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Molecular characterization of ethyl carbamate toxicity in *Caenorhabditis elegans*

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ABSTRACT

Ethyl carbamate is a common contaminant prevalent in fermented food with probable carcinogenic effects in animals. To date, other toxicological properties of ethyl carbamate are not well characterized. Using the genetic model *Caenorhabditis elegans*, we found that chronic exposure to ethyl carbamate during larval development impedes growth while exposure during adulthood inhibits reproduction, shortens lifespan, and promotes degeneration to dopaminergic neurons. Through whole-transcriptome RNA-sequencing, we found that ethyl carbamate invokes a widespread transcriptomic response inducing the differential expression of > 4,000 genes by at least 2-fold. Functional analysis of RNA-sequencing data revealed that up-regulated genes enrich to various neuron regulatory processes and xenobiotic defense. Gene expression analysis confirms that various genes encoding antioxidant enzymes and those functioning within phase I and II detoxification responses along with ABC transporters are highly up-regulated after ethyl carbamate exposure, suggesting the onset of oxidative stress. Overall, these findings report new toxicological properties of chronic ethyl carbamate exposure and provide new insights on its effects on transcriptome regulation in the *C. elegans* model.

1. Introduction

Ethyl carbamate (also known as urethane) is a common co-solvent used in various industrial applications including the manufacture of pesticides and cosmetics, and previously as an anesthetic agent for research laboratory animals [3,11]. Subsequent studies in mice revealed potential carcinogenic properties of ethyl carbamate as chronic exposure led to increased incidences of tumour formation across multiple organs [3]. In cancer research, ethyl carbamate has also been described as an inexpensive reagent to induce adenomas in mice [13]. Currently, ethyl carbamate is classified by the International Agency for Research on Cancer as a group 2 A carcinogen, a chemical with probable carcinogenic properties to humans. Outside of its industrial and research applications, ethyl carbamate is an emerging food contaminant that is naturally formed via the fermentation process and is commonly detected in alcoholic beverages and food products such as cheese and bread [41]. In Canada, ethyl carbamate is an identified food contaminant of concern with a maximum allowable range of 30–400 μg/kg in various alcoholic products. Ethyl carbamate has also been detected in cigarette smoke and smokeless tobacco products, providing an additional route of human exposure [14,27,39]. Upon entry into the human body, ethyl carbamate is metabolized by the liver microsomal esterases into ethanol, ammonia, and carbon dioxide via a hydrolysis reaction. However, a small fraction of ethyl carbamate can be processed by the cytochrome P450 2E1 protein to form vinyl carbamate epoxide that can adduct to DNA in a mutagenic manner [3,16]. In cell models, exposure to ethyl carbamate has also been shown to increase the production of reactive oxygen species, suggesting the induction of oxidative stress as a potential mechanism of its cytotoxicity [8].

In humans, dietary consumption of alcoholic beverages and fermented food products is the most common route of ethyl carbamate exposure [41]. Given the prevalence of these products, a potential concern is the unknown effects chronic ethyl carbamate exposure may have on other essential physiological processes such as growth and development, reproduction, aging, and neuromuscular function. These toxicological parameters have been historically challenging to monitor

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in mammals due to the relatively lengthy developmental period and lifespan of available research models. To address this, the nematode *Caenorhabditis elegans* provides a valuable alternative in vivo model to study these toxicological properties of ethyl carbamate. The *C. elegans* model has emerged as an advantageous complementary model for predictive toxicology due to the relative ease and rapidity for which chemical effects on larval development, aging, and reproduction can be consistently determined in 3–4 weeks [19,20]. Various developmental pathways and metabolic processes are highly conserved between *C. elegans* and mammals, and this is supported by studies demonstrating a correlation in toxicity ranking between *C. elegans* and mammals [19,20]. Furthermore, *C. elegans* has also proven to be a valuable model to study neurotoxic properties of various chemicals, given the ease at which individual neurons can be labelled with fluorescent proteins to enable in vivo live imaging of neuron morphology [29,36].

In this study, we set out to characterize the toxicological properties of ethyl carbamate using the *C. elegans* model to determine its impact on development, reproduction, aging, and neurological effects over a concentration range of 25–150 mM that was previously shown to induce toxicity in zebrafish and human cell models [8,40]. To investigate the molecular mechanisms of ethyl carbamate toxicity, we performed Next-Generation RNA-sequencing in combination with real-time PCR to systematically assess how *C. elegans* respond to ethyl carbamate toxicity at the transcriptome level.

2. Materials and methods

2.1. C. elegans strain

All *C. elegans* strains were cultured at 20 ºC using standard methods [6]. The following strains were used in this study: N2 Bristol wild-type, BZ555 *egIs1* [*dat-1p::GFP*], LX929 *vsIs48* [*unc-17p::GFP*], EG1285 *oxIs12* [*unc-47p::GFP + lin-15(+)*], and VP596 *dvIs19* [*(pAF15) gst-4p::GFP::NLS*] *III*.

2.2. Growth, reproduction, and lifespan assays

Assays to measure growth, reproduction, and lifespan were described previously in detail [29]. In brief, growth assays were performed by placing synchronized L1 stage *C. elegans* obtained via hypochlorite treatment on OP50 seeded nematode growth media (NGM) plates containing 0, 25, 50, 75, 100, 125, or 150 mM of ethyl carbamate for two days. Afterwards, *C. elegans* were imaged on an agar plate with an Olympus SZX61 stereomicroscope with a Retiga R3 camera. These concentration ranges were chosen as exposure to 25–100 mM of ethyl carbamate has been previously demonstrated to induce toxicity in human cell lines. ImageJ was used to calculate *C. elegans* body length to assess relative growth. Three independent growth assay trials totalling N = 33–109 worms were measured for each condition.

For reproduction assays, a single L4 stage *C. elegans* was placed on an OP50 seeded NGM agar plate containing 0, 50, 100, or 150 mM of ethyl carbamate. The number of offspring including eggs laid and hatched larvae was counted daily, and the single adult *C. elegans* was then moved to a new plate for offspring counting the next day. This process was repeated for five to six days or until reproduction had ceased, and the total number of offspring counted for all days was tallied to determine total brood size. Three independent reproduction assay trials totalling N = 11–15 worms were assessed for each condition.

For lifespan assays, synchronized L1 *C. elegans* were grown on OP50 seeded NGM agar plates for 48 h to develop into adults. One day old adults were then transferred to OP50 seeded NGM plates containing 0, 50, 100, or 150 mM of ethyl carbamate to initiate chemical exposure. Adult worms were transferred to new agar plates by daily picking during reproduction periods to avoid mixing the population with the F1 offspring. *C. elegans* were scored for survival every 1–2 days and were considered dead if they did not respond to gentle prodding with a metal pick. *C. elegans* with protruding vulva or intestine were recorded as censors. Three independent lifespan trials totalling N = 95–260 worms were assessed for each condition.

2.3. Fluorescent microscopy

To capture fluorescent images, *C. elegans* were first anesthetized with 2% sodium azide and mounted on a 2% agarose gel pad. Fluorescent images were captured with a Zeiss Axioskop 50 microscope with a Retiga R3 camera. Methodologies to score *C. elegans* neuronal integrity were previously described in detail [29]. To evaluate dopaminergic neuron integrity, three levels of *dat-1p*::GFP fluorescent pattern were categorized. Wild-type indicates neurons exhibit normal cephalic (CEP) sensilla dendrite in the anterior of the animal, blebbing indicates at least one abnormal punctae formation within the CEP dendrite, and breaks indicate discontinuation within the CEP dendrite. To quantify relative fluorescence of the *gst-4p*::GFP reporter, images were analyzed in ImageJ using the measure function with fluorescence determined after background subtraction of an area in the same image where the GFP signal was absent.

2.4. RNA extraction and RNA-sequencing

RNA was extracted from *C. elegans* using protocols previously described in detail [42]. In brief, synchronized worms at the L4/young adult stage were moved onto HT115(DE3) *E. coli* seeded NGM agar plates with or without 100 mM ethyl carbamate for 24 h. After ethyl carbamate exposure, worms were washed with M9 buffer and prepared for RNA extraction using the PureLink RNA Mini Kit (Thermo Fisher #12183025) with *C. elegans* lysis performed using a QSonica Q55 sonicator. Approximately 2,000 – 3,000 worms were used per RNA sample, with four samples prepared for each condition. Total RNA extracted from age synchronized control and 100 mM ethyl carbamate treated worms were sent to Novogene (Sacramento, CA) on dry ice for cDNA sequencing library preparation with oligo (dT) enrichment followed by RNA-sequencing. N = 3 samples for each condition were sequenced. Mapping of raw sequence to the WBcel235 genome was performed by HITSAT2 (v2.0.5), gene quantifications were performed via FeatureCounts (V1.5.0-p3), and differential gene expression analysis was performed via DeSeq2 [2,23,24]. Enrichment analysis of differentially expressed genes was performed via DAVID functional analysis [17]. Raw sequencing files and processed datasets are deposited to the NCBI Gene Expression Omnibus with the accession number GSE190099.

2.5. Real-time PCR

A portion of the purified RNA prepared for RNA-sequencing was aliquoted and prepared for qPCR. RNA was first treated with DNAse I followed by cDNA synthesis with the Applied Biosystems High-Capacity cDNA reverse transcription kits (#4368814). Real-time PCR (qPCR) was performed with the PowerUp™ SYBR™ Green Master Mix (#A25741) using primers listed in Table S1 [25,38]. Relative gene expression was normalized to the housekeeping *rpl-2* (ribosomal protein large subunit-2) gene. N = four samples for each condition were used for qPCR analysis.

2.6. Statistical analyses

The Graphpad Prism software (V7.04) was used to generate graphical data and perform statistical analysis. Means for data are shown in graphs with error bars indicating standard deviation. To compare two groups, a student's t-test was used to determine statistical significance. To compare more than two groups, One-way ANOVA with Dunnett's multiple comparisons was used. For lifespan analysis, the OASIS2 software (https://sbi.postech.ac.kr/oasis2/) was used to determine mean lifespan and to calculate statistical significance via the Log-rank test.

The Chi-square test was used to analyze the statistical significance of categorical data. To analyze the correlation between gene expression data obtained via qPCR and RNA-sequencing, linear regression analysis with the F-test was used. For analysis of RNA-sequencing data, false discovery rate (FDR) correction was applied to determine statistical significance. For all statistical tests, the following designations were used *P < 0.05, * *P < 0.01, and * **P < 0.001.

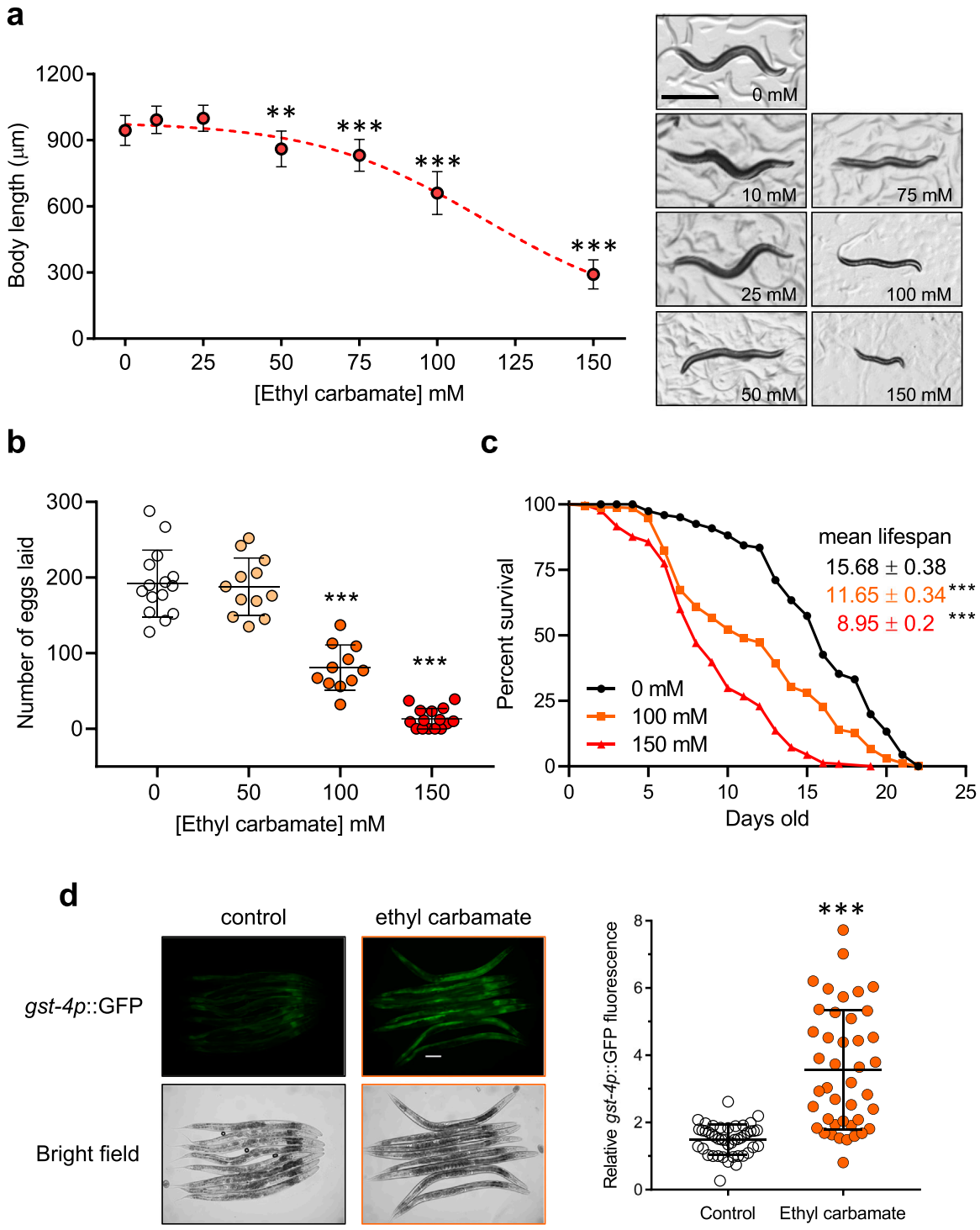


Fig. 1. Ethyl carbamate exposure is deleterious to *C. elegans* physiology. Effects of various concentrations of ethyl carbamate exposure on *C. elegans* **a**) larval growth, **b**) reproduction, and **c**) lifespan. Three independent trials were performed for each assay with a total of N = 33–109 worms measured for the growth assay, N = 11–15 worms measured for the reproductive assay, and N = 95–260 worms measured for the lifespan assay. **d**) Exposure to 100 mM of ethyl carbamate in *C. elegans* expressing the oxidative stress marker *gst-4p*::GFP. Three trials totalling N = 42–44 worms were analyzed. * *P < 0.01, * **P < 0.001 compared to control as determined by One-way ANOVA in **a**) and **b**), log-rank test in **c**), and student's t-test in **d**). Mean values are plotted in graphs with error bars indicating standard deviations. Scale bar indicates 500 µm in **a**) and 100 µm in **d**).

3. Results

3.1. Exposure to ethyl carbamate is deleterious to C. elegans physiology

To assess the toxicity of ethyl carbamate in an in vivo animal model, we exposed *C. elegans* to an increasing concentration of ethyl carbamate dissolved in solid agar media to assess its effects on various aspects of *C. elegans* physiology. First, we exposed *C. elegans* at the L1 larval stage to increasing concentrations of ethyl carbamate and allowed the animals to grow and develop for 48 h before assessing relative body size. Exposure starting at 50 mM caused a moderate but significant decrease of *C. elegans* body size by 9%, this is followed by a dose-response relationship as the exposure concentration increased to 75 mM, 100 mM, and 150 mM that led to a 12%, 30%, and 69% reduction in body size relative to the control respectively (Fig. 1a). Next, we sought to determine the potential reproductive toxicity of ethyl carbamate by initiating chemical exposure starting at the L4 larval stage that is approximately 12 h before the onset of embryo fertilization, to assess its effect on offspring production. Exposure at 50 mM had no significant impact on total brood size, whereas 100 mM and 150 mM exposure led to a 59% and 93% decrease in brood size relative to the control, respectively (Fig. 1b). To assess the long-term effects of chronic ethyl carbamate exposure, we performed lifespan assays with *C. elegans* grown continuously on agar plates containing 0 mM (control), 100 mM, or 150 mM of ethyl carbamate beginning at the L4 stage and monitored its lifespan. Exposure at both 100 mM and 150 mM significantly reduced the *C. elegans* lifespan, with exposure at 100 mM resulting in a 26% decrease and exposure at 150 mM resulting in a 43% decrease in mean lifespan compared to the control (Fig. 1c).

We then tested whether ethyl carbamate exposure induced oxidative stress by using a transgenic strain of worm expressing the promoter of the glutathione s-transferase-4 gene fused to a GFP protein (*gst-4p*::GFP). Exposure to 100 mM of ethyl carbamate for 24 h starting at the L4 stage led to a significant increase in the *gst-4p*::GFP fluorescence, indicating activation of the oxidative stress response (Fig. 1d). Overall, these results demonstrate that ethyl carbamate exposure induces oxidative stress and has deleterious effects on *C. elegans* development, reproduction, and aging physiology.

3.2. Ethyl carbamate induced transcriptome-wide gene expression alteration

Given that ethyl carbamate exposure activated the expression of *gst-4*::GFP, a well-characterized biomarker for oxidative stress in *C. elegans* [44], we performed RNA-sequencing to obtain an unbiased view on the effects of ethyl carbamate have on the transcriptome. Exposure to 100 mM of ethyl carbamate for 24 h led to drastic alterations to the *C. elegans* transcriptome, with 3,517 genes up-regulated by more than 2-fold and 569 genes down-regulated by more than 2-fold (Fig. 2a). Functional analysis of differentially expressed genes using DAVID GO revealed that up-regulated genes enrich various neuron-related functions, including axon guidance, synaptic transmission, and receptor signaling (Fig. 2b-e). Genes up-regulated are also highly clustered towards xenobiotic detoxification processes, including monooxygenase and oxidoreductase activity; this is further supported by enrichment towards KEGG pathways involving cytochrome P450 metabolism and ABC transporters (Fig. 2b-e). Meanwhile, genes down-regulated by ethyl carbamate enrich to cellular processes including protein folding, metabolism, and collagen/cuticle-related maintenance functions (Fig. 2b-d).

We then used qPCR to validate the expression changes of 20 genes shown to be up-regulated by RNA-sequencing after ethyl carbamate exposure that function in neuron regulation and xenobiotic defense. Fold-change comparison of the 20 genes between RNA-sequencing and qPCR showed a correlation coefficient of 0.96, suggesting that gene expression changes observed in RNA-sequencing are reliably reproduced by qPCR (Fig. 2f).

3.3. Chronic exposure to ethyl carbamate induced dopaminergic neurodegeneration

Functional analysis of genes up-regulated after ethyl carbamate exposure revealed enrichment towards KEGG pathway involved in neuroactive ligand-receptor interaction (Fig. 2e). Using qPCR, we first confirm that genes encoding the dopamine receptor (*dop-1*) and glutamate metabotropic receptors (*mgl-1*) are up-regulated after ethyl carbamate exposure (Fig. 3a). We then sought to determine whether chronic ethyl carbamate exposure causes morphological changes to cholinergic, GABAergic, and dopaminergic neurons, which are three major classes of neurons that control neuromuscular activity in *C. elegans* [28,31,33]. Exposure to 100 mM of ethyl carbamate for 24 h did not alter the morphology of cholinergic or GABAergic neurons (Fig. 3b). Extended exposure up to 4–6 days also showed similar results, suggesting that ethyl carbamate exposure did not alter the morphology of either cholinergic or GABAergic neurons (Fig. S1). A maximal exposure of 6 days was chosen as worms begin to die from ethyl carbamate toxicity after this period (Fig. 1c). For analysis of the cholinergic neuron, evaluation was restricted to 4 days as lethality to 100 mM of ethyl carbamate was observed in this strain at day 6 of exposure.

In contrast to cholinergic and GABAergic neurons, we observed clear morphological alterations to the dopaminergic neurons after 4 and 6 days of ethyl carbamate exposure (Fig. 3c). These morphological changes are characterized by increases in blebbing as evident by the accumulation of GFP signals in punctae shapes, along with breaks as evident by the discontinuation of normally smooth GFP signal along the CEP dendrites (Fig. 3c). After 4 days of exposure, a significant increase in the number of *C. elegans* exhibiting blebbing to the dopaminergic neuron was observed, this was followed by the onset of the more severe breaks observed at 6 days of exposure (Fig. 3c).

Together, these results reveal that prolonged and continuous exposure to ethyl carbamate in *C. elegans* led to the onset of dopaminergic neurodegeneration before lethality.

3.4. Broad activation of xenobiotic stress response followed ethyl carbamate exposure

RNA-sequencing results indicated that ethyl carbamate exposure activated various genes involved in xenobiotic detoxification. We then used qPCR to specifically measure the expression of various stress-responsive genes involved in xenobiotic detoxification, antioxidant defense, and protein misfolding chaperone response. Exposure to ethyl carbamate led to the up-regulation of various genes encoding phase I detoxification enzymes with oxidation and reduction functions, including alcohol/aldehyde dehydrogenases (*sodh-2*, *alh-5*), flavin monooxygenases (*fmo-2, fmo-5*), and cytochrome P450 enzymes (*cyp-13A12, cyp-13B1, cyp-32B1*) (Fig. 4a). Consistently, genes encoding various phase II detoxification enzymes with thiol conjugating activity including glutathione-s-transferases (*gst-5, gst-14, gst-21*) and UDP-glucuronosyl transferases (*ugt-9, ugt-33, ugt-37*) were also highly activated (Fig. 4b), along with up-regulation of genes encoding ABC transporter (P-glycoprotein related; *pgp-1*, *pgp-3*, *pgp-14*) that are involved with the transfer of xenobiotic molecules across cellular membranes (Fig. 4c) [15]. We then measured the expression of genes encoding key enzymes involved in antioxidant defense. Ethyl carbamate exposure up-regulated the expression of genes encoding superoxide dismutase (*sod-3, sod-5*) and catalase (*ctl-2*) but did not affect glutathione peroxidase enzymes (*gpx-3, gpx-4, gpx-5*) (Fig. 4d). Next, we measured the expression of genes encoding heat shock proteins involved in maintaining proteostasis under stress and found that the expressions of *hsp-16.49* and *hsp-70* were also significantly elevated (Fig. 4e). Together, these experiments suggest that ethyl carbamate exposure induces a broad stress response in *C. elegans* that involves activation of the xenobiotic detoxification and antioxidant defense, along with increased proteasome maintenance.

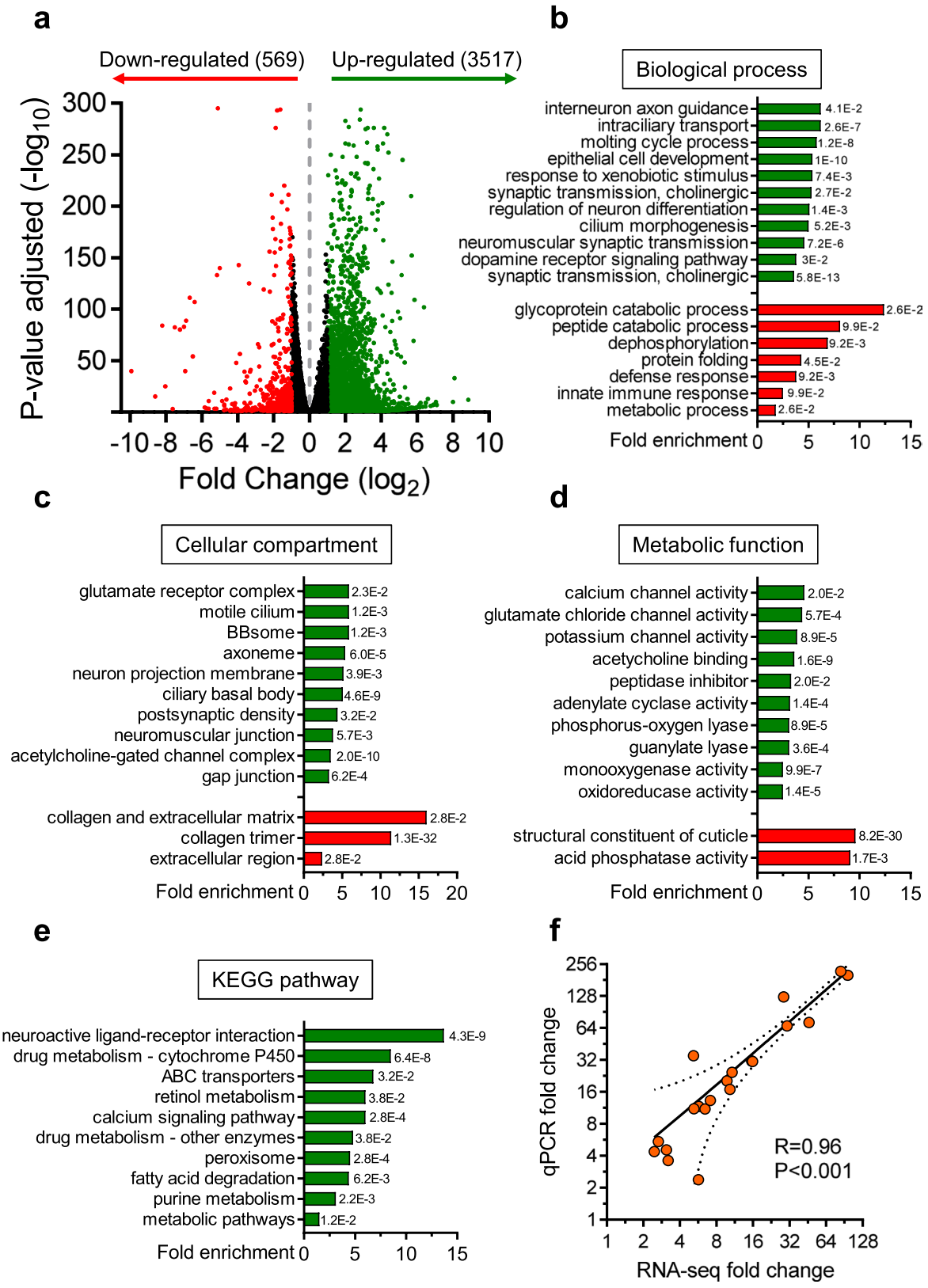
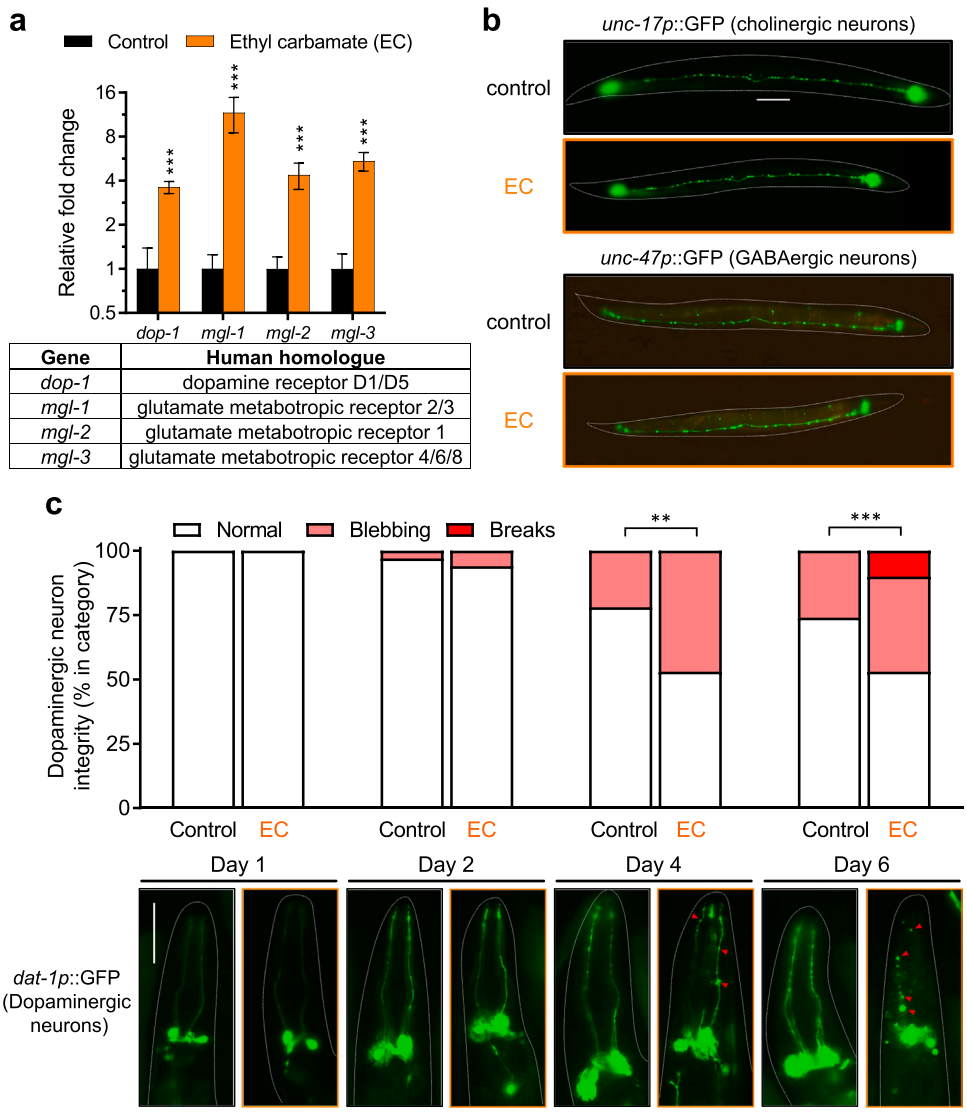


Fig. 2. Transcriptome-wide alterations to *C. elegans* gene expression are induced by ethyl carbamate. **a**) Volcano plot of gene expression changes in *C. elegans* after 24 h of 100 mM ethyl carbamate exposure. Differential gene expression highlighted in red (down-regulated > 2-fold) and green (up-regulated > 2-fold) with adjusted P-value < 0.05. DAVID GO Enrichment analysis of **b**) biological processes, **c**) cellular compartments, **d**) metabolic functions, and **e**) KEGG pathways of up-regulated (green) and down-regulated (red) genes after ethyl carbamate exposure. FDR values for each enriched category are shown next to the bar graph. **f**) Expression validation of 20 differentially expressed gene detected via RNA-sequencing with qPCR. Linear regression analysis of fold change values obtained from RNA-sequencing (x-axis) and qPCR (y-axis) ($P < 0.001$ as determined by the F-test).



Gene	Human homologue
dop-1	dopamine receptor D1/D5
mgl-1	glutamate metabotropic receptor 2/3
mgl-2	glutamate metabotropic receptor 1
mgl-3	glutamate metabotropic receptor 4/6/8

Fig. 3. Degeneration of dopaminergic neurons is evident after chronic ethyl carbamate exposure. **a**) Expression of dopaminergic and glutamatergic receptor genes after 24 h of 100 mM ethyl carbamate exposure as determined by qPCR. N = 4 samples analyzed per condition. Mean values are plotted with error bars indicating standard deviations. **b**) Representative images of cholinergic (*unc-17p*::GFP) and GABAergic (*unc-47p*::GFP) neuron integrity after 24 h of 100 mM ethyl carbamate exposure. **c**) Categorical scoring of dopaminergic (*dat-1p*::GFP) neuron integrity in *C. elegans* after 24 h of 100 mM ethyl carbamate exposure compared to control. N = 20 worms scored per condition per day. Representative images of *dat-1p*::GFP images are shown with red arrowheads indicating loci of neuronal blebbing or breaks. * *P < 0.01 and * **P < 0.001 as determined by student's t-test in **a**) and Chi-square test in **c**). Scale bar indicates 100 μm in b) and 50 μm in **c**).

Next, we compared the genes up-regulated by ethyl carbamate to two other environmental stressors, cadmium and hyperosmotic salt stress, which have previously been shown to highly activate the xenobiotic stress response [10,43]. Of the 859 genes up-regulated in *C. elegans* after cadmium exposure, 236 genes (27.4%) were similarly up-regulated after ethyl carbamate exposure. Functional analysis of these 236 genes revealed enrichment towards various xenobiotic responses (Fig. 4g). A comparison was also performed with osmotic stress-responsive genes where 403 out of 1014 genes (39.7%) up-regulated in *C. elegans* after hyperosmotic NaCl exposure were also similarly up-regulated after ethyl carbamate exposure (Fig. 4h). Genes up-regulated by both salt and ethyl carbamate exposure enriched to various cellular processes, including stress response functions such as oxidoreductase/monooxygenase activity and innate immunity, along with other functions including cilium morphogenesis, steroid hydroxylase activity, and iron ion binding (Fig. 4 g-h).

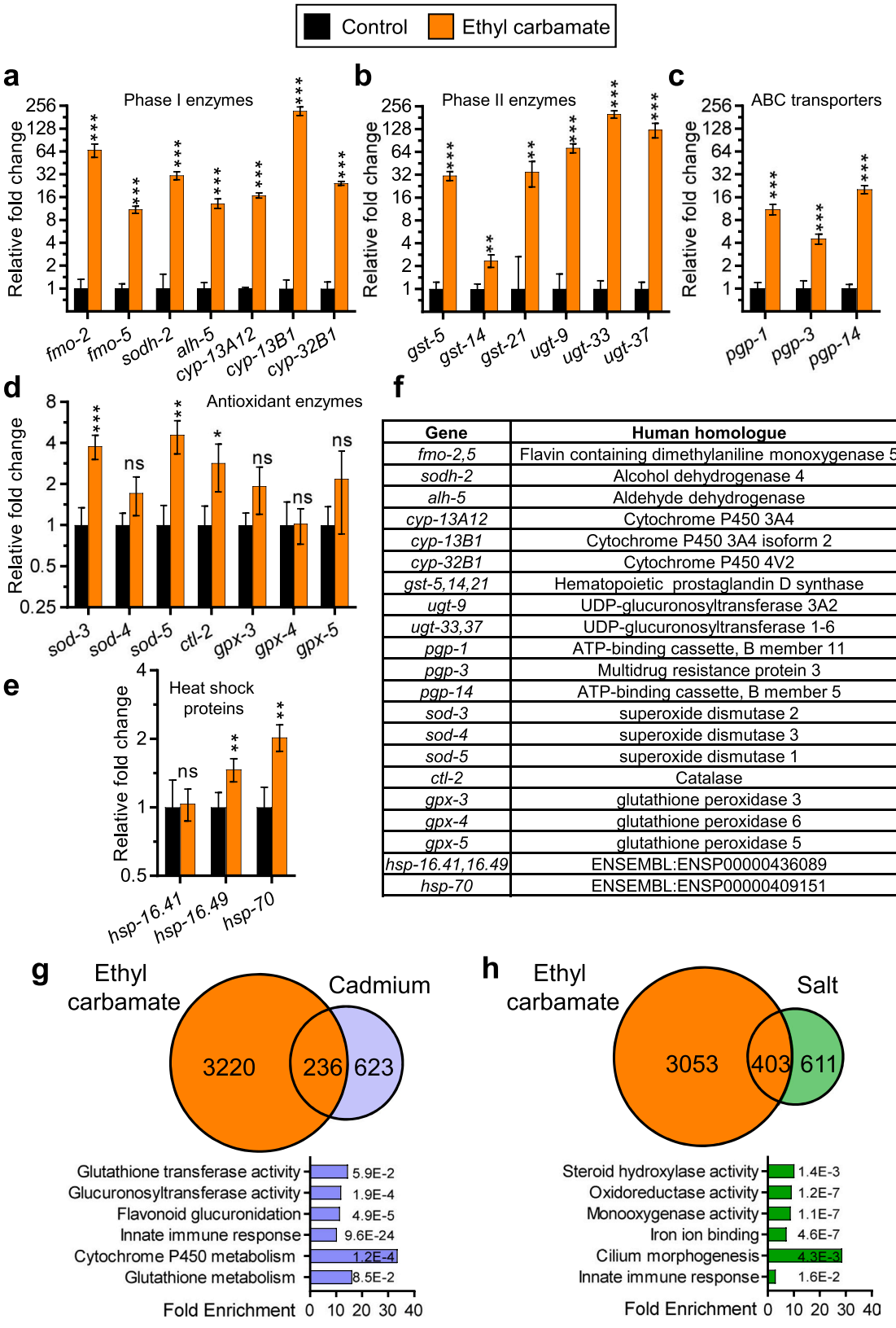
Overall, the results here demonstrate that *C. elegans* respond to ethyl carbamate exposure by mounting a widespread activation of conserved genes involved in xenobiotic detoxification. This mechanism is similar to stress responses activated by *C. elegans* after exposure to other environmental stressors, including heavy metal cadmium and hyperosmotic conditions.

4. Discussion

Ethyl carbamate is an emerging environmental contaminant that has been identified as a chemical of concern dues to its detectable presence in fermented food and tobacco products [39,41]. Beyond its probable carcinogenic effects, little is known about the possible harmful effects chronic ethyl carbamate exposure may have on other aspects of physiology. While the concentration of ethyl carbamate used in this *C. elegans* study is magnitudes higher than potential human exposure in the real world, we provide important mechanistic data describing the molecular mode of ethyl carbamate toxicity in provoking oxidative stress to activate the xenobiotic detoxification and antioxidant defense response (Figs. 2, 4), which are highly conserved between human and *C. elegans*. In addition, our study describes various ethyl carbamate toxicity endpoints that are of public concern, including developmental and reproductive toxicity (Fig. 1a-b), aging (Fig. 1c), and neurotoxicity (Fig. 3d). Together, these results highlight toxicity pathways of interest that can lead to deleterious effects in humans when ethyl carbamate exposure is sufficiently high.

4.1. Ethyl carbamate impairs development, reproduction, and lifespan

To date, the toxicological effects of chronic ethyl carbamate exposure on various aspects of development and reproductive physiology remain



Gene	Human homologue
fmo-2,5	Flavin containing dimethylaniline monoxygenase 5
sodh-2	Alcohol dehydrogenase 4
alh-5	Aldehyde dehydrogenase
cyp-13A12	Cytochrome P450 3A4
cyp-13B1	Cytochrome P450 3A4 isoform 2
cyp-32B1	Cytochrome P450 4V2
gst-5,14,21	Hematopoietic prostaglandin D synthase
ugt-9	UDP-glucuronosyltransferase 3A2
ugt-33,37	UDP-glucuronosyltransferase 1-6
pgp-1	ATP-binding cassette, B member 11
pgp-3	Multidrug resistance protein 3
pgp-14	ATP-binding cassette, B member 5
sod-3	superoxide dismutase 2
sod-4	superoxide dismutase 3
sod-5	superoxide dismutase 1
ctl-2	Catalase
gpx-3	glutathione peroxidase 3
gpx-4	glutathione peroxidase 6
gpx-5	glutathione peroxidase 5
hsp-16.41,16.49	ENSEMBL:ENSP00000436089
hsp-70	ENSEMBL:ENSP00000409151

Fig. 4. Xenobiotic detoxification response is activated after ethyl carbamate exposure. Exposure to 100 mM of ethyl carbamate for 24 h on the expression of genes encoding proteins functioning in **a**) phase I xenobiotic detoxification, **b**) phase II xenobiotic detoxification, **c**) ABC transporters, **d**) antioxidant enzymes, and **e**) heat shock proteins. Mean values are plotted in graphs with error bars indicating standard deviations. **f**) Human homologues of genes measured via qPCR. Venn diagram comparing ethyl carbamate up-regulated genes with genes up-regulated after **g**) cadmium exposure and **h**) hyperosmotic salt exposure. RNA-sequencing data in **g**) and **h**) were obtained from previously published datasets [10,43]. Enrichment analysis of overlapped genes is shown below each Venn diagram, with FDR values for each enriched category shown next to the bar graph.

limited. A recent study modelling ethyl carbamate toxicity using zebrafish showed that embryos exposed to ethyl carbamate had decreased survival rate, delayed rate of hatching, and increased frequency of hatchling body malformation [40]. This suggested that ethyl carbamate exposure induces developmental toxicity and is consistent with results from this study demonstrating the reduced body size of *C. elegans* upon exposure during larval development. Our study further shows that chronic ethyl carbamate exposure also suppresses *C. elegans* reproduction and shortens lifespan beginning at an exposure concentration of 50 mM. This demonstrates that chronic ethyl carbamate exposure is deleterious to development as well as reproduction and aging. While the concentration of ethyl carbamate used in this study is higher than the zebrafish study, this is attributed to the use of solid media for *C. elegans* experiments that can influence the relative bioavailability of chemical uptake compared to liquid media. *C. elegans* also possess a robust cuticle that serves as a barrier for peripheral chemical uptake, limiting ingestion as the primary route of substance entry [45]. At the highest ethyl carbamate concentration tested of 150 mM where severe growth and reproductive defects were observed, the mean lifespan of *C. elegans* was approximately nine days suggesting that this concentration is not acutely toxic.

4.1.1. Activation of xenobiotic detoxification in response to ethyl carbamate

In human cell models, exposure to 50–80 mM of ethyl carbamate increased the production of reactive oxygen species (ROS), suggesting that the onset of oxidative stress is a potential mechanism of ethyl carbamate toxicity [7]. Similar observations were reported in *C. elegans* and zebrafish models, where ethyl carbamate exposure increased ROS and superoxide anion levels [7,40]. This is supported by our data showing ethyl carbamate exposure activates the *gst-4*::GFP reporter that serves as a reliable fluorescent biomarker for oxidative stress induction [42,44]. Consistently, our RNA-sequencing results showed that an overwhelming number of genes up-regulated in response to ethyl carbamate exposure enrich to multiple cellular processes within the xenobiotic detoxification pathways. There were approximately 3,500 genes that showed at least a 2-fold up-regulation in gene expression, suggesting that ethyl carbamate exposure elicited a profound response at the transcriptome levels. A majority of the genes up-regulated function within xenobiotic detoxification and many of which are conserved between *C. elegans* and humans (Fig. 4f). For example, the *fmo-2* gene encoding flavin-containing monooxygenase was up-regulated by $>$ 50-fold in response to ethyl carbamate exposure and functions as a detoxification enzyme that catalyzes the addition of oxygen molecules to promote the solubilization and excretion of xenobiotic molecules [34]. In both *C. elegans* and mammalian cell models, overexpression of FMO protects against oxidative stress induced by a broad range of stressors, including paraquat and cadmium [18]. The activation of a xenobiotic detoxification response is commonly observed in *C. elegans* in response to environmental stress to provide cytoprotection (Fig. 4g, h), and *C. elegans* likely respond to ethyl carbamate toxicity using similar mechanisms by mounting an enhanced detoxification response to alleviate oxidative stress. However, the chronic presence of oxidative stress can eventually overwhelm the cellular detoxification capacity, causing irreversible damage to macromolecules, including lipids and proteins, leading to eventual cell death. Consistently, zebrafish exposed to ethyl carbamate show increased formation of apoptotic cells in a dose-dependent manner [40]. Mechanistically, induction of oxidative stress serves as a potential mechanism behind ethyl carbamate's various toxicological effects, as an imbalance in ROS levels has been shown to impair development and reproduction directly and is well implicated for its role in accelerating aging [1,26,9].

4.1.2. Neurotoxic property of ethyl carbamate

Elevated levels of oxidative stress have been directly linked to various neurodegenerative disorders in humans, including Alzheimer's disease and Parkinson's disease [23,24]. In the case of Parkinson's disease, selective degeneration of dopaminergic neurons is a hallmark feature associated with the onset of motor dysfunctions [5]. Our previous studies showed that chronic exposure to another food-borne contaminant acrylamide induced oxidative stress in *C. elegans* and caused degeneration of dopaminergic neurons [29,42]. This study showed that chronic ethyl carbamate exposure also resulted in the degeneration of dopaminergic neurons in *C. elegans* (Fig. 3c). The functional role of dopaminergic neurons is highly conserved between *C. elegans* and mammals in modulating dopamine neurotransmission to coordinate various locomotor functions [37]. The neurotoxicity of ethyl carbamate appears to be specific to dopaminergic neurons as the integrity of cholinergic or GABAergic neurons was unaffected. This could be attributed to various factors such as a potential specificity of ethyl carbamate in targeting dopaminergic neurons or differential properties between *C. elegans* neuron types such as anatomical locations or variability in neuron-specific stress defense capacity. It is also possible that ethyl carbamate may impact other neuronal functions that were not detected based on morphological changes characterized in this study.

Our investigation into the potential neurotoxic properties of ethyl carbamate was based on the up-regulation of various genes that function within neuronal regulation (Figs. 2, 3a). A previous study in mice observed that animals chronically treated with ethyl carbamate exhibit hyperactive behaviour, suggesting a potential effect on the nervous system [22]. Interestingly, evidence of hyperactive behaviour was also recently demonstrated in zebrafish, showing that ethyl carbamate treated fish had increased mobility and swimming velocity accompanied by evidence of neurodegeneration characterized by an increase in necrotic brain tissues [40]. While it remains to be determined whether ethyl carbamate's mechanism of neurotoxicity in *C. elegans* is directly linked to the increased expression of neuron regulating genes or as a consequence of increased levels of oxidative stress, our results provide initial evidence to support the neurotoxic potential of chronic ethyl carbamate exposure.

4.1.3. Mechanistic basis of ethyl carbamate toxicity

As briefly described in the introduction, human metabolism of ethyl carbamate can result in the formation of vinyl carbamate epoxide, which is a highly reactive electrophilic compound [32]. Electrophile chemicals are known to induce oxidative stress directly, and their cellular presence directly triggers the activation of the Nrf-2/SKN-1 transcription factor in human/*C. elegans* that is a master transcriptional regulator of antioxidant and xenobiotic defense [4]. This electrophilic toxicity of ethyl carbamate is consistent with our observation that several genes under the transcriptional control of SKN-1, including *gst* and *ugt* genes are highly activated by ethyl carbamate in *C. elegans* (Figs. 2, 4). Accumulation of vinyl carbamate epoxide has also been shown to form DNA adducts that serve as a key contributor to the carcinogenicity of this compound [12,30]. The formation of DNA adducts can interfere with DNA transcription to affect gene expression regulation. If the adduct accumulation persists, it can directly damage DNA by inducing site-specific mutations to the genome with carcinogenic outcomes [21]. While direct DNA damage was not examined in this study, ethyl carbamate exposure did induce a profound effect on the transcriptome by altering the expression of $>$ 4,000 genes by 2-fold ($\sim$20% of the *C. elegans* transcriptome; Fig. 2). A previous study in human cells has shown that levels of apoptotic cells dramatically increase upon exposure to ethyl carbamate [8]. This is consistent with the onset of DNA damage that activates the intrinsic programmed cell death response via apoptosis in removing cells with irreparable damage from the population [35].

To summarize, we characterized in this study the consequences of chronic ethyl carbamate exposure on various physiological functions and reported its effects on the transcriptome using the *C. elegans* model. We show that ethyl carbamate has deleterious effects on *C. elegans* growth, reproduction, and aging while also exhibiting neurotoxic effects

on the dopaminergic neurons. The activation of xenobiotic detoxification and antioxidant defense genes indicated that ethyl carbamate exposure induced oxidative stress, which may be a potential mechanism of its toxicological properties. Given the common occurrence of ethyl carbamate as a natural food contaminant, this study aims to broaden our basic knowledge to better understand the potential toxicity of chronic ethyl carbamate exposure may have in humans.

CRediT authorship contribution statement

JCC: Conceptualization, Investigation, Formal analysis; **SCC**: Investigation; **BMW** Investigation; **HT** Investigation; **CWW** Conceptualization, Resources, Writing – original draft/ Review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All datasets supporting this manuscript are found within the article and its supplementary files. RNA-sequencing data generated from this study (raw and annotated) are available on the NCBI GEO data repository GSE190099.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.03.029.

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