6-2-2021

Developmental ethanol exposure causes central nervous system dysfunction and may slow the aging process in a Drosophila model of fetal alcohol spectrum disorder

Khaoula Belhorma
San Jose State University

Nahed Darwish
San Jose State University

Elizabeth Benn-Hirsch
Ross University School of Medicine

Annalisa Duenas
San Jose State University

Hillary Gates
San Jose State University

See next page for additional authors

Recommended Citation

Khaoula Belhorma, Nahed Darwish, Elizabeth Benn-Hirsch, Annalisa Duenas, Hillary Gates, Navneet Sanghera, Jodie Wu, and Rachael L. French. "Developmental ethanol exposure causes central nervous system dysfunction and may slow the aging process in a Drosophila model of fetal alcohol spectrum disorder" *Alcohol* (2021): 65-73. https://doi.org/10.1016/j.alcohol.2021.03.006

Alcohol 94 (2021) 65–73

Contents lists available at ScienceDirect

Alcohol

journal homepage: http://www.alcoholjournal.org/

Developmental ethanol exposure causes central nervous system dysfunction and may slow the aging process in a Drosophila model of fetal alcohol spectrum disorder

Khaoula Belhorma, Nahed Darwish *, Elizabeth Benn-Hirsch ^, Annalisa Duenas, Hillary Gates @, Navneet Sanghera, Jodie Wu +, Rachael L. French*

Department of Biological Sciences, San José State University, 1 Washington Square, San José, California, 95192-0100, United States

ARTICLE INFO

Article history:
Received 5 June 2020
Received in revised form
25 March 2021
Accepted 31 March 2021

Keywords:
aging
fetal alcohol spectrum disorder
oxidative stress

ABSTRACT

Alcohol is a known teratogen, and developmental exposure to ethanol results in fetal alcohol spectrum disorder (FASD). Children born with FASD can exhibit a range of symptoms including low birth weight, microcephaly, and neurobehavioral problems. Treatment of patients with FASD is estimated to cost 4 billion dollars per year in the United States alone, and 2 million dollars per affected individual's lifetime. We have established *Drosophila melanogaster* as a model organism for the study of FASD. Here we report that mutations in *Dementin (Dmtn)*, the *Drosophila* ortholog of the Alzheimer's disease-associated protein TMCC2, convey sensitivity to developmental ethanol exposure, and provide evidence that *Dmtn* expression is disrupted by ethanol. In addition, we find that flies reared on ethanol exhibit mild climbing defects suggestive of neurodegeneration. Surprisingly, our data also suggest that flies reared on ethanol age more slowly than control animals, and we find that a number of slow-aging mutants are sensitive to developmental ethanol exposure. Finally, we find that flies reared on ethanol showed a persistent upregulation of genes encoding antioxidant enzymes, which may contribute to a reduced rate of central nervous system aging. Thus, in addition to the well-documented negative effects of developmental alcohol exposure on the nervous system, there may be a previously unsuspected neuroprotective effect in adult animals.

Introduction

Alcohol consumption is a double-edged sword. There is evidence for its having both harmful and beneficial effects on human health. Most research has focused on the negative health effects of excessive alcohol consumption; however, in recent years there has also been some focus on the benefits of moderate alcohol consumption.

One highly studied negative effect of alcohol consumption is its effect on development. Ethanol is a known teratogen that causes a range of symptoms, including neurobehavioral problems, microcephaly, and psychiatric comorbidities. These symptoms are collectively known as fetal alcohol spectrum disorder (FASD) (Banakar, Kudlur, & George, 2009).

In addition to effects on brain development and behavior, developmental alcohol exposure (DAE) causes oxidative stress via a variety of mechanisms, including increased production of reactive oxygen species (ROS) (Brocardo, Gil-Mohapel, & Christie, 2011; Heaton, Mitchell, & Paiva, 2000), dysregulation of fat metabolism (Harris, Trudell, & Mihic, 2008; Logan-Garbisch et al., 2015), and the downregulation of genes involved in ROS detoxification. In flies, these genes include *Peroxidase* (*Pxd*) and two glutathione-S-transferase (GST)-encoding genes (*GstD4* and *GstD8*). These changes diminish the fly's ability to respond to increased stress and contribute to an overall increase in sensitivity to oxidative stress (Logan-Garbisch et al., 2015). In response to this stress, *Drosophila*

* Corresponding author. Telephone: +1 408 924 4894
E-mail address: rachael.french@sjsu.edu (R.L. French).
* Synthego Corporation, 3565 Haven Ave. #1, Menlo Park, California 94025, United States.
^ Ross University School of Medicine, 2300 SW 145th Ave., Suite 200, Miramar, Florida 33027, United States.
@ Ligand Pharmaceuticals, 1440 O'Brien Drive, Menlo Park, California 94025, United States.
+ University of Minnesota Medical School, 420 Delaware St. SE, Minneapolis, Minnesota 55455, United States.

https://doi.org/10.1016/j.alcohol.2021.03.006

larvae undergoing DAE upregulate the oxidative stress response genes *Catalase* (*Cat*), *Glutathione synthetase 1* (*Gss1*), and *Superoxide dismutase 1* (*Sod1*) (Logan-Garbisch et al., 2015).

Oxidative stress is also known to be a contributor to aging. In animal models, decreased production of ROS contributes to increased longevity and decreased aging (Barja, Cadenas, Rojas, Perez-Campo, & Lopez-Torres, 1994; Dröge & Schipper, 2007; Sohal, 2002). There is considerable evidence that oxidative stress occurs in neurodegenerative diseases. In Alzheimer's disease, amyloid-beta peptides spontaneously generate ROS and free radicals, causing neuronal cell death and damage (Bennett, Grant, & Aldred, 2009; Gackowski et al., 2008). In addition, oxidative damage seems to precede the onset of symptomatic dementia. The isoprostane 8,12-*iso*-iPF$_{2\alpha}$-VI, a specific marker of *in vivo* lipid peroxidation that is elevated in Alzheimer's disease (AD), has been found to be a good predictor of AD in patients with mild cognitive impairment (Praticò, Clark, Liun, Lee, & Trojanowski, 2002).

Light-to-moderate ethanol consumption has also been shown to have neuroprotective effects. In epidemiological studies, the association between ethanol consumption and Alzheimer's disease (AD) and vascular dementia decreases with low levels of consumption and exponentially increases after a certain level, leading to a J-shaped curve. Compared to nondrinkers, light-to-moderate drinkers showed a decreased risk of Alzheimer's disease, vascular dementia, and cognitive decline regardless of the source of alcohol. Conversely, high alcohol consumption increased the risk of dementia, but not AD (Anstey, von Sanden, Salim, & O'Kearney, 2007; Luchsinger, Tang, Siddiqui, Shea, & Mayeux, 2004; Solfrizzi et al., 2007). Light-to-moderate alcohol consumption was found to be most strongly associated with reduced risk of dementia in individuals aged 55 or older (Ruitenberg et al., 2002).

From a genetic screen for mutations altering sensitivity to DAE (Lafler, 2015), we identified a loss-of-function allele of *Dementin (Dmtn)*. *Dmtn* is an ortholog of the human TMCC2 protein. In cultured human cells, TMCC2 and apolipoprotein E work together to affect the metabolism of amyloid beta precursor protein (APP) by increasing the levels of amyloid beta (Aβ) and both Aβ$_{40}$ and Aβ$_{42}$ fragments (Hopkins, Sáinz-Fuertes, & Lovestone, 2011). This relationship is conserved in flies, where *Dmtn* also alters the processing of the *Drosophila* APP homolog APP-like (APPL) (Hopkins, 2013). Adult flies homozygous for *Dmtn*^{1} exhibited Alzheimer's disease-like pathology. They exhibited all of the disease's hallmarks, including accumulation of abnormal APPL fragments, mislocalized microtubule-binding proteins, synaptic defects, neurodegeneration, and early death. In addition, *Dmtn* is required for normal brain development: *Dmtn*^{1} homozygotes exhibit developmental neurodegeneration, as well as AD-like pathology and adult neurodegeneration (Hopkins, 2013).

Here we show that flies mutant for *Dmtn* are sensitive to the lethal effects of DAE, and that *Dmtn* expression is a target of DAE. In addition, we find that DAE in wild-type flies results in reduced climbing ability, suggesting that DAE causes central nervous system dysfunction in flies. Further, we show evidence that DAE is protective against age-related declines in negative geotaxis behavior, suggesting a previously unsuspected neuroprotective effect of developmental ethanol exposure. We show persistent upregulation of oxidative stress genes after DAE, and hypothesize that this could result in protection against neurological aging.

Materials and methods

Fly strains and genetics

Fly stocks were maintained at 25 °C on standard cornmeal/molasses medium. All mutant alleles and transgenes were introgressed for five generations into our standard lab background (w^{1118}; Wild Type Berlin [*w; WTB*]), which is also our control strain, except for climbing assays, where we used WTB flies with wild-type eyes (because white-eyed flies do not see well and their defective phototaxis can interfere with normal negative geotaxis). y^{1} w^{1118}; $Dmtn^{A181}$/*TM3, Sb Ser*, y^{1} w^{67c23}; $Dmtn^{EY08071}$, w^{1118}; $Indy^{206}$ and *w; mth*^{1} were obtained from the Bloomington Drosophila Stock Center (Bloomington, Indiana, United States) (stock numbers 16060, 22314, 27901, and 27896).

Survival assays

Flies were acclimated for 24 hours at 25 °C in egg collection bottles capped with a petri dish lid filled with fly food. Flies were allowed to lay eggs for 24 hours, then 100 eggs per vial were transferred from the plates into vials containing either food with no ethanol (control conditions) or food containing 7% ethanol (experimental conditions). The vials were placed in baths of corresponding ethanol concentration (0 or 7%). The use of baths ensures that flies are exposed to a constant concentration of ethanol during development (10–16 days), as ethanol is volatile.

After vials had been in their corresponding water baths for 7 days, they were examined daily for wing spots (indicating that the flies are near to eclosion). All vials that contained at least one pupa with wing spots were retrieved from the bath and placed in a tray in a 25 °C incubator. At 10 days after egg laying, the number of flies that eclosed was recorded daily.

Negative geotaxis (climbing) assays

Climbing assays were performed according to Barone & Bohmann, 2013. The climbing assay was performed on 1–14-day-old flies (depending on the specific assay) in a dark room with artificial lighting placed directly above the vials. The apparatus used in the climbing assay was constructed by inserting half of a cotton vial plug into the bottom of one standard food vial. Adult flies were placed inside this vial, and a second vial was placed over it and secured with transparent tape. To quantify climbing, flies were tapped gently to the bottom of the apparatus, and climbing was recorded in a 1-minute iPhone video.

To analyze the videos, flies that had crossed the line of demarcation between the two vials (and had thus climbed half the distance from the bottom to the top of the apparatus) were counted at 10-second intervals. To assess the effect of aging, we repeated the assay every 2 days until the flies were 14 days old. Upon completion of the assay, the flies were placed in fresh vials containing food and kept at 25 °C until the next climbing assay. The flies were not anesthetized prior to the assay.

Quantitative RT-PCR

Total RNA was extracted from 2–5-day-old adults or early third instar larvae using Trizol reagent (Life Technologies, Carlsbad, California, United States) according to the manufacturer's instructions, resuspended in RNase-free water, and stored at −80 °C until use. For qRT-PCR, 2 μg of total RNA was reverse-transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, California), according to the manufacturer's instructions. The resulting cDNA was analyzed in triplicate by quantitative, real-time PCR using MxPro QPCR software version 4.10 (Stratagene, LaJolla, California). Both no-template and DNAse-treated non-reverse-transcribed mRNA samples were used as negative controls. No significant amplification was observed in these samples. *Rp49* transcript levels were used as a normalization control for RNA samples. Relative mRNA abundance was calculated using the comparative ΔCt method (Schmittgen & Livak, 2008).

Primer sequences

Ethanol absorption assay

To test ethanol absorption, 25 young male flies per genotype were placed into empty standard food vials. The vial plugs were then replaced with plugs to which 1 mL of 80% ethanol had been added. Flies were exposed to evaporating ethanol for 5 minutes (sufficient to induce behavioral changes including hyperactivity, but not long enough for flies to sedate). At the end of the exposure period, flies were transferred to a 1.5-mL microcentrifuge tube and snap frozen on dry ice. For processing, flies were homogenized in 250 mL of 50 mM Tris–HCl (pH 7.5), and samples were then spun at 15,000 *g* for 20 minutes at 4 °C. 5 mL of supernatant (equivalent to ½ fly) was then prepared for colorimetric assay using the Sigma–Aldrich Ethanol Assay Kit (Sigma–Aldrich; St. Louis, Missouri, United States, catalog #MAK076), according to manufacturer's instructions.

Statistical analysis

Statistics were performed using Student's *t* test, one-way ANOVA, or one-way ANOVA with repeated measures as indicated.

Results

Dmtn mutant flies are sensitive to ethanol-induced developmental lethality

We carried out a P-element mobilization screen and generated approximately 850 novel autosomal P{GawB} insertions, which were screened for altered sensitivity to the lethality and developmental delay caused by developmental alcohol exposure (Lafler, 2015). From this screen, we recovered an allele of *Dementin (Dmtn),* the *Drosophila* ortholog of the human gene TMCC2. This allele, which we have designated $Dmtn^{RF61}$, results from an insertion of P{GawB} 86 base pairs into the 5′UTR of Dmtn (Fig. 1A). This allele displayed significantly reduced survival to eclosion when reared on ethanol-containing media (64% survival, compared to 80% survival of wild-type animals reared on ethanol, Fig. 1B). To determine whether ethanol sensitivity was a general phenotype associated with loss of function of *Dmtn*, we tested three additional *Dmtn* alleles (Fig. 1B). We found that homozygosity for both $Dmtn^{EY08071}$ and $Dmtn^{MI08519}$ led to ethanol sensitivity (26% and 51% relative survival, respectively), and, further, that heterozygosity for the lethal allele $Dmtn^{A181}$ resulted in a mean relative survival of 20%, suggesting that reduced *Dmtn* can lead to dominant ethanol sensitivity during development.

We confirmed that mutation of *Dmtn* is responsible for the ethanol sensitivity we observe through non-complementation. Flies of the genotype $Dmtn^{EY08071}/Dmtn^{RF61}$ show the same sensitivity to ethanol as homozygotes for each allele (36% relative survival compared with 68–74% relative survival in heterozygotes) (Fig. 1C).

Cat:	Left primer: 5′-GAATTCTCGACGCAGTCACA-3′
	Right primer: 5′-CTGCAGCAGGATAGGTCCTC-3′
Gss1:	Left primer: 5′-AGTTCACGGCCAATCTGTTC-3′
	Right primer: 5′-ATCCTGACCACGATCCTCAC-3′
Sod:	Left primer: 5′-TTGCCATACGGATTGAAGTG-3′
	Right primer: 5′-CGAACAGGAGGTGAGAATCC-3′
Dmtn1:	Left Primer: 5′-TGCCAATGCCGATGTTTTGG-3′
	Right primer: 5′-ATTCGCTGCCATTGTCACTG-3′
Dmtn2:	Left Primer: 5′- TCTCGCAGCTGCAGAAAAAG-3′
	Right primer: 5′-TCGTCTGGAACTGGTGATTCTG-3′
Rp49:	Left primer: 5′ - ACGTTGTGCACCAGGAACTT - 3′
	Right primer: 5′ - CCAGTCGGATCGATATGCTAA - 3′

DAE causes a developmental delay in addition to increased developmental mortality (McClure, French, & Heberlein, 2011), and many mutant alleles that show increased lethality also display an increased development time relative to wild-type controls (Lafler, 2015), so it was a formal possibility that increased time spent in ethanol-containing media could explain the increased lethality. However, we have three lines of evidence indicating that *Dmtn* mutants do not display increased lethality due to longer exposure to ethanol. First, *Dmtn* mutants do not display increased development time relative to wild-type controls when reared on ethanol. Wild-type flies grown in control media had a median development time of 10.5 days, and this was increased to 12.9 days in ethanol-reared animals, for a delay of 2.4 days. *Dmtn*-mutant animals had average delays ranging from 2.9 to 3.1 days (Supplementary Figure 1A). While this shows a consistent small increase in development time in *Dmtn* flies reared on ethanol, none of these increases were statistically significant ($p = 0.18$, one-way ANOVA).

Second, there is no consistent relationship between increased development time and increased mortality in ethanol-reared mutants. In fact, the two phenotypes are completely separable, as described in Lafler, 2015. Supplementary Fig. 1B and 1C show two examples of mutations isolated from our screen, both of which display significant increases in development time when ethanol-reared, but no increase in ethanol-induced lethality. In this experiment, the relative survival of control animals was 70.3% when reared on ethanol, while the ethanol-reared survival rates of flies homozygous for *RF534* and *RF831* were 77.2% and 64.2%, respectively ($p = -0.092$, one-way ANOVA). However, while ethanol rearing caused a 3.3-day delay in development of control flies, the ethanol-induced delay in *RF534* mutants was 4.5 days, while *RF831* mutants had a 5.7-day developmental delay ($p = 0.00014$, one-way ANOVA with Tukey HSD *post hoc* analysis). Thus, increased ethanol exposure due to increased developmental delay is insufficient to cause an increase in lethality.

It is unlikely that increased development time is responsible for increased developmental lethality, because the two phenotypes map to different developmental stages, suggesting independent causes (McClure et al., 2011).

Lastly, we tested the ethanol absorption of flies homozygous for $Dmtn^{RF61}$, $Dmtn^{EY08071}$, and $Dmtn^{MI08519}$ and found them to be indistinguishable from wild-type animals (Supplementary Figure 2), indicating that the observed ethanol sensitivity is likely not due to changes in ethanol absorption or metabolism.

Developmental alcohol exposure alters Dmtn expression

Since mutation of *Dmtn* increases ethanol-induced developmental lethality, we decided to test whether DAE affects *Dmtn* transcript levels. We performed reverse transcriptase-mediated quantitative PCR (RT-qPCR) to assess transcript levels in third-instar larvae and adult w; WTB flies reared on ethanol. In larvae, we found that DAE results in a 4-fold decrease in third-instar larval *Dmtn* transcript levels (Fig. 2A) ($n = 6$; $p < 0.0001$, Student's *t* test). Intriguingly, we saw the opposite effect on adult transcript levels. In adult flies previously exposed to DAE (but not exposed to any ethanol for at least 2 days prior to RNA extraction), *Dmtn* transcript levels are increased 4.6-fold (Fig. 2B) ($n = 3$, $p < 0.0001$, Student's *t* test). This suggests a complex interaction between DAE and *Dmtn* expression.

Developmental alcohol exposure causes central nervous system dysfunction

In animal models, DAE causes abnormally high levels of apoptosis in the developing CNS, resulting in reduced brain mass

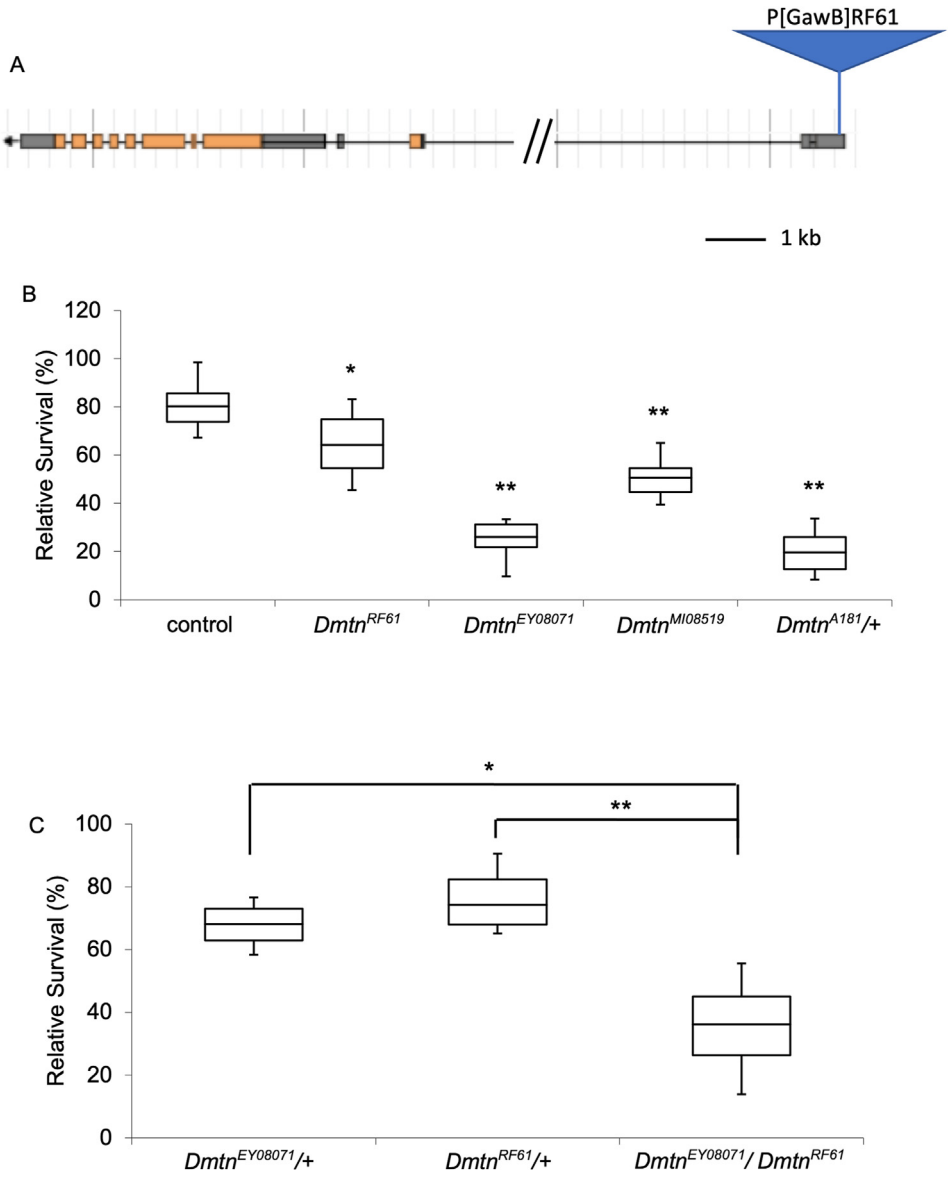

Fig. 1. ***Dmtn*** **mutant flies are sensitive to ethanol-induced developmental lethality. A)** Schematic diagram of the *Dmtn* gene. This figure was modified from the "collapsed" Dmtn J-Browse map on Flybase.org (Larkin et al., 2021). *Dmtn* encodes five transcripts and three polypeptides that differ in their amino termini. All five transcripts are shown in this format, compacted into a single representative map. *P{GawB}RF61* is inserted in the *Dmtn* 5′ UTR, 86 base pairs from the 5′ end of all five transcripts. Solid boxes represent exons; lines represent introns. Gray boxes represent UTR; orange boxes represent translated sequence. 7500 base pairs of elided intron sequence are represented by the hash marks. **B)** Multiple alleles of *Dmtn* display sensitivity to DAE. Relative survival in DAE of flies homozygous for $Dmtn^{RF61}$, $Dmtn^{EY08071}$, and $Dmtn^{MI08519}$ is reduced to 37–83% of that of wild-type flies, while survival of flies heterozygous for the lethal $Dmtn^{A181}$ heterozygotes is reduced to 25% of wild type (n = 4–24; $*p < 0.05$, $**p < 0.01$, one-way ANOVA with Tukey's HSD *post hoc* analysis). Survival has been normalized to genotype-matched controls reared on ethanol-free food. **C)** $Dmtn^{RF61}$ fails to complement $Dmtn^{EY0807}$. The relative survival in DAE of $Dmtn^{EY0807}/Dmtn^{RF61}$ compound heterozygotes is 36%, compared to 68% in $Dmtn^{EY0807}/+$ heterozygotes and 74% in $Dmtn^{RF61}/+$ heterozygotes (n = 4; $*p < 0.05$, $**p < 0.01$, one-way ANOVA with Tukey's HSD *post hoc* analysis). Centerlines show the sample median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

(Ikonomidou et al., 2000). In addition, small head size is observed in severe cases of fetal alcohol syndrome, suggestive of reduced brain size in infants, and this has been confirmed with brain imaging studies (Spadoni, McGee, Fryer, & Riley, 2007). We have previously shown that, in *Drosophila*, DAE leads to reduced larval brain size (McClure et al., 2011). In order to investigate whether this reduced brain size translates into altered CNS function in adult animals, we tested the climbing behavior of ethanol-reared flies. Negative geotaxis (climbing) assays have been established as a reliable assessment of central nervous system (CNS) health in flies (Ali, Escala, Ruan, & Zhai, 2011; Barone & Bohmann, 2013). In addition, climbing assays can also be used to assess aging by allowing the measurement of the natural loss of climbing ability in flies due to age (He & Jasper, 2014; Sun et al., 2013). Thus, negative geotaxis can be used as a proxy for age-induced neurodegeneration.

Dmtn is expressed in both neurons and glia, and mutations in the gene are reported to trigger neurodegeneration. *Dmtn* is also required for normal brain development (Hopkins, 2013). Because DAE alters *Dmtn* transcript levels, and *Dmtn* mutants are sensitive to the deleterious effects of ethanol on development (Fig. 2), we decided to test the climbing behavior of *Dmtn*-mutant animals as well.

We performed climbing assays on control flies, flies reared on ethanol, and *Dmtn*-mutant flies. We repeated the assay every 2 days

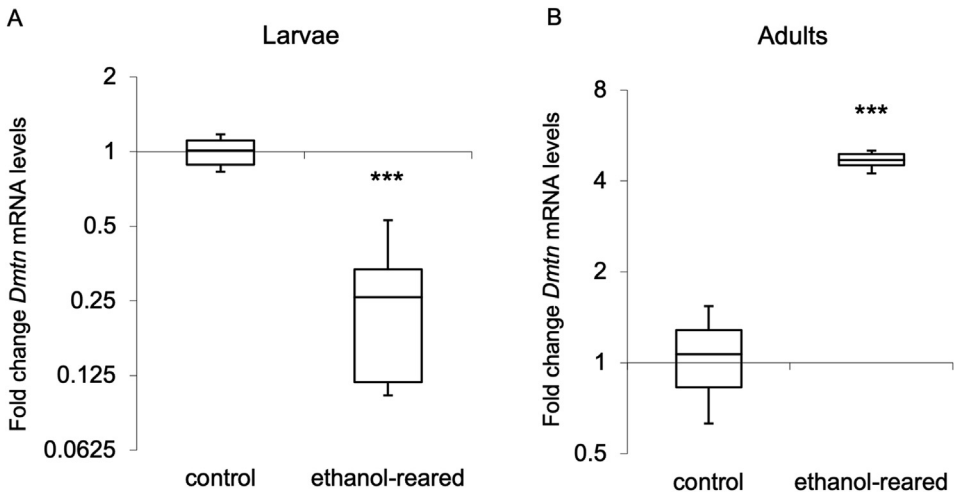

Fig. 2. DAE alters *Dmtn* expression. A, B) *Dmtn* transcript levels are reduced in ethanol-reared third-instar larvae (**A**, n = 6, $p < 0.0001$, Student's *t* test), but increased in ethanol-reared adult flies 2–5 days after removal from ethanol-containing media (**B**, n = 3, $p < 0.0001$, Student's *t* test). Centerlines show the sample median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

for 12 days to observe the effect of aging on the animals' climbing abilities. Our results demonstrate that DAE causes climbing defects in young flies. While 82% of 1–4-day-old wild-type flies were able to climb above the center line of the test apparatus (approximately 95 mm) within 20 seconds of the start of the assay, only 66% of age-matched ethanol-reared animals were able to do so (Fig. 3) (n = 12, $p < 0.0001$, Student's *t* test). This suggests that DAE results in CNS dysfunction in young *Drosophila*.

We found that homozygosity for $Dmtn^{EY08071}$ had no effect on climbing behavior. Eighty-four percent of 1–4-day-old $Dmtn^{EY08071}$ mutant flies were able to climb at least halfway up the test apparatus within 20 seconds, making them indistinguishable from wild type (Fig. 3). Thus, it appears that the reported neurodegeneration associated with mutation in *Dmtn* does not affect adult climbing behavior as a measure of CNS function. These results and their connection to neurodegeneration must be interpreted with caution, however, as our climbing assays were performed on a different allele of *Dmtn* ($Dmtn^{EY08071}$) than the one for which neurodegenerative phenotypes were reported $Dmtn^{e01970}$ (Hopkins, 2013). We were unable to test climbing in $Dmtn^{e01970}$ flies, as there are no publicly available stocks of this allele, and we were unable to obtain it from the original source.

Developmental ethanol exposure slows down age-related declines in climbing ability

Normally, flies' ability to climb diminishes with age (He & Jasper, 2014; Sun et al., 2013). We find that climbing ability begins to decline in wild-type flies at around 1 week of age, and consistently diminishes through 2 weeks of age (Fig. 4A). Intriguingly, we find that wild-type flies reared on ethanol do not lose climbing ability as rapidly as control flies (Fig. 4B). More than 81% of young (1–6-day-old) control flies climbed above the centerline within 20 seconds of the start of the assay (Fig. 4A). By 7–10 days of age, this had diminished to approximately 54% of flies (n = 4; $p < 0.002$, one-way repeated-measures ANOVA with Tukey's HSD *post hoc* analysis), representing a 33% loss in climbing ability. However, flies reared on ethanol fully retain their climbing ability at that age, with no significant declines until at least 11 days of age (Fig. 4B, n = 3; one-way repeated-measures ANOVA with Tukey's HSD *post hoc* analysis). We found no consistent effect of *Dmtn* mutation on climbing ability at any age (not shown).

Slow-aging mutants are sensitive to ethanol-induced developmental lethality

Because of our data suggesting that ethanol rearing can lead to slower aging, we asked whether altered sensitivity to DAE is a common feature of slow-aging mutants, which would suggest a common molecular mechanism underlying both slow aging and ethanol sensitivity. We tested loss-of-function mutations in two additional genes: *I'm not dead yet* (*Indy*), which encodes a membrane protein that transports Krebs cycle intermediates, and, when mutant, functions as a caloric restriction mimetic; and *methuselah* (*mth*), a G protein-coupled receptor that triggers insulin release coupled to nutrient status in flies (Araújo et al., 2013; Delanoue et al., 2016). Mutation of both *Indy* and *mth* result in extended longevity in flies (and, for *Indy*, in *C. elegans*; Rogina & Helfand, 2013), due at least in part to reduced insulin signaling and

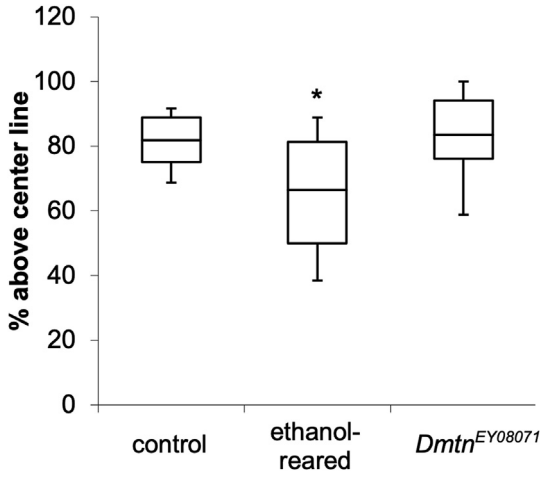

Fig. 3. DAE causes CNS dysfunction in wild-type, but not *Dmtn*, flies. Ethanol-reared wild-type flies (2–4 days old) exhibit reduced ability to perform negative geotaxis, compared to age-matched controls that were not reared on ethanol, while flies homozygous for $Dmtn^{EY08071}$ (not ethanol-reared) show climbing ability indistinguishable from wild-type controls (n = 12; $p = 0.0089$, one-way ANOVA with Tukey HSD *post hoc* analysis). Centerlines show the sample median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

concomitant reductions in the production of reactive oxygen species. Because DAE results in changes to both insulin signaling and the production of reactive oxygen species (Logan-Garbisch et al., 2015; McClure et al., 2011), these seemed like good candidates for mutations that might alter DAE sensitivity.

Flies homozygous for loss-of-function mutations in both genes ($Indy^{206}$ and mth^{1}) were sensitive to developmental ethanol-induced lethality (Fig. 5A). Only 25% of $Indy^{206}$ homozygotes and 45.6% of mth^{1} homozygotes survived DAE, compared to 71% of wild-type control animals (n = 7–8, $p < 0.0001$, Student's *t* test). As with the *Dmtn* mutant alleles, neither of these mutations shows any changes in ethanol absorption (Supplementary Figure 2).

Developmental ethanol exposure results in persistent upregulation of antioxidant genes

We previously showed that the antioxidant genes *Superoxide dismutase-1 (Sod1)*, *Glutathione synthetase 1 (Gss1)*, and *Catalase (Cat)* were upregulated as a result of ethanol exposure during development (Logan-Garbisch et al., 2015). Because aging is exacerbated by accumulation of oxidative stress (Barja et al., 1994; Dröge & Schipper, 2007; Sohal, 2002), we hypothesized that the slower aging we observed in flies reared on ethanol could be due to sustained upregulation of the same genes into adulthood.

We performed RT-qPCR to examine the expression of *Sod1*, *Gss1*, and *Cat* in adult flies after ethanol rearing and found that both *Sod1* and *Gss1* showed persistent upregulation (3.2-fold for *Sod1* and 3-fold for *Gss1;* Fig. 5B, n = 3 biological replicates, $p < 0.01$ for both *Sod1* and *Gss1*, one-way ANOVA with Tukey *post hoc* analysis). *Cat* expression was also elevated (1.8-fold over control levels), but this did not achieve statistical significance. These results provide support for the hypothesis that slow aging in ethanol-reared flies is due to persistent upregulation of antioxidant enzymes.

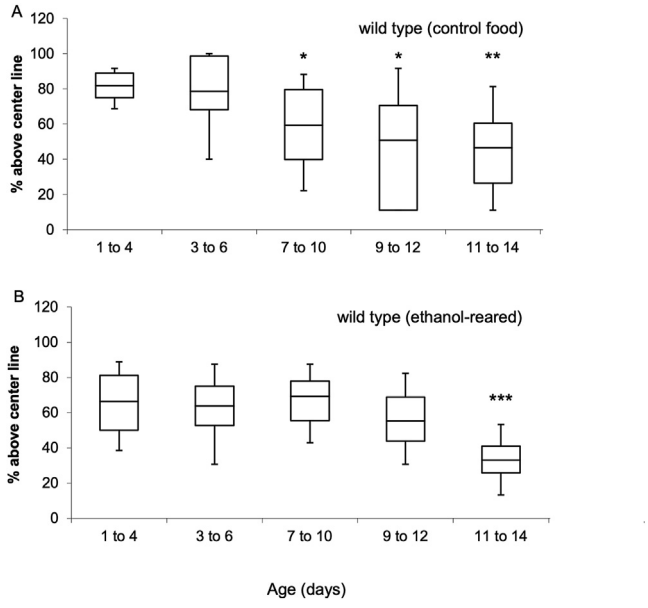

Fig. 4. DAE reduces age-related declines in negative geotaxis. A) Wild-type flies show significant reductions in their ability to perform negative geotaxis by 7–10 days of age (n = 11–12, $p = 0.013$, one-way repeated measures ANOVA with Tukey HSD *post hoc* analysis). **B)** Wild-type flies reared on ethanol do not climb as well as control flies when young (see also Fig. 3), but do not begin to lose climbing ability until 9 to 12 days of age, and do not show a significant decline until 11 to 14 days of age ($p = 0.63$ for 7–10-day-old flies; $p = 0.077$ for 9–12-day-old flies; $p = 0.00021$ for 11–14-day-old flies, n = 11–12, one-way repeated-measures ANOVA with Tukey HSD *post hoc* analysis). Centerlines show the sample median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

Discussion

Here we report that mutation of *Dmtn*, the *Drosophila* ortholog of TMCC2, leads to sensitivity to the lethal effects of ethanol during development. In addition, we find that DAE causes CNS dysfunction in flies: young ethanol-reared wild-type animals show an 18% reduction in climbing ability relative to unexposed, age-matched control flies. Unexpectedly, we also found that, despite the initial CNS dysfunction, flies that have been exposed to ethanol during development do not lose their ability to climb as rapidly as control animals, suggesting that DAE may delay some aspects of CNS aging. We attribute this protective effect of ethanol to an upregulation of antioxidant genes during development that persists during adulthood. In addition, we find that two slow-aging mutants, *Indy* and *mth*, are sensitive to ethanol-induced developmental mortality, suggesting a general connection between DAE sensitivity and slow aging. We found a persistent increase in expression of antioxidant genes in ethanol-reared animals, which may explain their slowed aging. There is a complex relationship between DAE and *Dmtn* transcript levels: *Dmtn* transcripts are downregulated in ethanol-reared larvae, but *Dmtn* expression is *increased* in adult flies after ethanol rearing.

In humans, alcohol intake shows a complex relationship with dementia and cognitive decline, with high consumption increasing the risk of dementia, while light-to-moderate intake is associated with a decreased risk of Alzheimer's disease and general cognitive decline (Anstey et al., 2007; Luchsinger et al., 2004; Solfrizzi et al., 2007). Here, we describe the first evidence for a protective effect of ethanol on CNS aging in *Drosophila*, while also demonstrating the complex relationship between CNS health and ethanol exposure.

We hypothesize that the persistent upregulation of *Superoxide dismutase-1 (Sod1)* and *Glutathione synthetase 1 (Gss1)* (Fig. 5) contribute to the retention of climbing ability seen in ethanol-reared animals. Evidence from mammals shows that superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) activity are lower in the brains of mammals compared with other organs (reviewed in Brocardo et al., 2011), while overexpression of human *Sod1* in motor neurons, but not muscles, has been shown to extend lifespan in *Drosophila* (Parkes et al., 1998). However, a more recent study showed that ubiquitous overexpression of Sod1, rather than expression in neurons or muscle, resulted in lifespan extension but not age-related locomotor impairment (Martin, Jones, & Grotewiel, 2009). Thus, it remains unclear whether antioxidant upregulation due to DAE is sufficient to confer neuroprotective effects against aging. This hypothesis can be tested by evaluating the climbing abilities of *Sod1* and *Gss1* mutant flies after ethanol rearing. If upregulation of *Sod1* and *Gss1* are responsible for the slow-aging phenotype, then flies mutant for these genes should not display slow aging when reared on ethanol. In addition, if the effects of DAE on aging are due to persistent upregulation of *Sod* or *Gss1* in the nervous system, we would expect that expressing RNAi constructs targeting these genes specifically in adult animals (through the use of a GAL-80^{ts} transgene combined with the pan-neuronal *elav-GAL4* driver and *UAS-RNAi* constructs targeting *Sod1* or *Gss1*) should reduce the anti-aging effects of DAE. These experiments are underway.

Indy flies are thought to age slowly due to reduced production of reactive oxygen species (Neretti et al., 2009), and *mth* flies are resistant to paraquat, suggesting enhanced antioxidant capabilities (Lin, Seroude, & Benzer, 1998), and both strains are sensitive to DAE. However, this coupling of oxidative stress resistance with sensitivity to DAE is unexpected, given that we have previously shown that transgenic upregulation of antioxidant genes results in

resistance to the lethal effects of DAE (Logan-Garbisch et al., 2015). However, it should be noted that we have previously seen a similar effect with mutation of the gene *urate oxidase* (*uro*) (Logan-Garbisch et al., 2015), so these results may hint at a complex relationship between DAE, oxidative stress, and cellular antioxidant pathways.

There are two potential confounding factors that influence interpretation of our negative geotaxis data. First, it is possible that the climbing ability of ethanol-reared flies does not decline rapidly as they age because they already have a mild climbing deficit at eclosion – it is not necessarily the case that the overall rate of decline should be the key measurement, as opposed to a finite "amount" of climbing ability. In that case, we would expect to see ethanol-reared animals begin to decline only after they have aged to the point where control animals are equivalent to ethanol-reared animals. While we cannot definitively rule out this possibility, 7–10-day-old ethanol-reared animals perform better than their control counterparts (Fig. 4B), which would never be expected. Thus, we think it is reasonable to conclude that there is a delay in the onset of declining negative geotaxis.

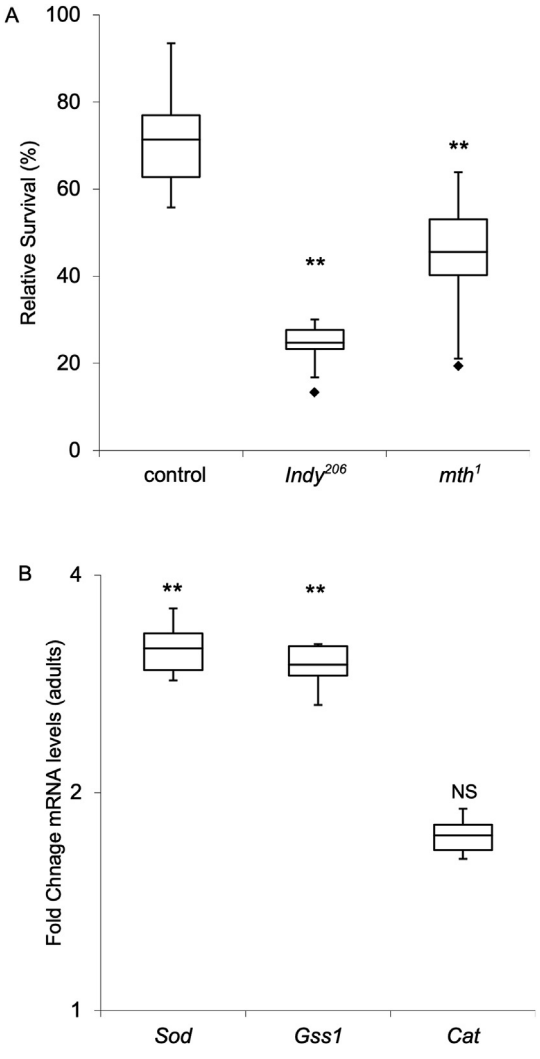

Fig. 5. Slow-aging mutants are sensitive to ethanol-induced developmental lethality, and antioxidant genes are persistently upregulated after DAE. A) Flies mutant for *Indy* and *mth* are sensitive to DAE. Relative survival of ethanol-reared $Indy^{206}$ homozygotes is reduced to 25%, compared with 71% in wild-type flies. Similarly, only 45.6% of mth^{1} homozygotes survive when reared on ethanol (n = 7–12, $^{**}p < 0.01$, one-way ANOVA with Tukey's HSD *post hoc* analysis). Survival has been normalized to genotype-matched controls reared on ethanol-free food. **B)** *Sod1* and *Gss1* transcript levels remain elevated in ethanol-reared adult flies for at least 5 days after removal from ethanol. *Cat* also shows a consistent but statistically insignificant increase (n = 3, $^{**}p < 0.01$, NS: not significant, one-way ANOVA with Tukey's HSD *post hoc* analysis). Centerlines show the sample median; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Diamonds represent outliers.

Second, as described previously (McClure et al., 2011), ethanol rearing results in a developmental delay due to increased time spent in early larval stages, which, in this study, means that ethanol-reared animals were an average of 2.4 *developmental days* "older" than their age-matched controls. Aging studies in *Drosophila* typically describe the flies' age in terms of number of days since eclosion, i.e., adult life, but it is possible that the climbing deficiencies we see in young (1–4-day-old) ethanol-reared flies are due to aging that took place before eclosion (that is, it is possible that a 4-day-old ethanol-reared fly is equivalent to a 6-day-old control fly). We think that this is unlikely, as 3–6-day-old control animals show no diminishment in climbing ability, as would be expected if the deficiencies seen in ethanol-reared animals were due to their being the equivalent of 2 days older than control animals (Fig. 4A). We acknowledge that this argument is weakened by the overlap in age between the two "bins", however.

In addition, as noted above, by the time ethanol-reared animals are 7–10 days old, they are performing better than their control counterparts (Fig. 4A & B). However, as these differences are not statistically significant, we cannot formally rule out the possibility that the CNS dysfunction we see in young ethanol-reared flies is due to their being developmentally older as a result of increased time in larval development. We are currently testing developing larvae for markers of cellular aging in order to determine whether these markers begin to change prior to eclosion.

The persistent upregulation of *Sod* and *Gss1* in adult animals after ethanol rearing is suggestive of an epigenetic effect of DAE on the regulation of these genes. In support of this hypothesis, several recent studies in mammals show that DAE results in epigenetic changes to gene regulation. For example, in rats, maternal alcohol consumption for a short period right at the time of conception is sufficient to impair glucose tolerance and decrease insulin sensitivity in male and female adult offspring. In the same study, DNA methyltransferases1, 3a, and 3b were shown to be upregulated in fetal livers exposed to DAE, consistent with epigenetic downregulation of genes involved in insulin sensitivity (Gårdebjer, Anderson, Pantaleon, Wlodek, & Moritz, 2015).

Another study found that expression of genes downregulated by DAE was restored by the administration of metformin, a diabetes drug that appears to influence the activity of a number of epigenetic modifying enzymes (Tunc-Ozcan, Wert, Lim, Ferreira, & Redei, 2018). Metformin rescued the expression of several genes that were downregulated by DAE and restored fetal alcohol exposure-induced fear memory deficit in rats, which are specifically associated with Dnmt1 (Tunc-Ozcan et al., 2018).

In *Drosophila*, many epigenetic regulators are affected by ethanol exposure, though these effects have been demonstrated only in adult animals. The H3K9 histone methyltransferase G9a (dG9a) functions as a key regulator for starvation-induced behaviors. Sucrose sensitivity in response to starvation conditions increases when dG9a levels are low. DG9a was also found to regulate the locomotion activity by controlling the expression of *insulin-like peptide* genes (Shimaji et al., 2017). In addition, Sirt1 (a histone deacetylase), the histone acetyltransferase Nej/CBP, and the histone demethylases NO66, KDM3, LID, and HSPBAP1 all appear to regulate long-term gene expression changes in response to ethanol exposure. *Sirt1* is downregulated by ethanol exposure, and mutations in *Sirt1* cause insulin resistance in flies (Engel et al., 2016;

Palu & Thummel, 2016; Pinzón et al., 2017; Ramirez-Roman, Billini, & Ghezzi, 2018).

It is of particular interest that most of the epigenetic effects described above affect insulin signaling and sensitivity. We have previously shown that DAE reduces insulin signaling in flies (McClure et al., 2011), and reduced insulin signaling leads to increased longevity in flies, *C. elegans*, and mice (Blüher, Kahn, & Kahn, 2003; Lin, Hsin, Libina, & Kenyon, 2001; Tatar et al., 1988). It is therefore possible that an additional effect of reduced insulin signaling in DAE-exposed larvae is slowed aging in adult animals. We are currently examining the role of *Sirt1*, *dG9a*, and other epigenetic regulators in mediating the developmental response to ethanol and the long-term changes in gene expression we see in ethanol-reared flies.

In conclusion, we have demonstrated that DAE causes CNS dysfunction in flies, which had not been shown previously. We also find that DAE results in resistance to at least one measure of CNS aging, and this is accompanied by long-term changes in gene expression. In addition, we show that flies mutant for *Dmtn*, a gene implicated in the health of the central nervous system, are sensitive to ethanol during development. Finally, we show that at least two slow-aging mutants are sensitive to ethanol during development, raising the possibility that ethanol sensitivity is a general feature of such mutants. We propose that flies may be aging more slowly due to the complex epigenetic regulation of antioxidant genes.

Funding

This research was supported by grants from the National Institutes of Health National Institute of General Medical Sciences (5SC3GM103739) and National Institute on Alcohol Abuse and Alcoholism (1R15AA027678) to RLF, a Project Development Grant from the California State University Program for Education and Research in Biotechnology to RLF, Howell-CSUPERB Research Scholars Program fellowships to both AD and JW, and an NIH Research Training Initiative for Student Enhancement (RISE) fellowship to EB.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

We thank members of the French lab for their helpful suggestions regarding experimental design and for critical readings of the manuscript, and Katherine Wilkinson and Miri VanHoven for insightful discussions and suggestions for experiments.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.alcohol.2021.03.006.

References

Ali, Y. O., Escala, W., Ruan, K., & Zhai, R. G. (2011). Assaying locomotor, learning, and memory deficits in Drosophila models of neurodegeneration. *Journal of Visualized Experiments, 49*, 2504. https://doi.org/10.3791/2504

Anstey, K. J., von Sanden, C., Salim, A., & O'Kearney, R. (2007). Smoking as a risk factor for dementia and cognitive decline: A meta-analysis of prospective studies. *American Journal of Epidemiology, 166*(4), 367–378. https://doi.org/10.1093/aje/kwm116

Araújo, A. R., Reis, M., Rocha, H., Aguiar, B., Morales-Hojas, R., Macedo-Ribeiro, S., et al. (2013). The Drosophila melanogaster methuselah gene: A novel gene with ancient functions. *PloS One, 8*(5), e63747. https://doi.org/10.1371/journal.pone.0063747

Banakar, M. K., Kudlur, N. S., & George, S. (2009). Fetal alcohol spectrum disorder (FASD). *Indian Journal of Pediatrics, 76*(11), 1173–1175. https://doi.org/10.1007/s12098-009-0239-2

Barja, G., Cadenas, S., Rojas, C., Perez-Campo, R., & Lopez-Torres, M. (1994). Low mitochondrial free radical production per unit O2 consumption can explain the simultaneous presence of high longevity and high aerobic metabolic rate in birds. *Free Radical Research, 21*(5), 317–327. https://doi.org/10.3109/10715769409056584

Barone, M. C., & Bohmann, D. (2013). Assessing neurodegenerative phenotypes in Drosophila dopaminergic neurons by climbing assays and whole brain immunostaining. *Journal of Visualized Experiments, 74*, e50339. https://doi.org/10.3791/50339

Bennett, S., Grant, M. M., & Aldred, S. (2009). Oxidative stress in vascular dementia and Alzheimer's disease: A common pathology. *Journal of Alzheimer's Disease, 17*(2), 245–257. https://doi.org/10.3233/JAD-2009-1041

Blüher, M., Kahn, B. B., & Kahn, C. R. (2003). Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science, 299*(5606), 572–574. https://doi.org/10.1126/science.1078223

Brocardo, P. S., Gil-Mohapel, J., & Christie, B. R. (2011). The role of oxidative stress in fetal alcohol spectrum disorders. *Brain Research Reviews, 67*(1–2), 209–225. https://doi.org/10.1016/j.brainresrev.2011.02.001

Delanoue, R., Meschi, E., Agrawal, N., Mauri, A., Tsatskis, Y., McNeill, H., et al. (2016). Drosophila insulin release is triggered by adipose Stunted ligand to brain Methuselah receptor. *Science, 353*(6307), 1553–1556. https://doi.org/10.1126/science.aaf8430

Dröge, W., & Schipper, H. M. (2007). Oxidative stress and aberrant signaling in aging and cognitive decline. *Aging Cell, 6*(3), 361–370. https://doi.org/10.1111/j.1474-9726.2007.00294.x

Engel, G. L., Marella, S., Kaun, K. R., Wu, J., Adhikari, P., Kong, E. C., et al. (2016). Sir2/Sirt1 links acute inebriation to presynaptic changes and the development of alcohol tolerance, preference, and reward. *Journal of Neuroscience, 36*(19), 5241–5251. https://doi.org/10.1523/JNEUROSCI.0499-16.2016

Gackowski, D., Rozalski, R., Siomek, A., Dziaman, T., Nicpon, K., Klimarczyk, M., et al. (2008). Oxidative stress and oxidative DNA damage is characteristic for mixed Alzheimer disease/vascular dementia. *Journal of the Neurological Sciences, 266*(1–2), 57–62. https://doi.org/10.1016/j.jns.2007.08.041

Gårdebjer, E. M., Anderson, S. T., Pantaleon, M., Wlodek, M. E., & Moritz, K. M. (2015). Maternal alcohol intake around the time of conception causes glucose intolerance and insulin insensitivity in rat offspring, which is exacerbated by a postnatal high-fat diet. *Federation of American Societies for Experimental Biology Journal, 29*(7), 2690–2701. https://doi.org/10.1096/fj.14-268979

Harris, R. A., Trudell, J. R., & Mihic, S. J. (2008). Ethanol's molecular targets. *Science Signaling, 1*(28), re7. https://doi.org/10.1126/scisignal.128re7

Heaton, M. B., Mitchell, J. J., & Paiva, M. (2000). Amelioration of ethanol-induced neurotoxicity in the neonatal rat central nervous system by antioxidant therapy. *Alcoholism: Clinical and Experimental Research, 24*(4), 512–518.

He, Y., & Jasper, H. (2014). Studying aging in Drosophila. *Methods, 68*(1), 129–133. https://doi.org/10.1016/j.ymeth.2014.04.008

Hopkins, P. C. (2013). Neurodegeneration in a Drosophila model for the function of TMCC2, an amyloid protein precursor-interacting and apolipoprotein E-binding protein. *PloS One, 8*(2), e55810. https://doi.org/10.1371/journal.pone.0055810

Hopkins, P. C., Sáinz-Fuertes, R., & Lovestone, S. (2011). The impact of a novel apolipoprotein E and amyloid-β protein precursor-interacting protein on the production of amyloid-β. *Journal of Alzheimer's Disease, 26*(2), 239–253. https://doi.org/10.3233/JAD-2011-102115

Ikonomidou, C., Bittigau, P., Ishimaru, M. J., Wozniak, D. F., Koch, C., Genz, K., et al. (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science, 287*(5455), 1056–1060. https://doi.org/10.1126/science.287.5455.1056

Lafler, J. (2015). Expanding a *Drosophila* model of fetal alcohol spectrum disorder: A forward genetic screen for developmental ethanol exposure phenotypes. *Master's Theses, 4593*. https://doi.org/10.31979/etd.ab38-z9gk

Larkin, A., Marygold, S. J., Antonazzo, G., Attrill, H., dos Santos, G., Garapati, P. V., et al. (2021). FlyBase: Updates to the Drosophila melanogaster knowledge base. *Nucleic Acids Research, 49*(D1), D899–D907. https://doi.org/10.1093/nar/gkaa1026

Lin, K., Hsin, H., Libina, N., & Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nature Genetics, 28*(2), 139–145. https://doi.org/10.1038/88850

Lin, Y. J., Seroude, L., & Benzer, S. (1998). Extended life-span and stress resistance in the Drosophila mutant methuselah. *Science, 282*(5390), 943–946. https://doi.org/10.1126/science.282.5390.943

Logan-Garbisch, T., Bortolazzo, A., Luu, P., Ford, A., Do, D., Khodabakhshi, P., et al. (2015). Developmental ethanol exposure leads to dysregulation of lipid metabolism and oxidative stress in Drosophila. *G3 (Bethesda), 5*(1), 49–59. https://doi.org/10.1534/g3.114.015040

Luchsinger, J. A., Tang, M. X., Siddiqui, M., Shea, S., & Mayeux, R. (2004). Alcohol intake and risk of dementia. *Journal of the American Geriatrics Society, 52*(4), 540–546. https://doi.org/10.1111/j.1532-5415.2004.52159.x

Martin, I., Jones, M. A., & Grotewiel, M. (2009). Manipulation of Sod1 expression ubiquitously, but not in the nervous system or muscle, impacts age-related parameters in Drosophila. *FEBS Letters, 583*(13), 2308–2314. https://doi.org/10.1016/j.febslet.2009.06.023

McClure, K. D., French, R. L., & Heberlein, U. (2011). A Drosophila model for fetal alcohol syndrome disorders: Role for the insulin pathway. *Disease Models & Mechanisms, 4*(3), 335–346. https://doi.org/10.1242/dmm.006411

Neretti, N., Wang, P. Y., Brodsky, A. S., Nyguyen, H. H., White, K. P., Rogina, B., et al. (2009). Long-lived Indy induces reduced mitochondrial reactive oxygen species production and oxidative damage. *Proceedings of the National Academy of Sciences of the United States of America, 106*(7), 2277–2282. https://doi.org/10.1073/pnas.0812484106

Palu, R. A., & Thummel, C. S. (2016). Sir2 acts through hepatocyte nuclear factor 4 to maintain insulin signaling and metabolic homeostasis in Drosophila. *PLoS Genetics, 12*(4), e1005978. https://doi.org/10.1371/journal.pgen.1005978

Parkes, T. L., Elia, A. J., Dickinson, D., Hilliker, A. J., Phillips, J. P., & Boulianne, G. L. (1998). Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons. *Nature Genetics, 19*(2), 171–174. https://doi.org/10.1038/534

Pinzón, J. H., Reed, A. R., Shalaby, N. A., Buszczak, M., Rodan, A. R., & Rothenfluh, A. (2017). Alcohol-induced behaviors require a subset of Drosophila JmjC-domain histone demethylases in the nervous system. *Alcoholism: Clinical and Experimental Research, 41*(12), 2015–2024. https://doi.org/10.1111/acer.13508

Praticò, D., Clark, C. M., Liun, F., Lee, V. Y. M., & Trojanowski, J. Q. (2002). Increase of brain oxidative stress in mild cognitive impairment: A possible predictor of alzheimer disease. *Archives of Neurology, 59*(6), 972–976. https://doi.org/10.1001/archneur.59.6.972

Ramirez-Roman, M. E., Billini, C. E., & Ghezzi, A. (2018). Epigenetic mechanisms of alcohol neuroadaptation: Insights from Drosophila. *Journal of Experimental Neuroscience, 12*, Article 1179069518779809. https://doi.org/10.1177/1179069518779809

Rogina, B., & Helfand, S. L. (2013). Indy mutations and Drosophila longevity. *Frontiers in Genetics, 47*. https://doi.org/10.3389/fgene.2013.00047

Ruitenberg, A., van Swieten, J. C., Witteman, J. C., Mehta, K. M., van Duijn, et al. (2002). Alcohol consumption and risk of dementia: The rotterdam study. *Lancet, 359*(9303), 281–286. https://doi.org/10.1016/S0140-6736(02)07493-7

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols, 3*(6), 1101–1108. https://doi.org/10.1038/nprot.2008.73

Shimaji, K., Tanaka, R., Maeda, T., Ozaki, M., Yoshida, H., Ohkawa, Y., et al. (2017). Histone methyltransferase G9a is a key regulator of the starvation-induced behaviors in Drosophila melanogaster. *Scientific Reports, 7*(1), 14763. https://doi.org/10.1038/s41598-017-15344-2

Sohal, R. S. (2002). Role of oxidative stress and protein oxidation in the aging process. *Free Radical Biology & Medicine, 33*(1), 37–44. https://doi.org/10.1016/s0891-5849(02)00856-0

Solfrizzi, V., D'Introno, A., Colacicco, A. M., Capurso, C., Del Parigi, A., Baldassarre, G., et al. (2007). Alcohol consumption, mild cognitive impairment, and progression to dementia. *Neurology, 68*(21), 1790–1799. https://doi.org/10.1212/01.wnl.0000262035.87304.89

Spadoni, A. D., McGee, C. L., Fryer, S. L., & Riley, E. P. (2007). Neuroimaging and fetal alcohol spectrum disorders. *Neuroscience & Biobehavioral Reviews, 31*(2), 239–245. https://doi.org/10.1016/j.neubiorev.2006.09.006

Sun, Y., Yolitz, J., Wang, C., Spangler, E., Zhan, M., & Zou, S. (2013). Aging studies in Drosophila melanogaster. *Methods in Molecular Biology, 1048*, 77–93. https://doi.org/10.1007/978-1-62703-556-9_7

Tatar, M., Kopelman, A., Epstein, D., Tu, M. P., Yin, C. M., & Garofalo, R. S. (1988). A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science, 292*(5514), 107–110. https://doi.org/10.1126/science.1057987

Tunc-Ozcan, E., Wert, S. L., Lim, P. H., Ferreira, A., & Redei, E. E. (2018). Hippocampus-dependent memory and allele-specific gene expression in adult offspring of alcohol-consuming dams after neonatal treatment with thyroxin or metformin. *Molecular Psychiatry, 23*(7), 1643–1651. https://doi.org/10.1038/mp.2017.129