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Cover Page Footnote

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The Effects of Alcohol on the Developing *Drosophila* Nervous System

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ABSTRACT

Ethanol is the most common human teratogen, contributing to fetal alcohol syndrome (FAS) when effects are the most severe. Key effects of fetal alcohol syndrome are observed in the nervous system. The high prevalence of prenatal alcohol exposure necessitates novel treatment and prevention methods. However, ethical issues prevent researching humans in utero. For this reason, the fruit fly Drosophila melanogaster has emerged as a model organism for studying FAS. Because Drosophila is a small and non-placental organism, its environment can be easily controlled, allowing for specific doses and time periods of ethanol exposure to be studied. This review discusses findings related to the impact of alcohol on the developing Drosophila nervous system. Findings related to reactive oxygen species (ROS) exposure, a consequence of prenatal alcohol exposure due to the metabolism of alcohol, are emphasized. Impacts of ethanol on insulin signaling and epidermal growth factors are also mentioned. Further research on Drosophila nervous system development under ethanol exposure may prove beneficial in the treatment and prevention of FAS.

INTRODUCTION

Ethanol is the most common human teratogen, contributing to fetal alcohol spectrum disorder (FASD) in mild to moderate cases and fetal alcohol syndrome (FAS) when effects are the most severe (Denny et al., 2017; Joya et al., 2015). Because ethanol consumption during pregnancy is so common, there is a clear need for novel treatment and prevention methods. Ethanol's potency can perhaps be attributed to its tendency to accumulate in amniotic fluid, where it may be swallowed by a fetus. Once alcohol has entered the fetal system through the placenta, it is metabolized preferentially by CYP2E1, which generates damaging reactive oxygen species (ROS). Both alcohol dehydrogenase, an enzyme for which alcohol has a lower affinity, and CYP2E1 concentrations are much lower in the fetus than in adults (Gupta et al., 2016). This suggests that fetal alcohol metabolism is much slower compared to adult metabolism, giving toxic byproducts more time to accumulate.

The major effects of FAS occur in the central nervous system. FAS patients often have a smaller overall brain, suffer from nonfebrile seizures, and have poor cognitive and self-regulatory abilities. Craniofacial abnormalities are also present (Denny et al., 2017), but this review will focus on the central nervous system (CNS) and some findings in the peripheral nervous system. Given the importance of the central nervous system and the severity of the illness, in addition to ethical issues regarding research on humans in utero, model organisms have been used to elucidate the sources of CNS and PNS damage due to prenatal alcohol exposure. The fruit fly Drosophila Melanogaster has emerged as a model organism for studying FAS. Because Drosophila is a small and non-placental organism, its environment can be easily controlled, allowing for specific doses and time periods of ethanol exposure to be studied (Scepanovic and Stewart, 2019).

Many of the phenotypes seen in alcohol-reared Drosophila reflect those seen in flies reared in ethanol-free environments and exposed to alcohol as adults. For instance, non-ethanol reared flies respond to ethanol exposure with an initial increase in locomotor activity followed by a decrease until they are sedated (Heberlein et al., 2004). Ethanol-reared flies have higher basal locomotion (Guevara et al., 2018), mimicking typical behavior at low levels of ethanol exposure, but are resistant to sedation when exposed to ethanol as adults (McClure et al., 2011). Human genetic analyses have revealed that the Drosophila orthologues of human genes implicated in sedation are active. Some of the proteins involved in sedation of both species are active in cortex glia (Lee et al., 2019; Schmitt et al., 2019). Another advantage of using Drosophila to study the effects of developmental ethanol exposure on the nervous system is that, like humans, there is natural variation in flies that determines how their nervous systems respond to ethanol exposure, including variation in the epidermal growth factor receptors (EGFRs) (Morozova et al., 2018).

Many changes to the Drosophila nervous system through ethanol exposure act via reactive oxygen species (ROS) (Logan-Garbisch et al., 2014), as they do in humans. Changes to EGF and fibroblast growth factor (FGF) signaling (Corl et al., 2009; Dos Santos et al., 2019; King et al., 2014), and changes to the insulin pathway are also important. Namely, expression of the insulin-like peptide dilp2, which is related to human insulin and comes from the insulin-producing cells of the fly brain, is reduced in larvae. This causes developmental delay (Guevara et al., 2018; McClure et al., 2011). There is a need for studies that observe these changes in ethanol-reared organisms, as opposed to inducing mutations or inhibiting expression via RNAi. Nevertheless, the few studies that do observe these changes in ethanol-reared flies strongly implicate these mechanisms in ethanol-induced nervous system damage, and therefore in human FAS.

REACTIVE OXYGEN SPECIES AND NEURAL DEVELOPMENT

It is well known that one of the causes of fetal alcohol syndrome is the presence of reactive oxygen species from alcohol metabolism (Joya et al., 2015; Logan-Garbisch et al., 2014). Logan-Garbisch et al. (2014) discussed a mechanism, at the time somewhat novel, through which ethanol causes FAS. Eggs were exposed to the teratogen via an ethanol bath or transfer to vials containing food laced with ethanol. Flies fed with ethanol-containing food exhibited an eclosion delay and hydrogen peroxide (a reactive oxygen species) had the same effect, indicating that damage via ROS was taking place. Finally, expression of mRNAs related to fatty acid metabolism and synthesis were disrupted, and because of this the authors expected ethanol-induced ROS would result in lipid accumulation through preventing the metabolism of long-chain fatty acids. For instance, transcription of eight triglyceride lipases was lowered by factors ranging from 5 to 200-fold in ethanol-reared flies. (Logan-Garbisch et al., 2014).

Lipid accumulation in response to ROS was also observed in glial cells. This accumulation preceded neurodegeneration in juvenile flies that had mutations in genes affecting mitochondrial function. Jun-N-terminal Kinase (JNK) signaling was also triggered in response to ROS, activating downstream genes that contribute to the lipid accumulation. However, the lipid droplets were not permanent and disappeared during aging. When JNK signaling was reduced, lipid droplets did not form, and neurodegeneration was delayed. The authors of this study believed lipid droplets contributed to neurodegeneration (Liu et al., 2015), but in a later study on flies with the human APOE gene suggested that lipid droplets are a protective factor against nerve damage. Flies expressing an APOE allele known as APOE4, which in humans is a risk factor for Alzheimer's-related neurodegeneration, accumulated fewer lipid deposits and had more photoreceptor disruption than flies with the APOE3 allele, which confers an average risk for Alzheimer's and caused flies todevelop lipid deposits in response to reactive oxygen species. (Liu et al., 2017). This, combined with the data from Logan-Garbisch et al., suggests that lipid deposits do play an important role in neurodegeneration, but their purpose is unclear. There are too many outside factors that arise when mutating specific genes, as they may exert effects on multiple pathways. More study on lipid accumulation in Drosophila glia is needed, using ethanol as a stressor in order to determine whether lipid accumulation may be responsible for some of the effects of human FAS.

Many behavioral changes can be observed in Drosophila exposed to ROS. Similar to how sufferers of FAS may have seizures (Denny et al., 2017), flies with mutations in the SOD2 (superoxide dismutase 2) gene are highly sensitive to mechanical stress, such as banging the vial that houses the mutant on a table. Flies will be temporarily paralyzed, mimicking a seizure. Superoxide dismutase is active in the mitochondria, where it converts ROS byproducts of respiration into oxygen and hydrogen peroxide. The SOD2 mutation reduces the activity of the enzyme, and in addition to seizures, mutants have shorter lifespans, are sensitive to high oxygen levels, and have abnormal brain morphology. The brain morphology changes were attributed to excess cell bodies in the neuropile, which usually lacks cell bodies in its interior (Celotto et al., 2012). Though ROS is often studied as a contributor to cell death in the central nervous system, this finding suggests that it may be responsible for aberrant placement of cells as well.

Reactive oxygen species also inhibit mitochondrial transport along axons. This occurs via the JNK pathway as well, and elevated intracellular Ca2+ levels also produce this effect. Mitochondria are sometimes transferred between neurons to accommodate for energy needs in other parts of an organism. ROS were found to increase Ca2+ levels, and when calcium was kept at a standard level, transport increased, albeit still at lower levels than in neurons not exposed to ROS. As in the study by Logan-Garbisch et al, JNK signaling was triggered under the presence of ROS. In addition, it was found to regulate axonal transport of mitochondria. Specifically, when JNK was upregulated without ROS, axonal transport in the anterograde direction (away from the cell body) decreased. However, transport did not decrease any further when ROS were added, suggesting ROS must act on the JNK pathway to produce this effect (Liao et al., 2017). The JNK pathway may be of interest in research on alcohol and the Drosophila central nervous system. Inhibiting this pathway may at least partially rescue

nervous system defects, which could be crucial as human alcohol consumption during pregnancy is at an all-time high (Denny et al., 2017).

EFFECTS ON EGF AND FGF SIGNALING

During Drosophila development, and especially during the larval stage, cells must respond to external signals that regulate stages and locations of cell division, apoptosis, and quiescence (Miyares and Lee, 2019). Epidermal growth factor (EGF) and fibroblast growth factor (FGF) signaling have been found to regulate the development of the mushroom body, which contributes to ethanol-induced locomotion in developmentally normal flies (King et al., 2011). Though the relationship between EGF/FGF signaling and the developing Drosophila nervous system has seldom been explored, similar locomotor effects are seen in flies exposed to ethanol during development and flies exposed to ethanol as adults (Guevara et al., 2018; King et al., 2014).

Guevara et al. noted that developmental ethanol exposure increased the average distance traveled by flies (2018). In a similar vein and building on previous findings that adult flies exposed to alcohol exhibit increased locomotion at low levels (Heberlein et al., 2004), King et al. observed that EGFR signaling suppressed the locomotion effect in ethanol-exposed adult flies, and FGFR signaling promoted it. Overexpression of the fibroblast growth factor receptor gene htl increased locomotion caused by ethanol exposure; thus, FGFs may be involved in the increased locomotion in flies that developed under ethanol exposure. In addition, when flies were heterozygous for a gain-of-function mutation on an EGFR ligand spitz, their mushroom body lobes exhibited axon overgrowth (King et al., 2014). These findings indicate that ethanol could affect regulation of axonal growth in the mushroom body, and the changes it produces are likely responsible for the increased locomotion seen in ethanol-reared flies. Other changes, such as those affecting the memory, may also be possible since the mushroom body has been implicated in forming ethanol-based memories. Flies exposed to ethanol alone experienced distinct changes in mRNA transcript levels in the mushroom body compared to flies exposed to humid air alone. These changes were believed to be the result of RNA splicing, since knockdown of splice factors prevented the formation of odor-induced ethanol memories (Petruccelli et al., 2020). If this finding can be replicated in flies that have been reared in the presence of ethanol, it may suggest a memory component to fetal alcohol syndrome.

Given that flies exposed to ethanol during development exhibit a suppressed ethanol-induced locomotion phenotype as adults, and that EGF signaling suppresses ethanol-induced locomotion, it is likely that EGF signaling is increased in ethanol-reared flies. One EGFR inhibitor is a kinase known as happyhour (hppy). The hppy gene was discovered using a forward genetics approach involving random insertions of a transposable element. Flies with the insertion mutation in hppy were more resistant to ethanol-induced sedation, which was measured by observing how flies reacted when exposed to a high concentration of ethanol. Sedation was measured by observing the loss of the righting reflex, which corrects body position when an organism is no longer upright. This modified response was also observed when the mutated happy gene was selectively expressed in CNS neurons alongside a GFP reporter. To investigate the connection with the EGF pathway, the EGF pathway was inhibited using an RNAi-producing transgene. These flies were more sensitive to ethanol-induced sedation, consistent with previous findings (King et al., 2014). When the RNAi-transgene was only expressed in the neurons of flies that contained hppy insertion mutations, flies were rescued from sedation sensitivity. (Corl et al., 2009). The hppy gene therefore may be an important mediator of FAS-like phenotypes in Drosophila, and it likely determines ethanol sensitivity by functioning downstream of the EGF pathway. When flies are reared in ethanol, hppy expression may be decreased.

The axon overgrowth found by King et al. was not an isolated observation, as it was also observed in hppy mutants. Specifically, this overgrowth occurred in type II neuromuscular junctions, which are octopaminergic, and it occurred by excess branching. When hppy was mutated, EGFR signaling was not repressed and this led to an inhibition of target selection. Synapses at incorrect locations were formed in addition to synapses at the usual locations. When happyhour was expressed only in the octopaminergic neurons of happyhour mutants, less ectopic synapses were formed, rescuing the mutant phenotype. In addition, overexpression of EGFR in these same neurons led to a substantial decrease in Drosophila muscle 12 cells with Type II neuromuscular junctions. As Gurken is an epidermal growth factor, it was also studied to determine if it was involved in this change. RNAi inhibition of Gurken caused an increase in ectopic neuromuscular junctions on muscles 6 and 7, and overexpression prevented the standard junctions from forming at muscle 12 (Naylor and DiAntonio, 2012). This further suggests that the hppy gene could be involved in the phenotypes that are observed in flies reared in ethanol. In addition, the identification of a specific EGF, Gurken, as a contributor to neuromuscular junction formation is a finding that could be explored further in alcohol-reared flies.

An additional mediator of the EGF signaling pathway is arouser. It is activated by EGF signaling and has been shown to reduce ethanol sensitivity, as was seen in King

et. al. When subjected to an insertion mutation, flies exhibit an increased number of synaptic terminals and are more sensitive to ethanol-induced sedation. This contrasts with ethanol-reared flies, which are less sensitive to ethanol-induced sedation (King et al., 2014; McClure et al., 2011). The extra synaptic terminals appear in multiple locations, but a key one is the pigment dispersing factor (PDF) neurons in the visual medulla. Interestingly, EGFR signaling in dopaminergic neurons does not activate arouser, as indicated by knockdown of arouser here. This had no effect on ethanol sensitivity or synaptic terminal number. The authors suggest that though EGFR signaling does regulate ethanol sensitivity in some neurons via arouser activation, it does not contribute to an increased synapse number. A different pathway, PI3K/Akt, is believed to activate arouser in those neurons that experience increased synapse number, including dopaminergic neurons. Another key finding is that when adult arouser mutants were subjected to social isolation, the excess synaptic terminal phenotype was reduced in PDF neurons (Eddison et al., 2011).

It is reasonable to assume that flies reared in EGFR will activate arouser in some of their neurons, leading to synaptic terminal overgrowth. The latter result from Eddison et al. suggests that some of this overgrowth that occurred in the larval stage can be reversed. In addition, the fact that a pathway besides EGFR is responsible for axon overgrowth in EGFR mutants can explain why EGFR expression in octopaminergic neurons (similar to dopaminergic neurons) leads to axon overgrowth in the mushroom body (King et al., 2014), but EGFR expression in PDF neurons does not lead to axon overgrowth.

FGF signaling also regulates nervous system development and ethanol sensitivity, albeit in a different mechanism than EGF signaling. As stated previously, overexpression of the FGF receptor gene htl increased ethanol-induced locomotion (King et al., 2014), and flies exposed to ethanol during development moved farther than flies reared in standard conditions (Guevara et al., 2018). FGF signaling is also responsible for axonal branch targeting in mechanosensory neurons, in addition to affecting the development of mushroom body neurons as found by King et. al. Loss of function in FGF receptors in the mechanosensory neurons led to the loss of axonal branches, whereas overexpression led to ectopic branches being created, similar to the ectopic branching seen in studies on EGFRs (King et al., 2014; Naylor and DiAntonio, 2012) and the excess neuropile cell bodies in ROS studies (Celotto et al., 2012). The FGF-mediated targeting errors were observed in the left and right posterior scutellar (pSc) neurons, and the receptors btl and htl were studied. Knockdown of both genes decreased secondary and tertiary axon branches, and both overexpression and knockdown caused axonal branches to be directed towards inappropriate locations. Additionally, a cleaning reflex that originates from the pSc neurons and pSc bristles was inhibited in both overexpression and knockdown mutants (Dos Santos et al., 2019). Since FGF signaling is likely decreased in ethanol-reared flies, they may exhibit reduced axonal branching in the neurons where this signaling occurs, such as in the pSc neurons. This is different from how EGF signaling seems to change in ethanol-reared flies. Given that epidermal growth factors and fibroblast growth factors are crucial for cell signaling and development in multicellular organisms, including humans, further study of these proteins may lead to a better understanding of the effects of prenatal alcohol exposure. In addition, further research at the genetic level may help physicians predict the severity of alcohol-related phenotypes.

CHANGES IN DROSOPHILA INSULIN-LIKE PEPTIDE AND NEUROPEPTIDE F SIGNALING

The family of insulin homologues in Drosophila melanogaster are Drosophila Insulin-Like peptides, or Dilps, and McClure et al. noted that Dilp expression is reduced in ethanol-reared flies. Dilp2 was expressed less in the insulin producing cells (IPCs) in the brains of these flies. Expression of the Dilp receptor was reduced as well. Overall brain size was reduced in a dosage-dependent manner, due to reduced cell division in the larval central nervous system (McClure et al., 2011). Other findings related to Dilp2 include that it mediates PI3-kinase activation, which is required for neuroblast growth in the brain and the development of glia in the cortex (Yuan et al., 2020), and that it is necessary for proper dendrite growth in the Dm8 neurons of the optic lobe (Luo et al., 2020). The Drosophila insulin receptor, InR, acts upstream of the p70 S6 kinase, which when phosphorylated can be used to measure nervous system activity and resistance to ethanol-induced sedation. More phosphorylation indicates the kinase is active (Acevedo et al., 2015), therefore the p70 S6 kinase may have more phosphorylation in ethanol-reared flies, leading to sedation insensitivity. These findings suggest that alcohol exposure, both during development and during adulthood, changes insulin signaling pathways and may lead to structural changes at the brain and neuronal levels. Structural changes such as these may be responsible for the behavioral phenotypes seen in alcohol-reared flies.

The insulin-producing neurons in the fly brain are activated by a variety of neuropeptide F known as short neuropeptide F (sNFP). Overexpression of this peptide reduces lifespan and increases resistance to starvation, while a reduction in sNFP levels extends lifespan and reduces resistance to starvation (Kapan et al., 2012). Guevara et. al (2018) hypothesized that NPF signaling

might be increased in ethanol-reared flies since ethanol is a rewarding substance, and it may be protective against the reduction in feeding caused by ethanol rearing. Guevara et. al observed that ethanol-reared flies with reduced NPF signaling ate less than ethanol-reared flies with normal signaling, who ate less than non-ethanol-reared flies. Similar issues with feeding are seen in human FAS patients, who are frequently hospitalized for this reason (Kvigne et al., 2004). Another study supports this idea, as lower Drosophila Dilp signaling due to starvation for 12 hours promoted feeding via neuropeptide F. This was because insulin signaling decreased slowly in starved flies, and constant low-level insulin signaling was necessary to activate neuropeptide F and promote feeding (Sudhakar et al., 2020).

In contrast to adult brain signaling, NPF suppresses insulin signaling in the prothoracic gland during the third instar larval stage. When expression of the NPF receptor (NPFR) is reduced in the PG, flies pupariated later. These flies also exhibited increased body size (Kannangara et al., 2020), which is interesting given that ethanol-reared flies tend to be smaller (Castaneda and Nespolo, 2013). This difference could be attributable to the authors' decision to reduce NPFR expression only in the PG cells as opposed to all cells. Alternatively, changes in insulin signaling may not act at the level of NPF, instead beginning after NPF activates insulin-producing cells. However, NPF may produce different changes outside of this gland, since it is also expressed in the dorso lateral peptidergic neurons during development and the peptide is partially responsible for reduced pupariation when NPF signaling is low (Megha et al., 2019).

CONCLUSION

Drosophila melanogaster is a useful model organism for studying changes in the developing nervous system due to ethanol exposure and for studying how those changes affect adult behaviors. Ethanol-rearing studies of Drosophila appear to be increasing in frequency, and a few major studies have produced specific genetic and nervous system changes that have been described in flies with induced mutations, suppressed gene expression, and exposure to other ROS-inducing chemicals. Some causes of FAS in humans are already known—the creation of reactive oxygen species for instance (Gupta et al., 2016; Joya et al., 2015). Because the developmental environment and genome of Drosophila melanogaster can be easily manipulated, known causes of FAS can be studied in greater depth than they can in humans. In the case of reactive oxygen species, the JNK pathway is a common theme in ROS research that should be studied in ethanol-reared flies.

Growth factors are essential in drosophila nervous development, as seen in the development of systems such as the mushroom body (King et al., 2014) and neuromuscular junctions (Naylor and DiAntonio, 2012). Changes to the learning and memory-mediating mushroom body could explain why ethanol-reared flies respond differently to ethanol than flies reared without ethanol, and ethanol memory is a key concept that could be studied in humans.

Perhaps the most unusual change to the ethanol-reared Drosophila nervous system is the reduction in insulin signaling and subsequent reduction in feeding, though NPF signaling prevents extreme reduction in feeding and seems to help flies survive despite nervous system damage (Guevara et al., 2018). Other adaptive changes to the nervous system should be explored, and genetic variation in the ability of Drosophila to adapt to developmental ethanol exposure may be useful in determining which human FAS patients would be most at risk for adverse outcomes.

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