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The Effects of Oxidative Stress on RNA Editing with Insight into the Relationship Between Air Pollution and Alzheimer's Disease

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ABSTRACT

Human interactions with the environment today add pressure on the world's resources and ecosystems, which in turn harm human health. Many anthropogenic environmental changes, including climate change, biodiversity loss, and pollution, have accelerated morbidity and mortality. Research across multiple countries has found correlations between anthropogenic air pollution exposure and neurological degeneration, particularly with Alzheimer's disease (AD). RNA editing induced by oxidative stress is one possible mechanism in which air pollution exposure increases the risk of AD. To study this further, RNA adenosine-to-inosine (A-to-I) alterations in the central nervous system (CNS) were analyzed to identify a mechanism linking air pollution to neurological degeneration. Adenosine deaminase acting on RNA (ADAR) enzymes mediate A-to-I alterations and have been associated with AD pathways. The A-to-I occurrence frequency may increase in polluted environments due to increased oxidative stress. To test this hypothesis, wildtype (WT) and ADAR knockout (adr-2(-)) Caenorhabditis elegans in their first larval (L1) developmental stage were exposed to juglone solution for 0, 15, and 60 minutes to model a control of no oxidative stress exposure, a treatment of acute exposure, and a treatment of chronic exposure. Quantitative Real Time PCR (qRT-PCR) was used to measure the expression of three genes: gst-4, sel-12 (an Alzheimer's associated gene), and adr-2 normalized to the expression of gpd-3 (the housekeeping gene that corrected for systemic errors). Based on the results, the change in gst-4 gene expression indicated that the method successfully induced oxidative stress in the treatment groups. The expression of the Alzheimer's gene, sel-12, significantly decreased only when worms without adr-2 were acutely exposed to oxidative stress. Reduced sel-12 expression downregulates endoplasmic reticulum calcium levels and results in neurodegeneration (Sarasija et al., 2018). However, adr-2 expression in WT worms did not significantly change under oxidative stress. This suggests that ADR-2 binding, independent of adr-2 editing, plays a significant role in preventing neurodegeneration during acute exposure to air pollution. The data also shows that acute exposure to oxidative stress-inducing air pollution during early developmental stages increases the risks of neurodegeneration. Future studies are needed to explore the mechanism of adr-2 in promoting sel-12 expression.

KEYWORDS: *RNA editing, Alzheimer's disease, air pollution, oxidative stress*

INTRODUCTION

Alzheimer's Disease and Air Pollution

Alzheimer's disease (AD) and other dementias are currently the sixth leading cause of death in America (National Center for Health Statistics, 2020). Although medicine can minimize a patient's symptoms, there is no cure for AD and related dementias. Advancing age is the greatest risk factor for AD, and the US population is growing older (Association, Ph.D., & Bleiler, 2013). By 2030, all baby boomers will be older than 65 years of age (Jordan, 2020). One in five Americans will be of retirement age (Jordan, 2020). This is a concern for Americans because an aging population will add stress to healthcare systems. By 2025, the demand for adult primary care services is projected to rise by 14%, while vascular surgery will increase by 31%, cardiology by 20%, and neurological surgery, radiology, and general surgery by 18% each (Dall et al., 2013). If the healthcare system does not develop more specialists in these fields soon, the physicians currently available will not be able to keep up with the demand.

Within the past few decades, studies have found significant connections between air pollution and neurological degeneration. Four independent studies that were conducted in different countries by different researchers resulted in similar conclusions: air pollution exposure increased neurological decline (Chen et al., 2017; Younan et al., 2020; Zhang, Chen, & Zhang, 2018).

In the American study, researchers identified a positive correlation between fine particulate matter (PM2.5) exposure and brain changes that resulted in memory problems (Younan et al., 2020). These PM2.5 particles came from traffic exhaust, smoke, and dust--many human-caused sources. In the Canadian study, researchers analyzed whether living near major roadways was associated with higher incident cases of dementia, Parkinson's disease (PD), and multiple sclerosis (MS) (Chen et al., 2017). After ten years of data collection, the researchers concluded that living near high traffic roads was significantly associated with a higher incidence of dementia, but not PD or MS (Chen et al., 2017). In the Chinese study, researchers from China and America analyzed a Chinese data set and found that long-term exposure to air pollution correlated with decreased performance on verbal and math tests (Zhang et al., 2018). Lastly, in the English study, researchers determined that adults living in areas with high annual air pollution concentrations were 1.4 times more likely to get dementia, specifically AD, compared to people living in lower air pollution concentrations (Carey et al., 2018). Based on these epidemiological studies, it appears as though air pollution exposure is correlated with neurodegeneration. However, randomized studies are needed to determine causality.

Alzheimer's Disease and RNA Editing

To identify a causal link between air pollution and AD, the effects of RNA editing should be examined. RNA editing frequently occurs in genes expressed in the central nervous system (CNS) and plays an important role in neurological development and brain function (Yang, Okada, & Sakurai, 2021). RNA editing is an enzyme-mediated process that alters individual bases in double-stranded RNA. The impact of RNA editing depends on the location of the alteration. For example, alterations impact mRNA translation and protein function when they occur in coding and exon regions, but when they occur in non-coding regions, they can stabilize secondary structures (Christofi & Zaravinos, 2019). RNA can also be edited in many ways. One mechanism involves ADAR (adenosine deaminases acting on RNA) binding proteins, which are essential for mammalian development. Knockouts of the ADAR gene in mice are embryonically lethal (Hartner et al., 2004; Higuchi et al., 2000). ADAR binding proteins catalyze the deamination of adenosine to inosine (A-to-I) (Nishikura, 2016). Inosine mimics guanosine and will base pair with cytosine, but adenosine base pairs with uridine (Nishikura, 2016). This single base substitution frequently causes RNA codons to base pair with different anti-codons and introduce different amino acids into a protein's primary sequence during translation (Lodish, Berk, Zipursky, & et al, 2000; Nishikura, 2016). These recording events change the protein's structure and impact its function, as well as its intra- and intermolecular interactions (Ivanov et al., 2013).

In past research, irregular RNA editing has been associated with the pathologies of neurological and psychiatric disorders (Yang et al., 2021). For example, RNA editing influences the progression of Amyotrophic Lateral Sclerosis (ALS). When ADAR RNA editing occurs in glutamate receptors, the receptor becomes impermeable to calcium ions (Maas, Kawahara, Tamburro, & Nishikura, 2006). Unedited glutamate receptors remain permeable to calcium ions (Maas et al., 2006). This difference occurs because RNA editing changes one CAG codon to a CIG codon, replacing the amino acid glutamine with arginine (Maas et al., 2006). Glutamine and arginine are different sizes and have different charges. The edited receptor has a different channel shape and ion affinity, which prevents calcium ions from passing through (Maas et al., 2006). In ALS, unedited glutamate receptors allow excess calcium to flow into neurons, resulting in overstimulation and cell death (Maas et al., 2006). However, edited receptors help maintain calcium homeostasis (Maas et al., 2006). In this case, RNA editing is necessary to remain healthy. Perhaps RNA editing can explain the AD pathology.

Three previous studies have shown that patients with AD have significant changes in RNA editing levels. In the first study, Gardner et. al. (2019) found that editing levels across both African-American and non-Hispanic White ethnicities significantly differed in genes involved in immune regulation, inflammatory, and endocytic processes when the patient had AD. Differentially edited sites significantly disrupted microRNA (miRNA) binding and induced deleterious nonsynonymous coding changes in AD associated genes (Gardner et al., 2019). Based on these findings, RNA editing levels differ in AD and affect known AD-related genes in important AD pathways (Gardner et al., 2019).

In the second study, Khermesh et. al. (2016) quantified and compared ADAR A-to-I editing levels in target sites between AD patients and controls. In RNA samples from the hippocampus and temporal lobe, RNA editing levels were reduced in AD patients (Khermesh et al., 2016). Reduced RNA editing was also observed in glutamate receptors in AD patients, which contributes to progressive AD and ALS (Khermesh et al., 2016). In this study, the hypo-editing pattern exceeded ADAR expression patterns, suggesting that editing occurred independent of enzyme levels (Khermesh et al., 2016), but still directly impacted AD processes.

A third study, analyzed hippocampal tissues in late onset AD (LOAD) patients, Parkinson's Disease (PD) patients, and healthy patients (Annese et al., 2018). RNA expression and RNA editing were significantly reduced in LOAD patients (Annese et al., 2018). This study replicated the second study with findings of down-regulated editing levels in several glutamate receptors essential for neuronal function. Based on this literature, RNA editing levels tend to decrease in AD patients.

Air Pollution and RNA Editing

RNA editing is also highly sensitive to environmental stress (Zaidan et al., 2018). Air pollution changes an individual's environment and exposes them to toxins that induce oxidative stress. This stress significantly alters A-to-I RNA editing in the CNS, suggesting RNA editing could be a possible mechanism by which air pollution exposure induces AD prognosis.

Recent studies have used C. elegans as a model organism to explore the effects of air pollution and oxidative stress on growth and development. In one study, Haghani et. al (2019) exposed C. elegans at developmental (L1) and adult life stages (L4) to airborne PM produced by traffic emissions to analyze the effects of chronic exposure on genes involved in stress responses and development. The effects of exposure were dose-dependent (Haghani et al., 2019) and more severe when the worms were exposed at the earlier stage of development (L1). Haghani et. al (2019) examined the effects on three Alzheimer's homolog genes, apl-1, sel-12, and lrp-1, and found that they all significantly decreased in the L1 worms immediately after the one-hour exposure.

For this experiment, Haghani et. al (2019) was used as a guide. Caenorhabditis elegans (C. elegans) were selected to be the model organism. C. elegans are small, simple nematodes. They have highly conserved biological pathways and short generation periods (Kaletta & Hengartner, 2006). Eggs develop for 2.5 hours in utero; then about 9 hours after being laid, the nematodes hatch and enter the first larval (L1) stage (Altun & Hall, 2009). Worms reach adulthood within 38-42 hours from L1 (Altun & Hall, 2009). These worms are also transparent, which allows for fluorescent reporters to be effectively used to determine when and where certain genes are expressed (Kaletta & Hengartner, 2006). C. elegans are hermaphroditic and generate large amounts of offspring without mating (Altun & Hall, 2009). Most importantly, ADAR knockouts in C. elegans are viable (Tonkin et al., 2002). C. elegans were used in this controlled, randomized experiment to determine how ADAR processes affect development.

Based on the Haghani et. al (2019) study, sel-12 was selected to be the gene examined for this experiment. Sel-12 is also the homolog of Presenilin 2 (PSEN2), an aspartyl protease in humans that is a subunit of γ-secretase (Cai et. al., 2015). γ-secretase cleaves amyloid precursor proteins (APP) and produces β-amyloid peptides (Aβ) (Cai et al., 2015). Aβs tend to aggregate and form plaques, a pathological characteristic of AD (Cai et al., 2015). Mutations in PSEN2 can increase γ-secretase activity and increase Aβ production, leading to more plaques and more severe AD (Cai et al., 2015). In C. elegans, decreased sel-12 expression might generate a physiological response similar to increased γ-secretase activity. Overall, PM exposure to L1 worms delayed development and decreased Alzheimer's associated gene expression, suggesting that chronic exposure to air pollution can alter physiology and result in potentially negative health impacts.

Other literature uses oxidative stress to explore the effects of air pollution on growth and development. Oxidative stress occurs when free radicals outnumber antioxidants in the body (Lobo et al., 2010). Free radicals are atoms with unpaired electrons that can readily react with other atoms, disrupting cellular processes (Lobo et al., 2010). One of the most reactive compounds is a hydroxyl radical, an oxygen atom bound to a hydrogen atom with an unpaired electron (Lobo et al., 2010). This molecule is

easily produced when pollutants such as various oxides react with water molecules (Lobo et al., 2010). Once hydroxyl radicals form, they quickly react with other cellular processes and cause extensive damage (Lobo et al., 2010). Since water makes up 60% of the human body, radical formation upon air pollution inhalation occurs rapidly and abundantly (Lodovici & Bigagli, 2011). As a result, oxidative stress causes aging, the greatest risk factor for AD (Liguori et al., 2018). To assess sensitivity to oxidative stress, a past study used C. elegans as a model to measure survival after exposure to juglone, a reactive oxygen species (ROS)-generating compound (Senchuk et al., 2017). Survival after exposure was dose-dependent (Senchuk et al., 2017). As the concentration of juglone increased, the percent survival decreased (Senchuk et al., 2017). Therefore, physiological changes due to oxidative stress responses can be lethal in model organisms and pose concerns for human responses to air pollution exposure.

The current literature suggests air pollution exposure and oxidative stress responses induce physiological changes that delay development, decrease sel-12 expression, and reduce survival. To further develop this literature, this experiment assessed whether ADAR-mediated processes affect C. elegans' oxidative stress response by exposing wildtype (WT) and adr-2 knockout (adr-2(-)) C. elegans to a juglone solution, and then comparing the expression of the sel-12, gst-4, gpd-3, and adr-2 genes using quantitative Real-Time PCR (qRT-PCR). Sel-12 is the human homolog of PSEN2, a commonly mutated gene in patients with AD. Monitoring sel-12 expression will suggest whether Alzheimer's associated genes are impacted by oxidative stress and/or ADAR proteins, gst-4 expression will confirm whether oxidative stress occurred, and adr-2 expression will indirectly measure ADAR editing. Since transcription and translation of the adr-2 gene produce the only A-to-I editing enzyme in C. elegans, it may be inferred that more adr-2 expression can lead to increased RNA editing. To minimize systemic errors, all data will be normalized to gpd-3, a housekeeping gene that should not change in expression upon oxidative stress or loss of adr-2. If ADARs significantly impact physiological responses, then there will be significant differences between the WT and adr-2(-) strains in the 15 minute and 60 minute treatments.

METHODS

Oxidative Stress in Worm Strains

The following strains were used in this study: (WT) HH43 [rab3p::rfp::C35E7.6 3' UTR, rab3p::gfp::unc-54 (3' UTR), unc-119 genomic rescue] and (adr-2(-)) HH45 [adr-2(ok735); rab3p:: rfp::C35E7.6 (3' UTR), rab3p::gfp::unc-54 (3' UTR), unc-119 genomic rescue]. These worms were transgenically made by fusing a fluorescent reporter gene to a known edited gene (C35E7.6) under the control of a neuronal-specific promoter (rab3) (Hundley et al., 2008). Strain 43 was the wild type and functioned as a control. Strain 45 was transgenically altered to knock out the adr-2 gene.

Worm strains were maintained by growth on small NGM plates seeded with Escherichia coli OP50. Worms were picked onto five new plates twice a week, on Monday and Friday. Worms used for oxidative stress treatments were starved for 7-10 days, then chunked onto large seeded NGM plates and grown for 72 hours. After 48 hours, worm food was added to avoid extended starvation. After 72 hours, worms were bleached and left to hatch overnight, synchronizing all worms to the first larval (L1) developmental stage. After 24 hours, approximately 100,000 worms were randomly assigned to one of the control or treatment groups. For each strain, the control group was exposed to 0 minutes of juglone solution, one treatment group was exposed to 15 minutes of juglone solution, and the second treatment group was exposed to 60 minutes of juglone solution. Juglone solution was prepared fresh for each use as previously described by Senchuk et. al (2017). A 38mM juglone stock solution was made by dissolving 0.165g Juglone in 25mL of 100% ethanol and stirred in the dark for 1 hour, in a cold room (4°C). A 1:10 dilution of the juglone solution was made in liquid NGM and used immediately as toxicity was lost with time. 10µL of the 1:10 solution was added to the 15 minute and 60 minute treatment groups. After treatments, Trizol was added to the worms, which were then frozen with liquid nitrogen in preparation for RNA isolation. Three biological replicates of the experiment were conducted.

RNA Analysis

Total RNA was isolated from worms using Trizol (Invitrogen). RNA was further treated with Turbo DNase (Ambion) to remove DNA contamination and then isolated using the RNeasy Extraction kit (Qiagen).

Reverse-transcription was performed with random hexamer primers and oligo-dt using Superscript III Reverse-transcriptase (Invitrogen). Incubating at 85°C for 10 minutes stopped the reverse-transcription reaction and the remaining DNA/RNA hybrids were removed by treatment with RNaseH (NEB). Complementary DNA (cDNA) was amplified by quantitative Real-Time PCR (qRT-PCR) using SYBR Green on the Thermo Quant Studio software. The expressions of the sel-12 and adr-2 genes were quantified and compared between the samples. Additionally, gst-4 and gpd-3 expressions were analyzed in the qRT-PCR assay. Gst-4 expression molecularly confirmed oxidative stress occurred and all values were normalized to the housekeeping gene gpd-3, which was not expected to change in levels under the genetic or stress conditions. Three technical replicates were conducted for each biological sample.

Statistical Analyses

Prism 9 software was used to conduct statistical analyses of all the data collected in this experiment. The expressions of gst-4, sel-12, and adr-2 were all normalized to the expression of gpd-3, a housekeeping gene that did not change with adr-2 knockout and oxidative stress. The expression of gst-4 was statistically analyzed with two-tailed, paired ratio t-tests, using a 95% confidence interval and a p-value of 0.05. When the sel-12 expression levels were normalized to the 0 minute control, the data was analyzed with ordinary, one-way ANOVA and Fisher's least significant difference (LSD) tests, using an alpha value of 0.05. When the sel-12 expression levels were normalized to the WT strain, the data were analyzed using two-tailed, unpaired t-tests with 95% confidence levels and a p-value of 0.05. The adr-2 expression levels were analyzed with ordinary, one-way ANOVA and Fisher's LSD tests, using an alpha value of 0.05.

RESULTS & DISCUSSION

Establishment of the Oxidative Stress Assay

The goal of exposing nematodes to the juglone solution was to induce an oxidative stress response. This exposure assay had never been done before in the Hundley lab, so it was important to use gst-4 to confirm oxidative stress occurred. The gst-4 gene f

facilitates glutathione transferase activity, which detoxifies oxidative stress environments (Park et al., 2009). Figure 1 showed that gst-4 expression was directly proportional to juglone exposure in both worm strains: as juglone exposure increased, gst-4 expression increased. This confirmed that oxidative stress occurred in both worm strains during the experiment, indicating that the exposure assay worked.

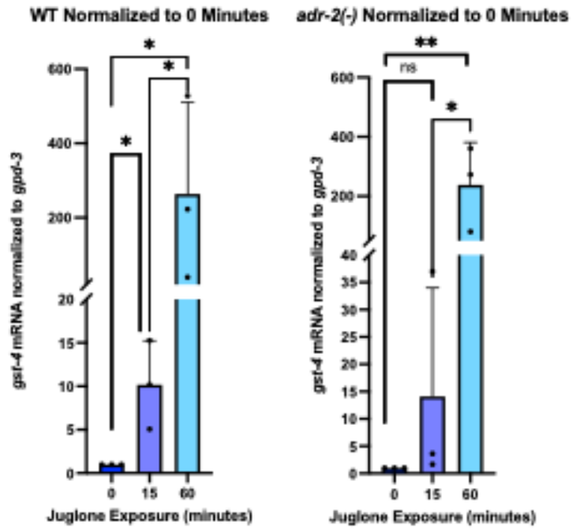

Figure 1. *gst-4 expression normalized to gpd-3 expression compared across the treatment groups in each worm strain. Data was further normalized to the control in each worm strain, 0 minute juglone exposure. Figure 1a shows data for WT. Figure 1b shows data for adr-2(-).*

The raw data set showed no significant difference between the WT and adr-2(-) strains, but the adr-2(-) strain consistently reported higher gst-4 levels (Figures 2 and 3). This suggests that lack of adr-2 induced more oxidative environments. Without significance, this could have occurred by chance, but the pattern was repeated in each treatment, suggesting that the lack of significance was due to large standard deviations between biological replicates. These large variations could have been the result of making the juglone solution fresh for each replicate and stirring the solution at 4°C instead of room temperature. Any slight difference in the concentration would have dramatically altered the amount of oxidative stress the worms were exposed to. Additionally, during waste disposal of the solution, clumps of solid juglone were still observed, indicating that the solution did not mix thoroughly. Next time, the solution should be stirred at room temperature to improve reliability.

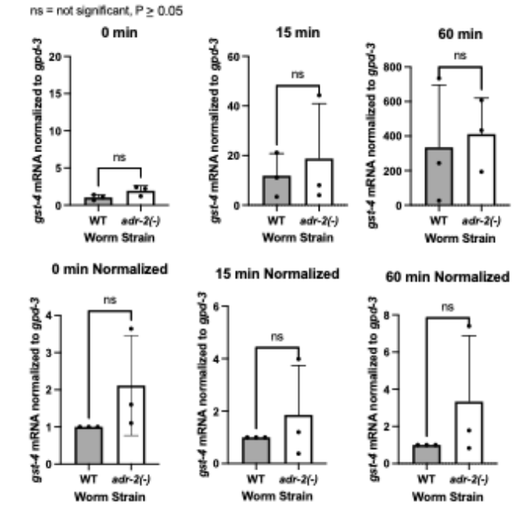

Figure 2. *gst-4 expression normalized to gpd-3 expression compared between worm strains in each treatment group. Raw data appear in the upper row and data normalized to the control in each worm strain appear in the bottom row. Individual graphs are labeled in the top row, left to right: 2a, 2b, 2c; and in the bottom row, left to right: 2d, 2e, 2f.*

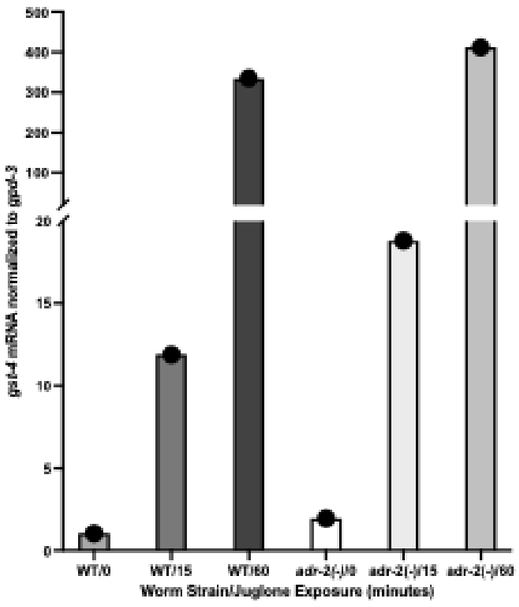

Figure 3. *gst-4 expression normalized to gpd-3 to represent the mean fold changes between each treatment group.*

The gst-4 expression significantly increased in the WT strain as the juglone exposure increased (Figure 1). However, in the adr-2(-) strain, gst-4 expression only significantly increased after chronic exposure (Figure 1). This was partially due to the large standard deviations in the adr-2(-) 15 minute and 60 minute treatment groups. It was difficult to show significance because the differences between the replicants in the same treatment were larger than the differences between the two treatments (Figure 1). Nonetheless, the mean expression at the 15 minute treatment dramatically increased from the control by approximately 10-fold (Figure 2). Important biological changes in gene expression can be as little as 2-fold differences. The oxidative stress response is a very robust effect: gst-4 expression increased by 10+ fold in each treatment. Perhaps, the 60 minute treatment was too harsh since the data greatly fluctuated, ranging from 40-fold to greater than 500-fold increases. Regardless, since gst-4 expression increased as the juglone exposure increased, this assay effectively induced oxidative stress in C. elegans.

Impact of Oxidative Stress and ADAR on Expression of sel-12

Sel-12 expression was measured to determine the effect of oxidative stress on associated Alzheimer's genes. The WT strain demonstrated no significant changes between each of the treatment groups (Figure 4a). Alternatively, sel-12 expression in the adr-2(-) strain significantly decreased after acute exposure (Figure 4b). This suggests that adr-2 is involved in sel-12 expression during the acute oxidative stress response. After chronic exposure, sel-12 expression returned to a level comparable to what was observed at the controlled, 0 minute exposure (Figures 4a and 4b). This occurred in both worm strains, suggesting that adr-2 is not as important for chronic oxidative stress responses. It also suggests that after 15 minutes of exposure, the nematodes underwent a physiological change to help them adapt and survive in their oxidative stress environment, like how humans switch from anaerobic to aerobic pathways while exercising for prolonged periods. Future studies are needed to examine this potential physiological change.

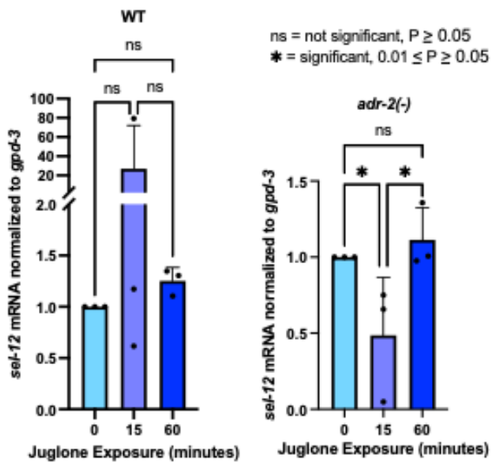

Figure 4. *sel-12 expression normalized to gpd-3 expression compared across the treatment groups in each worm strain. Data was further normalized to the control in each worm strain, 0 minute juglone exposure. Figure 4a shows data for WT. Figure 4b shows data for adr-2(-).*

As described above, sel-12 is the nematode homolog gene to human Presenilin 2 (PSEN2). PSEN2 is a component in γ-secretase, which is involved in forming β-amyloid peptide (Aβ) plaques, a common Alzheimer's disease trait (Cai et al., 2015). This is known as the amyloid hypothesis (Sarasija et al., 2018). Loss of function mutations in PSEN2 increases plaque formation (Cai et al., 2015). However, Sarasija et. al. (2018) claim there is no correlation between plaque load and dementia severity. Many postmortem examinations of AD patients with severe cognitive decline lacked plaque formation, suggesting a different pathway was responsible for AD deterioration (Sarasija et al., 2018).

Sarasija et. al. (2018) explained that C. elegans do not encode for Aβ. Instead, C. elegans use a different pathway: sel-12 modulates endoplasmic reticulum (ER) regulation of calcium (Sarasija et al., 2018). Loss of function or expression of sel-12 dysregulates ER calcium levels and causes mitochondrial disorganization that diminishes health (Sarasija et al., 2018). By disrupting calcium regulation, loss of sel-12 increases oxidative stress-mediated neurodegeneration (Sarasija et al., 2018). In another study that analyzed mice, Calvo-Rodriguez et. al. (2020) found associations between increased mitochondrial calcium levels and neuronal degeneration. Increased mitochondrial calcium levels were associated with plaque formation and neuronal degeneration in transgenic mice (Calvo-Rodriguez et al., 2020). This paper links the pathway Sarasija et. al. (2018) discovered and the sel-12 pathway with the amyloid hypothesis currently dominating AD discussions. However, PSEN2 differs from sel-12. Overexpression of PSEN2 mutants induces Alzheimer's disease pathology, while under expression of sel-12 induces more oxidative stress-mediated neurodegeneration (Cai et al., 2015; PSEN2 presenilin 2 [Homo sapiens (human)], 2021).

Based on Figures 4b and 5e, adr-2(-) worms significantly reduced ¬sel-12 expression after acute exposure. According to Sarasija et. al. (2018), significant loss of sel-12 expression should have also significantly increased gst-4 expression if it induced more oxidative stress. Although Figure 1b did not show significant changes in gst-4 expression between 0 and 15 minute treatment groups, Figure 3 showed that the mean gst-4 expression was seven times greater in adr-2(-) compared to WT after acute exposure. These findings suggest that adr-2 is essential for sel-12 expression under acute exposure and support Sarasija et al. (2018)'s discovery that loss of sel-12 increases oxidative stress. The data suggest that adr-2 knockouts produce phenotypes like what would be expected from sel-12 loss of function mutations. Therefore, adr-2 knockouts induced greater oxidative stress-mediated neurodegeneration.

The Haghani et. al. (2019) study was used as a model for this experiment, but the results were not replicated. In the Haghani et. al (2019) study, the three Alzheimer's associated genes significantly decreased after 60 minutes of exposure to particulate matter (PM). If the results were replicated, sel-12 expression should have significantly decreased after chronic exposure in the WT worms. Instead, sel-12 expression was slightly elevated after chronic exposure. One reason for this is that the traffic-related air pollution used in Haghani et. al. (2019)'s study could have induced both oxidative stress and inflammatory responses, but the juglone solution used in this experiment only induced an oxidative stress response. Perhaps, the

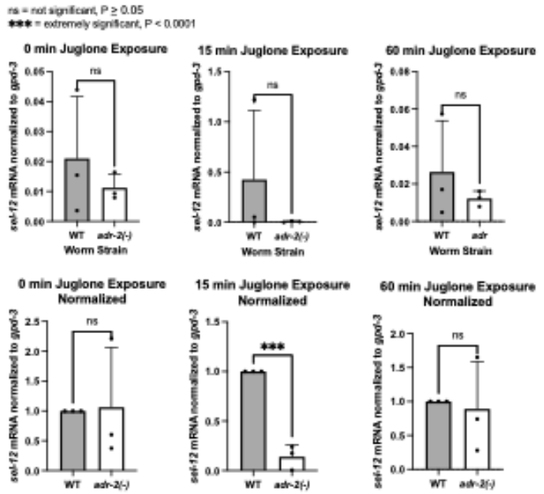

Figure 5. *sel-12 expression normalized to gpd-3 expression compared between worm strains in each treatment group. Raw data appears in the upper row and data normalized to the control in each worm strain (0 minute juglone exposure) appears in the bottom row. Individual graphs are labeled in the top row, left to right: 5a, 5b, 5c; and in the bottom row, left to right: 5d, 5e, 5f.*

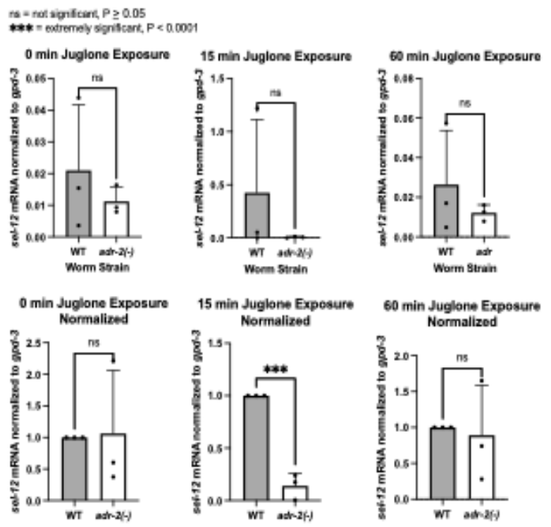

Figure 5. *adr-2 expression normalized to gpd-3 expression compared across the treatment groups in the WT worm strain. Data was further normalized to the control, 0 minute juglone exposure.*

inflammatory response plays a role in decreasing the expression of Alzheimer's associated genes. Additionally, the worms used in this experiment were transgenically made, which distinguishes them from the worms used in the Haghani et. al. (2019) experiment. Since not every variable was identical, the results were not reproducible. Nonetheless, the significant decrease in sel-12 expression in adr-2(-) worms at acute exposure suggests that adr-2 is necessary for sel-12 expression.

One possible application of this research would be to assess PSEN2 and mitochondrial calcium levels in various populations, some living near high traffic and high polluted regions compared to others living in remote, eco-friendly areas, to determine a possible correlation between whether air pollution exposure impacts Alzheimer's disease pathology via the amyloid hypothesis, the mitochondrial calcium hypothesis, or both.

Impact of Oxidative Stress on RNA Editing Enzyme Levels

Lastly, adr-2 expression was only measured in WT worms since the adr-2(-) strain did not express adr-2. The adr-2 expression did not significantly change between the different treatment groups (Figure 6). Although the mean adr-2 expression was approximately 9 times greater in the 15 minute treatment than in the 0 minute treatment, the variation between samples was too large to deem significant (see Table 10 in Appendix). This variation could have been due to slight differences in concentrations of the juglone solution as it was made fresh for each biological replicate.

Interestingly, one replicate appeared to be an outlier in the 15 minute treatment in both adr-2 and sel-12 expression. Both "outliers" were in the second biological replicate and produced abnormally high values (see Table 11 in Appendix). This data point remained included in the graphs and the data analysis because it presented a pattern of observation: adr-2 expression directly correlated with sel-12 expression. When adr-2 expression was high, sel-12 expression was also high, supporting the conclusion that adr-2 was involved in sel-12 expression, especially at acute exposure.

The lack of significant change in adr-2 expression upon oxidative stress suggests that RNA editing levels may not change under oxidative stress. It might also suggest that regulating factors of editing levels, which often function independently of enzyme levels, may be responsible for abnormal editing (Rajendren et al., 2021). In a previous study, Wahlstedt et al. (2009), showed that increased A-to-I editing during mammalian brain development was observed without ADAR expression and directly correlated with the observed changes in editing levels. Additionally, recent studies demonstrate how mammalian ADARs play editing-independent roles by binding to mRNAs (Anantharaman et al., 2017; Bahn et al., 2015; Wang et al., 2013). Since adr-2 expressions did not change significantly, ADR-2 binding was likely the primary regulator of sel-12 expression. Future studies should analyze ADAR protein binding regulation in gene expression.

Although human PSEN2 pathways may differ from C. elegans sel-12 pathways, mutations in these genes result in metabolic interferences that induce neurological degeneration. Sarasija et. al. (2018) and Calvo-Rodriguez et. al. (2020) have studied these two pathways independently and proposed parallel pathologies that rely on increased calcium levels to facilitate neuronal degeneration. Additionally, ADAR binding proteins play a role in calcium regulation. Higuchi et al. (2000) showed that mice lacking ADAR2 died of epileptic seizures because hypo-editing

in glutamate receptors resulted in excess calcium influx and neuronal excitotoxicity. Therefore, adr-2 genes play a role in neuronal degeneration by producing enough ADR-2 binding proteins to regulate calcium levels.

Other Future Applications

In other literature, down-regulation and reduced levels of RNA editing were reported in AD patients (Annese et al., 2018; Khermesh et al., 2016). However, these studies have not analyzed the effects of loss of ADAR on Alzheimer's gene expression since ADAR mutants would be lethal. One way to assess the role of ADARs in population studies would be to determine how ADAR levels correlate with PSEN2 levels in oxidative stress conditions. Maybe this could be done upon molecular analyses of blood samples from participants. If this study could be conducted, the possible results could resemble the pattern seen in the "outlier" reported by the second replicate for the 15 minute treatment: high ADAR expression should correlate with high PSEN2 expression.

Based on the data collected, sel-12 gene expression only decreased in the adr-2(-) worms at 15 minute exposure. Moving forward, studies should determine if editing of sel-12 is important for gene regulation. To do this, the same experiment could be conducted by swapping adr-2(-) worms for worms that produce ADR-2 but cannot edit. If RNA editing is not important, then the data should be reproducible, and would strongly suggest that ADAR-2 binding regulates sel-12 expression. This result would support the conclusion that the decreased sel-12 expression in adr-2(-) was independent of A-to-I RNA editing enzyme levels.

If the absence of adr-2 reduced sel-12 expression and loss of sel-12 expression induces more oxidative mediated neurological degeneration via excess mitochondrial calcium, then calcium regulation should be explored as a possible treatment for AD patients. If calcium levels can be monitored to minimize unwanted influxes, then AD prognosis could be improved.

Other future studies can replicate this experimental design to determine oxidative stress effects on other known Alzheimer's associated genes. This experiment can also be adjusted so that the worms are exposed to the same treatments, fed to develop into adults, then frozen for RNA extraction to identify how early exposure impacts physiology in adults. Other variations of this idea could occur by exposing worms for the entire duration of their development from L1 to adulthood. Additionally, if this experiment was repeated and adjusted to switch juglone solution with air pollution extracted from car exhausts, then researchers would be able to explore the impacts of both inflammatory and oxidative stress responses simultaneously rather than just the impacts of oxidative stress. To evolve this methodology, an editing essay could also be conducted to directly measure the amount of A-to-I editing in the nervous system. To do this, a fluorescent reporter gene fused to a known edited gene (C35E7.6) with a neuronal-specific promoter could be used. Once RNA is isolated from the entire worm, any changes to the A-to-I alteration frequency can be observed if oxidative stress interferes with RNA editing, linking air pollution and Alzheimer's disease. Furthermore, studies could explore the rates of A-to-I alterations across people varying in their degree of pollution exposure and prevalence of Alzheimer's disease to provide additional insight into the role of air pollution in neurological degeneration.

CONCLUSION

ADR-2 binding, independent of adr-2 editing, plays a significant role in sel-12 expression during acute oxidative stress exposure. Similarly, ADAR binding in humans plays a role in regulating Alzheimer's disease pathologies and calcium levels. Past literature suggests that earlier and more frequent exposure to air pollutants induces more physiological damage and the experimental data collected from this experiment presents the risks of acute exposure at early developmental stages. Based on these results, society must embrace the precautionary principle: take preventative action in the face of risk and uncertainty. Air pollutants are toxic at very low concentrations, which makes minimal exposure dangerous. Urbanization increases air pollution exposure, but mitigation approaches minimize physiological consequences. As populations continue to migrate to urban centers, city management, and municipal councils can devote resources to developing, remodeling, and implementing sustainable urban cities that include green spaces, electric transport, energy-conscious construction, renewable energy sources, and improved waste management. However, many of these factors, especially waste management at the producer and consumer levels, require education and government laws for proper regulation and enforcement. Change cannot occur without proper structure. However, every individual can control their behavior. If everyone lives with the precautionary principle in mind and minimizes their exposure to air pollution, then systemic changes will occur.

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APPENDIX

Gene/Type	Primer (5'->3')
adr-2 HH1016	GCATGACAATCTATGAGAGCTGTTCCATCG
adr-2 HH1017	GCGAAATTTTATGAACTTTGTAAGAACAAC
sel-12 HH2803	CTTATTCATGGATGGCTTATTGTCAGCAGT
sel-12 HH2804	CATTCCGAGAACTCCATAGTTACCCAGT
gst-4 HH2808	GTCGATGGAGAACAATTCGGTCAGTCAATG
gst-4 HH2809	CCATAGAAAACGGAAGAAGTGAATTGACGG
gpd-3 HH315	GGAGGAGCCAAGAAGGTC
gpd-3 HH316	AAGTGGAGCAAGGCAGTT

Table 1. *Primers used for each gene.*

Treatment Comparisons (minutes)	P value	P value summary	Significantly Different (P<0.05)?	One-Tailed or Two-Tailed P value	T, df	Numbers of pairs	Confidence Intervals
15 vs 0	0.0203	*	Yes	Two-tailed	t = 6.919, df = 2	3	95%
60 vs 15	0.0224	*	Yes	Two-tailed	t = 6.570, df = 2	3	95%
60 vs 0	0.0214	*	Yes	Two-tailed	t = 6.721, df = 2	3	95%

Table 2. *Primers used for each gene. Gst-4 expression in WT worms normalized to 0 minute control was analyzed with a two-tailed, paired ratio t-test. Statistical analysis for Figure 1a.*

Treatment Comparisons (minutes)	P value	P value summary	Significantly Different (P<0.05)?	One-Tailed or Two-Tailed P value	T, df	Numbers of pairs	Confidence Intervals
15 vs 0	0.1929	ns	No	Two-tailed	t = 1.933, df = 2	3	95%
60 vs 15	0.0303	*	Yes	Two-tailed	t = 5.618, df = 2	3	95%
60 vs 0	0.0075	**	Yes	Two-tailed	t = 11.50, df = 2	3	95%

Table 3. *Gst-4 expression in adr-2(-) worms normalized to 0 minute control was analyzed with a two-tailed, paired ratio t-test. Statistical analysis for Figure 1b.*

WT vs adr-2(-) comparisons per treatment	P value	P value summary	Significantly Different (P<0.05)?	One-Tailed or Two-Tailed P value	T, df	Numbers of pairs	Confidence Intervals
0 minute	0.2210	ns	No	Two-tailed	t = 1.757, df = 2	3	95%
15 minute	0.7988	ns	No	Two-tailed	t = 0.2905, df = 2	3	95%
60 minute	0.3401	ns	No	Two-tailed	t = 1.242, df = 2	3	95%

Table 4. *Raw data of gst-4 expression compared between both worm strains was analyzed with a two-tailed, paired ratio t-test. Statistical analysis for Figures 2a, 2b, and 2c.*

WT vs adr-2(-) comparisons per treatment	P value	P value summary	Significantly Different (P<0.05)?	One-Tailed or Two-Tailed P value	T, df	Numbers of pairs	Confidence Intervals
0 minute	0.2219	ns	No	Two-tailed	t = 1.752, df = 2	3	95%
15 minute	0.7976	ns	No	Two-tailed	t = 0.2923, df = 2	3	95%
60 minute	0.3404	ns	No	Two-tailed	t = 1.241, df = 2	3	95%

Table 5. *Gst-4 expression normalized to WT strain and compared between both worm strains across the different treatments was analyzed with a two-tailed, paired ratio t-test. Statistical analysis for Figures 2d, 2e, and 2f.*

Treatment comparison (minutes)	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Individual P value	
0 vs. 15	-26.00	-78.15 to 26.16	No	ns	0.2683	A-B
0 vs. 60	-0.2522	-52.42 to 51.91	No	ns	0.9909	A-C
15 vs. 60	25.75	-26.41 to 77.91	No	ns	0.2725	B-C

Test Details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
0 vs. 15	1.000	27.00	-26.00	21.32	3	3	1.220	6
0 vs. 60	1.000	1.252	-0.2522	21.32	3	3	0.01183	6
15 vs. 60	27.00	1.252	25.75	21.32	3	3	1.208	6

Table 6. *Sel-12 expression in WT worms normalized to 0 minute control was analyzed with ordinary, one-way ANOVA and Fisher's LSD test. Statistical analysis for Figure 4a.*

Treatment comparison (minutes)	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Individual P value
0 vs. 15	0.5146	0.01163 to 1.018	Yes	*	0.0463
0 vs. 60	-0.1132	-0.6162 to 0.3897	No	ns	0.6016
15 vs. 60	-0.6278	-1.131 to 0.1249	Yes	*	0.0224

Test Details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
0 vs. 15	1.000	0.4854	0.5146	0.2055	3	3	2.503	6
0 vs. 60	1.000	1.113	-0.1132	0.2055	3	3	0.5509	6
15 vs. 60	0.4854	1.113	-0.6278	0.2055	3	3	3.054	6

Table 7. *Sel-12 expression in adr-2(-) worms normalized to 0 minute control was analyzed with ordinary, one-way ANOVA and Fisher's LSD test. Statistical analysis for Figure 4b.*

WT vs adr-2(-) comparisons per treatment	P value	P value summary	Significantly Different (P<0.05)?	One-Tailed or Two-Tailed P value	T, df	Confidence Intervals
0 minute	0.4701	ns	No	Two-tailed	t = 0.7969, df = 4	95%
15 minute	0.3525	ns	No	Two-tailed	t = 1.051, df = 4	95%
60 minute	0.4261	ns	No	Two-tailed	t = 0.8851, df = 4	95%

Table 8. *Raw data of sel-12 expression compared between both worm strains was analyzed with a two-tailed, unpaired t-test. Statistical analysis for Figures 5a, 5b, and 5c.*

WT vs adr-2(-) comparisons per treatment	P value	P value summary	Significantly Different (P<0.05)?	One-Tailed or Two-Tailed P value	T, df	Confidence Intervals
0 minute	0.9196	ns	No	Two-tailed	t = 0.1075, df = 4	95%
15 minute	0.0002	**	Yes	Two-tailed	t = 12.32, df = 4	95%
60 minute	0.7990	ns	No	Two-tailed	t = 0.2721, df = 4	95%

Table 9. *Sel-12 expression normalized to WT strain and compared between both worm strains across the different treatments was analyzed with a two-tailed, unpaired t-test. Statistical analysis for Figures 5d, 5e, and 5f.*

Treatment comparison (minutes)	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Individual P value
0 vs. 15	-7.644	-23.15 to 7.860	No	ns	0.2731
0 vs. 60	-0.2065	-15.71 to 15.30	No	ns	0.9751
15 vs. 60	7.437	-8.066 to 22.94	No	ns	0.2849

Test Details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
0 vs. 15	1.000	8.644	-7.644	6.336	3	3	1.206	6
0 vs. 60	1.000	1.207	-0.2065	6.336	3	3	0.0326	6
15 vs. 60	8.644	1.207	7.437	6.336	3	3	1.174	6

Table 10. *Adr-2 expression in WT strain was analyzed with ordinary, one-way ANOVA and Fisher's LSD test. Statistical analysis for Figure 6.*

	adr-2* Expression Normalized to *gpd-3			***adr-2* Expression Normalized to *gpd-3***		
0 minute	1	1	1	1	1	1
15 minute	1.16	24.16	0.61	1.17	79.22	0.62
60 minute	1.36	1.12	1.061	1.31	1.10	1.35

Table 11. *WT data normalized to 0 minute treatment control. Second biological replicate in 15 minute exposure treatment in both adr-2 and sel-12 data showed extremely high expression.*

ABOUT THE AUTHOR

mcrf0621@gmail.com **Melanie Forbes**

Melanie Forbes graduated from IU Bloomington in May 2022 with a Master of Science in Healthcare Management. A year prior, she completed an Honors Bachelor of Science in May 2021, majoring in Human Biology and minoring in Chemistry. Throughout her undergraduate studies, Melanie grew curious about the link between air pollution and Alzheimer's disease at the molecular level. She further studied this connection in her pursuit of becoming the first student to complete the human biology honors thesis: a research study that requires both humanitarian and scientific research components. Since graduating, Melanie has been playing professional soccer in the top divisions in Portugal and Iceland. After her soccer career, Melanie hopes to return to school and pursue medicine, where she can continue to be a part of the soccer community as a professional healthcare provider.

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