Microbiota and diapause-induced neuroprotection share a dependency on calcium but differ in their effects on mitochondrial morphology

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Title: Microbiota and diapause-induced neuroprotection share a dependency on calcium but differ in their effects on mitochondrial morphology.

Abbreviated title: Effects of microbiota and diapause on neuroprotection.

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Abstract

The balance between the degeneration and regeneration of damaged neurons depends on intrinsic and environmental variables. In nematodes, neuronal degeneration can be reversed by intestinal GABA and lactate-producing bacteria, or by hibernation driven by food deprivation. However, it is not known whether these neuroprotective interventions share common pathways to drive regenerative outcomes. Using a well-established neuronal degeneration model in the touch circuit of the bacterivore nematode Caenorhabditis elegans, we investigate the mechanistic commonalities between neuroprotection offered by the gut microbiota and hunger-induced diapause. Using transcriptomics approaches coupled to reverse genetics, we identify genes that are necessary for neuroprotection conferred by the microbiota. Some of these genes establish links between the microbiota and calcium homeostasis, diapause entry, and neuronal function and development. We find that extracellular calcium as well as mitochondrial MCU-1 and reticular SCA-1 calcium transporters are needed for neuroprotection by bacteria and by diapause entry. While the benefits exerted by neuroprotective bacteria requires mitochondrial function, the diet itself does not affect mitochondrial size. In contrast, diapause increases both the number and length of mitochondria. These results suggest that metabolically-induced neuronal protection may occur via multiple mechanisms.
Calcium signaling and mitochondrial function have recently been suggested to promote axonal growth following neuronal damage, but the underlying mechanisms and physiological significance are unclear. Combining transcriptomics, genetics and cell biological approaches in a simple animal model of axonal degeneration and regeneration, we demonstrate that neuronal repair conferred by two different metabolic processes occurs in diverse ways, requiring differential changes in mitochondrial function and calcium homeostasis. Furthermore, this work shows that neuroprotection can be additive, providing a new conceptual framework for developing therapeutic interventions in neurodegenerative conditions that leverage the intersection of metabolism, microbiota and mitochondrial function.

Introduction

Axonal degeneration underlies many neuropathies and neurodegenerative diseases (Coleman and Perry, 2002) that involve a controlled dismantling of neuronal morphological extensions (Saxena and Caroni, 2007). This process in the nematode *C. elegans* can be counteracted by developmental arrest induced by food deprivation (Calixto et al., 2012; Caneo et al., 2019) as well as by exposure to specific intestinal microbiota (Urrutia et al., 2020). The mechanisms that promote neuronal protection in both of these conditions are unclear. Is neuroprotection achieved actively as neuronal repair or merely the result of diminished neurodegenerative processes? Moreover, it is unknown whether ingested microbiota and metabolic state act similarly in this context.

Given the pivotal role of gene expression in the link between environment and cellular phenotype (Davidson and Levin, 2005; van Kesteren et al., 2011; Chen et al., 2015; Lu et al.,
identifying transcriptional changes at various stages of recovery may hold the key to understanding novel mechanisms and pathways for neuroprotection.

The microbiota composition and health are intimately interconnected with diet (Scott et al., 2013; Portune et al., 2017; Illiano et al., 2020; Jackson and Theiss, 2020). Through this relationship, metabolites produced by the microbiota have been linked to the development of neurodegenerative disorders (Chandra et al., 2020; Goyal et al., 2021; Milošević et al., 2021; Zhang et al., 2022). Dysbiosis of the gut microbiota is associated with inflammation, Reactive Oxygen Species (ROS) increment, and mitochondrial dysfunction (Yardeni et al., 2019; Jackson and Theiss, 2020).

Oxidative damage is central to neurodegeneration, and mitochondria are key to the neutralization of oxidative stress (Kim et al., 2015; Singh et al., 2019; Ballard and Towarnicki, 2020). The effects associated with failure in the neutralization of ROS include impairment of synthesis and transport of lipids, and Ca\(^{2+}\) transport (Vance, 2014). Loss of mitochondrial homeostasis has been linked to degenerative processes in different animal and cell models (Pathak et al., 2013; Blaszczyk, 2020). In mice, intracellular calcium transporters, such as the Mitochondrial Calcium Uniporter, have been shown to contribute to degeneration (Calvo-Rodriguez et al., 2020; Calvo-Rodriguez and Baeskai, 2021). However, mitochondria maintain cellular homeostasis by also modulating energy production and capture of calcium (Beal, 1996; Shvartsman et al., 2007; Ferrer, 2009; Picard and McEwen, 2014). Calcium signaling in microdomains is important in determining whether neuronal regeneration or degeneration occurs (Xu et al., 2001; Berridge, 2006; Yan and Jin, 2012), and involves the participation of the endoplasmic reticulum (ER) and mitochondria.
To gain mechanistic insight on the commonalities between microbiota and diapause-induced neuro-regeneration, we use a genetically encoded insult to the touch circuit of *C. elegans* (Driscoll and Chalfie, 1991; Calixto et al., 2012), whereby a mutation triggers a loss of selectivity of the MEC-4 channel (Shi et al., 2018), leading to an increase in cytoplasmic calcium and the subsequent energetic collapse of the neuron (Driscoll and Chalfie, 1991; Xu et al., 2001; Bianchi et al., 2004; Shi et al., 2018; Shi et al., 2018). Using this model, we investigate here the contribution of gene expression, mitochondria morphology and the need for calcium in these two interventions.

**Materials and Methods**

**C. elegans growth and maintenance**

Wild type, transgenics and mutant *C. elegans* were maintained at 20°C as reported previously (Brenner, 1974). Nematode strains used were:

- wild type (N2),
- TU2773 \[uls31(Pmec-17mec-17::gfp); mec-4d(e1611)]
- WCH6 \[uls31[Pmec-18sid-1; Pmyo-2mcherry],  uls31[Pmec-17mec-17::gfp],  sid-1[pk3321],  mec-4d[e1611]]
- js609 \[jsIs609:Is[Pmec-4::MLS::gfp]]
- WCH42 \[jsIs609:Is[Pmec-4::MLS::gfp];  mec-4d [e1611]]

All animals were maintained in *E. coli* OP50 before using or feeding with other bacteria.

**Bacterial growth**
E. coli OP50 and E. coli HT115 bacteria were grown overnight on Luria-Bertani (LB) plates at 37°C from glycerol stocks kept at -80°C. The next morning, a large amount of the bacterial lawn was inoculated in LB broth and grown for 6 hours on agitation at 200-220 rpm at 37°C. 300 μL of bacterial culture was seeded onto 60 mm NGM plates and allowed to dry overnight.

C. elegans mec-4d transcriptomic analysis

Total RNA was isolated from synchronized C. elegans mec-4d populations feeding on E. coli OP50, or E. coli HT115, at 12, 24, and 48 hours after hatching using RNA-Solv® Reagent (Omega Biotek). Spectrophotometric quantification and integrity of RNA were determined on the Agilent 2100 Bioanalyzer (Agilent Technologies). mRNA libraries were prepared with the IlluminaTruSeq™ RNA sample preparation kit (Illumina) according to the manufacturer's protocol. The quality and size distribution of the libraries were evaluated with the Agilent 2100 Bioanalyzer using a DNA 1000 chip (Agilent Technologies) and quantified using the KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems), on the Step One Plus Real-Time PCR System (Applied Biosystems). The libraries were sequenced using the HiSeq paired-end protocol (2x 100 bp). High-quality reads were selected using Trimmomatic v0.36 and mapped to the C. elegans reference genome (Wormbase release ws235) using Tophat v2.0.9. (Trapnell et al., 2012). The resulting bam files were transformed for visualization of mapping results in the UCSC genome browser (https://genome.ucsc.edu). The mapping results and HTSeq-count v0.6.0 were applied with the intersection non-empty argument to get the more permissive count. Based on the counts, a DE analysis in R v3.3.2 was performed, between conditions at the different time points using both DESeq2 (Love et al., 2014) and edgeR (Robinson et al., 2010). The threshold for differential expression analysis cutoffs was defined as...
log10|FC| > 1, and adjusted P value (adj Pvalue) <0.05. Result data for the differentially expressed genes are displayed in heatmaps in Extended Data Figure 1-1, and data mining scripts and processing is displayed in a repository in the following link (https://github.com/ArlesUrrutia/mec4dvsOPvsHT115). Results for adj Pvalue and Log2FC were cleaned for unmeasurable genes and then analyzed in Python3.9 for Volcano plot observations of both DE analysis (Extended Data Figure 1-1). Result data and data mining for the differentially expressed genes are displayed in Extended Data Table 1-2.

Enrichment analysis: Gene Ontology (GO) analysis was performed using the enrichment tool in wormbase.org (Angeles-Albores et al., 2016; Angeles-Albores et al., 2018). Gexplore (Hutter et al., 2009; Boeck et al., 2016) was used to find among our set of RNAi positive genes, those that are highly expressed in the dauer stage.

Cultures without Calcium

To obtain calcium free media, CaCl₂ was not added to the NGM media. Bacteria used to feed animals was grown over-day with the addition of EGTA 500 mM (Winkler) on LB broth (Calixto et al., 2012).

Dauer synchronization

After the detection of a few dauers by direct observation, plates were washed using 1% SDS and the liquid containing mixed stages animals was placed on an Eppendorf tube. The tube was centrifuged for 2 minutes at 2000 rpm and the supernatant removed. The pellet of nematodes was washed for 15 minutes with a solution of SDS 1% with 2,5 µg/mL of Carbenicillin.
(PhytoTechnology Laboratories) and 25 μg/mL of Amphotericin B (Fungizone, Gibco/Thermofisher). Afterwards, animals were washed three more times with sterile distilled water and antibiotics. The pellet was placed on NGM media plates and after one hour the portion of agar where the pellet was placed, was cut off using a stainless-steel spatula and then washed with the same mixture of sterile distilled water with antibiotics. Animals were centrifuged for 2 minutes at 4500 rpm and placed in Cell Culture Plates (TrueLine®) with distilled water and antibiotics as mentioned before. The media was replaced every 2 weeks.

**RNA interference (RNAi) by feeding**

Bacterial clones from Ahringer Library were taken from glycerol stocks and grown overnight on LB plates containing tetracycline (PhytoTechnology Laboratories) (12.5 μg/mL). The next morning, a chunk of bacterial lawn was grown on liquid LB containing carbenicillin (PhytoTechnology Laboratories) (50 μg/mL) for 8 hours. NGM plates were prepared to add 1mM of IPTG and 400 μL of bacterial growth was seeded. As control for RNAi, *unc-22* dsRNA was used, which renders animals uncoordinated (Unc).

**RNAi in developing animals**

Thirty to sixty newly hatched (0-2 hours post hatching) L1s were placed on plates containing dsRNA expressing *E. coli* HT115 bacteria. Larvae and adults were removed with M9 from plates full of laid embryos. Embryos remained attached to the plates and were allowed to hatch for 2 hours. 0-2 hour post hatching L1 larvae were picked with a mouth pipette in M9 and placed on experimental NGM plates seeded with the desired bacterial clones. Silencing was tested on the
TU2773 strain (systemic, non-neuronal RNAi), and WCH6 (TRN specific RNAi, (Calixto et al., 2010; Calixto et al., 2012). The morphology of the AVM was scored at 72 hours post-hatching.

RNAi in dauers

To avoid contamination, thirty to ninety animals resulting from hypochlorite treatment of gravid hermaphrodites were seeded on 5 plates per RNA condition. By direct observation, we detected the first day of dauers on plates. Nematodes were synchronized on day 2 or 3 depending on the number of dauers required for observation. We followed the protocol described above as dauer synchronization. After some animals crawled out of the drop, 25 individuals were picked for observation under the microscope.

Scoring of neuronal integrity

For morphological evaluation, worms were mounted on 2% agarose pads. Dauers were mounted and paralyzed with 20 mM levamisole and developing animals with 1 mM levamisole. Morphological categories were assigned using the same criteria as in Urrutia et al. (Urrutia et al., 2020). Neurons with full-length axons, as well as those with anterior processes that passed the point of bifurcation to the nerve ring, were classified as AxW. Axons with only a process connected to the nerve ring were classified as AxL, and those that did not reach the bifurcation to the nerve ring were classified as AxT. Lack of axon and soma only was classified as AxØ, and the total absence was indicated as AxØ-S. For simplicity, all graphs show only the AxW category.

Microscopy and photography
Images were taken using DSLR Remote Pro (Breeze Systems Ltd., UK), a Canon Rebel T3i camera, and a fluorescence microscope (Nikon Eclipse Ni-U). Configuration was set up at 1/10 exposure time and ISO correction at 200 for fluorescence images.

Quantitative measurements of mitochondrial morphology

Thirty to sixty newly hatched js609 or WCH42 animals were placed on plates seeded with *E. coli* OP50 or *E. coli* HT115, and images of the mitochondria of AVM axons of L2 animals were taken after 24 hours. Dauers were obtained one week later from the same plate by 1% SDS treatment, placed on sterile NGM plates devoid from bacteria to allow dauers to crawl away from the 1% SDS drop. Living dauers were mounted on 2% agarose pads and photographs were taken at different focal points to register all mitochondria in each AVM. Each set was measured separately using ImageJ, and each mitochondrion was registered independently. First, we used a Neubauer chamber to standardize the observed length in pixels from the image. Then, using the line tool from ImageJ we measured the number of pixels that each mitochondrion had in the image. With a scale entered in ImageJ we obtained the length in micrometers. Each value was recording in excel, and from that, the average value and the number of mitochondria in each axon was calculated.

Mitochondria shorter than 2µm were classified as fragmented, between 2 and 4 µm as intermediate, and longer than 4µm mitochondria as filamentous (Neve et al., 2020).

Experimental design and statistical analyses

*Functional validation by RNAi*
The functional validation of the genes identified as upregulated was tested by feeding animals with dsRNA (RNAi). RNAi experiments used *unc-22* dsRNA as control, which produces twitching in nematodes. When the plate has over 70% of Unc animals, the experiment is considered valid. Statistical analysis is done using an *E. coli* HT115 that does not express dsRNA as control (One-way ANOVA).

**Calcium depletion**

Calcium chelators such as EGTA can reduce the degeneration rate in *mec-4d* animals (Calixto et al., 2012). Animals cultured in absence of environmental calcium were compared in a two-way ANOVA test to animals grown in NGM (CaCl$_2$ 1mM) at 72 hours after hatching, given the same bacterial diet. Similar comparisons are done for 1 to 2-week-old dauers in absence of calcium using the same type of test. The role of intracellular calcium transporters like *sca-1* and *mcu-1* in neuroprotection was tested using RNAi and compared to an *E. coli* HT115 control for developing (72 hours post hatching) and dauer animals. For developing animals, one-way ANOVA was used and for dauers, a Two-way ANOVA.

**Mitochondrial Measurements**

Analysis of mitochondrial number and length was done using one-way ANOVA comparisons in wild type and *mec-4d* genetic backgrounds. Specific comparisons included evaluating animals at the same developmental stage (L2 or dauer) in different bacterial diets and comparing L2 with dauers within the same diet. For the functional testing of mitochondrial genes, RNAi was performed in TRN-specific and systemic strains carrying the *mec-4d* mutation and the scoring was done at 72 hours post hatching. The *E. coli* HT115 diet served as the control for each gene.
One-way ANOVA was used to assess both the treatment and control groups. Pearson’s correlation analysis was used to examine the relationship between mitochondrial number and length, with the percentage of wild-type axons (AxW) observed in mec-4d animals L2 and dauers.

All experiments were done at least 3 times (three biological replicas, started in different days and from different parental nematodes). Each biological replica contained a triplicate (three technical replicas). Statistical evaluation was done by a One or Two-way ANOVA with post-hoc analyses.

Criteria for data exclusion. We excluded experimental replicas when there was contamination with unwanted bacteria or fungi on the nematode plates, or when bacteria had been almost or completely consumed.

**Results**

Transcriptomic profiling of diet induced neuroprotection in developing *C. elegans*.

Intestinal bacteria and their metabolites can define the degeneration rate of touch receptor neurons (TRNs) of developing *C. elegans* expressing the mec-4d degenerin (Figure 1A, Urrutia et al., 2020). Specifically, GABA and lactate producing *E. coli* HT115 are protective in contrast with *E. coli* OP50, which does not produce the metabolites (Figure 1B). To identify the nematode genes that may underlie diet-induced neuroprotection we performed RNAseq of mec-4d animals feeding on *E. coli* HT115 and *E. coli* OP50 at 12-, 24-, and 48-hours post-hatching. Differential expression analysis was done comparing animals fed on *E. coli* HT115 with those fed on *E. coli* OP50 at each time point. Ninety-three genes were differentially expressed, most of which were found in earlier development (12 and 24 hours, Extended Data Figure 1-1,
Extended Data Table 1-1). Forty genes were upregulated in animals fed *E. coli* HT115 (Figure 1C) while fifty-three were downregulated (Figure 1D). Upregulated genes were phenotypically enriched (Angeles-Albores et al., 2016) in neuronal development, axonal pathfinding and axonal outgrowth as well as in diapause formation (Extended Data Figure 1-2), and enriched in the Gene Ontology (GO) terms calcium binding, neuronal development, the unfolded protein response, and biotic interactions, among other (Extended Data Table 1-2). This shows that *E. coli* HT115 diet increases expression of genes involved in neuronal processes that can be detected even when the pool of RNAs used for the analysis is from the whole nematode and not a neuron-specific transcriptome. The comparison of our results with previous published data showed little overlap, probably due to differences in the nematode genotype and the developmental stage of RNA collection (adult wildtype (MacNeil et al., 2013) and adult *glp-4* mutants (Revtovich et al., 2019) in contrast with our analysis done in *mec-4d* at three different larval stages (L1, L2-L3 and L4). Moreover, GO and phenotype enrichment of previous data did not contain any neuronally enriched functions (Extended Data Table 1-3), suggesting that the protective diet increased expression of ad hoc genes required for repair in *mec-4d* background. On the other hand, downregulated genes in *E. coli* HT115 were associated with body morphology phenotypes (Extended Data Figure 1-2) and with GO terms such as ATP synthesis and other functions that reside in the mitochondria, in addition to cuticle development (Extended Data Figure 1-2). Interestingly, previous transcriptomics, coincide with enrichment in mitochondrial processes, although the genes are not shared (MacNeil et al., 2013; Revtovich et al., 2019), suggesting that *E. coli* HT115 has a role over mitochondrial function regardless of genetic background.
We tested whether genes that increase their expression in mec-4d fed on \textit{E. coli} HT115 contribute to neuroprotection \textit{in vivo}. This can be functionally tested by targeted silencing using RNAi interference (Fraser et al., 2000; Kamath et al., 2003). We used \textit{E. coli} HT115 expressing dsRNA for 31 of the 40 upregulated genes to study their contribution to neuroprotection in mec-4d animals. The remaining 9 clones were not available in the feeding RNAi library or caused embryonic lethality (Fraser et al., 2000; Kamath et al., 2003). To dissect whether the function of these genes is required in the touch neurons or systemically, we used strains with TRN-specific and systemic RNAi (Calixto et al., 2010). Fifteen dsRNAs caused a decrease in neuroprotection when silenced in non-neuronal tissues (Figure 1E and G). Of those, seven were needed for protection specifically in the TRNs (Figure 1F and G). A large proportion of genes necessary for neuroprotection in mec-4d animals are neuronally expressed including the TRNs (Table 1 and (Taylor et al., 2021) and show functional clustering (Angeles-Albores et al., 2016) in neuronal phenotypes (Figure 1H). We compared the functional enrichment provided by genes only required systemically with those required in the TRNs and found a large overlap in neuronal phenotypes despite having come from different gene pools (Figure 1I). Clustering by GO of RNAi positive genes also showed enrichment in neuronal categories (Extended Data Figure 1-3). GO enrichment showed TRN-specific axon guidance and axon projection terms, while systemic-specific categories were enriched in stress responses to biotic stimulus and to incorrectly folded proteins. The systemic and TRN enrichment in GO terms overlapped in neuronal development and immune defenses to microbes (Extended Data Figure 1-3). This is coherent with a systemic contribution to lowering cellular stress while neuronal autonomous processes are clustered in categories related to neuronal repair.
Diapause entry is also strongly neuroprotective (Caneo et al., 2019). We compared the transcriptomic results shown above to available data on dauer gene expression obtained by others (Hutter et al., 2009; Boeck et al., 2016). Four of the fifteen genes required for neuroprotection are also upregulated in dauers: sax-2, hsp-16.11, T22B7.3, and T12D8.5 (Boeck et al., 2016), suggesting that there is a common neuroprotective gene pool. These genes cluster in categories mainly related to neurogenesis and the unfolded protein response, probably to regulate cellular homeostasis after the early stress response (Extended Data Figure 1-4). Genes that are not shared with dauers include categories mostly related to axonal guidance, development and body morphology development and interspecies communication (Extended Data Figure 1-4) suggesting there are functional distinction between the two gene pools.

**Calcium depletion and neuroprotection**

Transcriptomics analysis (Figure 1) revealed that several genes related to calcium homeostasis are differentially expressed in neuroprotective conditions. This prompted us to look further into the calcium contribution to neuroprotection induced by diet and by diapause. E. coli HT115-fed mec-4d nematodes growing in media lacking calcium showed a significantly lower percent of wildtype TRN axons (AxW) at 72 hours after hatching (Figure 2A), suggesting that calcium also contributes to axonal regrowth. In contrast, AxW counts in animals on the standard E. coli OP50 diet did not differ in low calcium. We next asked whether calcium plays a similar role in diapause-induced regeneration. E. coli OP50 and HT115 fed worms were induced to enter diapause by bacterial food exhaustion. Calcium removal in both dauer populations caused a significant decrease in wild type axons (Figure 2B). Notably, the extent of axonal regrowth and reduction in control and calcium depleted conditions, respectively, did not differ between the two
groups of dauers, suggesting that feeding on *E. coli* HT115 did not mask or attenuate diapause benefits. Taken together, the results support the idea that although both microbiota- and diapause-induced neuroprotective processes require calcium, the underlying mechanisms are not completely overlapping.

Mitochondrial and Reticular calcium transporters in TRNs are required for neuroprotection.

Given the observed dependency on calcium, we next asked whether calcium redistribution across different subcellular compartments by specific transporters plays a role in neuroprotection. For the *E. coli* HT115 diet during development and in diapause, we evaluated the requirement for *mcu-1*, the Mitochondrial Calcium Uniporter, and *sca-1*, the Sarco Endoplasmic Reticulum Calcium ATPase (SERCA) (Betzer et al., 2018; Csordás et al., 2018; Sarasija et al., 2018; Wang et al., 2019; Calvo-Rodriguez et al., 2020). These two transporters were silenced by feeding *mcu-1* and *sca-1* dsRNA expressing bacteria to synchronized L1 *mec-4d* worms and we scored neuronal integrity of the AVM TRN either 72 hours later for developing animals, (Urrutia et al., 2020) or after the second generation had become dauers (detailed protocol in Methods).

At 72 hours post dsRNA treatment during development, only the simultaneous silencing of *mcu-1* and *sca-1* caused a reduction in AxW in the TRN-specific RNAi strain (*Figure 2C* and *D*), suggesting that either transporter is sufficient for diet-induced neuroprotection. In dauers, on the other hand, silencing either *mcu-1* or *sca-1* impaired axonal regeneration with TRN-specific or systemic RNAi (*Figure 2E* and *F*). Taken together, these results suggest that either calcium transporter can compensate for the loss of the other in *E. coli* HT115-mediated neuroprotection whereas both are necessary systemically in dauer neuroprotection.
Diapause induces increase in mitochondrial number and size in mec-4d animals

Regeneration under a chronic degenerative stimulus can be energetically demanding (Caneo et al., 2019). Specifically, MEC-4d overactivity imposes an ionic imbalance that can only be repaired by active Na⁺ extrusion such as would occur through the NaK ATPase (Calixto, 2015; Davis et al., 1995). Because mitochondria are central to cellular energetic control and have a core role in neuronal cell death (Dawson and Dawson, 2017); and axonal degeneration in the mec-4d model (Calixto et al., 2012) we examined whether diet- and diapause-induced neuroprotection were associated with mitochondrial changes in TRNs. We measured mitochondrial number and length in the AVM neuron of animals expressing a fluorescent mitochondrial marker (jsIs609:Is[Pmec-4::MLS::gfp]) in both wild-type (Fatouros et al., 2012) and mec-4d backgrounds (Figure 3A). These quantifications were performed in one or two-week-old dauers and in L2 control animals because dauers have an L2d lineage (Karp and Ambros, 2012). Initially, we measured each mitochondrion and recorded each value separated by sample, then we examined mitochondrial values and categorized them into three distinct groups based on their size: filamentous, intermediate, and fragmented (Neve et al., 2020, Figure 3).

First, we noted that mec-4d L2 animals have significantly less mitochondria in the TRNs than wild types in either diet (Figure 3B). Second, regardless of genotype, mitochondria numbers are similar between diets (Figure 3C and D). These two observations indicate that E. coli HT115 does not exert its neuroprotective effects through rescuing mitochondria reductions. However, mec-4d dauers grown on E. coli OP50 but not E. coli HT115 have significantly more mitochondria than wild type animals (Figure 3B). Third, TRNs in wildtype dauers possess fewer mitochondria compared to their L2 controls (Figure 3C), supporting the idea that diapause has
lower metabolic demands under non-degenerative conditions. This decrease was observed regardless of diet in wildtype but varied in mec-4d dauers. TRNs in mutant dauers previously fed with *E. coli* OP50 but not *E. coli* HT115 have significantly more mitochondria than their respective wildtype dauers (Figure 3B) and L2 mutants (Figure 3D), suggesting that mitochondria increase may be a mechanism in dauers to alleviate homeostatic stress in mec-4d TRNs in non-protective diet.

Non-fragmented mitochondria are considered to be optimized for function and may protect against neuronal damage (Chen et al., 2007b; Kiryu-Seo and Kiyama, 2019; Wang et al., 2021). We wondered whether they play a role in diet or diapause-induced neuroprotection. Each mitochondrion in the AVM axons was classified as non-fragmented (filamentous, intermediate), or fragmented. We represented the percentage of animals with the largest mitochondria (non-fragmented) in each condition (Figures 3E-G). First, TRNs in L2 mec-4d mutants have smaller mitochondria than wild type TRNs in either diet, suggesting that diet does not compensate for mitochondrial size changes that are associated with mec-4d in developing animals (Figure 3E). In dauers however, the number of non-fragmented mitochondria is similar between genotypes (Figure 3E). Wildtype L2 and dauers have equal number of non-fragmented mitochondria, independent of the diet, suggesting that in absence of a prodegenerative stimulus TRN mitochondria do not change in size (Figure 3F). Importantly, mec-4d dauers have larger mitochondria than mec-4d L2 controls on *E. coli* OP50 (Figure 3G), and there is a similar trend on *E. coli* HT115 (adj p-value 0.06), supporting the idea that mitochondrial enlargement may account for diapause-mediated regeneration. We further evaluated the relationship between the percentage of wild type axons observed and mitochondrial parameters. Linear regression analysis revealed a strong relationship between AxW morphology and mitochondrial length for animals
on both diets (Figure 3H and 3I), although no clear relationship between mitochondrial numbers and axonal regeneration can be stated on E coli HT115 fed worms (Figure 3I). Thus far, our results support the idea that diapause- but not diet-induced neuroprotection is associated with mitochondrial changes in size and number.

To confirm the lack of association between diet and mitochondrial number and size, we further investigated whether HT115- induced protection requires genes involved in mitochondrial fusion (eat-3, fzo-1) (Kanazawa et al., 2008) -fission (drp-1, Lu et al., 2011; Navarro-González et al., 2017; Byrne et al., 2019) dynamics using TRN-specific and systemic RNAi strains as before. Consistent with our previous evaluations, there was no significant effect on neuronal protection for drp-1 or eat-3 dsRNA (Figure 3G and H). However, silencing of fzo-1, known to be important for external membrane fusion of mitochondria, significantly decreased the number of wildtype axons. Interestingly, fzo-1 is the ortholog of mitofusins, which in addition to mitochondrial fusion, stabilize the connection between mitochondria and ER, to for example, transport calcium from the ER to the mitochondria (Decuypere et al., 2011; Rodríguez-Arribas et al., 2017; Herrera-Cruz and Simmen, 2017; Michel and Kornmann, 2012). Thus, fzo-1 loss could be affecting axonal regrowth through these processes.

We tested the relevance of two other genes involved in mitochondrial metabolism, cts-1 (citrate synthase, Tricarboxylic Acid Cycle) and icl-1 (isocitrate lyase, Glyoxylate cycle), in axonal regrowth. Both of them are required for neuroprotection induced by diet in the TRN-specific RNAi strain (Figure 3G and H), showing that metabolic function of mitochondria is critical for dietary neuronal protection in a cell autonomous manner.
Taken together, our results suggest that diet-induced neuroprotection requires mitochondrial function in metabolism while diapause-induced neuroprotection may increase mitochondrial number and size.

Discussion

Food availability and microbiota composition affect neuronal integrity and survival (Caneo et al., 2019; Liu et al., 2020; Urrutia et al., 2020; Shandilya et al., 2022; Zhang et al., 2022; Urquiza-Zurich et al., 2023). To find commonalities between these two neuroprotective conditions, we study gene expression, calcium contribution, and mitochondrial parameters in the AVM neuron of *C. elegans*. Transcriptomics analysis reveals that feeding on neuroprotective bacteria induces the expression of genes required for calcium dynamics, neurogenesis and neuronal function, and dauer formation. Removing extracellular calcium affects both diet and diapause induced neuroprotection. Interestingly, simultaneous silencing of both *mcu-1* and *sca-1* is necessary to prevent axonal regrowth induced by diet whereas perturbation of either calcium transporter is sufficient to disrupt diapause-conferred neuroprotection. Moreover, larger mitochondria in the TRNs are promoted by diapause but not by protective microbiota.

A neuroprotective gene pool?

The TRNs are highly regenerative cells capable of regrowth after axotomy (Wu et al., 2007) or under protective treatment in chronic models of damage such as the *mec-4d* degenerin (Caneo et al., 2019; Urrutia et al., 2020). Microbiota protection occurs early in development and is long-lasting (Urrutia et al., 2020) coherent with the early expression of neuroprotective genes found here. But how does the expression of these genes contribute to creating a protective
environment and promote neuronal regrowth? We hypothesize that there are two components working in parallel, one systemic and one in the TRNs. Based on our transcriptomics analysis, we think that the systemic component includes direct effects of bacteria in the intestine by secretion of specific metabolites (MacNeil et al., 2013), and bidirectional communication through immune genes such as clc-1; irg-5, cnc-4. It also includes the expression of genes that operate outside the TRNs to create a propitious environment for regeneration (crh-2; sax-2) and lowering redox stress and alleviate the energetic demand of repairing tissues (hsp-16.11). The cell autonomous effect relies on TRN expression of genes that promote neuronal growth in situ upon favorable conditions in the extracellular milieu (ten-1, hrg-2). Metabolites produced by \( E. coli \) HT115 such as GABA and lactate, could improve the stress status of the intestine of nematodes previously reared on \( E. coli \) OP50. For example, GABA production and extrusion through the GABA shunt (Feehily et al., 2013) is a mechanism used by bacteria to lower the acidic stress of the intestine of host where they colonize. \( E. coli \) HT115 unlike \( E. coli \) OP50 contains all enzymes required for GABA production and export (Urrutia et al., 2020). An interesting class of genes that appeared in our screen and others (Revtovich et al., 2019; MacNeil et al., 2013) are the \textit{hrg} (heme responsive genes). Free heme is highly reactive and can intercalate in lipid bilayers (Chen et al., 2012). HRG-2, a heme deficiency responsive membrane protein, regulates heme homeostasis and detoxification (Chen et al., 2012), alleviating cellular redox stress. This function is required for \( E. coli \) HT115 induced neuroprotection systemically and in the TRNs.

In parallel, \( E. coli \) HT115 metabolites could directly travel to the TRNs using specific transporters like UNC-47 or SNF-5; or trigger signaling cascades such as those initiated by the transcription factor DAF-16/ FOXO, or through specific GABA receptors like GAB-1 or LGC-
37, all of which are necessary for *E. coli* HT115 protection (Urrutia et al., 2020). Others studies have shown that *E. coli* HT115 lowers cellular stress in *C. elegans* by counteracting vitamin B12 deficiency and the toxic accumulation of propionate, most of which improves mitochondrial health (Revtovich et al., 2019). Bacterial metabolites and the induction of a nematode gene pool could then directly lower systemic mitochondrial stress (*hsp-16.11*) and promote specific functions in adjacent tissues to the TRNs. For example, adhesion molecules such as cadherins mediate cell signaling and neuroregeneration (Hirota et al., 2001; Kanemaru et al., 2013; Friedman et al., 2015; Yulis et al., 2018; Punovuori et al., 2021). The teneurins, are transmembrane proteins fundamental for the development of the nervous system (Drabikowski et al., 2005; Mörck et al., 2010; Tucker et al., 2012; Topf and Drabikowski, 2019) and also neuroprotective (Al Chawaf et al., 2007; Trubiani et al., 2007; Tessarin et al., 2019).

A few genes necessary for *E. coli* HT115-induced protection are also upregulated in dauers (Hutter et al., 2009; Boeck et al., 2016), suggesting that both signaling processes might share a common gene pool. Previous analysis shows that *C. elegans* diapause induces large transcriptional changes to accommodate periods of long-lasting starvation (Wang and Kim, 2003; Jones et al., 2001; Dalley and Golomb, 1992). Some of these changes include the overexpression of pro-regenerative genes such as *dlk-1* and the repression of anti-regenerative genes such as *efa-6* (Chen et al., 2011; Calixto, 2015). A similar scenario could be induced by the protective microbiota.

**Calcium contribution**

Active maintenance of calcium levels is required to promote regeneration, which directly involves the mitochondrial calcium uniporter *mcu-1* and the SERCA pump *sca-1* (Sarasija et al., 2023).
2018; Wang et al., 2019; Calvo-Rodriguez et al., 2020; Calvo-Rodriguez and Bacskai, 2021). The blockade of mcu-1 prevents cellular neuronal death in the context of Alzheimer’s Disease (Sarasija et al., 2018; Calvo-Rodriguez and Bacskai, 2021). Here we show that during development the silencing of both mcu-1 and sca-1 are required for a reduction in wild-type axons TRN-autonomously, suggesting they are redundant. It is also possible that an increase in intracellular calcium by itself is not damaging under a protective diet. Intraorganellar stress has been shown to trigger degeneration (Sarasija et al., 2018; Wang et al., 2019).

In diapausing animals, silencing of mcu-1 or sca-1 halted regeneration of TRNs. Our results contrast with the simple idea that calcium by itself only damages the cell. Silencing of sca-1 and mcu-1 induces higher cytoplasmic calcium concentration, but only regeneration is impaired, and an increment in neuronal death is not observed when calcium is present in the environment of diapausing animals. Earlier it was proposed that calcium toxicity may be related to an increment in mitochondrial calcium, which promotes oxidant-induced mitochondrial loss of function, ATP depletion, and mitochondrial bursting (Harman and Maxwell, 1995; Calvo-Rodriguez et al., 2020).

The reduction of intracellular calcium transporters does not simply generate an increment of calcium concentrations inside the neuron, but rather alters the subcellular pattern of calcium levels that it also prevents mitochondrial bursting (Sarasija et al., 2018; Calvo-Rodriguez et al., 2020; Calvo-Rodriguez and Bacskai, 2021), suggesting that mitochondrial or ER damage is more relevant for the outcome of the cell than the cytoplasmic concentration of calcium.

Regeneration actively requires calcium (Sun et al., 2014; Khachaturian, 1989; Chierzi et al., 2005; Ghosh-Roy et al., 2010). In E. coli HT115 calcium depletion impaired the protection
and regrowth of axons, as is observed in dauer-induced regeneration. This suggests that calcium may be crucial during axonal maintenance generally.

Mitochondria and neuroprotection

The role of mitochondrial function in neuronal protection has been linked to the microbiota-metabolites-brain axis (Saint-Georges-Chaumet and Edeas, 2016; Nurrahma et al., 2021). The mitochondria of animals that enter diapause become larger and more numerous in mec-4d animals under prodegenerative pressure. Since energy production is associated with mitochondrial fusion (Skulachev, 2001), longer mitochondria may be key to the correlations found in our work and previous reports (Chen et al., 2007a; Knott et al., 2008; Chen and Chan, 2010; Chang et al., 2019; Wang et al., 2019).

Intermittent fasting improves cognitive traits and mitochondrial function (Liu et al., 2020). Dauers have reduced metabolic rates and elevated levels of heat shock proteins; are resistant to oxidative stress, and exhibit a low metabolic rate compared with other larval stages (Anderson, 1982; O’Riordan and Burnell, 1990; Burnell, 1989; Dalley and Golomb, 1992; Penkov et al., 2020). Most of these characteristics are associated with increased mitochondrial functions, such as more ATP availability or a reduction of toxicity (Fariss et al., 2005; Labbadia et al., 2017). mec-4d dauers have longer and more mitochondria suggesting these traits help maintain axons (Knott et al., 2008; Chang et al., 2019). Diapause induction affects mitochondrial physiology (Artal-Sanz and Tavernarakis, 2009; Lourenço et al., 2015; Lourenço and Artal-Sanz, 2021), suggesting the regeneration of dauers might be a consequence of an increased buffering capacity and improved energy management by larger mitochondria.
drp-1 or eat-3 silencing and consequently impaired mitochondrial fission/fusion did not affect neuroprotection by diet, consistent with our observation that animals fed with *E. coli* HT115 do not increase the number or size of mitochondria. However, the loss of *fzo-1/mitofusin-1* which initiates the fusion of the external mitochondrial membrane caused a dramatic reduction in AxW morphology, suggesting that mitochondrial structure influences the regenerative processes. Unsurprisingly, the silencing of genes required for mitochondrial metabolic function *cts-1* (citrate synthase) and *icl-1* (isocitrate lyase) affect neuroprotection in a TRN autonomous manner, which directly impacts in ATP production of the cells and their capacity to response to stress (Zampese et al., 2022). This supports that changes in the metabolism induced by the microbiota, can cause the neuronal protection to be independent of an increase in mitochondrial number or size.

References


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Table 1. Genes confirmed by RNAi to be required for HT115-induced neuroprotection

<table>
<thead>
<tr>
<th>Gene</th>
<th>TRN Systemic</th>
<th>Systemic Function</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrg-2</td>
<td>*** *****</td>
<td>Heme binding activity</td>
<td>TRN, Hypodermis</td>
</tr>
<tr>
<td>T12D8.5</td>
<td>**** *****</td>
<td></td>
<td>TRN, Head mesodermal cell, intestine, and neurons</td>
</tr>
<tr>
<td>ten-1</td>
<td>*** ****</td>
<td>Neuronal and epidermal development</td>
<td>TRN, Body muscle cell, gonad, hypodermis, neurons</td>
</tr>
<tr>
<td>T22B7.3</td>
<td>* ****</td>
<td></td>
<td>AFD and ASER neurons, germline precursor cell, and hypodermis</td>
</tr>
<tr>
<td>irg-5</td>
<td>* ***</td>
<td>Defense response to Gram + bacterium.</td>
<td>Intestine, pharyngeal muscle and motor neurons</td>
</tr>
<tr>
<td>rips-1</td>
<td>**** ****</td>
<td>S-adenosylmethionine (SAM)-dependent methyltransferase</td>
<td>AVB, Intestine</td>
</tr>
<tr>
<td>clec-125</td>
<td>** ****</td>
<td>Carbohydrate binding activity</td>
<td>Sensory neurons</td>
</tr>
<tr>
<td>hsp-16.11</td>
<td>***</td>
<td>Involved in endoplasmic reticulum UPR</td>
<td>TRN, Amphid and motor neurons</td>
</tr>
<tr>
<td>cdh-4</td>
<td>****</td>
<td>Axon guidance in the ventral cord</td>
<td>TRN, QL, QR, and VD neurons, muscle, and rectal gland cell</td>
</tr>
<tr>
<td>sax-2</td>
<td>****</td>
<td>Neuron projection development</td>
<td>TRN, head, tail and ventral nerve cord neurons</td>
</tr>
<tr>
<td>cnc-4</td>
<td>****</td>
<td>Involved in defense response</td>
<td>Hypodermis, intestine, and seam cell</td>
</tr>
<tr>
<td>Gene</td>
<td>Expression</td>
<td>Function and Location</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td>H01G02.1</td>
<td>****</td>
<td>GABAergic and dopaminergic neurons, excretory cell, intestine, and pharyngeal muscle</td>
<td></td>
</tr>
<tr>
<td>H02F09.2</td>
<td>**</td>
<td>ASER neuron and intestine</td>
<td></td>
</tr>
<tr>
<td>K09F6.6</td>
<td>***</td>
<td>Defense response to Gram+ bacterium; epithelial cell-cell adhesion; and innate immune response</td>
<td></td>
</tr>
<tr>
<td>clc-1</td>
<td>****</td>
<td>Pharynx</td>
<td></td>
</tr>
<tr>
<td>cts-1</td>
<td>***</td>
<td>Citrate synthase activity (TCA) TRN, neuron, intestine, hypodermis, body wall muscle</td>
<td></td>
</tr>
<tr>
<td>icl-1</td>
<td>***</td>
<td>Isocitrate lyase activity Intestine, hypodermis, muscle cell, pharynx</td>
<td></td>
</tr>
</tbody>
</table>

**Legends**

**Fig 1.** Gene expression analysis of microbiota induced neuronal protection and in vivo validation of neuroprotective gene candidates.

**A.** Upper panel: Photography of a wild type nematode expressing gfp in all the touch receptor neurons (TRN). Bottom panels: mec-4d animals expressing gfp in the AVM with different degrees of protection/ degeneration. Wild type axons (AxW) are considered the protected category. **B.** Time course of wild type axonal morphology (AxW) in wild type and mec-4d animals grown on *E. coli* OP50 and *E. coli* HT115 diets at 12, 24, 48, and 72 post-hatching (N=3; Two-way ANOVA). **C-D.** Heatmaps of upregulated and downregulated (D) genes in *E. coli* HT115 diet compared to *E. coli* OP50 at 12-, 24-, and 48- hours post-hatching. Darker blue indicates higher levels of expression. **E-F.** Percentage of AxW morphology in animals feeding on
double stranded RNA (dsRNA)-expressing bacteria of upregulated genes in a systemic strain (E) and TRNs specific strain (F), after 72-hours post-hatching (One-way ANOVA, N=3 or more). G. Venn diagram showing the common and unique genes between those functionally required for *E. coli* HT115 protection systemically and TRNs specifically. H. Gene Enrichment for phenotype of all genes required for neuroprotection. I. Venn diagram showing the common and unique phenotypical enrichment categories of genes required systemically and TRN specifically. ****p < 0.0001, *** < 0.001, **p < 0.005, *p < 0.05. Heatmaps and Volcano plots of transcriptomics analysis can be found in Extended Data Figure 1-1. Additional enrichment analyses are shown in Extended Data Figure 1-2, 1-3 and 1-4. Differentially expressed genes are listed in Extended Data Table 1-1. All genes used for enrichment analysis and their statistics is shown in Extended Data Table 1-2 and Extended Data Table 1-3.

**Figure 2. Mitochondrial and ER calcium transporters are required TRN-autonomously for neuroprotection induced by diet and diapause-induced regeneration.** A. Percentage of AxW morphology in *mec-4d* animals at 72-hours post-hatching in *E. coli* OP50 and *E. coli* HT115 with and without calcium (N=3; Two-way ANOVA). B. Percentage of AxW morphology in dauer *mec-4d* animals cultured before synchronization in *E. coli* OP50 and *E. coli* HT115 with or without calcium (N=3; Two-way ANOVA). C-D. Percentage of AxW morphology in *mec-4d* animals on *sca-1* and *mcu-1* dsRNA expressing bacteria 72 hours post-hatching in systemic (C) and TRN RNAi specific strains (D) (N=4; One-way ANOVA). E-F. Percentage of AxW morphology in *mec-4d* animals feeding on *sca-1* and *mcu-1* dsRNA expressing bacteria in dauers in systemic (E) and TRN specific strains (F) (N=4; One-way ANOVA). ****p < 0.001, **p < 0.005, *p < 0.05.
Figure 3. Diapause improves mitochondrial defects caused by a degenerin mutation in the TRNs. A. Representative images of wild type or mec-4d dauers previously fed on *E. coli* OP50 expressing *mec-4p::MLS-GFP*. Inset shows mitochondria of different sizes in a section of the AVM axon. Bottom panel are representative images of filamentous, intermediate, and fragmented mitochondria expressing mitochondrial *gfp*. Filamentous and intermediate mitochondria are referred to as non-fragmented. B-D. Number of mitochondria normalized by axonal length in TRNs growing in *E. coli* OP50 and *E. coli* HT115 in wild type (B, C) and mec-4d (B, D) strains in L2 and early dauer stages (N=3; One-way ANOVA). E-G. Percentage of the population that exhibits non-fragmented mitochondria in TRNs wild type (E, F) and mec-4d animals (E, G) growing *E. coli* OP50 or *E. coli* HT115 in L2 and early dauers (N=3; One-way ANOVA). H-K. Percentage of AxW morphology in animals feeding on dsRNA-expressing bacteria of mitochondrial genes in a TRN (H, J) or systemic (I, K)-specific strain, 72 hours post-hatching (N=3 or 4; One-way ANOVA). **p<0.005, *p<0.05. L, M. Correlation between mitochondrial length and number of mitochondria normalized by the length in mec-4d animals growing in *E. coli* OP50 (L) and *E. coli* HT115 (M) in L2 and dauers.

Table 1. Genes confirmed by RNAi to be required for HT115-induced neuroprotection. P values indicate when the gene is required systemically and/or TRN autonomously for neuroprotection. ****p < 0.0001, *** < 0.001, **p < 0.005, *p < 0.05

Extended Data
Figure 1-1. A. Heatmap of genes differentially expressed (DE) in *E. coli* HT115 compared to *E. coli* OP50 at different times during development. DeSeq and EdgeR analysis are shown. B. Volcano plot for DE analysis on each developmental timepoint.

Figure 1-2. Enrichment analysis of genes differentially expressed in *E. coli* HT115 diet.

A-D. Phenotype (A, C) and Gene Ontology (B, D) enrichment of genes upregulated (A, B) and downregulated (C, D) in *E. coli* HT115 compared to *E. coli* OP50. Statistical analyses are shown on Extended Data Table 1-3.

Figure 1-3. Enrichment analysis of genes required for neuroprotection conferred by *E. coli* HT115.

(A) and Gene Ontology of genes that are required for *E. coli* HT115 neuroprotection. B. Venn diagram of Gene Ontology categories of genes required systemically and in the TRNs for neuroprotection. Statistical analyses are shown on Extended Data Table 1-3.

Figure 1-4. A-B. Enrichment analysis of genes upregulated in dauers that are shared with genes required for neuroprotection in *E. coli* HT115. Gene ontology enrichment associated to genes shared between *E. coli* HT115 RNAi positive clones and dauers (A), and those not shared with dauers (B). Statistical analyses are shown on Extended Data Table 1-3.

Table 1-1. Genes differentially expressed in animals feeding on *E. coli* HT115 compared to *E. coli* OP50. Number of reads per replica are shown for each condition as well as the differential expression analysis done by DeSeq and EdgeR.
Table 1-2. Lists, enrichment analyses and statistics of genes differentially expressed in mec-4d animals feeding *E. coli* HT115 compared to *E. coli* OP50.

Table 1-3. Lists, enrichment analyses and statistics of genes overexpressed in animals feeding *E. coli* HT115 compared to *E. coli* OP50 in previous works by others (MacNeil et al., 2013; Revtovich et al., 2019).
Figure 2

A) 72 hours post hatching

B) dauers

C) Systemic RNAi

D) TRN-RNAi

E) Systemic RNAi dauers

F) TRN-RNAi dauers