recognized for NRA-2-mediated retention. We infer that NRA-2 recognizes an aberrant conformation of a MEC channel complex, rather than a specific, abnormally folded subunit on its own.

An NRA-2-related complex might influence channel biogenesis across species

It is noteworthy that reported regulated surface expression of mammalian channel ASIC1a shares striking features with the MEC-10(d) data we observe: concentration in ER at a site surrounding the nucleus and differential trafficking of the ASIC1a subunit (for ASIC1a, under serum or insulin deprivation; but co-expressed ASIC2a is not impacted) to enhance channel activity (Chai, Li et al. 2010). Regulated ER retention may thus underlie differential DEG/ENaC subunit expression across species.

NRA-2 is a resident ER protein that is most homologous to human nicalin, and more distantly related to the nicastrin subunit of the mammalian γ -secretase complex. Nicastrin can promote maturation and trafficking of other proteins in the γ -secretase complex and has been suggested to act as a nucleation center to regulate inclusion of specific components of the complex (Shah, Lee et al. 2005). Mammalian nicalin works in a complex with NOMO (Haffner, Frauli et al. 2004) and TMEM147 (Dettmer, Kuhn et al. 2010), and can modulate Nodal/TGF β growth factor signaling in Zebrafish (Haffner, Frauli et al. 2004) via an unclear mechanism that does not appear to involve protease activity.

Human nicalin can partially rescue *nra-2* defects in *C. elegans* muscle, and NOMO-homologous NRA-4 co-IPs along with NRA-2 when AChR is targeted (Almedom, Liewald et al. 2009). Moreover, *nra-4* mutants have similar defects to *nra-2* mutants and to *nra-2; nra-4* double mutants in *C. elegans* muscle, supporting roles in same

process and activity in the same complex. Considering structural and functional similarities along with our clear demonstration of NRA-2 influence on MEC-10(d) surface expression, we speculate that the mammalian Nicalin/NOMO complex might also influence DEG/ENaC surface expression (and possibly other channels). If so, this step might be considered for therapeutic modulation of DEG/ENaC channel activity in cystic fibrosis (Hobbs, Da Tan et al. 2013), renal disease (Alvarez de la Rosa, Navarro-Gonzalez et al. 2013), pain management, and possibly ischemic stroke (Kweon and Suh 2013).

Materials and Methods

C. elegans strains

Nematodes were maintained according to standard procedures {Brenner, 1974 #1372}. Transgenic strains were generated as described {Fire, 1986 #6857}.

Strains/alleles used:

N2 (wild type);

RB1480: *nra-2(ok1731)*

ZB4038: *zdlIs5[P_{mec-4}GFP]; mec-4(u231)*

ZB4039: *zdlIs5[P_{mec-4}GFP]; mec-4(u231); nra-2(ok1731)*

Transgenic strains used:

ZB4031: [*P_{mec-18::sid-1}*; *P_{sng-1}YFP*]; *Is[P_{mec-4}mec-10(d)::GFP*; *P_{mec-4}mCherry*]. Generated from strain TU3312 (*P_{mec-18}Sid-1*; *P_{sng-1}YFP*) and integrated by X-irradiation, according to standard procedures.

ZB4032: *Is[mec-10(d)]*; *P_{mec-4}mec-10(d)::GFP*; *P_{mec-4}mCherry*

ZB4033: *Is[mec-10(d)]*; *nra-2(ok1731)*

ZB4034: *zdlIs5[P_{mec-4}GFP]*; *mec-4(u231)*

ZB4035: *bzIs141[P_{mec-4}mec-4::GFP]*

ZB4036: *bzIs142 [P_{mec-4}mec-10::GFP]*;

ZB4037: *P_{mec-4}mec-10(d)GFP*; *nre-1(hd20)*; *lin-15B(hd126)*

ZB4045: *bzIs141[P_{mec-4}mec-4::GFP]*; *nra-2(ok1731)*

ZB4046: *bzIs142[P_{mec-4}mec-10::GFP]*; *nra-2(ok1731)*

Cell death assay

% TRN degeneration was scored as described before (Zhang, Bianchi et al. 2008).

Briefly, mCherry-labeled TRNs were counted in *Is[mec-10(d)]* and *nra-2(ok1731)*;

Is[mec-10(d)] strains using a Zeiss Imager.D1m microscope and a Zeiss

PlanApochromat 20X NA 0.8 objective. Average number of surviving TRNs per

animal was calculated as the total number of TRNs counted divided by the total

number of animals counted.

***C. elegans* cell culture**

C. elegans embryonic cells were cultured following published procedures {Christensen, 2002 #1947; Sangaletti, 2013 #6741; Sangaletti, 2013 #6848}. Briefly, gravid adults were cultured on enriched peptone plates (8P plates) seeded with *Escherichia coli* strain Na22. Eggs were isolated by lysing animals with a solution containing fresh bleach and 10N NaOH. Eggs were separated from animal carcasses by treatment with 30% of sucrose dissolved in egg buffer solution (NaCl 118 mM, KCl 48 mM, CaCl₂ 2 mM, MgCl₂ 2 mM, Hepes 25 mM, pH 7.3, 340 mOsm). The eggshell was removed by treatment with 2 mg/ml chitinase (Sigma Aldrich, catalog #. C6137) and cells were dissociated by manual pipetting through a 27 gauge needle, resuspended in culture media (L-15 + 10 % FBS + 45 mM sucrose + Penicillin 100 IU and Streptomycin 100 µg/ml) and plated on glass coverslips previously covered with peanut lectin (0.5 mg/ml). Cells were seeded at a density of ~230,000 cells/cm² and used 4-6 days after seeding.

Strains used for all microscopy were *Is[mec-10(d)]* and *nra-2(ok1731); Is[mec-10(d)]*.

Immunofluorescence staining and microscopy

Fluorescent photographs of GFP *C. elegans* were taken using a Zeiss Axiovert 200M microscope equipped with GFP filter and a QImaging Rolera e-mc² camera with Metamorph software. For MEC-4::GFP and MEC-10:GFP quantification, photographs were taken using the same exposure time (300 ms) and quantifications were performed in ImageJ.

C. elegans primary embryonic cell culture was set up in 35mm glass bottom cell culture dishes (Bioexpress, cat. # T-2881-16). Primary embryonic cells from *C. elegans* were fixed in 4% paraformaldehyde for 20 minutes, and permeabilized for 10 minutes with M9+0.1% Tween20. Cells were blocked in 2% BSA for 1 hour at RT, incubated with primary antibody for 1 hour at RT, washed 3X with M9+0.01% Tween20, incubated with secondary antibody for 1 hour at RT, washed 3X with M9+0.01% Tween20 and imaged on a Zeiss Axiovert microscope with a 63X NA 1.3 objective. Images were taken at 250ms exposure, at 1x1 binning. Images were deconvolved using Autoquant X3. Measurements were made on thresholded images using MetaMorph software.

Primary antibodies used: mouse polyclonal α -EEA-1 at 1 ug/ml, mouse mAb SQV8 (Developmental Studies Hybridoma Bank - dshb.biology.uiowa.edu) at 1 ug/ml. Secondary antibodies used: anti-mouse Cy5 (Jackson ImmunoResearch cat. # 115-175-146) at 1:5,000 dilution

ER Tracker and LysoTracker staining: *C. elegans* primary embryonic cell culture was set up as described above. ER tracker Blue White DPX (Life Technologies cat. # MP12353) or LysoTracker Blue DND-22 (Life Technologies cat. #L-7525) were incubated at 1uM concentration in M9+0.01% Tween20 for 30 minutes and washed 3X with M9+0.01% Tween20 prior to imaging.

TIRF microscopy

C. elegans primary embryonic cell culture was set up using *Is[mec-10(d)]* and *nra-2(ok1731); Is[mec-10(d)]* strains as described above.. Cells were imaged live on an

Olympus IX70 microscope using an Olympus PlanApo 60X NA 1.45 TIRFM objective. Touch neurons in cell culture were identified by mCherry expression using standard wide-field microscopy. TIRF imaging was done using a Melles Griot Ar488 laser (Melles Griot Laser Group, Carlsbad, CA) and an Olympus IX2 Laser Combiner. A micrometer screw was used to change the incident beam angle to 61° for TIRF mode imaging. All images were taken at 500ms exposure using iVision software (BioVision Technologies Inc. Exter, PA). Image quantification was done in ImageJ. Images were thresholded, an ROI was drawn around the cell body and mean intensity of fluorescent signal was measured. Statistical analysis was performed using GraphPad Prism 6.

Electrophysiology

Complementary RNAs (cRNA) were synthesized using T7 mMESSAGE mMACHINE kit, purified and run on denaturing agarose gels to check for size and integrity. cRNA quantification was performed spectroscopically. Stage V–VI oocytes were selected among multistaged oocytes dissected by 2-h collagenase treatment (2 mg/ml in Ca²⁺-free OR2 solution) from *Xenopus laevis* ovaries. Oocytes were injected with 10 ng/oocyte of each cRNA and incubated in OR2 medium + 500 μM amiloride. OR2 solution consisted of 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 0.5 g/l polyvinyl pyrrolidone, and 5 mM HEPES (pH 7.2), supplemented with penicillin and streptomycin (0.1 mg/ml) and 2 mM Na-pyruvate at 20°C for 2–3 days before recording. Currents were measured using a two-

electrode voltage-clamp amplifier (GeneClamp 500B; Axon Instruments) at room temperature. Electrodes (0.2– 0.5 M Ω) were filled with 3 M KCl, and oocytes were perfused with a physiological NaCl solution containing (in mM) 100 NaCl, 2 KCl, 1 CaCl₂, 2 MgCl₂, and 10 HEPES, pH 7.2. We used the pCLAMP suite of programs (Axon Instruments) for data acquisition and analysis. Currents were filtered at 200 Hz and sampled at 1 kHz. Currents are expressed as control minus 500 μ M amiloride (Goodman, Ernstrom et al. 2002, Bianchi, Gerstbrein et al. 2004).

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Author contribution

SK designed and performed experiments, analyzed data and wrote the manuscript, SY performed experiments, LB designed and performed experiments, analyzed data and wrote the manuscript. MD designed experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. *nra-2* disruption enhances *mec-10(d)*-induced necrosis in *C. elegans*

(A) Screen strategy for identification of enhancers of *mec-10(d)*-induced necrosis

Touch receptor neuron channel subunit *mec-10(d)* encodes an amino acid substitution in the pore that induces low levels of necrosis in TRNs, as measured by number of surviving GFP⁺ cells. Enhancer loci are identified as those that confer increased levels of necrosis after RNAi knockdown, diminishing the number of apparent GFP-expressing neurons.

(B) RNAi disruption of *nra-2* in strains sensitized for neuronal RNAi enhances *mec-10(d)*-induced necrosis. % TRN necrosis in *P_{mec-18}sid-1* expressing nematodes subjected to *nra-2*(RNAi) or empty vector control L4440. We scored surviving touch neurons by GFP signal, % degeneration was determined by the formula $((1 - (x/6)) \times 100)$ where x is the number of surviving neurons. Error bars, s.e.m.; average of 3 repeat trials (each with at least 35 L4 animals), * p=0.0261, Student's t-test.

(C) *nra-2* deletion enhances *mec-10(d)*-induced necrosis. We scored % degeneration as in (B). *nra-2(Δ)* is *nra-2(ok1731)*; rescue transgene is *Ex[P_{mec-4}*nra-2*; P_{ttx-3}RFP]*. Error bars, s.e.m.; average of 3 repeat trials (each with at least 30 L4 animals), ** p=0.0033, one-way ANOVA.

Figure 2. *nra-2*(Δ) does not induce significant changes in MEC-4 and MEC-10 distribution and is associated with a modest change in overall touch sensation

(A) MEC-4::GFP (puncta in processes and dispersed in soma) shows similar patterns in wild type and *nra-2*(Δ) backgrounds. Images are representative of 30 individual cell observations (Scale bar = 5 μ m).

(B) MEC-10::GFP (soma restriction) shows similar patterns in wild type and *nra-2*(Δ) backgrounds. Images are representative of 30 individual cell observations (Scale bar = 5 μ m).

(C) MEC-4::GFP intensity in soma in wild type and *nra-2*(Δ) backgrounds, n =30 for each strain.

(D) MEC-10::GFP intensity in soma in wild type and *nra-2*(Δ) backgrounds, n =30 for each strain.

(E) Touch sensitivity was recorded as the fraction of nematodes that respond to the first five touches with an eyelash pick. Error bars, s.e.m. n=3 (30 animals per experiment), **** p<0.0001, one-way ANOVA.

(F) % TRN necrosis in *mec-4(d)* vs. *nra-2(Δ)*; *mec-4(d)* is not significantly different. We scored surviving touch neurons by GFP signal, % degeneration was determined by the formula $((1-(x/6))*100$ where x is the number of surviving neurons. Error bars, s.e.m.; 3 trials; n>35 L4 animals.

Figure 3. *nra-2(Δ)* lowers the proportion of MEC-10(d) present in the ER

(A-H) Representative examples of distribution of MEC-10d::GFP relative to compartment reporters for endosomes (EEA-1 Ab staining (WT A and *nra-2(Δ)* B), SQV-8 mAb staining (WT C and *nra-2(Δ)* D), LysoTracker staining (WT E and *nra-2(Δ)* F), ER Tracker staining (WT G and *nra-2(Δ)* H). Thresholded images were measured for colocalization of red organelle marker with green MEC-10(D)::GFP.

I). Quantification of MEC-10(d)::GFP accumulation in subcellular compartments. Deconvolved thresholded images were measured for colocalization using Metamorph. Error bars, s.e.m. n>20. * p<0.05, Student's t-test.

Figure 4. TIRF imaging suggests that more MEC-10(d) is situated at the cell surface in *nra-2(Δ)* mutants.

(A) Representative images of *nra-2(+)* and *nra-2(Δ)* neurons that express *mec-10(d)::GFP* fluorescence in culture. MEC-10(d)::GFP fluorescence is visualized under

TIRF conditions and cell boundary is traced in red outline. All images were taken using a 60X NA 1.45 Zeiss objective.

(B) Quantification of mean surface channel expression measured by TIRF microscopy. Exposure time for all images was 500 ms. Error bars, s.e.m. n=50 cells. **** p<0.0001, Student's t-test.

Figure 5. NRA-2 suppresses MEC-10(d) – induced currents in *Xenopus* oocytes.

(A) and **(B)** Representative traces of Na⁺ current in *Xenopus* oocytes induced by expression of MEC channel subunits containing MEC-10(d) without NRA-2 (A) and with co-injection of NRA-2 (B). Voltage protocol was from -160 mV to + 100 mV from a holding potential of -30 mV.

(C) Current amplitude histogram of Na⁺ current recorded at -100 mV in oocytes with and without co-injection of NRA-2 (n=9). ** p<0.01, Student's t-test.

(D) % current suppression of MEC-4(d) and/or MEC-10(d) – induced Na⁺ current by NRA-2 co-injection in oocytes. p values indicate significance of suppression over controls not injected with NRA-2, by Student's t-test.

Figure 6. A model of NRA-2 function in *C. elegans* TRNs.

A working model of NRA-2 interaction with a mechanosensory channel composed of wild type and MEC-10(d) subunits. Top half: NRA-2 binding in ER results in sorting of mechanosensory channels, with the MEC-10(d) – containing channels

preferentially retained in the ER. Bottom half: In an *nra-2*(Δ) background, there is no retention of degenerative channels in the ER, and the resultant accumulation of toxic channels leads to enhanced neurotoxicity.

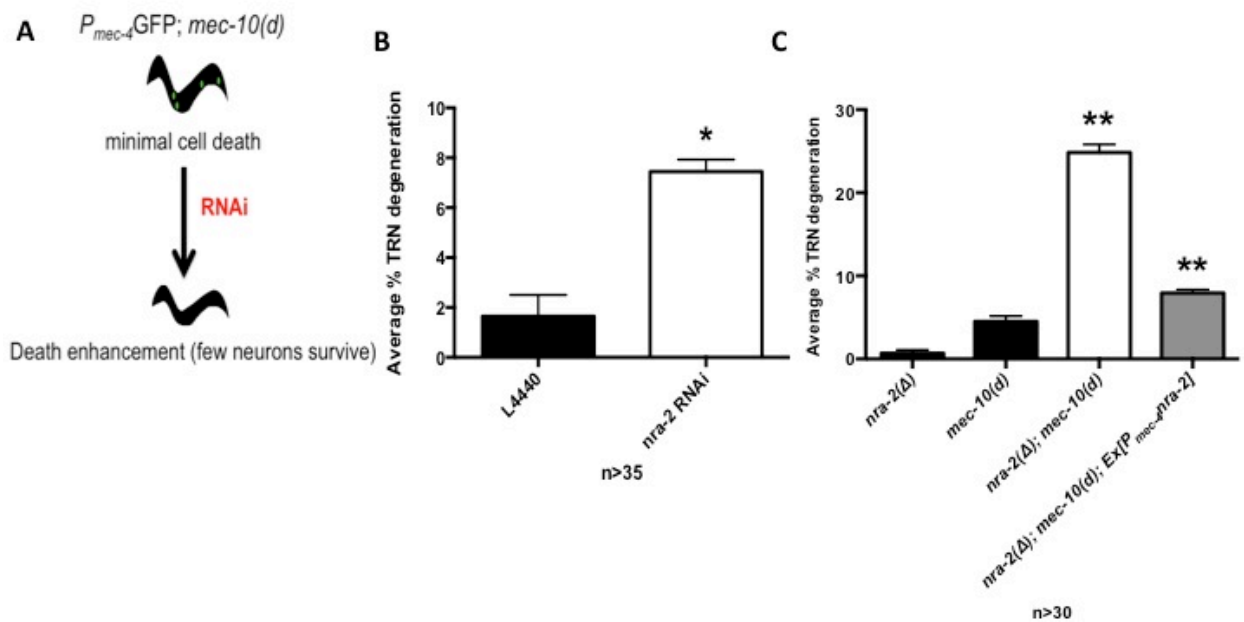


Figure 1

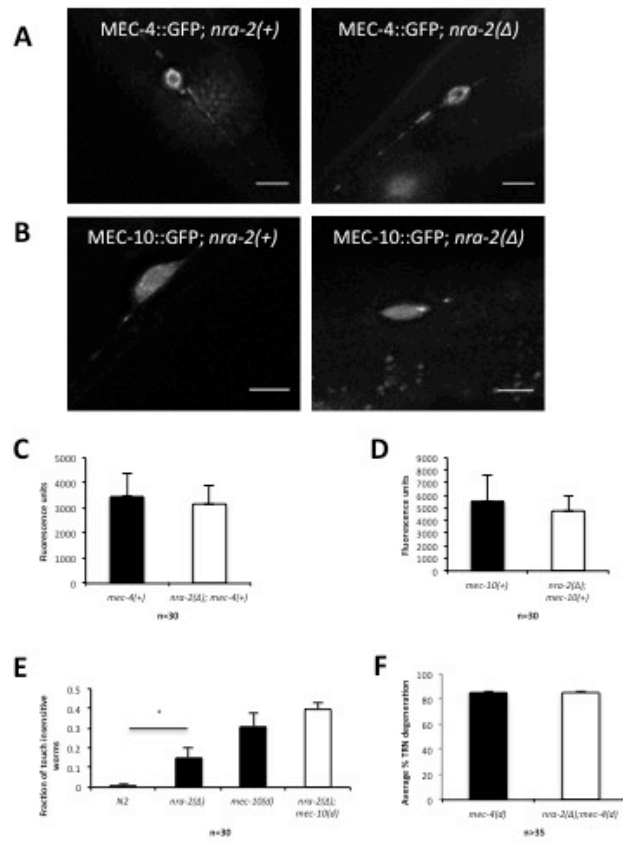


Figure 2

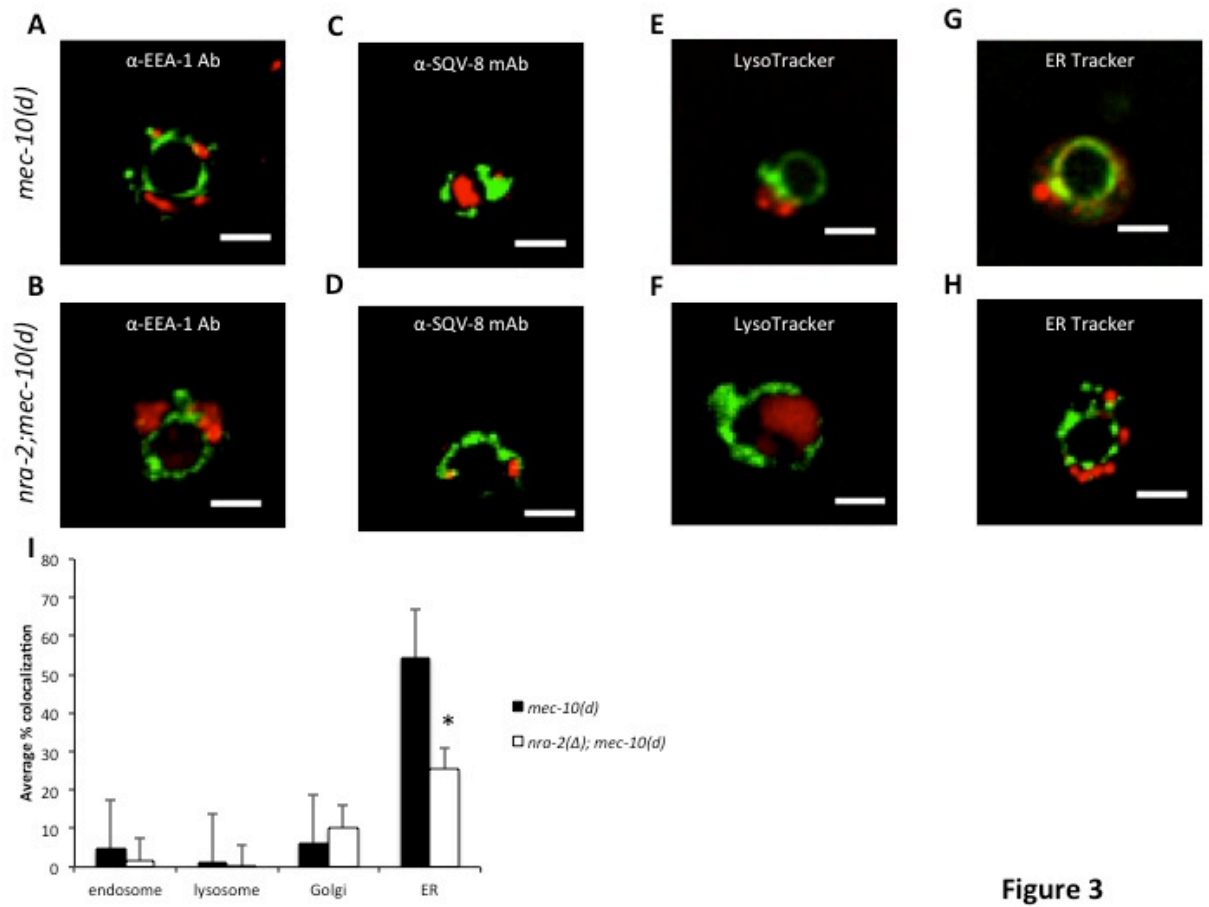


Figure 3

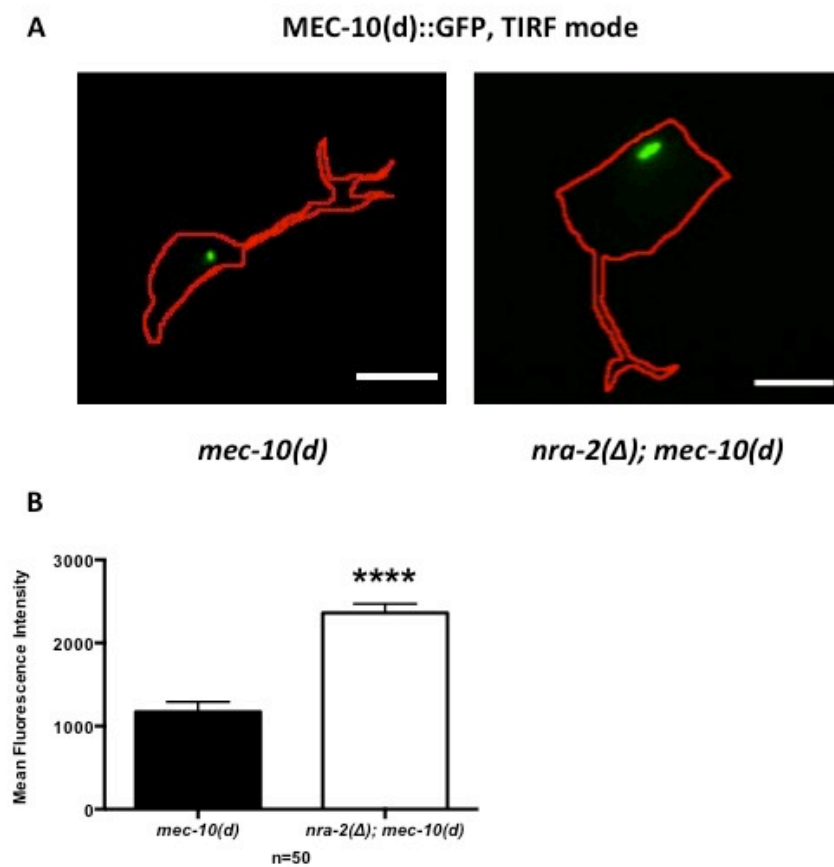


Figure 4

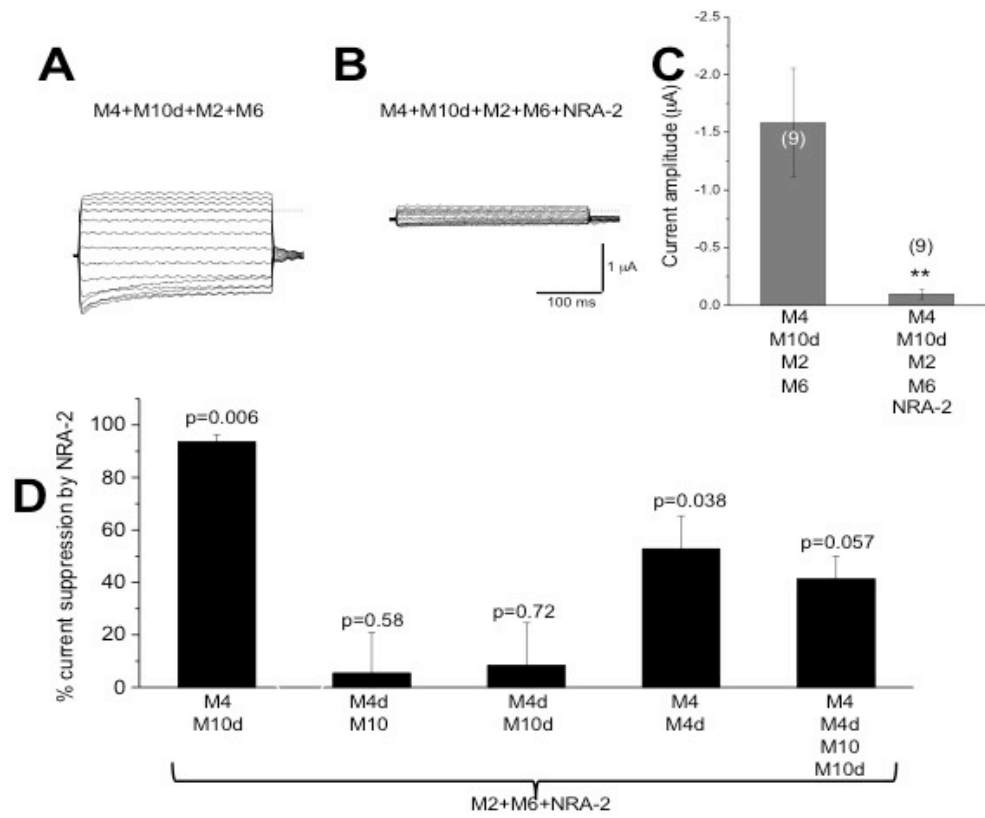


Figure 5

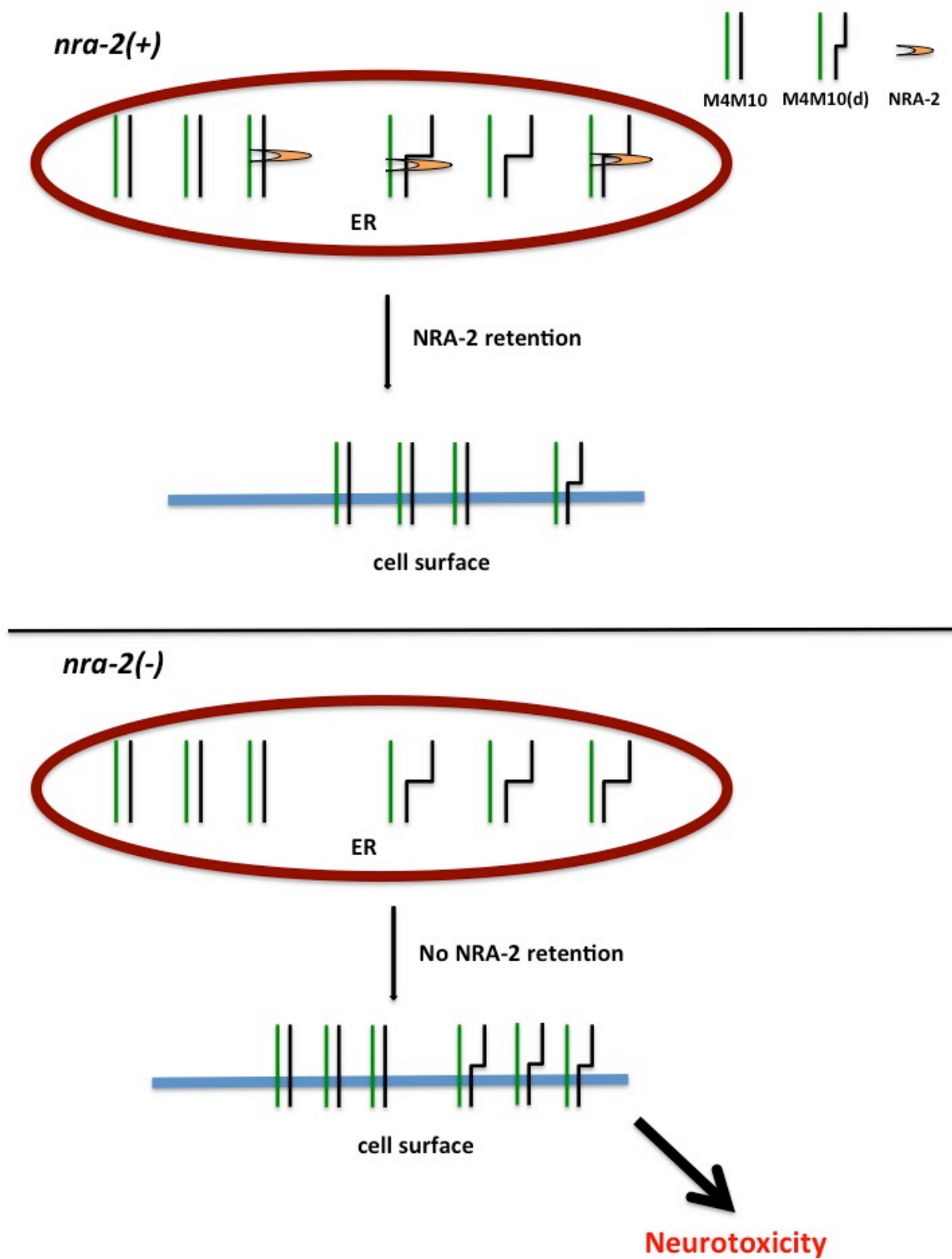


Figure 6

**A plant-derived PPAR α agonist regulates energy metabolism and
extends health span in *C. elegans***

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Shrutika Yeola, Ilya Raskin and Monica Driscoll

Abstract

Plant species are a rich source of bioactive compounds that impact metabolism and show beneficial effects on health and longevity. Here we describe the effects of 20-hydroxyecdysone (20HE), a polyhydroxylated sterol that improves locomotory prowess and extends median life span in *C. elegans*.

Treatment with 20HE for 1 week of adult life yielded a dramatic improvement in locomotory performance of the poorest performers in a population of wild type *C. elegans* and lowered AGE pigment levels, total fat content and ROS accumulation, while increasing mitochondrial respiration. This effect was independent of *daf-16* but required *hsf-1*. The *C. elegans* PPAR α ortholog NHR-49 was required for this benefit, and NHR-49 – regulated gene *fat-7* was upregulated. Furthermore, HeLa cells treated with 20HE for 24 hours showed an upregulation of PPAR α protein levels. We propose that 20HE acts as a PPAR α agonist and promotes longevity and health by modulating energy metabolism and inducing mitohormesis.

Introduction

Understanding the basic cellular mechanisms underlying longevity and health is the focus of a growing body of work in diverse model systems. Work on *C. elegans* has provided key insights into the role of metabolic pathways in regulating health and life span. The insulin-IGF-1 signaling pathway (IIS) and dietary restriction pathways have been shown to be major factors in determining longevity of *C. elegans* (Guarente and Kenyon 2000, Lapierre and Hansen 2012, Tissenbaum 2012, Wilkinson, Taylor et al. 2012). In addition, fat metabolism is being increasingly scrutinized for its relationship to ageing and longevity (Ackerman and Gems 2012, Hansen, Flatt et al. 2013).

Hitherto the focus of ageing research has been to quantify longevity directly by measuring the duration of life of adult *C. elegans*, or to quantify biomarkers such as advanced glycation end products (AGE pigments) in *C. elegans* tissues to provide insight into the nature of the ageing process (Gerstbrein, Stamatas et al. 2005, Kenyon 2010). Ageing is associated with a number of morphological abnormalities as well as functional decline (Herndon, Schmeissner et al. 2002). A behavioral assay that can quantify functional decline can provide insight into the nature of the ageing process as well as provide clues about the metabolic regulation underlying good health. We attempted to use locomotory vigor as a quantifiable behavioral measure of health and to study the effects of a bioactive compound on locomotory prowess of a population of age-matched *C. elegans* in order to provide clues to its efficacy. The

locomotory assay thus provides us with a starting point to a comprehensive study on the effects of bioactive compounds on metabolic health in *C. elegans*. To this end, my work relies on the use of computer vision program developed in our lab called CeleST (*C. elegans* swim test) that quantitates 10 aspects of swim posture (Restif et al, submitted).

Plants synthesizing bioactive compounds have been used for centuries in traditional recipes to promote vigor and delay ageing. Recent research on these plant species has led to the identification of a number of bioactive compounds that act on key metabolic processes, from ATP-generating catabolic processes to immune modulation to antioxidant capabilities (Dong, Guha et al. 2012). Ecdysteroids (analogs of the insect molting hormone ecdysone) comprise one group of bioactive phytochemicals that has been studied for over forty years for its beneficial effects on mammalian physiology, in addition to its effects on insect development (Dinan and Lafont 2006). 20-hydroxyecdysone (20HE) is one of the most commonly synthesized ecdysteroid in plants and often the most predominantly expressed out of the milieu of bioactive compounds. 20HE has been shown to bind the insect ecdysone receptor (EcR), a nuclear hormone receptor that heterodimerizes with Ultraspiracle (USP) to modulate insect development and innate immunity (Beckstead, Lam et al. 2005). Recent studies have elucidated some of the anabolic effects of 20HE on mammalian models (Bathori, Toth et al. 2008, Gorelick-Feldman, Cohick et al. 2010), and also highlighted its role in ameliorating symptoms of metabolic syndrome such as hyperglycemia and weight gain (Kizelsztejn, Govorko et

al. 2009). However, the underlying mechanism by which 20HE exerts its numerous effects on mammalian physiology remains elusive.

We attempted to use *C. elegans* to identify the pathways that were affected by 20HE treatment and to outline a mechanism that explains the diverse metabolic changes that occur as a result of 20HE treatment. *C. elegans* possesses numerous genes encoding nuclear hormone receptors (Antebi 2006). However, like mammalian systems, *C. elegans* does not possess an ecdysone receptor. We developed a locomotory assay as a first indicator of healthy metabolism and organ function. We tested whether 20HE treatment had any effects on locomotory performance and found a subtle but distinct phenotype that 20HE significantly increased locomotory performance in 10-day old *C. elegans*, an effect that was magnified in “eliminating” the poorest performing individuals in a population and insignificant in the best performing individuals. That is to say that 20HE increased the general locomotory health of a population by elevating the lowest level of performance. Our observation suggests that functional decline in homogenous populations may be the result of subtle variations in metabolic function. We hypothesize that there may be conserved function between *C. elegans* nuclear hormone receptors and their mammalian counterparts. We identified *nhr-49*, a PPAR α ortholog, as a key mediator of this locomotory phenotype. Finally, we show that 20HE can upregulate mammalian PPAR α and suggest that the beneficial effects of 20HE on locomotory performance and other age-related biomarkers may be due to an adaptive stress

response to increased mitochondrial respiration caused by a NHR-49/PPAR α – mediated increase in fatty acid catabolism.

Results

20HE treatment extends multiple aspects of *C. elegans* healthspan

20HE is reported to confer health benefits in humans (Dinan and Lafont 2006), but the mechanisms by which 20HE acts to promote robustness is unknown. To date, 20HE effects in invertebrate models highly amenable to mechanistic dissection has not been used to further understanding of pathways that contribute to 20HE effects. To test for potential healthspan extension effects of 20HE on *C. elegans*, we reared age-synchronized cultures on plates -/+ 20HE and scored survival over adult life (Fig. 1A). We found that animals reared in the presence of 500uM 20HE lived longer than untreated controls ($p < 0.0001$ by Mantel-Cox test). Notably, we found that the median survival was markedly extended, with a 20 day median lifespan for 20HE as compared to a 16 day median lifespan for untreated animals, ($p < 0.0001$, two trials, $n = 100$ per trial). Maximum lifespan, however, was not significantly changed.

We also assayed the effects of 20HE treatment on AGE pigment levels in *C. elegans*. AGE pigments include naturally fluorescent glycosylated lipids (Advanced Glycation End-products) and heterogenous cross-linked materials referred to as lipofuscin that accumulate in lysosome-like compartments over time in the *C. elegans* gut as well as in non-dividing mammalian tissues (Gerstbrein, Stamatatos et al. 2005). The

accumulation of age pigments is thought to impair lysosomal function. There is an inverse correlation between levels of accumulated AGE pigments and old age health in wild type *C. elegans* i.e., high age pigment scores correlate with short life expectancy and accelerated locomotory decline (Gerstbrein, Stamatas et al. 2005). We measured AGE pigment fluorescence at 430nm, normalizing scores to tryptophan fluorescence, which does not change with age. We found that untreated animals exhibited a normalized AGE/TRP ratio of 1.08 while 20HE-treated animals showed a ratio of 0.77, a decrease of nearly 40% ($p=0.0395$, Figure 1B). Thus 20HE treatment limits accumulation of age pigments in aging animals.

As in humans, *C. elegans* locomotory capacity declines with age via a muscle decline that resembles human sarcopenia. To test the effect of 20HE on locomotory decline, we measured swim behavior in late middle age, -/+ 20HE. We assayed swimming by measuring the number of head thrashes made in liquid medium over a period of 30 seconds using a software platform developed in-house for tracking and measuring *C. elegans* locomotory performance. At 10 days of life, we recorded a significant difference between treated and untreated animals, with 20HE-fed animals exhibiting a higher mean thrashing rate (Figure 1C, $p=0.0014$ by unpaired t-test). Interestingly, our analysis of dot plots suggested amelioration seen in the overall population score was a consequence of improved the swimming of the weakest swimmers (Figure 1D). For example, if we compared swim scores of the lowest 25% of the population at day 10 (bottom quartile), we found a three fold difference in swim capacity (Fig. 1E). In contrast, when we compared the top two

swim score quartiles of the treated vs. untreated populations we found no significant difference in locomotory performance (Figure 1F). In sum, 20HE treatment confers beneficial effects on *C. elegans* life span and healthspan, with an apparent impact of limiting the decline of the weakest sector of the population.

20HE may act via insulin signaling, with a strong dependence on downstream *hsf-1*, rather than *daf-16*.

Given that 20HE confers distinctive healthspan effects on aging *C. elegans*, we sought to define the molecular pathway(s) that it might engage to confer benefits.. The insulin-IGF-1 like signaling (IIS) pathway is a highly conserved pathway that influences longevity across phyla (Hsieh, DeFord et al. 2002, Masternak, Panici et al. 2009, Kenyon 2011). Reduction-of-function of the *C. elegans* insulin receptor DAF-2 results in a long-lived phenotype, whereas knockdown of the downstream transcription factor DAF-16/FOXO results in diminished life span and poor health (Lin, Hsin et al. 2001). To determine whether 20HE might exert its effects via insulin signaling, we first performed *daf-2(RNAi) +/- 20HE*. We measured swimming performance at 7 days of adult life. We observed that *daf-2(RNAi)* increased the average number of head thrashes/30s in the untreated cohort, confirming the previously described effects of *daf-2* knockdown on locomotory healthspan (Lin, Hsin et al. 2001)(Figure 2A). The addition of 20HE to the culture did not further improve locomotory performance above *daf-2(RNAi)* levels. Our data are consistent with a model in which 20HE acts via *daf-2* IIS to extend locomotory healthspan.

To further investigate the potential role of IIS in 20HE action, we tested for the requirements for two major transcription factors that act downstream of *daf-2* IISR, *daf-16* and *hsf-1*. We found that *daf-16(RNAi)* decreases swim scores across quartiles. The addition of 20HE treatment to *daf-16(RNAi)* animals, however, results in a significant improvement in swim performance, restoring swim scores close to wild type levels without 20HE treatment (Figure 2A). Our data support that some of 20HE benefits can occur via a *daf-16* independent mechanism. That swimming prowess does not reach levels conferred by 20HE in a wild type animal, suggests that *daf-16* could be responsible for some of the 20HE benefits.

HSF-1 is also activated under conditions of low insulin signaling (Chiang, Ching et al. 2012). We observed that *hsf-1(RNAi)* caused significant locomotory impairment in older *C. elegans*. Moreover, 20HE treatment had no effect on locomotory performance of *hsf-1(RNAi)* animals (Figure 2A). Our data identify *hsf-1* as a transcription factor required for 20HE benefits, that might be activated under conditions of low insulin signaling. Thus, our studies implicate the insulin signaling pathway in 20HE benefits via *hsf-1* but in part independently of *daf-16*.

20HE engages some genes that mediate dietary restriction in a *pha-4*/FOXO-dependent, but *skn-1*-independent, pathway.

Another major conserved pathway for healthspan and lifespan involves dietary restriction, which can be mediated somewhat independently of insulin pathway

components, although cross talk can occur and some common machinery can be used (Steinkraus, Smith et al. 2008, Shimokawa and Trindade 2010). We sought first indication that 20HE might engage DR by testing whether 20HE treatment could further extend locomotory benefits of the *eat-2(ad1116)* mutant, which is impaired for feeding (McKay, Raizen et al. 2004) and lives longer via a DR mechanism (Figure 2B). We find that 20HE does not further improve older age swimming prowess above baseline in the *eat-2* mutant, consistent with the possibility that 20HE might engage pathways in common with DR to promote healthspan.

To further probe potential DR pathways in 20HE effects, we knocked down FoxA transcription factor *pha-4* and the bZip transcription factor *skn-1*, needed for longevity in specific DR paradigms (Panowski, Wolff et al. 2007, Paek, Lo et al. 2012). We found that *pha-4*(RNAi) abrogated the effects of 20HE treatment (Figure 2B), revealing an essential role for this transcription factor in 20HE benefits. In contrast, addition of 500uM 20HE to the media fully rescued *skn-1*(RNAi)-induced impairment of old age swimming (Figure 2B). Thus, 20HE benefit is independent of *skn-1*. Overall, our data support that 20HE may have some DR mimetic capacity, using PHA-4/FOXA transcription factor to confer benefits.

20HE benefit requires NHR-49

20HE is a polyhydroxylated sterol, and thus we hypothesized that 20HE binds to a steroid hormone receptor. Previous studies in mammalian systems have implicated

vertebrate homologs of the insect ecdysone receptor (EcR) such as liver X receptor (LXR) and farnesoid X receptor (FXR) as potential targets of 20HE (Dinan and Lafont 2006), but the receptor that binds 20HE has remained elusive. The *C. elegans* genome encodes 284 nuclear hormone receptors, with most of the diversity of *C. elegans* nuclear hormone receptors thought to arise from gene duplication of an ancestral HNF4 (hepatocyte nuclear factor) gene (Antebi 2006). 15 *C. elegans* nuclear hormone receptors share closest homology with human nuclear hormone receptors. Of the human-related *C. elegans* nuclear hormone receptors, *nhr-49* encodes a nuclear hormone receptor that regulates fatty acid metabolism in *C. elegans* and is proposed to be the *C. elegans* functional ortholog of the mammalian peroxisome-proliferator-activated receptor (PPAR α) (Van Gilst, Hadjivassiliou et al. 2005). We therefore tested the effect of *nhr-49*(RNAi) on 20HE-treated *C. elegans* and observed that *nhr-49*(RNAi) completely abrogated 20HE locomotory benefit (Figure 3A). We repeated this experiment with *nhr-49* deletion allele *nhr-49(nr2041)* and confirmed a requirement for *nhr-49* in 20HE-conferred locomotory benefits (Figure 3B).

Since NHR-49 is considered to be a functional ortholog of PPAR α , we decided to test whether 20HE treatment in a mammalian system causes a detectable change in protein levels of PPAR α . We therefore treated HeLa cells with vehicle control (0.07% ethanol) or 50uM 20HE for 24 hours and performed a western blot on the lysates. We observed that an anti-PPAR α monoclonal antibody recognized a band of 52 kDa size, which is the expected size of PPAR α (Figure 3C). Densitometric analysis

revealed an increase in the size of the band for the 20HE-treated HeLa cells compared to the control ($p < 0.05$ by paired two-tailed t-test – Figure 3D). Our data suggests that 20HE treatment upregulates PPAR α in HeLa cells. Given prior work on *nhr-49* (Van Gilst, Hadjivassiliou et al. 2005) our finding also suggests that the primary mechanism of 20HE benefit might be via modulation of fat metabolism.

20HE influences fat metabolism in *C. elegans*

NHR-49 has been implicated in regulation of fat metabolism in *C. elegans* (Van Gilst, Hadjivassiliou et al. 2005) by regulating the expression of enzymes modulating lipid composition and enhancing β -oxidation (Van Gilst, Hadjivassiliou et al. 2005). If 20HE activates NHR-49, we expect to see a decrease in fat accumulation in *C. elegans* in treated animals. To quantify fat accumulation in animals treated with 20HE, we performed Oil Red O staining in 3-day old *C. elegans* that were cultured in NGM media containing 500uM 20HE. Oil Red O is a lysochrome dye that stains neutral triglycerides and lipids. The intensity of Oil Red O staining has been shown to positively correlate with triglyceride levels measured biochemically (O'Rourke, Soukas et al. 2009). We observed a significant decrease in fat staining in 3-day old adults fed 500uM 20HE compared to controls (Figure 4A, 4B). Furthermore, if 20HE were to activate NHR-49 we would expect to see an activation of its downstream targets. One of the most strongly regulated targets of NHR-49 is the steroyl-CoA desaturase FAT-7 (Van Gilst, Hadjivassiliou et al. 2005). We cultured *C. elegans* expressing P_{fat-7}::GFP and found that GFP levels were significantly increased in 3-day

old *C. elegans* fed 500uM 20HE (Figure 4C, 4D). Together, our results indicate that 20HE treatment causes upregulation of *C. elegans* genes that promote fat breakdown and leads to a decrease in fat accumulation

20HE-induced health benefit involves mitohormetic response

How might activation of fat metabolism increase longevity and locomotory vigor in *C. elegans*? One hypothesis is that increase in β -oxidation causes an increase in mitochondrial respiration and accumulation of reactive oxygen species, which then triggers an adaptive response known as mitohormesis (Ristow and Zarse 2010). Features of mitohormesis include a long-term reduction in reactive oxygen species, the induction of the mitochondrial unfolded protein response, and mitophagy (Owusu-Ansah, Song et al. 2013). To ask whether 20HE might induce mitohormesis, we tested whether 20HE increased basal and maximal oxygen consumption rates (OCR) in *C. elegans*. Basal OCR is a measure of the steady-state rate of respiration of mitochondria. Maximal OCR is a measure of the upper limit of mitochondrial respiration rate, which occurs upon exposure to the ionophore FCCP that neutralizes the mitochondrial inner membrane potential. We found that injection of 500uM 20HE into a mixed culture of *C. elegans* caused a transient increase in basal oxygen consumption rate (OCR) over controls by a peak value of 15% within 8 minutes of treatment (Figure 5A). Addition of the oxidative phosphorylation uncoupler FCCP caused a 42% increase in OCR in the control population and a 75% increase in the 20HE-treated population, indicating that 20HE was associated with a significant increase in maximal OCR (Figure 5A). These results suggest that even a

transient exposure to 20HE may significantly increase mitochondrial respiration rate and could induce mitohormesis.

If a mitohormetic response were involved we would expect to see an overall decrease in the accumulation of reactive oxygen species (ROS) due to activation of antioxidant enzymes such as superoxide dismutase. Cell-permeable dyes like dichlorodihydrofluorescein diacetate (DCF-DA) can be used to test levels of reactive oxygen species as they get oxidized within the cell to emit green fluorescence whose intensity can be measured with a plate reader. To test our hypothesis we measured the oxidation of DCF-DA over 4 hours in *C. elegans* treated with vehicle control (0.07% ethanol) or 500uM 20HE for 48 hours. We observed a 50% decrease in accumulated ROS in 20HE-treated *C. elegans*, ($p=0.0346$, Figure 5B). Finally, we knocked down the *C. elegans* mitochondrial superoxide dismutase *sod-3* by RNAi and observed that 20HE-treated *sod-3* *C. elegans* did not exhibit any significant increase in bottom quartile locomotory performance over controls, indicating that 20HE benefit requires *sod-3* (Figure 5C). These results together suggest that 20HE treatment increases mitochondrial respiration rate and that this may result in the activation of a mitohormetic response that is dependent on *sod-3* activation.

Discussion

Overview

Our study demonstrates the effect of a plant-derived ecdysteroid on an evolutionarily conserved metabolic network that modulates fat metabolism and prolongs health. We show that 20-hydroxyecdysone (20HE) binds to the conserved nuclear hormone receptor PPAR α *in vitro*. The activation of the 20HE pathway in *C. elegans* engages genes involved in fatty acid catabolism, like *fat-7*, leading to lowered fat accumulation and an increase in mitochondrial respiration. This increase in mitochondrial respiration provokes the mitohormetic response, an adaptive stress response that promotes cellular health. The result is that 20HE treatment causes a significant improvement in aging and fitness biomarkers in *C. elegans*, shifting these away from the poorest performers evident in an untreated population. Our study mirrors the results seen in mammalian models showing that PPAR activation reduces weight gain and hyperglycemia, suggesting that 20HE treatment causes global health benefits in diverse species.

Bioactive properties of ecdysteroids

Ecdysteroids (analogs of the insect molting hormone ecdysone) exert numerous effects on invertebrate as well as on vertebrate physiology. Ecdysteroids have been

widely studied for their effects on insect metamorphosis (Beckstead, Lam et al. 2005, Kirilly, Wong et al. 2011), on innate immunity (Rus, Flatt et al. 2013) and on their favorable anabolic, non-androgenic effects in mammalian models (Dinan and Lafont 2006). In mammals, ecdysteroids have been shown to have therapeutic benefit for the treatment of metabolic syndrome and obesity (Kizelsztejn, Govorko et al. 2009). In our study, we describe a mechanism for the action of the ecdysteroid 20-hydroxyecdysone (20HE), one of the most common ecdysteroids synthesized by plants, in the nematode *C. elegans*. *C. elegans* lacks the biochemical machinery to synthesize 20HE from cholesterol, and does not possess an obvious homolog of the ecdysone receptor (EcR), that binds 20HE (Chitwood and Feldlaufer 1990). Here we show that the bioactivity of 20HE in nematodes as well as in mammals, involves an interaction with the peroxisome proliferator activated receptor alpha (PPAR α) and modulation of the biochemical pathways that determine fat metabolism.

PPAR α and fat metabolism

PPARs belong to a family of adopted orphan nuclear receptors that bind to dietary lipids with low affinity and form heterodimers to regulate downstream gene transcription. PPAR δ activation leads to displacement of the nuclear co-repressor (NCoR1) complex from the promoter region of PPAR δ target genes (Fredens, Engholm-Keller et al. 2011). Strongly regulated targets of PPAR α include enzymes mediating fat breakdown such as lipoprotein lipase (Schoonjans, Peinado-Onsurbe et al. 1996), fatty acid catabolism such as acyl CoA oxidase and carnitine palmitoyl

transferase 1 (CPT1). *C. elegans* orthologs of these enzymes are regulated by NHR-49 (Van Gilst, Hadjivassiliou et al. 2005, Fredens and Faergeman 2012), making it a functional ortholog of PPAR α .

C. elegans NHR-49 shares homology with the mammalian hepatocyte nuclear factor HNF4. It is plausible that *nhr-49*, along with several other *C. elegans* nuclear hormone receptors, may have evolved by gene duplication of an ancestral HNF4-like gene. The HNF4 ligand binding domain has not been shown to bind steroid ligands; however, functional studies have shown that genes regulated by PPAR α in mammalian systems have homologs in *C. elegans* that are regulated by *nhr-49*, making it a functional ortholog of PPAR α (Van Gilst, Hadjivassiliou et al. 2005). Further studies, including detailed binding assays in cell models will need to be performed to confirm the affinity of 20HE to NHR-49/PPAR α .

Mammalian studies have shown that PPAR α activation causes an increase in lipolysis and β -oxidation of fatty acids. PPAR α agonists like fenofibrate are used as serum lipid-lowering agents in patients with dyslipidemia and metabolic syndrome. Over the past decade, several PPAR agonists have been used in clinical trials for the treatment of type II diabetes and metabolic syndrome. However, nearly all of these drugs have been shown to possess side effects of treatment, including severe reduction of HDL cholesterol (in the case of PPAR α agonists), heart failure or tumorigenesis (in the case of PPAR γ agonists). A PPAR agonist such as 20HE that

lowers risk factors for cardiovascular disease with minimal side effects is therefore an attractive target for drug development.

Differential benefit of 20HE in *C. elegans* populations

A key observation of our study was the differential benefit offered by 20HE treatment to different quartiles within a homogenous, age-matched population of *C. elegans*. On the whole, 20HE treatment induced a small but statistically significant locomotory benefit in treated populations compared to controls. What was more significant was that this locomotory benefit was more dramatic among the 1st quartile, or poorest performers in the group when compared to 1st quartile performers in the control group. This observation allows for two conclusions – either the benefit induced by 20HE treatment specifically affects only those individuals that have compromised performance, or that 20HE benefit is reduced in individuals that are metabolically healthier. The latter explanation is more likely, as other assays performed in our study indicate a significant difference between control and treated populations even when they are not sorted by quartiles. This would indicate that 20HE likely benefits all individuals in a population, but the effects are more dramatic among those individuals that seem compromised in locomotory performance and other aging biomarkers. One question would then be why does 20HE show more dramatic effects between individuals in a population? A plausible answer is that regulation of pathways affected by 20HE treatment is more robust in healthier individuals in a population. There may be a greater relative

activation of NHR-49 in these individuals, or the rate of mitochondrial respiration may be higher (either due to more efficient fat catabolism or other factors). There is a well-established link between glucose and fatty acid metabolism underlying the pathology of type II diabetes and metabolic syndrome (Randle, Garland et al. 1963) that suggests that rates of glucose oxidation or fatty acid oxidation depend partially on existing plasma concentrations of these metabolites. It is possible that healthier individuals may be managing their carbohydrate and fat metabolism more efficiently to begin with, and that this efficiency may underlie the health benefits seen later in life. More experiments will need to be performed to assess NHR-49 levels and mitochondrial oxygen consumption in 1st quartile vs. 4th quartile individuals to determine whether there is an existing bias in efficacy of fat metabolism in a population, regardless of 20HE treatment.

20HE and insulin signaling

Insulin–IGF-1 signaling (IIS) is one of the strongest metabolic regulators of health span and longevity in diverse animal models. Binding of insulin/IGF-1 to the insulin receptor (*daf-2*) results in a downstream signaling cascade mediated by PI3 kinase (*age-1*) that results in the phosphorylation and nuclear exclusion of the FOXO transcription factor (*daf-16*). Inhibition of insulin signaling leads to translocation of *daf-16* to the nucleus and transcriptional upregulation of genes resulting in longevity. Our study demonstrates that 20HE treatment is dependent on *daf-2* but not on *daf-16*. One plausible explanation for this phenotype is that reduction of

insulin signaling may confer greater benefits to health than activated PPAR α , in a manner that masks the effect of PPAR α signaling. *daf-2* mutants display a metabolic profile that shows an upregulation in genes involved in gluconeogenesis. The elevated fat content in *daf-2* mutants and the upregulation of gluconeogenic genes suggests a mechanism whereby the primary substrate for gluconeogenesis is acetyl-CoA derived from stored lipids (McElwee, Schuster et al. 2006). The effect of gluconeogenic upregulation may thus mask the effect of PPAR α -induced increase in fat metabolism on health. Moreover, several gluconeogenic genes have multiple DAF-16 Binding Elements (DBEs), suggesting that they can be strongly regulated by *daf-16*. However, the loss of *daf-16* does not abrogate 20HE-induced health benefit, indicating that 20HE action might not involve gluconeogenetic upregulation or downstream insulin signaling effectors.

20HE and HSF-1

The heat shock transcription factor HSF-1 is an important mediator of longevity induced by IIS or inhibition of TOR signaling (Seo, Choi et al. 2013). HSF-1 is required for longevity benefits arising from reduction of IIS, dietary restriction, or inhibition of TOR signaling, making it an important downstream effector of health span. We observed that *hsf-1* RNAi abrogates 20HE benefit. We also demonstrate that 20HE benefit is independent of *daf-16*, indicating that the *hsf-1* requirement for 20HE benefit likely does not come from its association with the insulin signaling pathway.

It is plausible that HSF-1 may be required for 20HE benefit due to its association with DR pathways, and we observe that DR conditions abrogate 20HE benefit though they induce a health benefit of their own. DR is thought to activate autophagy, and HSF-1 potentiates starvation or rapamycin-induced autophagy (Dokladny, Zuhl et al. 2013). The transcription factor TFEB that regulates lysosomal biogenesis and autophagy is induced by starvation and exerts global transcriptional control over lipid catabolism by activation of PPAR α (Settembre, De Cegli et al. 2013). We have not yet determined whether 20HE treatment induces autophagy, but it is plausible that *hsf-1* requirement for 20HE benefit may arise from the close association between heat shock proteins and effectors of autophagy.

Mitochondrial effects of 20HE

We show that 20HE treatment in *C. elegans* increases basal and maximal oxygen consumption rates, which are measures of cellular respiration. PPAR α agonists such as fenofibrate promote β -oxidation in tissues, which causes an increase in mitochondrial respiration. This effect can be described as a genomic effect that requires new protein synthesis and is not immediately observed upon introduction of the agonist. Anti-diabetic drugs like metformin and PPAR agonists like thiazolidenediones (TZDs) also show a nongenomic mode of action by directly binding to Complex I in mitochondria, causing a decrease in mitochondrial

respiration (Brunmair, Staniek et al. 2004). Treatment with 20HE caused a rapid and significant increase in mitochondrial respiration rate, indicating that 20HE action at high concentrations might have a nongenomic component. However, treatment with rotenone abrogated 20HE effect on mitochondrial respiration rate, indicating that this action is not mediated via Complex I. Further experiments will need to be performed to determine the kinetics of possible direct binding of 20HE to mitochondria.

Materials and Methods

C. elegans strains used

N2: *C. elegans* wild type

STE68: *nhr-49(nr2041)*

BC15777: *dpy-5(e907); sEx15777[P_{fat-7}::GFP]*

DA1116: *eat-2(ad1116)*

All *C. elegans* cultures were synchronized by bleaching gravid hermaphrodites

Locomotory assay

7-day old adult hermaphrodites were placed under a standard stereoscope on a glass slide in 50ul of M9. Thrashing movements were recorded for 30s using a Qimaging Rolera Fast camera at 18fps. Frames were saved as bitmaps. Worm measures were calculated by the *C. elegans* Swim Tracker (CeleST), developed by Christophe Restif. Statistical analysis on locomotory assay measures was performed using GraphPad Prism software.

AGE pigment assay

AGE pigment analysis was performed as described before (Gerstbrein, Stamatas et al. 2005)

Life span analysis

N2 *C. elegans* were grown to adulthood on NGM plates seeded with OP50-1. On day 1 of adulthood, 100 N2 *C. elegans* were placed on a seeded NGM plate containing 500uM 20HE, the other population of *C. elegans* was placed on control plates containing 0.07% ethanol. Worms were transferred every other day for 1 week and then every other day until all the individuals comprising the population were recorded as dead. Survival curves were prepared on GraphPad software.

HeLa cell culture

HeLa cells were cultured in DMEM/F12 Ham basal medium (Invitrogen) supplemented with 10% FBS (BioWest) in a standard CO₂ incubator at 37°C. Cells were grown to 50% confluence and plated on 35mm tissue culture dishes (BD Bioscience). Cells were incubated with vehicle control (0.07% ethanol) or 50uM 20HE for 24 hours at 37°C and then harvested in cold PBS using a cell scraper. Cell pellets were resuspended in 1X RIPA buffer with Complete Mini Protease Inhibitor Cocktail tablets (Roche) and sonicated using a Microson Ultrasonic Cell Disruptor

(Misonix). Cell lysates were centrifuged at 14,000 rpm for 15 minutes. Protein estimation was done by Bradford assay.

PAGE and western blotting

PAGE was performed according to standard procedures, using 20ug/well of cell lysate. Western transfer was performed according to standard procedures. Blocking solution used was 2% nonfat milk. 1° antibody: monoclonal anti-PPAR α antibody from clone 3B6 (Pierce # MA-1822) at 1:2,000 dilution. 2° antibody: goat anti-mouse IgG-HRP (Jackson ImmunoResearch # 115-035-062) at 1:100,000 dilution. Bands were visualized using ECL (WesternBright Quantum - BioExpress)

Oil Red O staining

Oil Red O dye was prepared and assay was performed as described before (Heestand, Shen et al. 2013). Briefly, 100 N2 Day 1 adults were transferred to seeded NGM plates containing 0.07% ethanol or 500uM 20HE for 48 hours, then washed off plates using M9+0.01% Tween20, fixed using 50% isopropanol for 15 minutes and incubated in Oil Red O working solution overnight at room temperature. Worms were washed in M9+0.01% Tween20, mounted and visualized under a 20X NA 0.8 objective on a Zeiss Imager.D1m upright compound microscope. Images were acquired using iVision software (BioVision Technologies) and quantification was done using ImageJ software.

P_{fat-7}::GFP fluorescence

1 day old adults expressing P_{fat-7}::GFP were transferred to seeded NGM plates containing 0.07% ethanol or 500uM 20HE for 48 hours, then transferred to a 96-well plate containing 50ul M9+50uL 20mM sodium azide. GFP fluorescence was quantified using a plate reader.

OCR measurements

OCR measurements were performed using an XF-24 Flux Analyzer (Seahorse Bioscience). A mixed culture of *C. elegans* was grown on seeded NGM plates for 5 days. Worms were washed 3X in M9 and 500ul aliquots of worms were transferred to the XF microcapture plate. Basal OCRs were recorded for 12 minutes (3 reads, 4 minutes each) at which point worm cultures were injected with either control vector (0.1% DMSO) or 500uM 20HE. OCRs were then recorded for another 80 minutes, after which a mitochondrial stress test was performed, using 1uM oligomycin, 1uM FCCP and 1uM rotenone/antimycin (12 minutes, 3 reads/treatment).

ROS assay

1-day old adult *C. elegans* were transferred to seeded NGM plates containing 0.07% ethanol or 500uM 20HE for 48 hours. 50 worms/trial were picked into individual wells of a 96-well plate containing 50ul M9. 50ul 100uM DCF-DA (Sigma) was then added to the wells. Fluorescence signal per well was recorded immediately after addition of DCF-DA and 4 hours after addition of DCF-DA using a plate reader. The 96-well plate was kept protected from light on a gentle shake between readings.

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Figure legends

Figure 1: 20HE treatment prolongs life span and locomotory performance of wild type

C. elegans

(A) Life span curves of N2 *C. elegans* cultured at 20°C on NGM plates (control – solid black line) and on NGM plates with 500uM 20HE (dashed black line). error bars, s.e.m. $p < 0.0001$ by Mantel-Cox test. Median lifespan: control – 16 days; 500uM 20HE – 20 days. Data from 2 trials, $n=100$ per trial.

(B) 20HE reduces AGE pigment accumulation in 10-day old N2 animals by ~40%. Average AGE pigment fluorescence detected at 430nm. Error bars, s.e.m., 3 trials, $n=50$ per trial. $p=0.0395$ by unpaired t-test.

(C) Average number of head thrashes per 30s for 10-day old N2 animals treated with 500uM 20HE (white bars) and untreated controls (black bars). Error bars, s.e.m. $n=100$, $p=0.0014$ by unpaired t-test.

(D) Average number of head thrashes per 30s for the bottom half of the population from A. Error bars, s.e.m. $n=50$, $p < 0.0001$ by unpaired t-test.

(E) Average number of head thrashes per 30s for the 1st quartile of the population from C. Error bars, s.e.m. $n=25$, $p < 0.0001$ by unpaired t-test.

(F) Average number of head thrashes/30s among top 50% of animals tested.

Control – black bars, 500uM 20HE – white bars. n=50. Error bars, s.e.m.

Figure 2: 20HE benefit requires some members of the insulin signaling and DR pathways

(A) 20HE benefit on locomotory healthspan may act via *daf-2* and *hsf-1* but is *daf-16*-independent. We measured locomotory performance as average number of head thrashes/30s in 10-day old N2 *C. elegans* fed empty vector L4440 (black bars), *daf-2* RNAi (dotted bars), *daf-16* RNAi (white bars), or *hsf-1* RNAi (striped bars). Error bars, s.e.m. n>50, p<0.0001 by one-way ANOVA.

(B) 20HE benefit may be conferred via a mechanism shared with that activated in *eat-2* mutants that is dependent on *pha-4* but not *skn-1*. We measured locomotory performance as the average number of head thrashes/30s in 10-day old N2 *C. elegans* fed empty vector L4440 (black bars), *eat-2(ad1116)* (dotted bars), or *pha-4(RNAi)* (striped bars) or *skn-1(RNAi)* (white bars). n>50, p<0.001 by one-way ANOVA.

Figure 3: 20HE benefit requires *nhr-49*

- (A) Bottom quartile locomotory performance of 10-day old wild type *C. elegans* raised on control NGM plates (black bars) or plates containing 500 uM 20HE (white bars) and fed *nhr-49* RNAi. Error bars, s.e.m. n=30, p=0.7554 by unpaired t-test.
- (B) Bottom quartile locomotory performance of 10-day old *nhr-49(nr2041)* deletion mutant raised on 500 uM 20HE (white bars) compared to control (black bars). Error bars, s.e.m. n=10. p=0.4975.
- (C) Representative Western Blots using anti-PPAR α mAb -/+ 20HE. HeLa cells after 24 hours of treatment with vehicle control (0.07% ethanol) or 50 μ M 20HE.
- (D) Densitometry quantification of western blots of HeLa cell lysate $\pm$ 50 μ M 20HE (p=0.0472 by unpaired t-test).

Figure 4: 20HE treatment causes changes in *C. elegans* fat metabolism

- (A) Representative images of Oil Red O staining of 3-day old adult *C. elegans* after 48 hours on control NGM plates or NGM plates containing 500uM 20HE.

(B) Intensity of Oil Red O staining in 3-day old wild type *C. elegans* after 48 hours on control NGM plates (black bars) or NGM plates with 500uM 20HE (white bars). Error bars, s.e.m., n=32 each condition, $p < 0.0001$ by paired t-test.

(C) Representative images of $P_{fat-7}::GFP$ gut staining in 3-day old *C. elegans* raised on control NGM plates (black bars) or NGM plates with 500uM 20HE (white bars).

(D) Average intensity of $P_{fat-7}::GFP$ signal in 3-day old *C. elegans* raised on control NGM plates (black bars) or NGM plates with 500uM 20HE (white bars). Error bars, s.e.m. N=3, 50 animals per trial. $p = 0.0312$ by unpaired one-tailed t-test.

Figure 5: 20HE benefit involves mitohormetic response

(A) 20HE treatment causes an increase in basal and maximal respiration rate.

Animals were washed 3X in M9 and plated on to Seahorse XF-24 Flux Analyzer plates. Basal OCR was measured for 12 minutes followed by injection of 500uM 20HE (black circles) or vehicle control (70% ethanol – white circles). OCRs were then recorded for 80 minutes, followed by a mitochondrial stress test that included subsequent injections of 1uM oligomycin, 1uM FCCP and 1uM antimycin/rotenone. Error bars, s.e.m. 4 trials, n=50.

(B) Reactive Oxygen Species (ROS) accumulation measured by DCF-DA. N2 *C. elegans* treated with vehicle (70% ethanol – black bars) or 500uM 20HE in

70% ethanol (white bars) for 48 hours were incubated with DCF-DA for 4 hours at 20°C. Error bars, s.e.m., 3 trials, n=50 per trial. $p=0.0346$ by paired t-test.

(C) Bottom quartile locomotory performance of 10-day old wild type *C. elegans* fed empty vector L4440 or *sod-3* dsRNA, with or without 500uM 20HE. Black bars – L4440. White bars – L4440+500uM 20HE. Dark grey bars – *sod-3(RNAi)*. Light grey bars, *sod-3(RNAi)*+500uM 20HE. Error bars, s.e.m. n=12. $p<0.0001$ by one-way ANOVA.

Figure 1

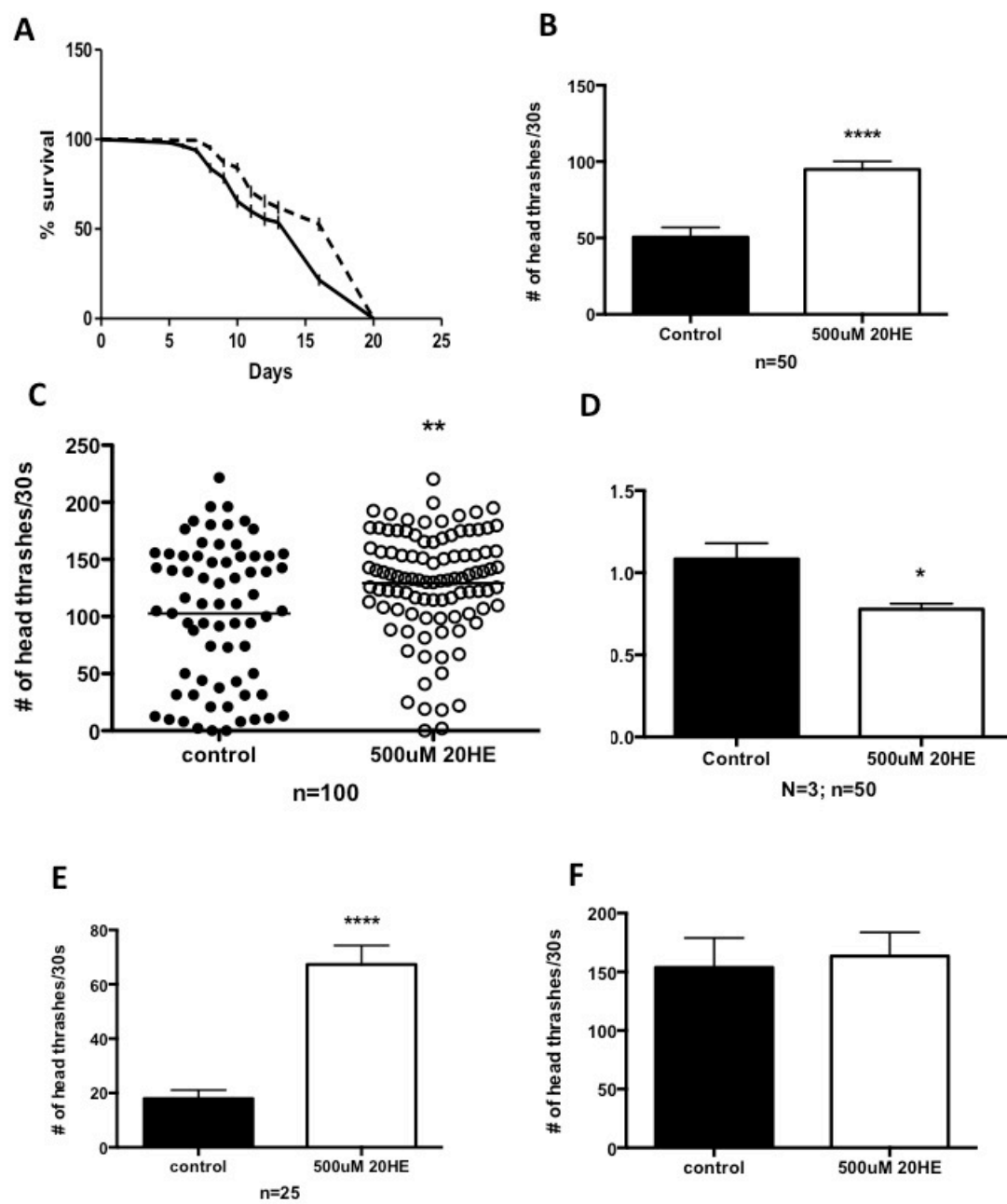


Figure 2

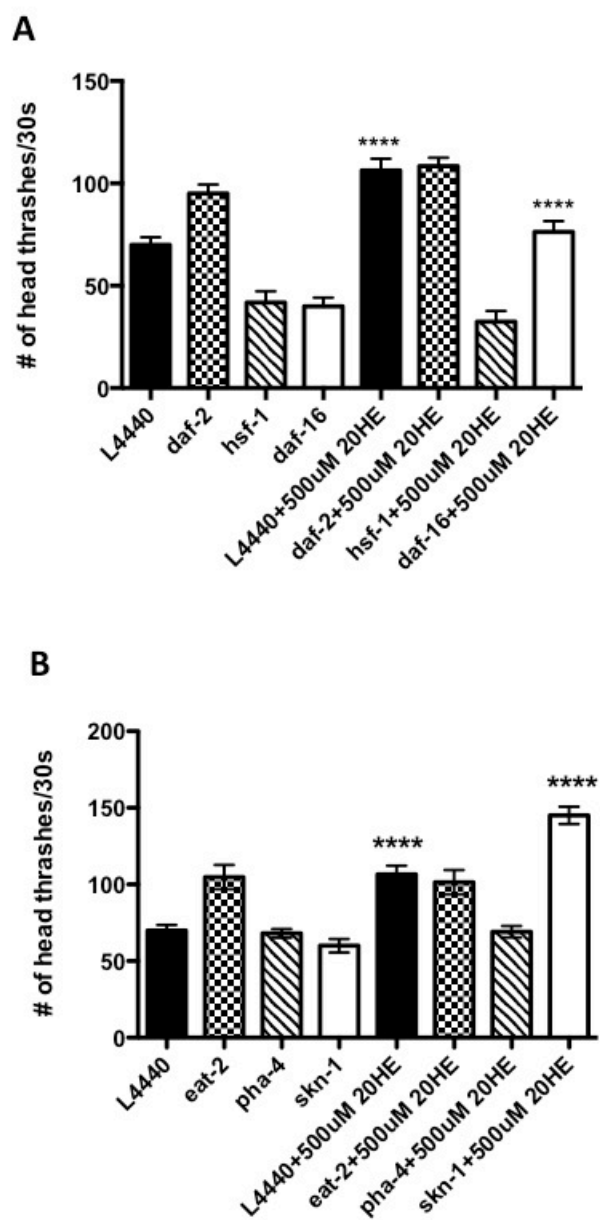


Figure 3

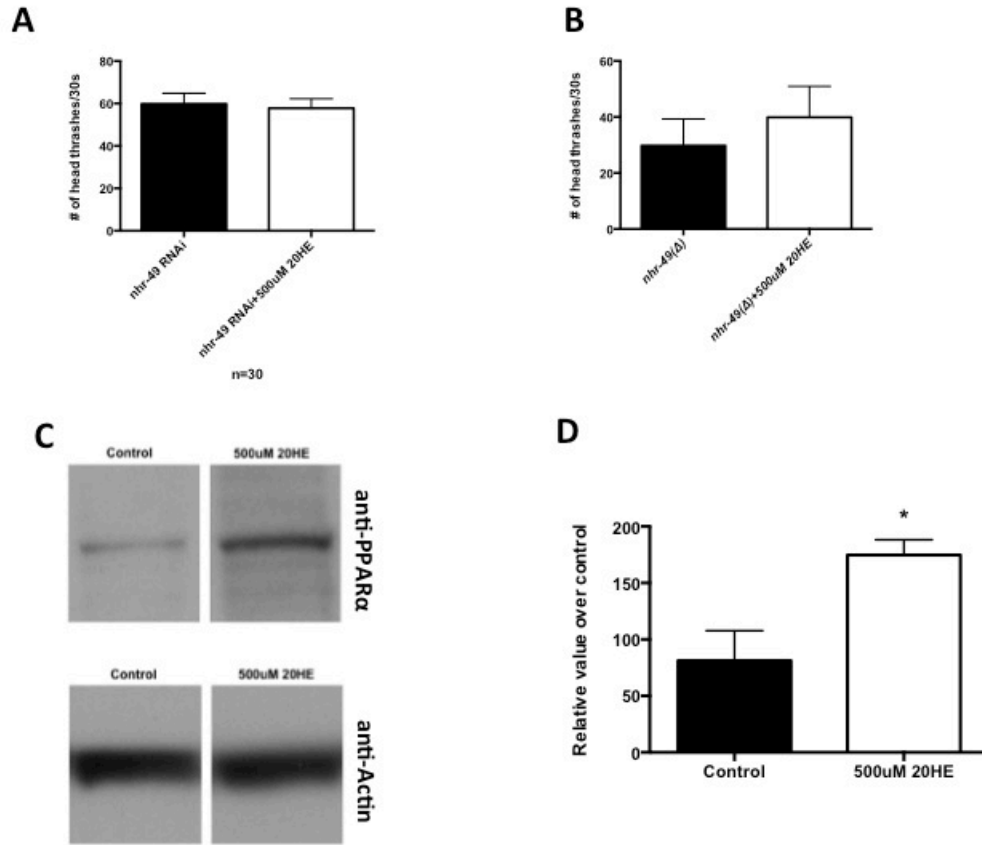


Figure 4

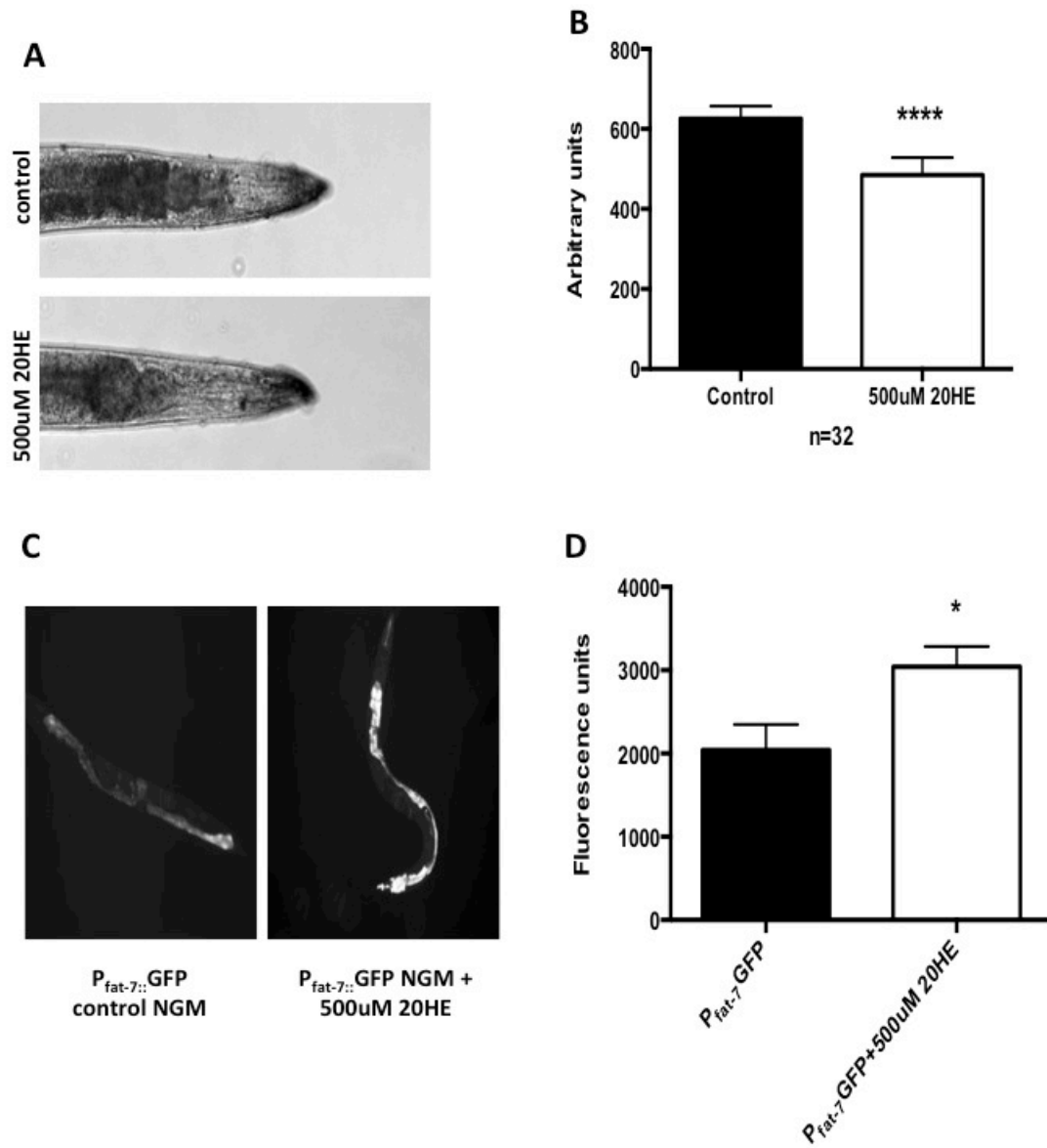
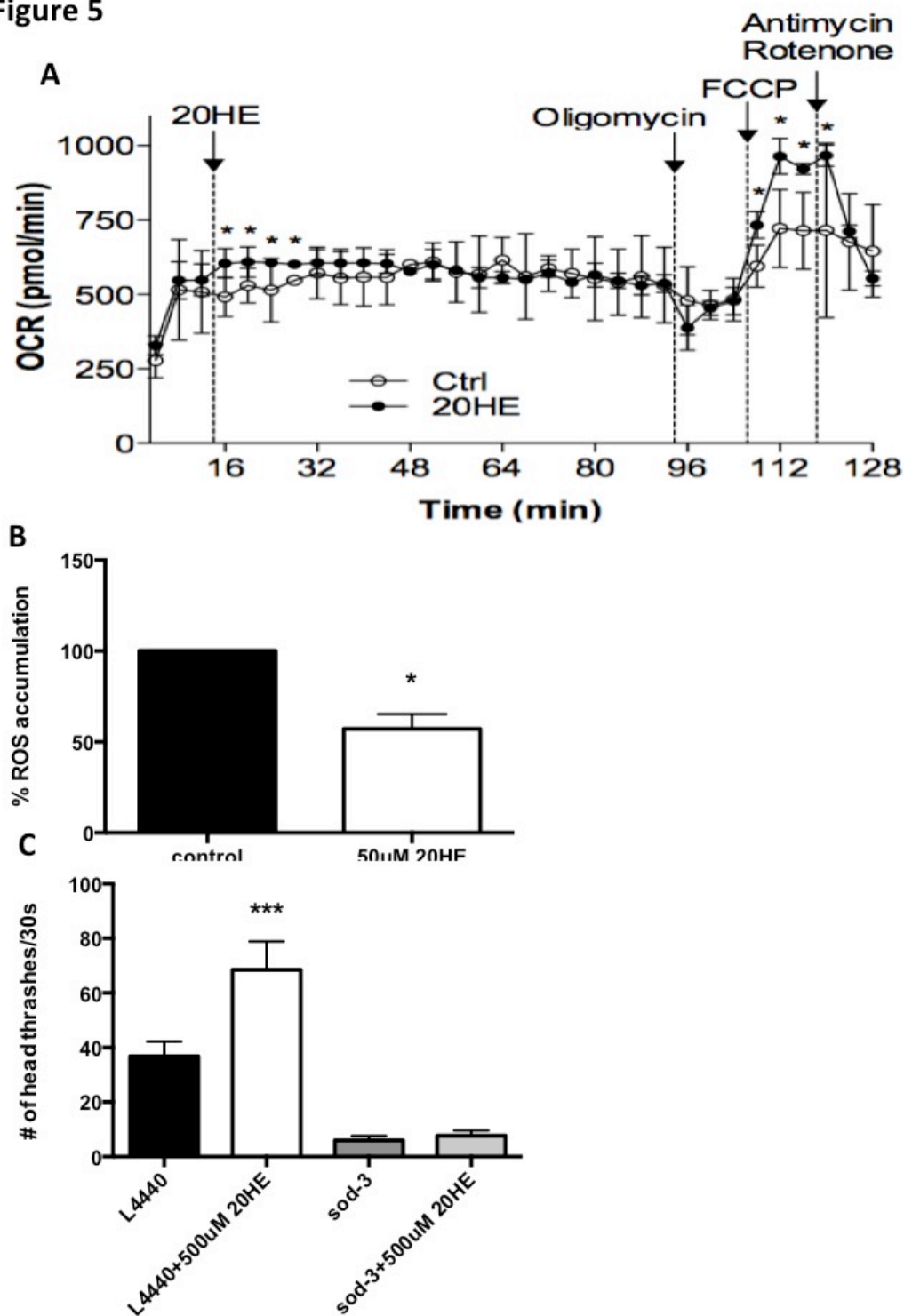


Figure 5



Summary and concluding remarks

Part 1: NRA-2/nicalin regulates neuronal death by controlling the localization of toxic DEG/ENaCs.

Two important conclusions can be drawn from the study described in Chapter 2:

A) ER chaperone NRA-2 regulates cell surface levels of MEC-10(d) and thereby modulates the impact of the necrotic insult on the cell.

B) MEC-10(d) localization is unique and appears to be disparate from the localization of its partner MEC-4(d). The implications are discussed below:

- 1) Although the study shows that the loss of function of *nra-2* enhances *mec-10(d)* - induced necrosis, it does not address whether this enhancement extends to other necrotic inducers that also form aberrant channels i.e. *deg-1(d)*, *deg-3(d)* or *unc-8*. These will need to be tested by measuring the TRN survival rate of *nra-2(Δ)* mutants in *deg-1(d)*, *deg-3(d)* and *unc-8* backgrounds. Furthermore, *nra-2* has been shown to act in association with *nra-4*, the *C. elegans* homolog of NOMO. We will need to assay the effect of a double knockout of *nra-2* and *nra-4* on *mec-10(d)* – induced necrosis.
- 2) MEC-10 has been shown to require MEC-4 in order to form a functional channel. To determine whether MEC-10(d) localization to the single spot in

the soma requires MEC-4, we will need to image MEC-10(d)::GFP in a *mec-4(Δ)* background.

- 3) We do not yet have an understanding of how NRA-2 might be sensing the conformation of an assembled DEG/ENaC channel. There is no known domain in NRA-2 that appears to function as a protein-protein interacting domain. However, near the N-terminus of the protein there is an aminopeptidase domain that is poorly conserved and lacks catalytic activity. It is possible that this domain may detect amide groups in certain conformations. 3D models of MEC-4 channel structure posit that the ion selectivity of the channel stems from the co-ordination of cations in the lumen of the channel with carbonyl oxygen atoms in the TMII helix of the MEC-4/MEC-10 protein, which contains the (d) residue. The model posits that the bulky side chains of the (d) residue do not allow this oxygen atom to interact with cations in the lumen, leading to a constitutively open channel (Gessman et al. 2010). It is therefore plausible that during assembly of the mechanosensory channel, the NRA-2 aminopeptidase domain may play a role in the recognition of a mutated TMII helix in MEC-4 or MEC-10. In order to test this hypothesis we will need to test the effects of transgenic *nra-2* lacking aminopeptidase domain on *mec-10(d)* – induced necrosis.

Part 2: A plant-derived ecdysteroid improves health span and regulates fat metabolism in *C. elegans* in an NHR-49/PPAR α – dependent manner.

The study in Chapter 3 is a characterization of the effects of 20-hydroxyecdysone (20HE) in *C. elegans*. The results point toward 20HE – induced upregulation of fat metabolism in a manner dependent on the *C. elegans* nuclear hormone receptor NHR-49. The study also suggests that NHR-49 is the *C. elegans* ortholog of mammalian PPAR α . However, some key questions are unanswered:

- 1) The effects of 20HE treatment in *nhr-49*(Δ) background suggest that it is required for 20HE benefit. If this is true, western blots of *C. elegans* lysates probed with an antibody against PPAR α should show an increase in NHR-49 levels in wild type *C. elegans* treated with 20HE, but no band in *nhr-49*(Δ) *C. elegans*. This experiment will need to be done.
- 2) If NHR-49 is activated/upregulated by 20HE, then we should observe an increase in levels of transcripts or proteins regulated by NHR-49. While we see an upregulation of *fat-7*, other NHR-49-regulated genes will need to be assayed by RT-PCR or promoter-driven GFP expression in *C. elegans* to make a stronger case. Candidate genes include *cpt-1* (carnitine palmitoyl transferase), *acs-2* (acyl CoA synthetase) and *ech-2* (enoyl-CoA hydratase). These transcripts should be increased in a wild type background but not in *nhr-49*(Δ).

- 3) We will need to show binding of 20HE to PPAR α at physiological concentrations. NMR experiments are currently being performed to address this question.
- 4) We do not have any understanding of the precise nature of the interaction of 20HE with PPAR α . This can be addressed by looking for physical interaction of 20HE with PPAR α constructs expressing only the ligand binding domain (LBD). NMR experiments can reveal what groups in 20HE are undergoing an interaction with the protein, and with what residues within the LBD.