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Insulin Signal Transduction Mediates Ethanol-Induced Feeding Dysfunction in a Fly Model of Fetal Alcohol Spectrum Disorder

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Biography

Growing up, Manae was always interested in understanding the concepts behind how things work. This led to her passion for Biology. As a first-generation student, she is working hard to earn her bachelor's degree in hopes of pursuing her PhD in molecular biology and genetics. Manae hopes to become a scientific researcher - studying the epigenetic effects of nutrition. She hopes to establish novel results to aid in prevention and mitigation of diseases and disorders related to nutrition. Manae is a proud member of Dr. Rachael French's Behavioral Genetics lab. She really enjoys being a part the research lab as it relates to what she hopes to do in the future. For leisure, Manae likes to take her cute dog on adventures - especially hiking and road trips. Most of the time however, she just likes to stay in bed, watch videos on her phone, and do absolutely nothing.

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ABSTRACT

Fetal alcohol spectrum disorder (FASD) is the leading cause of congenital intellectual disabilities in the Western World, with a worldwide prevalence of 2-11% of all births. FASD is preventable but recent epidemiological studies suggest that public awareness campaigns have reached the limits of their effectiveness. Therefore, research is shifting from prevention to treatment and mitigation of symptoms. No biological treatments for FASD exist, due in part to the fact the cellular mechanisms of alcohol toxicity are not well-understood. Developmental alcohol exposure (DAE) causes a variety of deleterious effects in both vertebrates and invertebrates, including increased mortality, slow growth, learning and memory deficits, and behavioral changes including feeding abnormalities. In this study, we use the common fruit fly, *Drosophila melanogaster*, as a model to study the effects of developmental alcohol exposure (DAE) on feeding behavior. We have previously shown that DAE causes feeding dysfunction in fly larvae, consistent with phenotypes seen in mammalian models, and that these effects are mediated by the reward molecule neuropeptide F (NPF). In addition, we have shown that DAE reduced insulin signal transduction. Here, we investigate the interaction between reduced insulin signaling and feeding changes in flies exposed to ethanol during development.

INTRODUCTION

Fetal Alcohol Spectrum Disorder (FASD) is a preventable disorder induced by alcohol consumption during pregnancy; those affected by developmental alcohol exposure (DAE) experience reduced feeding motivation as well as other cognitive and developmental issues (Guevara et al., 2018). Half of all women in the United States drink, and an estimated 50% of all pregnancies are unplanned (Riley and McGee, 2005). 32% of women report that they would continue to drink while trying to conceive (Peadon et al., 2011) and recent evidence suggests that the periconceptual period is a particularly sensitive time for the programming of future disease generally, and for the programming of metabolic syndrome particularly (Gardebjer et al., 2015; McMillen et al., 2008). Thus, focusing on prevention alone is ineffective, so focus has turned to treatment. There is no approved medical treatment for FASD, and relatively little is understood about the molecular mechanisms of developmental alcohol toxicity. Development of effective treatments to mitigate the symptoms of FASD will depend on a thorough understanding of the underlying molecular causes of each symptom.

We previously demonstrated that developmental alcohol exposure (DAE) leads to a dramatic reduction in insulin and insulin-like growth factor (IGF) signaling in 3rd instar *Drosophila* larvae (McClure et al., 2011), and this result is consistent with the effects of DAE in mammalian systems (Breese et al., 1993; de la Monte et al., 2005; Gatford et al., 2007; McGough et al., 2009; Singh et al., 1996). More recently, our group showed that DAE leads to reduced larval feeding and that ethanol induces an elevated expression of neuropeptide F (NPF; NPY in mammals). NPF/NPY expression results in increased feeding, which partially compensates for the anorexic effects of ethanol (Guevara et al., 2018). Finally, in larvae, NPF release can be negatively regulated by insulin (Wu et al., 2005). Therefore, we hypothesized that altered insulin signal transduction as a result of DAE results in increased NPF signaling and protection against the effects of DAE on feeding.

To test this hypothesis, we tested the ethanol-modified feeding behavior of larvae defective in insulin signaling. We predicted an increase in NPF signaling in these mutants, resulting in a partial or complete rescue of larvae back to normal eating levels in DAE conditions. However, our

data suggest a more complex relationship between insulin and feeding in ethanol-reared flies. Flies mutant for the insulin receptor display the expected rescue of ethanol-induced feeding defects. However, flies with a complete loss of ilp2, the primary fly insulin-like peptide, show the opposite phenotype. Our results indicate that ethanol, insulin, and feeding interact in a complex fashion during development.

MATERIALS AND METHODS

Fly Stocks, Genetics, and Husbandry

Fly stocks were maintained at 25°C on standard corn meal and molasses medium. Fly strains were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Indiana) and the strains used were: InR^{GC25}/TM3, Sb (Bloomington Stock 9554), InR^{E19}*/TM2, Ubx* (Bloomingston Stock 9646), and w^{1118}*;Tl{Tl}Ilp2* Wildtype controls were our standard laboratory stock strain (w^{1118}*, Wild-Type Berlin (w:WTB)).*

Ethanol Rearing

Adult flies were placed in egg-laying bottles, capped with Petri plates containing standard fly food with no ethanol and left to acclimate for a day. On the second day, the plates were replaced with food plates containing 7% ethanol (made by adding 7 mL of ethanol to 93 mL of food) with a small amount of yeast paste in the center. The flies were allowed to lay overnight, then the plates were transferred to a closed Tupperware container containing 1 L of 7% ethanol (experimental conditions). Control plates (food with no ethanol) are collected the same way and ultimately are transferred to an identical closed Tupperware container containing 1 L of deionized water (control conditions).

Feeding Assays

For larval feeding assays, young third instar larvae were collected at approximately 72h after egg- laying (AEL) (control conditions), or 96 AEL (experimental conditions). The difference is due to the approximately 24-hour developmental delay caused by ethanol-rearing (McClure et al, 2011). This ensures that all larvae are age-matched. Larvae were kept food-deprived for 2 h prior to feeding, while first instar larvae were not starved. 30 larvae were placed onto 3% agarose plates and allowed to feed on yeast

paste containing 0.5% v/v FD&C Blue Dye #1 for 20 min. A larva was considered to have eaten by the presence of blue dye in 3/4 its length.

Statistical Analysis

All statistical analyses were conducted using two-way ANOVA with a Tukey HSD *post-hoc* analysis unless otherwise indicated.

RESULTS

Insulin Receptor Loss of Function Is Protective Against DAE-Induced Feeding Abnormalities

We previously showed that DAE causes reduced feeding, and that this effect is exacerbated by reduced signaling through the neuropeptide F (NPF; NPY in mammals) reward pathway. In addition, we demonstrated that NPF signaling appears to be increased in ethanol-reared larvae, and we hypothesize that this increase serves to compensate for reduced feeding caused by ethanol though an as-yet-unidentified pathway (Guevara et al., 2018). Insulin signal transduction also affects larval feeding. Specifically, NPF makes animals more likely to eat noxious food under conditions of food deprivation, and insulin signaling appears to inhibit the activity of NPF receptor (NPFR1)-expressing cells, such that, when animals are well-fed, DILP signaling leads to reduced feeding (and reduced acceptance of noxious foods) (Wu et al., 2005). We hypothesized that insulin similarly regulates NPF in ethanol-reared animals, such that the DAE-induced reduction in *InR* expression leads to increased release of NPF in the brains of ethanol-reared larvae. This hypothesis predicts that mutations leading to reduced insulin signaling should be protective against the DAE-induced feeding deficits.

To test this hypothesis, we reared animals heterozygous for InR^{GC25} or InR^{E19} (loss of function alleles that reduce InR proteins levels to 57% or 50% of wildtype, respectively) in ethanol and compared their feeding to that of wild type ethanol-reared animals. As shown in Figure 1, ethanol-rearing resulted in wild type larvae being less likely to feed: only 58% of wild type ethanol-reared larvae fed, compared with 76% of control animals. This effect completely disappeared in InR^{GC25} heterozygotes, however: InR^{GC25} heterozygotes grown in control food ate slightly less than wild type animals (61% fed), and there was no effect of ethanol-rearing on InR^{GC25}

heterozygotes (57% fed). Similarly, InR^{E19} heterozygotes showed reduced feeding relative to wild type (44% fed), but this phenotype disappeared when InR^{E19} heterozygotes were ethanol-reared (69% fed).

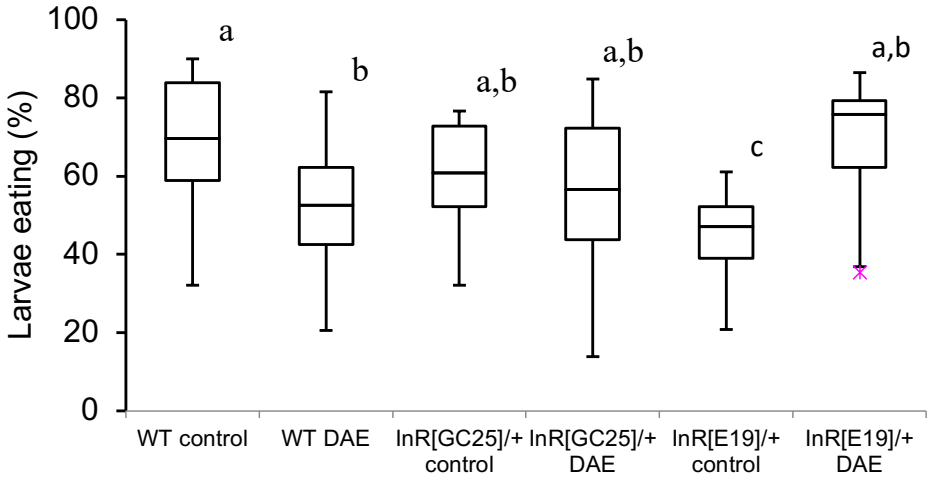

Figure 1: *InR* heterozygotes are resistant to the effects of DAE on feeding behavior. Percentage of early third instar larvae that ate within a 20-min interval after 2 h of food deprivation (N = 8-16. P = 0.0162 for the effect of genotype, P = 0.23 for the effect of DAE, P = 0.0001 for the interaction between DAE and genotype, two-way ANOVA with Tukey's post-hoc analysis). WT: wildtype. Center lines show the sample mean; 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. Boxes sharing the same letter do not differ significantly, while boxes with different letters are significantly different (P < 0.05). Stars indicate outliers.

Loss of ilp2 Exacerbates DAE-Induced Feeding Abnormalities

We have previously demonstrated that both InR and the primary fly insulin, ilp2, are reduced by 50-75% in ethanol-reared animals (Mcclure et al., 2011). To examine the effects of mutation of ilp2 on feeding in DAE animals, we reared larvae homozygous for a complete loss-of-function of ilp2 in ethanol and compared their feeding to that of wild type ethanol-reared animals. Unexpectedly, we found that loss of ilp2 has the opposite effect as reduction of InR. As shown in Figure 2, 73% of control animals fed during the assay, while only 59% of ethanol-reared controls fed. Complete loss-of-function of ilp2 does not affect feeding in the absence of ethanol (68% of ilp2/ilp2 animals fed), but ilp2/ilp2 larvae are profoundly

sensitive to the effects of DAE on feeding, with only 35% feeding. These results are surprising, because a receptor and its primary ligand would be expected to have the same loss-of-function phenotype, suggesting a complex relationship between DAE, insulin signaling, and feeding behavior in *Drosophila*.

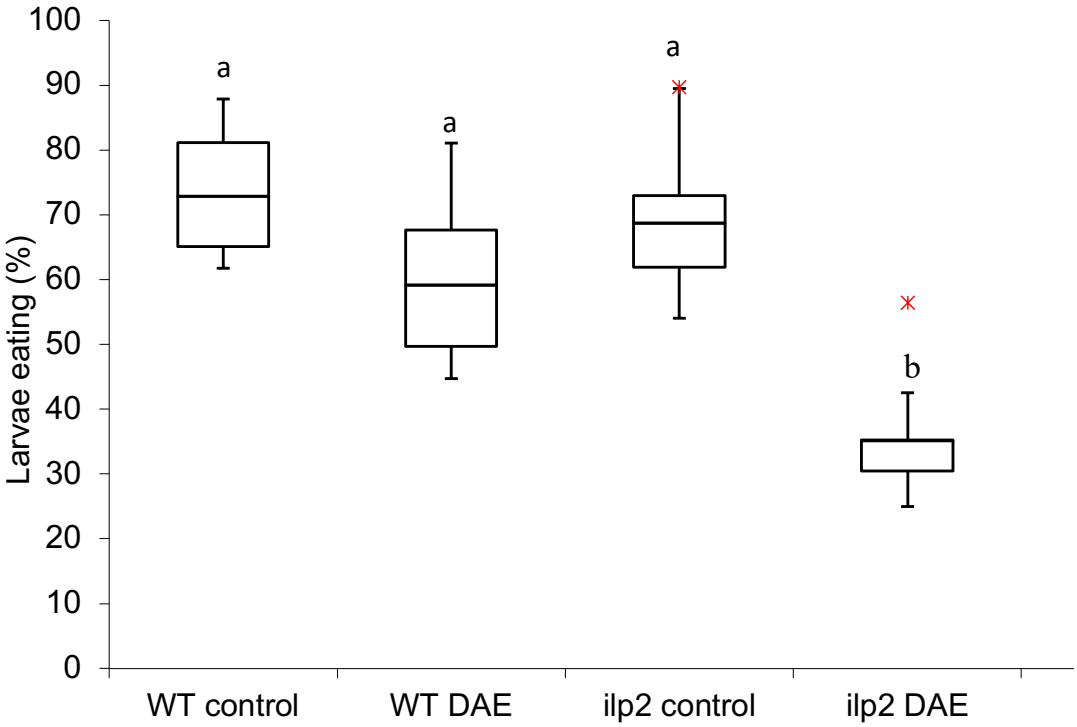

Figure 2: *ilp2* larvae are sensitive to the effects of DAE on feeding behavior. Percentage of early third instar larvae that ate within a 20-min interval after 2 h of food deprivation (N = 7-8. P = 0.0019 for the effect of genotype, P < 0.0001 for the effect of DAE, P = 0.0098 for the interaction between DAE and genotype, two-way ANOVA with Tukey's post-hoc analysis). Center lines show the sample mean; 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. Boxes sharing the same letter do not differ significantly, while boxes with different letters are significantly different (P < 0.05). Stars indicate outliers.

DISCUSSION

The goal of this project was to explore further the mechanism by which developmental exposure to ethanol changes feeding behavior in *Drosophila*. We previously demonstrated that ethanol-rearing alters feeding behavior, and that signaling by neuropeptide F (NPF) is protective against these changes (Guevara et al., 2018). We have also shown that insulin signal transduction is affected by DAE (McClure et al., 2011), and others have shown that insulin signaling regulates feeding through interaction with NPF-responsive cells (Wu et al., 2005; Wang et al., 2016). We therefore investigated the effects of reduced insulin signal transduction on ethanol-induced anorexia in *Drosphila* larvae.

Our results suggest a complex relationship between DAE, insulin, and feeding behavior. Reducing expression of the insulin receptor (InR) results in rescue of ethanol-induced feeding deficits, as predicted (Figure 1). However, complete elimination of the primary *Drosophila* insulin-like peptide, ilp2, has the opposite effect: ilp2 mutant animals are sensitive to the effects of DAE on feeding (Figure 2).

There are several possible explanations for these results. First, our insulin receptor mutants reduce protein levels by about half, and it is not possible to reduce InR levels further, as all mutations in this gene are homozygous lethal. On the other hand, there is no ilp2-mediated signaling in *ilp2/ilp2* mutants. One explanation for this disparity may be positive feedback in the insulin receptor pathway, such that there is a compensatory increase in signaling or expression in animals with a partial loss-of-function, and this explains the rescue seen in the InR heterozygotes.

Another possibility lies in the fact that there are seven fly insulin-like peptides, all of which signal through the same receptor. Our results may reflect a differential requirement for the InR and some of its ligands with regard to feeding behavior. If true, this would be an exciting result, because, to date, there has been no demonstration of distinct behavioral functions for the different ilps. We are currently testing null mutations in all of the remaining six ilps in order to get a full characterization of the role of ilps in feeding behavior in ethanol-reared flies. These experiments are ongoing, but preliminary data suggest that flies with a complete loss of *ilp3* are resistant to DAE-induced anorexia, similar to InR heterozygotes (not shown).

Finally, our results may reflect differences in the functions of ilp2 and the InR in different parts of the feeding circuitry during larval development. Insulin signaling_promotes hunger and feeding behavior through activation of both octopamine and dopamine signaling, in addition to its effects on reducing feeding through inhibition of NPF signaling (Wang et al., 2016), and dopamine and octopamine receptor expression are both reduced by DAE (Logan-Garbisch et al., 2011). We are currently carrying out experiments to elucidate the signaling relationships between insulin, NPF, octopamine, and dopamine during feeding in ethanol-reared larvae.

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