The Anticonvulsant Ethosuximide Disrupts Sensory Function to Extend C. elegans Lifespan

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Abstract
Ethosuximide is a medication used to treat seizure disorders in humans, and we previously demonstrated that ethosuximide can delay age-related changes and extend the lifespan of the nematode Caenorhabditis elegans. The mechanism of action of ethosuximide in lifespan extension is unknown, and elucidating how ethosuximide functions is important for defining endogenous processes that influence lifespan and for exploring the potential of ethosuximide as a therapeutic for age-related diseases. To identify genes that mediate the activity of ethosuximide, we conducted a genetic screen and identified mutations in two genes, che-3 and osm-3, that cause resistance to ethosuximide-mediated toxicity. Mutations in che-3 and osm-3 cause defects in overlapping sets of chemosensory neurons, resulting in defective chemosensation and an extended lifespan. These findings suggest that ethosuximide extends lifespan by inhibiting the function of specific chemosensory neurons. This model is supported by the observation that ethosuximide-treated animals displayed numerous phenotypic similarities with mutants that have chemosensory defects, indicating that ethosuximide inhibits chemosensory function. Furthermore, ethosuximide extends lifespan by inhibiting chemosensation, since the long-lived osm-3 mutants were resistant to the lifespan extension caused by ethosuximide. These studies demonstrate a novel mechanism of action for a lifespan-extending drug and indicate that sensory perception has a critical role in controlling lifespan. Sensory perception also influences the lifespan of Drosophila, suggesting that sensory perception has an evolutionarily conserved role in lifespan control. These studies highlight the potential of ethosuximide and related drugs that modulate sensory perception to extend lifespan in diverse animals.

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Introduction
Pharmacological compounds that extend lifespan could delay the progression of age-related degenerative changes and age-related illnesses such as Alzheimer’s disease and cardiovascular disease. In addition, the characterization of drugs that extend lifespan can elucidate endogenous mechanisms involved in lifespan determination, since the targets of these drugs are likely to influence normal aging. The short lifespan and rapid aging of invertebrates makes them powerful models for the identification of drugs that extend lifespan and for the characterization of the mechanism of action of these drugs [1–3]. The free-living soil nematode Caenorhabditis elegans has been a leading system for studying genetic and pharmacologic influences on lifespan. Four categories of compounds have been reported to extend C. elegans lifespan: a variety of antioxidant compounds [4–9]; complex mixtures derived from plants [10,11]; resveratrol, a potential modulator of Sir2 activity [12,13]; and medications such as heterocyclic anticonvulsant medications that may act by affecting neural activity [14–17]. Compounds that extend the lifespan of vertebrates have not been well characterized. However, a recent report showing that resveratrol can extend the lifespan of a short-lived fish suggests that compounds that extend invertebrate lifespan may be relevant to vertebrate biology [18].

By screening 19 drugs from different structural and functional classes that are FDA-approved for human use, Evason et al. (2005) discovered that ethosuximide can extend the lifespan of C. elegans [14]. Ethosuximide is a small, heterocyclic ring compound of the succinimide class that is approved for human use as an anticonvulsant [19]. Trimethadione is a structurally related anticonvulsant that is a member of the oxazolidinedione class, and trimethadione also extends C. elegans lifespan [14]. Ethosuximide is commonly used in clinical practice whereas trimethadione is rarely used due to the potential for adverse side effects. Ethosuximide extended the mean adult lifespan of wild-type animals grown on agar dishes by 17% [14]. The effect is dose dependent, and at high doses ethosuximide causes toxicity. In addition, ethosuximide extends the span of time that animals display fast body movement and pharyngeal pumping, demonstrating that ethosuximide delays age-related functional declines in addition to extending lifespan. Ethosuximide has been shown to affect the activity of multiple ion channels in vertebrate cultured cells, including T-type calcium channels [20–22]. The relationship between these activities in cultured cells and the anticonvulsant activity in whole animals has yet to be defined fully. Furthermore, the mechanism of action for lifespan extension in worms is not well characterized.

To elucidate the mechanism of action of ethosuximide, we conducted a genetic screen for mutations that cause resistance to the drug. Screening for drug resistance is a well-established approach in C. elegans [23]. A mutation can cause resistance to a drug for several different reasons such as altering the molecular
Aging is a major factor that contributes to disease and disability in humans, but no medicines have been demonstrated to delay human aging. We previously conducted a screen for FDA-approved drugs that can extend the lifespan of the nematode worm *C. elegans*, resulting in the identification of ethosuximide, a medicine used to treat epilepsy. To elucidate the mechanism of action of ethosuximide in lifespan extension, we conducted a genetic screen for *C. elegans* mutations that cause resistance to ethosuximide. Here, we describe the identification of genes that are critical for ethosuximide sensitivity. These genes are necessary for the function of neurons that mediate sensory perception. Furthermore, ethosuximide treatment caused defects in sensory perception. These results indicate that ethosuximide affects lifespan by inhibiting neurons that function in the perception of sensory cues. These studies highlight the importance of sensory neurons in lifespan determination and demonstrate that a drug can act on specific cells within the nervous system to extend lifespan. Sensory perception also modulates *Drosophila* lifespan, suggesting this is an evolutionarily conserved relationship. Our results indicate that sensory perception may be a promising target for pharmacological extension of lifespan in a variety of animals.

**Author Summary**

To characterize the mechanism of action of ethosuximide, we conducted a genetic screen and identified mutations in two genes, *che-3* and *osm-3*, that cause resistance to ethosuximide-mediated toxicity. Mutations in *che-3* and *osm-3* cause defects in overlapping sets of chemosensory neurons and can extend lifespan [32]. These findings indicate that ethosuximide extends lifespan by inhibiting a subset of chemosensory neurons. Here we present results that strongly support this model. Ethosuximide treated wild-type animals displayed numerous phenotypic similarities with mutants that have chemosensory defects, indicating that ethosuximide inhibits chemosensory function. Importantly, the long-lived *osm-3* mutants did not respond to lifespan extending doses of ethosuximide. These studies demonstrate a novel mechanism of action for a lifespan extending drug and demonstrate the potential of pharmacologically targeting the sensory system as a means to extend animal lifespan.

**Results**

The *C. elegans* T-type Calcium Channel, CCA-1, Is Not Required for Ethosuximide to Extend Lifespan or Stimulate Egg-Laying

Studies using vertebrate cultured cells have led to the proposal that T-type calcium channels may be the molecular target of ethosuximide in controlling seizures [20,22,35]. To determine if ethosuximide inhibits T-type calcium channels to extend lifespan, we analyzed a null mutation in the gene encoding the *C. elegans* orthologue of the mammalian T-type calcium channel, *cca-1*(gk30) [36,37]. If ethosuximide inhibits T-type calcium channels to extend lifespan, then we predict that (1) a *cca-1* loss-of-function mutant will be long-lived and (2) a *cca-1* loss-of-function mutant will not respond to the lifespan extension caused by ethosuximide. *cca-1*(gk30) mutants displayed a mean adult lifespan of 15.2 days, which was not significantly different from the 16.1 day mean adult lifespan of wild-type animals (Figure 1A). *cca-1*(gk30) hermaphrodites treated with ethosuximide displayed a robust lifespan extension, indicating that ethosuximide does not require T-type calcium channels to extend *C. elegans* lifespan (Figure 1A).

In addition to extending lifespan, ethosuximide also stimulates the rate of egg-laying [14]. To determine if ethosuximide requires T-type calcium channels to stimulate egg-laying, we utilized a well-described assay that involves counting the eggs laid by a young adult hermaphrodite that is transferred from a Petri dish with abundant food to M9 liquid culture with no food [38]. We analyzed a second putative *cca-1* null allele, *cca-1*(ad1650) [36,37]. Wild-type and *cca-1*(ad1650) hermaphrodites transferred to M9 buffer laid zero eggs in 90 minutes (Figure 1B), demonstrating that egg-laying is strongly inhibited by these conditions. Wild-type and *cca-1*(ad1650) hermaphrodites displayed a similar, dose-dependent increase in egg-laying in response to ethosuximide (Figure 1B), indicating that T-type calcium channels are not required for ethosuximide stimulated egg-laying. Collectively, these results suggest that ethosuximide modulates lifespan and egg-laying in *C. elegans* by acting on molecular targets distinct from the T-type calcium channel CCA-1.

**A Genetic Screen for Ethosuximide Resistant Mutants Implicates Ciliated Neuron Function in Ethosuximide Sensitivity**

The identification and characterization of mutants that are resistant to the activity of a drug has been a useful approach for elucidating the mechanism of drugs in *C. elegans* [24,39–41]. The analysis of candidate genes, including *cca-1* and genes that influence longevity [14], did not clearly identify the target of ethosuximide. Therefore, we conducted a genetic screen for mutations that cause resistance to ethosuximide. To identify a phenotype for conducting a genetic screen that is more suitable than lifespan, we performed a dose response analysis. The optimal dosage for lifespan extension by ethosuximide was 2–4 mg/ml in the culture media [14]. Concentrations of ethosuximide greater than 4 mg/ml caused dose dependent larval lethality (data not
Ethosuximide resistance in these strains is caused by a mutation in one gene. In all cases the mutant strains displayed partially penetrant survival in 12 mg/ml ethosuximide (Table 1 and data not shown), indicating that these mutations cause a shift in the dose response to ethosuximide and do not abrogate sensitivity to this drug. Here we describe six of these mutations that represent two complementation groups.

To characterize the mutations that cause ethosuximide resistance, we positioned these mutations on the C. elegans genetic and physical maps. The mutations am178, am165, and am162 exhibited tight linkage to a single nucleotide polymorphism (SNP) marker positioned at the center of Chromosome I (see Materials and Methods). Three-factor mapping positioned am178 between dpy-5 and unc-75 on Chromosome I (Figure 2). High resolution mapping positioned am178 between SNP markers uCEI-952 and snp_T01G91, a ~250 kB interval on Chromosome I (Figure 2).

To identify the gene affected by am178, we analyzed candidate genes in the interval by measuring the ability of existing mutations to cause resistance to ethosuximide lethality. To quantitatively assess the drug resistance of a mutant, we exposed embryos to 12 mg/ml ethosuximide for five to six days and measured the number of surviving animals that developed past the L1 larval stage. che-3(e1124) mutants displayed resistance to 12 mg/ml ethosuximide that was 31 percent penetrant (Table 1). Furthermore, che-3(e1124) failed to complement am178 for resistance to ethosuximide, indicating that am178 is a mutation in the che-3 (see Materials and Methods). To test the prediction that the am178 mutation reduces the activity of the che-3 gene, we generated

![Figure 1. Ethosuximide treatment extended lifespan and stimulated egg laying in cca-1 null-mutants.](image)

**Table 1. Mutants with defects in cilia structure were resistant to ethosuximide toxicity.**

| Genotype | Amphid Dye-Filling* | Percent Ethosuximide Resistant† (n) |
|----------|---------------------|------------------------------------|
| WT       | +                   | 0 (783)                            |
| che-3(am178) | +              | 30.1 (199)                         |
| che-3(am165) | Dyf              | 26.1 (413)                         |
| che-3(am162) | Dyf              | 10.2 (525)                         |
| che-3(e1124) | Dyf              | 30.8 (668)*                         |
| osm-3(am161) | Dyf              | 43.4 (426)                         |
| osm-3(am177) | Dyf              | 41.1 (451)                         |
| osm-3(am172) | Dyf              | ND                                 |
| osm-3(p802) | Dyf              | 79.6 (621)                         |
| che-13(p105) | Dyf              | 62.0 (756)                         |
|daf-10(e1387) | Dyf              | 22.6 (729)                         |
| osm-5(p813) | Dyf              | 36.6 (209)*                         |

*Animals cultured at 20°C were classified as normal (+) or defective (Dyf) for amphid neuron dye filling (see Materials and Methods).

†Experiments were done with 12 mg/ml ethosuximide. The differences in percent resistance between che-3 alleles were not statistically significant except that che-3(am162) was significantly different from che-3(am178), che-3(am165) and che-3(e1124) (p = 0.0001). The differences in percent resistance between osm-3 alleles were not statistically significant except that osm-3(p802) was significantly different from osm-3(am161) and osm-3(am177) (p = 0.0001).

The dye-filling phenotype of these mutants was described previously [28]. These values may underestimate the fraction of animals that were resistant to ethosuximide, since we observed multiple animals that were mature, indicating they were ethosuximide resistant, but they were desiccated on the side of the dish and not included in the data. We have observed that mutants with severe chemotaxis defects have a propensity to leave the agar surface.

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selected 56 ethosuximide resistant, non-Dpy self-progeny. From
are shown below. From
cause visible phenotypes and SNP markers are shown above; map units
represents a portion of Chromosome I. Genes that can be mutated to
markers
positioned the recombination events in the intervals shown below. The
double-headed arrow indicates a 250 kbp interval between the SNP
markers uCE-952 and snp_T01G9[1] that contains am178.
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transgenic am178 mutants containing 32 kB of genomic DNA in a
fosmid clone that included the che-3 gene. This wild-type copy of
the che-3 gene rescued the am178 mutant phenotype (see Materials
and Methods). To identify the molecular lesion, we determined the
DNA sequence of the predicted che-3 exons and splice junctions
using DNA from am178 mutants. We identified a single base
change that affects the splice junction following exon 9 (the 5'
slice site was changed from GTATG in wild-type to GTATA in
am178 mutants). Analysis of transcripts demonstrated that am178
mutants produce an aberrantly spliced che-3 mRNA that is
predicted to encode a truncated CHE-3 protein, suggesting that
am178 is a loss-of-function mutation of the che-3 gene.

che-3 encodes a cytoplasmic dynein heavy-chain [42]. Dynein is
a component of the intraflagellar transport (IFT) machinery, and
this machinery builds and maintains the structure of ciliated
neurons in C. elegans. Mutations that disrupt IFT components, such
as CHE-3 dynein, result in defects in the structure of ciliated
neurons [43]. C. elegans contains 60 ciliated neurons, and these
neurons function in the perception of chemical and mechanical
cues [28–31,44]. The most extensively characterized ciliated
neurons are present in the amphid organ, a bilaterally symmetrical
neural structure located in the anterior of the animal. Some
amphid neurons have ciliated dendrites that are exposed to the
external environment, since they extend through a channel in the
cuticle [28,45]. Since these neurons are exposed to the
environment, treating animals with lipophilic dyes such as DiO
specifically stains these neurons [Figure 3A][28,46]. Mutants with
severe defects in the structure of these neurons display no staining,
a phenotype referred to as dye-filling defective (Dyf) (Figure 3D)
[28,31]. Many che-3 mutations cause a robust Dyf phenotype [42].

To characterize che-3(am178) and two candidate che-3 alleles
that were positioned near the center of Chromosome I, am162 and
am165, we performed the dye-filling assay. am162 and am165 both
caused a Dyf phenotype [Table 1], and both mutations failed to
complement che-3(e1124) for dye-filling defects (Materials and
Methods), indicating that am162 and am165 are alleles of che-3.
che-3(am178) mutants cultured at 20°C displayed a significant
defect in DiO staining that was not significantly different from wild-type (Table 1, Table 2 and
Figure 3B). However, che-3(am178) mutants cultured at 27°C
displayed a highly penetrant dye-filling defective phenotype (Table 2), indicating that the am178 mutation partially reduces the activity of the che-3 gene at the permissive temperature of 20°C and strongly reduces the activity of the che-3 gene at the restrictive
temperature of 27°C. To determine the temporal requirement for
che-3 gene activity, we cultured hermaphrodites at 20°C until the
L4 stage of development and then shifted the animals to 27°C for
24 hours before scoring DiO staining. Using this regimen che-
3(am178) mutants displayed a significant defect in DiO staining,
indicating that che-3 function is necessary after the L4 stage to
maintain cilia structure (Table 2). The findings that che-3(am178)
mutants cultured at 20°C displayed robust resistance to ethosux-
imide toxicity but did not display dye-filling defects indicate that
gross morphological defects in amphid neuron structure are not
required for che-3 mutants to be resistant to ethosuximide.

Three Mutations that Cause Ethosuximide Resistance
Affect the osm-3 Gene that is Necessary for Ciliated
Neuron Structure

Since che-3 mutations cause both ethosuximide resistance and
DiO staining defects, we characterized dye-staining of the
remaining ethosuximide resistant mutants. Three mutations that
exhibited tightest linkage to a marker positioned at –3.7 on
Chromosome IV, am161, am172 and am177, caused defects in DiO

![Figure 2. Positioning the am178 mutation.](Image)
The horizontal line represents a portion of Chromosome I. Genes that can be mutated to
cause visible phenotypes and SNP markers are shown above; map units
are shown below. From dpy-5 am178/CB4856 hermaphrodites, we
selected 56 ethosuximide resistant, non-Dpy self-progeny. From am178
unc-75/CB4856 hermaphrodites, we selected 72 ethosuximide resistant,
non-Unc self-progeny. An analysis of SNP markers in these strains
positioned the recombination events in the intervals shown below. The
double-headed arrow indicates a 250 kbp interval between the SNP
markers uCE-952 and snp_T01G9[1] that contains am178.
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![Figure 3. Ethosuximide treated animals and che-3(am178) mutants cultured at 20°C did not display dye-filling defects.](Image)
Animals were incubated in the lipophilic dye DiO and observed using
fluorescence microscopy at 200 × magnification. Each panel illustrates
the anterior tip of the animal (top) to the base of the bi-lobed pharynx
(bottom). DiO stains amphid neuron processes (arrows) and cell bodies
(arrowheads). Wild-type (WT) animals displayed robust staining that was
not affected by treatment with 4 mg/ml ethosuximide (WT+ETH4). che-
3(am178) mutants cultured at 20°C displayed robust staining, whereas
osm-3(p802) mutants displayed no detectable staining (the Dyf
phenotype).
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![Figure 3B.](Image)
staining (Table 1). We investigated the possibility that these are osm-3 alleles because osm-3 is located at -2.2 on Chromosome IV and can be mutated to produce defective DiO staining (Figure 3D). am161, am172, and am177 each failed to complement the osm-3 reference allele p802 for DiO staining defects, suggesting that each mutation affects the osm-3 gene (see Materials and Methods). Furthermore, the osm-3(p802) mutation caused 80 percent resistance to ethosuximide (Table 1), consistent with the model that am161, am172, and am177 are osm-3 alleles.

Like the CHE-3 dynein, the OSM-3 kinesin is a component of the IFT machinery that is essential for the formation of ciliated nerve endings. The OSM-3 kinesin has been divided into four domains: motor, neck, rod, and tail (Figure 4) [47]. Previous molecular genetic studies identified two missense mutations that affect the motor region and one missense mutation that affects the neck region, thus identifying a residue in the IFT machinery that is essential for the formation of ciliated neurons [47].

**Table 2. The che-3(am178) mutation caused temperature-sensitive defects in DiO staining of amphid neurons.**

| Genotype   | Temperature (°C) | % Dyf | N  |
|------------|------------------|-------|----|
| WT         | 20               | 0     | 22 |
| che-3(am178) | 20               | 1.3   | 76 |
| WT         | 27               | 0     | 47 |
| che-3(am178) | 27               | 93    | 121|
| WT         | 20–20            | 0     | 64 |
| che-3(am178) | 20–20            | 0     | 83 |
| WT         | 20–27            | 0     | 51 |
| che-3(am178) | 20–27            | 44    | 72 |

*For lines 1–4, hermaphrodites were cultured from conception to L4 at the indicated temperature. For lines 5–8, hermaphrodites were cultured from conception to L4 at 20°C and then shifted to the indicated temperature for approximately 24 hours. doi:10.1371/journal.pgen.1000230.t002*

indicating that ciliated neurons may be an important cellular focus of ethosuximide function. The che-3, che-13, daf-10, and osm-5 genes are necessary for the structure or activity of all 60 ciliated neurons [28]. By contrast, the osm-3 gene is necessary for only a subset of ciliated neurons. osm-3 expression is restricted to amphiad, phasmid and inter labial neurons that function in chemosensation, and osm-3 loss-of-function mutants are specifically defective in behaviors mediated by these neurons [28,48,49]. These observations define a subset of chemosensory neurons that are defective in osm-3 mutants as critical for the response to ethosuximide toxicity.

**osm-3 Mutants Responded to the Egg-Laying Stimulation Caused by Serotonin and Ethosuximide, Indicating that These Mutants Absorbed These Drugs**

In principle, mutations that disrupt cilium structure could cause resistance to ethosuximide toxicity because they affect the molecular target, the cellular target or the metabolism of the drug. Mutations that disrupt cilium structure prevent the lipophilic dye DiO from staining the membranes of ciliated neurons. This raises the possibility that these mutations also disrupt absorption of water soluble drugs, such as ethosuximide [24]. If this were the case then these mutants are predicted to be resistant to a wide variety of drugs. To investigate this possibility, we analyzed resistance to serotonin, a well-characterized compound that stimulates egg-laying [38]. We compared the resistance of wild-type hermaphrodites and osm-3(p802) mutants. osm-3(p802) has been extensively characterized using behavioral assays and electron microscopic analyses [28]. Genetic studies indicate that osm-3(p802) is a strong loss-of-function mutation, and molecular studies demonstrate that the allele contains a nonsense change at codon 346 [47] (Figure 4). Our analysis also supports the
Mutations that Impair Ciliated Neurons and Treatment with Ethosuximide Cause Similar Defects in Chemotaxis

Our findings suggest that ethosuximide affects the activity of chemosensory neurons. To directly investigate this model, we compared the effects of ethosuximide treatment and mutations that cause defects in chemosensory neurons. Animals with defects in amphid neurons resulting from mutations or physical ablation do not respond properly to chemical cues from the environment and display defective chemotaxis [29]. Untreated wild-type animals displayed a similar, dose-dependent increase in the rate of egg-laying in the presence of ethosuximide (Figure 5B), indicating that ethosuximide stimulates egg-laying in these conditions. These results demonstrate that osm-3 mutants have a normal response to ethosuximide-stimulated egg-laying, indicating that the entry of ethosuximide into the animal is normal in these mutants.

Ethosuximide Treatment Caused Daf-c and Daf-d Phenotypes, Similar to Mutations that Disrupt Chemosensory Function

Amphid neurons play important roles in the developmental decision between forming an L3 larva that matures to a reproductive adult or a dauer larva that persists until environmental conditions improve. Laser ablation studies indicate that specific chemosensory amphid neurons are necessary to inhibit dauer formation [ASI, ADF, and ASG], and to promote dauer formation [ASJ] [50,51]. Consistent with these observations, mutations that cause defects in multiple chemosensory neurons...
Ethosuximide Disrupts C. elegans Sensory Function

Figure 6. Ethosuximide disrupted chemotaxis toward the volatile attractants isoamyl alcohol and diacetyl. Wild-type animals grown from conception to adulthood on NGM plates containing 2 mg/ml ethosuximide or no drug were transferred to the center of a 10 cm chemotaxis plate containing 4 mg/ml of ethosuximide or no drug, respectively. A chemotaxis index (CI) to isoamyl alcohol or diacetyl was determined. (A) Chemotaxis to isoamyl alcohol. The mean CI±SD for untreated and ethosuximide treated animals was 0.8±0.2 and 0.3±0.2, respectively (p<0.0001, n=8) (Student T-test). (B) Chemotaxis to diacetyl. The mean CI±SD for untreated and ethosuximide treated animals was 0.8±0.14 and 0.2±0.2, respectively (p=0.0009, n=6) (Student T-test). Error bars represent standard error of mean.

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Table 3. Ethosuximide inhibited dauer formation in a high-density, starved population at 25°C.

| Genotype Ethosuximide (mg/ml) | Dauer ± SD (Number/Dish) | N (Dishes) |
|-------------------------------|--------------------------|------------|
| WT                            | 0                        | 250±110    | 13         |
| WT                            | 4                        | 16±15***   | 11         |
| osm-3(p802)                   | 0                        | 26±27***   | 10         |

***p<0.0001, comparisons are to WT with no drug treatment. p values determined by Student T-test.

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Table 4. Ethosuximide stimulated dauer formation in a low-density, well-fed population at 27°C.

| Genotype Ethosuximide (mg/ml) | Dauer (%) | N (animals) |
|-------------------------------|-----------|-------------|
| WT                            | 0         | 1310        |
| WT                            | 4         | 9.9***      | 1322       |
| osm-3(p802)                   | 0         | 6.6         | 637        |
| osm-3(p802)                   | 4         | 5.0         | 697        |

***p<0.0001, comparisons are to the same genotype with no drug treatment. p values determined by Student T-test.

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Mutations that Impair Chemosensory Neurons and Treatment with Ethosuximide Caused Similar Defects in L1 Arrest

Insulin/IGF-1 signaling is a critical regulator of dauer formation and an important modulator of adult lifespan [55–57]. daf-2 encodes a receptor tyrosine kinase similar to the insulin receptor [56], and age-1 encodes a phosphatidylinositol-3-kinase that functions downstream in the signaling pathway [58]. Loss-of-

have complex effects on dauer formation. These mutations can cause dauer defective (Daf-d) phenotypes or dauer constitutive (Daf-c) phenotypes depending on the environmental conditions [32,52–54]. To investigate the effects of ethosuximide on chemosensory neuron activity, we analyzed the effects of ethosuximide treatment on dauer formation in environmental conditions previously described to cause Daf-d and Daf-c phenotypes in animals with chemosensory neuron defects.

The combination of high temperature, high population density and low food availability promotes the formation of dauer larvae in wild-type animals [23]. Mutants with defects in chemosensory neurons display a Daf-d phenotype in this combination of culture conditions, indicating that these sensory neurons are necessary to respond to these environmental cues and promote dauer larval formation [54]. To determine if ethosuximide treatment causes a similar Daf-d phenotype, we analyzed animals cultured at the high temperature of 25°C, at a high population density, and with low food availability. The osm-3(p802) mutation significantly reduced the average number of dauer larvae by 10-fold, from 250 to 26 (p<0.0001) (Table 3), consistent with previous observations [54]. Ethosuximide treatment of wild-type animals significantly reduced the average number of dauer larvae by 16-fold, from 250 to 16 (p<0.0001) (Table 3). These results indicate that ethosuximide inhibits chemosensory neurons that are necessary to promote dauer larval formation.

By contrast to the culture conditions described above, culturing animals in low density populations with abundant food at the extremely high temperature of 27°C reveals that chemosensory neuron function is necessary to inhibit dauer formation [32,52]. To determine if ethosuximide treatment and mutations that disrupt chemosensory neurons cause similar defects, we analyzed dauer larval formation at 27°C. The osm-3(p802) mutation significantly increased the fraction of dauer larvae from 0 to 7 percent (Table 4). Ethosuximide treatment of wild-type animals significantly increased the fraction of dauer larvae from 0 to 10 percent (p<0.0001) (Table 4), indicating that ethosuximide treatment and an osm-3 mutation caused similar defects in the ability to inhibit dauer formation. To investigate the relationship between the dauer promoting effects of ethosuximide and the osm-3(p802) mutation, we treated osm-3(p802) mutants with ethosuximide. Ethosuximide treatment did not enhance the dauer arrest caused by osm-3(p802) (Table 4), suggesting that ethosuximide and osm-3 mutations promote dauer arrest by a similar mechanism. Collectively, these results indicate that ethosuximide treatment can cause Daf-c and Daf-d phenotypes in specific environmental conditions, and this result supports the model that ethosuximide disrupts the activity of multiple chemosensory neurons.
function mutations in daf-2 and age-1 promote dauer formation. In standard culture conditions that do not promote dauer formation, 2 mg/ml ethosuximide treatment enhanced the dauer arrest phenotype of daf-2(e1370) mutants from 3.4 percent (N = 263) to 91.3 percent (N = 304), suggesting an interaction between ethosuximide and insulin/IGF-1 signaling.

In addition, the insulin/IGF-1 signaling pathway regulates the ability of animals that hatch in the absence of food to arrest at the L1 stage of development [59,60]. Loss-of-function mutations in daf-2 and age-1 cause animals to arrest at the L1 stage inappropriately when food is present [55,61]. The activity of chemosensory neurons modulates this L1 arrest, since mutations in genes such as osm-3 and che-3 enhance the L1 arrest phenotype of daf-2 and age-1 mutants [54,61]. To test the effects of ethosuximide treatment on L1 arrest, we treated daf-2 mutants with the lifespan-extending dose of 4 mg/ml ethosuximide. Treatment of daf-2(e1370) animals with ethosuximide significantly increased L1 arrest from 19.2 percent in untreated animals to 47.5 percent in drug treated animals (Table 5). The ability of mutations that affect chemosensory neurons to enhance the daf-2 L1 arrest phenotype requires the activity of daf-16, since the addition of a daf-16 mutation abrogates this effect [62]. Similarly, the daf-16(mu86) mutation significantly reduced the effect of ethosuximide treatment from 47.5 percent in daf-2(e1370) mutants to 2.3 percent in daf-2(e1370); daf-16(mu86) mutants (Table 5). These results indicate that ethosuximide treatment and mutations that disrupt chemosensory neurons have a similar effect on the L1 arrest phenotype of an insulin-signaling mutant, consistent with the model that ethosuximide inhibits the activity of chemosensory neurons. One interpretation of these findings is that daf-2(e1370) mutants are hypersensitive to ethosuximide toxicity, since wild-type animals treated with 12 mg/ml ethosuximide also arrest development at the L1 stage.

Table 5. Ethosuximide enhanced daf-2(e1370) L1 arrest.

| Genotype            | Ethosuximide (mg/ml) | L1 arrest (%) | N (animals) |
|---------------------|----------------------|---------------|-------------|
| WT                  | 0                    | 0             | 471         |
| WT                  | 4                    | 0.6           | 449         |
| daf-2(e1370)        | 0                    | 19.2          | 332         |
| daf-2(e1370)        | 4                    | 47.5***       | 284         |
| daf-16(mu86)        | 0                    | 0             | 497         |
| daf-16(mu86)        | 4                    | 2.7***        | 558         |
| daf-2(e1370)/daf-16(mu86) | 0            | 0.3           | 603         |
| daf-2(e1370)/daf-16(mu86) | 4            | 2.3*          | 469         |

*This value includes arrested L1 larvae and eggs that failed to hatch, as described by Gems et al. (1998) [55]. Unhatched eggs were only a small proportion of this total. Statistical comparisons are to the same genotype with no drug treatment. Numbers with no asterisks are not significant (P > 0.05); *, P < 0.05; **, P < 0.005; ***, P < 0.0001. P values determined by Student T-test. The 47.5 value in line four is significantly larger than the 2.3 value in line eight (P < 0.0001).

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osm-3 Mutants Display an Extended Lifespan and Are Resistant to the Lifespan Extension Caused by Ethosuximide

In addition to functioning in chemotaxis, dauer formation, and L1 larval arrest, chemosensory neurons play a role in adult lifespan determination [32–34]. The finding that ethosuximide treatment extends the adult lifespan and affects the activity of chemosensory neurons, suggests that ethosuximide extends adult lifespan by affecting the activity of chemosensory neurons. To investigate this model, we monitored the lifespan of osm-3(p802) hermaphrodites. osm-3 mutants display a significantly extended lifespan [32] (Figure 7 and Table 6). If ethosuximide extends the adult lifespan by modulating the activity of chemosensory neurons, then osm-3(+/) animals are predicted to be resistant to the lifespan extension caused by ethosuximide. Treatment of wild-type hermaphrodites with 2 mg/ml or 4 mg/ml ethosuximide significantly extended the mean lifespan by 16 percent or 13 percent, respectively, and the maximum lifespan by 29 percent or 21 percent, respectively (Figure 7 and Table 6). By contrast, treatment of osm-3(p802) hermaphrodites with 2 mg/ml or 4 mg/ml ethosuximide did not cause a statistically significant extension of mean or maximum lifespan (Figure 7 and Table 6). These results support the model that ethosuximide extends lifespan by inhibiting chemosensory neurons.

Discussion

Chemosensory Neurons Are the Cellular Target of the Lifespan-Extending Drug, Ethosuximide

A variety of drugs have been demonstrated to extend the lifespan of invertebrates, including compounds that are proposed to act as antioxidants [4–9], complex chemical mixtures derived from plants [10,11], resveratrol [12,13], histone deacetylase inhibitors [63,64], and compounds that influence the vertebrate nervous system [14–17]. In each case, the understanding of how these drugs act to extend lifespan is limited. In particular, the cellular target has not been established for any of these compounds. Advancing this understanding is challenging because of the complexity of the aging phenotype and the fact that many determinants influence lifespan. Characterizing the mechanism of these drugs and defining the cellular target of action is critical for elucidating the endogenous pathways that are affected by the drugs, and ultimately using these drugs in therapeutic applications. Here we demonstrate that chemosensory neurons are the cellular target of ethosuximide in lifespan extension. Two lines of evidence support this conclusion. First, ethosuximide treatment...
caused the same defects as mutations that result in structural defects in chemosensory neurons, including defective chemotaxis, abnormal dauer arrest, abnormal L1 arrest and extended lifespan. Second, osm-3 mutants that are defective in a subset of chemosensory neurons were resistant to the lifespan extension caused by ethosuximide treatment and the toxic effects of ethosuximide. Together these results suggest that ethosuximide inhibits chemosensory function to extend C. elegans lifespan. This conclusion is important because it defines a specific cellular target for a lifespan extending drug and it demonstrates for the first time that a drug can act on specific cells in the nervous system to extend lifespan.

Our previous studies of the related compound trimethadione demonstrated that osm-3 mutants are partially resistant to the mean lifespan extension caused by trimethadione and are fully resistant to the maximum lifespan extension caused by trimethadione [14]. These results are consistent with the conclusion that the lifespan extension caused by trimethadione is partly caused by inhibiting the function of chemosensory neurons. The finding that part of the lifespan extending activity of trimethadione was not suppressed by an osm-3 mutation raises the possibility that trimethadione functions by additional mechanisms to extend lifespan.

We demonstrated that resistance to ethosuximide lethality can be caused by mutations in several different genes that disrupt ciliated neuron structure and function, including che-3, osm-3, che-13, osm-5 anddaf-10. Ciliated neurons in the amphid sheath have access to the environment so that they can detect chemicals and function in sensory perception. Because these neurons are exposed to the environment the cell membranes can be stained with lipophilic dyes. These observations raise the possibility that ciliated neurons absorb water-soluble chemicals, like ethosuximide, from the environment and mutations that disrupt cilium structure reduce drug absorption [24]. However, several lines of evidence suggest that mutants with defects in ciliated neurons are normal for drug absorption. First, we demonstrated that che-3(am162) mutants were weakly resistant to ethosuximide lethality and strongly defective in DIO staining. By contrast, che-3(am178) mutants raised at 20°C were strongly resistant to ethosuximide lethality but displayed relatively normal DIO staining, indicating that the ciliated neurons in these mutants have access to the environment. The lack of a correlation between dye-filling defects and ethosuximide resistance suggests that the ethosuximide resistance of che-3 mutants is not caused by a defect in the ability of the drug to enter the animal. Second, we demonstrated that osm-3 mutants are not resistant to drugs in general. Cilium structure mutants are resistant to the effects of the nematicidal drug ivermectin [24]. Therefore, we directly tested the possibility that cilium structure mutants are resistant to drugs in general by analyzing sensitivity to a third compound, serotonin. osm-3 mutants and wild-type animals displayed similar sensitivity to egg-laying stimulation caused by serotonin, indicating that the mutation does not impair absorption of serotonin. In addition, screens for mutations that cause resistance to fluoxetine [27,39], levamisole [65], benzimidazole [66], aldicarb [67], nemadipine-A [41], BMS-192/964 [40], and α-amanitin [68] have been described. Despite extensive screening and the successful identification of mutations resistant to these compounds, none of these reports describe the isolation of mutations that disrupt the structure of ciliated neurons. While the failure to identify a class of mutations in a genetic screen is not a definitive finding, these results suggest that mutations that disrupt cilium structure do not cause significant resistance to these compounds. Third, we took advantage of our observation that ethosuximide treatment stimulates egg-laying and this effect requires the non-ciliated HSN neurons [14]. osm-3(p802) mutants that are highly resistant to the lethality and lifespan extension caused by ethosuximide were nonetheless sensitive to the egg-laying effects of the drug, indicating that these mutants are not defective in ethosuximide absorption. A simple model to explain our findings is that ethosuximide is absorbed by animals independent of the structure of ciliated neurons and the drug influences the activity of multiple neurons throughout the body. The activity of ethosuximide on HSN neurons that mediate egg-laying is intact in osm-3 mutants whereas the activity on ciliated neurons that mediate toxicity and lifespan extension is altered.

Chemosensory Neurons Control Lifespan

Chemosensory neurons have been extensively characterized in C. elegans to understand behavior, and Kenyon and colleagues demonstrated that chemosensory neurons play an important role in controlling adult lifespan [32,34]. Mutants that have defects in chemosensory neuron function display an extended lifespan [32], and ablation of specific neurons such as ASI, AWA, and AWC extends the adult lifespan [34]. Here we provide an independent line of evidence that these neurons control lifespan. Our results indicate that ethosuximide inhibits the activity of chemosensory neurons and thereby causes a lifespan extension. These results provide independent support the model that high levels of chemosensory neuronal activity promote a reduced lifespan and inhibiting these neurons causes a lifespan extension.

An important issue that is raised by these observations is how does the activity of chemosensory neurons influence lifespan? One possibility is that the chemosensory neurons regulate the activity of the insulin/IGF-1 signaling pathway. Amphid neurons are the site of expression of many insulin/IGF-1 ligands [69] raising the possibility that these neurons release ligand in response to environmental cues and thereby influence lifespan by an endocrine

Table 6. osm-3 mutants displayed an extended lifespan and were resistant to the lifespan extension caused by ethosuximide.

| Genotype | Ethosuximide (mg/ml) | Mean Lifespan ± SD (days) | Percent Change | p* | Maximum Lifespan ± SD (days) | Percent Change | p* | N* |
|----------|-----------------------|---------------------------|---------------|----|-----------------------------|---------------|----|----|
| WT       | 0                     | 18.7±3.7                  | -             | -  | 24.9±1.3                   | -             | -  | 164|
| 2        | 21.7±6.7              | +16                       | <0.0001       |    | 32.2±2.4                   | +29.3         | <0.0001 | 146|
| 4        | 21.2±5.6              | +13.4                     | <0.0001       |    | 30.2±2.0                   | +21.3         | <0.0001 | 158|
| osm-3(p802) | 0                | 22.5±6.4                   | -             | -  | 34.4±4.3                   | -             | -  | 128|
| 2        | 23.9±7.0              | +6.2                      | 0.10          |    | 35.6±3.6                   | +3.5          | 0.44 | 131|
| 4        | 22.1±6.2              | -1.8                      | 0.65          |    | 35.0±4.0                   | +1.7          | 0.71 | 122|

*Comparisons are to the same genotype with no drug treatment. p values determined by Student T-test.

Maximum adult lifespan is the mean lifespan of the 10% of the population that had the longest lifespan.

Number of animals observed in three independent trials.

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mechanism. Consistent with this model, ethosuximide enhanced L1 arrest in daf-2 mutants, similar to osm-3 mutations [54, 61]. An important test of this model is the dependence of these effects on DAF-16, a transcription factor that mediates effects of insulin/IGF-1 signaling and that ethosuximide may extend lifespan by disrupting this pathway. However, ethosuximide and osm-3 mutations extend the lifespan of daf-16 mutants, indicating loss of insulin/IGF-1 signaling cannot fully explain these lifespan extensions [14, 32]. Furthermore, ablation of AWA and AWC amphid neurons extends the lifespan of daf-16 mutants [34]. Therefore, our data support the model that ethosuximide acts through insulin-dependent and insulin-independently extend C. elegans lifespan.

Sensory Perception of Food May Accelerate Lifespan, and Inhibition of Sensory Perception May Be Sufficient to Extend Lifespan

Restricting dietary intake can extend lifespan in many animal models [70–73], and these observations document the critical role of food availability in controlling lifespan. An important question is how do animals assess food availability to control lifespan? In particular, is food availability determined metabolically, by monitoring nutrients that are ingested, or is food availability determined by neural sensation, by monitoring food-derived cues in the environment? Recent studies in Drosophila indicate that the effects of dietary restriction are mediated, in part, by the perception of food-derived cues [74]. Exposure of flies to food-derived odors can partially suppress the lifespan extension caused by dietary restriction. Therefore, mutations that extend lifespan by disrupting chemosensation may block perception of food-related chemical cues and thereby activate pathways that respond to dietary restriction.

Here we demonstrate that the anticonvulsant ethosuximide extends C. elegans lifespan by inhibiting sensory neurons that are hypothesized to mediate attraction to environmental food sources. Hermaphrodites treated with ethosuximide appear to ingest a normal amount of food since they do not display a diminished body size or progeny production, the characteristics of dietary restricted animals [72]. These results suggest that inhibiting the sensation of food is sufficient to extend lifespan even in the presence of normal food ingestion. These observations raise the exciting possibility that inhibiting the sensation of food is sufficient to extend lifespan even in the presence of normal food ingestion. These observations raise the exciting possibility that inhibiting the sensation of food may be a conserved mechanism of lifespan extension. In addition to controlling seizures, a common side effect of ethosuximide resistance was caused by a single mutation.

Materials and Methods

General Strains and Methods

C. elegans strains were cultured at 20°C on 6 cm Petri dishes containing nematode growth media (NGM) agar and a lawn of E. coli OP50 unless otherwise noted [76]. Unless otherwise noted, preparation and storage of dishes containing pharmacological compounds was performed as previously described [14].

We used the following C. elegans mutations that are described in Riddle et al. [23] or in this study: che-3(e1124) I, che-3(am165) I, che-3(am162) I, che-3(aka78) I, che-3(e1805) I, daf-16(mu66) I, unc-11(e47) I, dpy-5(e61) I, unc-29(e1072) I, unc-75(e350) I, daf-2(e1370) III, daf-2(m41) III, osm-3(p802) IV, osm-3(am161) IV, osm-3(am177) IV, osm-3(am172) IV, daf-10(e1387) IV, osm-5(p813) X, lin-15(n765) X, cca-1(ad1650) X, and cca-1(gk30) X.

The following well-characterized mutations were used in this study: che-3(e1124) Q2293stop, dpy-5(e61) Q473stop, and unc-29(e1072) Q346stop. Furthermore, ablation of AWA and AWB amphid neurons extends the lifespan of daf-16 mutants [34]. Therefore, our data support the model that ethosuximide acts through insulin-dependent and insulin-independently extend C. elegans lifespan.

Isolation and Genetic Analysis of Ethosuximide-Resistant Mutants

N2 hermaphrodites (P0) were mutagenized as described by Brenner [76] with either 50 mM EMS or 0.5 mM NBU. 100,000 F1 hermaphrodites were treated with hypochlorite, and F2 eggs were plated on NGM containing 10–12 mg/ml ethosuximide (Sigma, St Louis, MO). Since E. coli failed to form a thick lawn on plates containing 12 mg/ml ethosuximide, these plates were seeded with E. coli OP50 that had been concentrated 10-fold. F2 progeny that matured to the L4/adult stage were picked as ethosuximide resistant mutants, and populations derived from these individuals were retested for ethosuximide resistance. Forty-two mutant strains were backcrossed successfully, indicating that ethosuximide resistance was caused by a single mutation.

Positioning Mutations Relative to SNP Markers and Visible Markers

Single nucleotide polymorphism (SNP) mapping analysis was performed on a subset of resistant mutants by mating ethosuximide resistant mutants (P0) to the divergent CB4856 strain (P0), selecting F1 outcross progeny, and scoring F2 self-progeny for ethosuximide resistance. F2 animals that displayed resistance were judged to be homozygous for the mutation, and F3 progeny were harvested for DNA. SNPs distributed throughout the C. elegans genome [81] were scored using Pyrosequencing (Biotage Foxboro, MA) or direct DNA sequencing. Linkage values were calculated by determining the ratio of N2 DNA to CB4856 DNA at each polymorphism.

The am178 mapping experiment was tightly linked to a SNP marker at the center of Chromosome I. Three factor mapping experiments with visible markers yielded the following results. From am178/vec-11 dpy-5 hermaphrodites, 5/6 Unc non Dpy self progeny segregated am178 and 0/9 Dpy non Unc self progeny segregated am178; From am178/vec-5 unc-75 hermaphrodites, 3/8 Unc non Dpy self progeny segregated am178; From am178/vec-5 unc-29 hermaphrodites, 7/8 Dpy non Unc self progeny segregated am178. These results position am178 right of dpy-5, left of unc-75, and probably left of unc-29.

For high resolution SNP mapping of am178, we mated dpy-5(e61) am178 and am178 unc-75(e350) homzygotes to CB4856 males, picked F1 outcross progeny, and selected non-Dpy and non-Unc F2 self-progeny resistant to 12 mg/ml ethosuximide. We
prepared DNA from strains homozygous for the recombinant chromosome and scored SNP markers.

**Complementation and Transgenic Rescue**

Because che-3 and osm-3 mutations interfere with male mating, che-3(e1124) and osm-3(p802) were maintained over hT2 and nT1 myo-2::GFP balancer chromosomes, respectively. For complementation analysis with am178 and che-3(e1124), one am178 hermaphrodite was mated to five che-3(e1124)/hT2 males and outcross progeny were scored for ethosuximide resistance. Non-GFP, outcrossed am178/che-3(e1124) animals survived 12 mg/ml ethosuximide treatment, indicating that che-3(e1124) and am178 fail to complement for ethosuximide resistance. Neither che-3(e1124)/+ nor am178/+ animals survived 12 mg/ml ethosuximide treatment.

For complementation analysis with dye-filling defective alleles, ethosuximide resistant mutants that displayed a Dyf phenotype were mated to either che-3(e1124)/hT2 or osm-3(p802)/nT1 males, and non-GFP outcross progeny were subjected to dye-filling analysis. 9/9 am172/osm-3(p802) animals, 15/15 am177/osm-3(p802) animals and 20/25 am161/osm-3(p802) animals displayed a Dyf phenotype, indicating that these three alleles fail to complement osm-3(p802) for dye-filling. 14/15 am165/che-3(e1124) animals and 9/9 am162/che-3(e1124) animals displayed a Dyf phenotype, indicating that these two alleles fail to complement che-3(e1124) for dye-filling.

To analyze the ability of genomic DNA to rescue the mutant phenotype of am178, we generated transgenic animals containing extrachromosomal arrays using standard procedures [82]. Fosmid clone WRM0637cB0 contains the entire che-3 coding region and only one other entire gene, F18C12.4. To identify transgenic animals, we co-injected the fosmid clone WRM062bF09 that rescues the lin-15(n765ts) multi-vulval (Muv) phenotype (M. Nonet, personal communication). These two fosmids were co-injected at a ratio of 20 ng/μl each into am178; lin-15(n765ts) animals to generate the transgenic array amEx100. Hermaphrodites containing the amEx100 array transmitted it to about 73% of self-progeny, since 27% of self-progeny were Muv (n = 100). When self-progeny of am178; lin-15(n765ts) amEx100 hermaphrodites were plated on media containing 12 mg/ml ethosuximide, the only animals that survived to adulthood after 5–6 days were Muv (N = 59), indicating that they did not contain amEx100. These results indicate that the amEx100 array rescues the am178 resistance to ethosuximide phenotype, suggesting that the gene affected by am178 is contained in fosmid WRM0637cB0.

**DNA Sequence Analysis and RNA Analysis**

DNA sequencing was performed using standard procedures. We PCR amplified predicted exons and splice junctions (www.wormbase.org) from the che-3 and osm-3 genes using DNA from am178 and am177 mutant animals, respectively. The am178 strain contained a G to A change at the fifth base of intron 9. To analyze the che-3 messenger RNA, we prepared RNA from a population of am178 mutants (TRizol, Invitrogen), DNase treated the RNA (DNA-free, Ambion), and generated cDNA by performing reverse transcription (RevertAid, Ambion). We PCR amplified the junctions between exons 9 and 10 and determined the DNA sequence of the PCR product to infer the splicing pattern of the mRNA. The che-3 mRNA derived from am178 mutants displayed a deletion of 17 nucleotides (GTCAGCTTGGTCTTTCG) in exon 9.

**Dye-Filling Analysis**

Staining with DiO was performed using previously described methods [31,83]. L4 hermaphrodites were placed in 100 μL of M9 buffer containing 20 μg/ml DiO (Molecular Probes) in a microtiter dish and incubated for ~2 hrs at room temperature. To remove non-specifically bound DiO, we transferred the animals to a NGM dish seeded with E. coli OP50 and cultured for 20–30 minutes at room temperature. For analysis of strains in Table 1, five to fifteen animals were observed using a Zeiss Axioplan 2 microscope equipped for fluorescence microscopy at 400× magnification. Animals were categorized in the following classes: [I] no amphid neurons stained; [II] 1–2 amphid neurons stained; or [III] more than 2 amphid neurons stained. All the strains described in Table 1 displayed only the class I or only the class III pattern of staining. For analysis of che-3(am178) mutants in Table 2, DiO staining was observed using an Olympus SZX12 dissecting microscope equipped for fluorescence microscopy at 144× magnification. Animals were scored as Dyf if no amphid neurons stained robustly with DiO.

**Quantification of Ethosuximide Resistance**

To quantify the penetrance of the ethosuximide resistance phenotype, we adapted an assay method described by Rand and Johnson [84]. Eggs were picked to a Petri dish with agar containing 12 mg/ml ethosuximide and counted. Five to six days later we counted the number of animals that matured past the L1 stage. For resistant strains most animals were L4 larvae or adults. The remaining eggs produced animals that did not mature past the L1 stage or left the agar surface. The number of animals that matured past the L1 stage was divided by the total number of eggs to determine the percent resistant to ethosuximide.

**Chemotaxis to Volatile Compounds**

For pharmacological analysis of chemotaxis, dishes were prepared by adding powdered ethosuximide directly to 60 °C chemotaxis agar, agitating to dissolve the compound and dispensing to 10 cm Petri dishes. Chemotaxis assays were performed as described previously [85] with two minor modifications. First, to obtain a large number of well-fed animals for the assay, we cultured animals from conception to adulthood in the presence of ethosuximide or no drug on 6 cm Petri dishes containing OP50 that was concentrated 10-fold. Second, we added ethosuximide to the molten chemotaxis agar before dispensing into 10 cm Petri dishes and used these dishes after 3–8 hours. To conduct chemotaxis assays, we pipeted animals in a small volume of liquid onto the center of a 10 cm Petri dish that had been pre-treated with 1 μL of 1M Na azide, a paralyzing agent, at two diametrically opposed spots on the plate. At this time, we added 1 μL of volatile odorant to one azide treated spot and 1 μL of ethanol as a control to the other azide treated spot. Isoamyl alcohol and diacetyl were diluted in ethanol at concentrations of 1:10 and 1:1000, respectively. After 60 minutes we scored the number of paralyzed animals at each azide spot and the number of moving animals on the plate. The sodium azide spots were positioned at the edge of the dish, and animals that desiccated on the side of the dish at the positions of the sodium azide spots were attributed to those categories; otherwise such animals were attributed to the moving animal category. Chemotaxis index was determined using the following formula:

\[
\text{Chemotaxis Index} = \frac{(\text{Number of animals at odorant}) - (\text{Number of animals at ethanol})}{\text{Total number of animals on plate}}
\]

**Analysis of Egg-Laying**

The analysis of egg-laying was performed as described previously [85]. To determine the effect of ethosuximide on egg-laying, we picked L4 hermaphrodites, incubated them at 20 °C for approximately 20 hours on NGM plates with abundant food, individually placed them in 100 μL of M9 buffer in a microtiter
dish plus or minus ethosuximide, and counted the number of eggs laid in 90 minutes. We used a similar method to analyze serotonin, except animals were placed in 50 μL buffer.

Analysis of Dauer Larvae and Larval Arrest Phenotypes

To analyze dauer formation at 27°C, we allowed 8–10 gravid hermaphrodites to lay eggs on one Petri dish for 4–8 hours at room temperature and then incubated the dish at 27°C for precisely 44 hours [52]. We counted the total number of animals on the dish, flooded the dish with 1% SDS to kill all animals except for dauer larvae, and counted the number of live dauer larvae after 15 minutes. To analyze dauer formation at 25°C, we prepared NGM media without peptone, the main carbon source for the E. coli OP50. The omission of peptone from the media allowed us to provide a consistent amount of E. coli OP50 food, and made the experiment independent of the effects of ethosuximide on bacterial proliferation. We aliquoted 200 μL of an overnight E. coli OP50 culture that had been concentrated 10-fold on each dish. We placed two L4 hermaphrodites on each dish, incubated the animals at 25°C, and monitored the dishes daily for starvation [38]. Five days post-starvation we scored the number of dauer larvae by flooding the dishes with 1% SDS and counting the number of live animals after 15 minutes. Dauer formation of daf-2(m41) mutants was analyzed as described previously [16].

To analyze the L1 arrest phenotype, we cultured L4 hermaphrodites 1–2 days on NGM dishes with 4 mg/ml ethosuximide or no drug, transferred these adults to fresh dishes with 4 mg/ml ethosuximide or no drug for 4–8 hours at room temperature, and removed the adults. These dishes containing freshly deposited eggs were cultured at 25.5°C for 48 hours. We counted the number of eggs, L1 larvae, and older larval stages.

Lifespan Analysis

For a typical lifespan experiment, parental worms were cultured in the presence of the drug, and progeny were selected at the L4 stage for lifespan analysis. Thus, these progeny were exposed to drug from the time of conception until death. For measurements of lifespan, hermaphrodites were chosen for analysis at the L4 stage (defined as day 0) and analyzed every 1–2 days from day 3 until death. Approximately 13 hermaphrodites were cultured on each Petri dish. Hermaphrodites were transferred to fresh Petri dishes about every two days until the cessation of progeny production and about every week thereafter. Animals were scored as dead if they displayed no spontaneous movement or response when prodded. Dead worms that displayed internally hatched progeny, an extruded gonad or desiccation due to crawling off the agar were excluded from the data. Lifespan is the number of days from the L4 stage to the average of the last day a worm was observed to be alive and the first day a worm was observed to be dead.

Lifespan experiments involving pharmacological compounds were always done in parallel with a control group.

Statistical Methods

For each experimental group, comparisons were made to a control group maintained in the same incubator and analyzed at the same time points. Mean, standard error, P values, and other statistical parameters were calculated using InStat 2.03 software (Graphpad Software) or Microsoft Excel.

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Author Contributions

Conceived and designed the experiments: JJC KE CLP DLS. Analyzed the data: JJC KE CLP DLS KK. Wrote the paper: JJC KK.

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