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Developmental Effects of Nicotine Exposure in Drosophila Melanogaster

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DEVELOPMENTAL EFFECTS OF NICOTINE EXPOSURE IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

DEVELOPMENTAL EFFECTS OF NICOTINE IN *DROSOPHILA MELANOGASTER*

By Lisa Wong

Approximately 12%-20% of pregnant women smoke at some point during pregnancy, and 10% of pregnant women are reported to have smoked during the last 3 months of pregnancy. Smoking during pregnancy leads to developmental health risks for the fetus and child, including increased mortality, low birth weight, and developmental delays. The direct molecular targets of nicotine are nicotinic acetylcholine receptors (nAChRs) due to the similarities in structure between nicotine and acetylcholine. However, in many cases, it remains unclear what molecular events downstream of nAChRs lead to the deleterious effects of nicotine on development. We have established *Drosophila melanogaster* as a genetic model system to study the developmental effects of nicotine. So far, we have established that nicotine reduces survival and increases development time in a dose-responsive manner. In addition, we have evidence that developmental nicotine exposure may reduce adult body weight, and that ethanol and nicotine act in a non-additive fashion to reduce survival. Finally, we show that nicotine exposure does not appear to affect brain size in developing larvae. Our results show that the effects of nicotine on fly development are similar to those seen in mammals, and establish *Drosophila* as a model organism for the study of the deleterious effects of nicotine on development.

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First and foremost I want to thank Dr. French for her continued support of my masters and research. Her guidance, motivation, enthusiasm, and immense knowledge have helped me in my research and writing of this thesis. I would not have been able to accomplish this endeavor without her support or imagined a better PI for my masters.

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Introduction

Developmental Nicotine Exposure

Cigarettes contain thousands of harmful chemicals, and smoking causes many adverse health outcomes. Chief among these chemicals is nicotine, which is found naturally in tobacco and is very highly addictive (American Heart Association [AHA], 2015). Some of the risks associated with smoking are heart disease, stroke, and lung cancer (CDC, 2017). Although smoking is well-known to cause serious health risks, 15.1% of all adults still smoke (CDC, 2017). Even though smoking by pregnant women leads to additional health risks to the unborn child, there is a higher prevalence for maternal smoking in women aged 20-24 (13.0%) than other age groups and the highest rate is for American Indian or Alaska Native women (18.0%) (Curtin & Mathews, 2016). This prevalence of smoking is likely at least in part due to the highly addictive nature of nicotine (CDC, 2017).

Nicotine affects the mesolimbic pathway (also known as the reward pathway). Upon stimulation, this neural circuit releases the neurotransmitter dopamine, which is responsible for the pleasurable feelings associated with rewards (Adinoff, 2004). The mesolimbic pathway plays a role in reward behaviors and simple motor responses (Figure 1). Rewarding behaviors (e.g. sex, eating, or smoking) increase dopamine levels, leading to reinforcement of the behavior. Similarly, most drugs of abuse also stimulate the mesolimbic pathway to release dopamine (Blum et al., 2012).

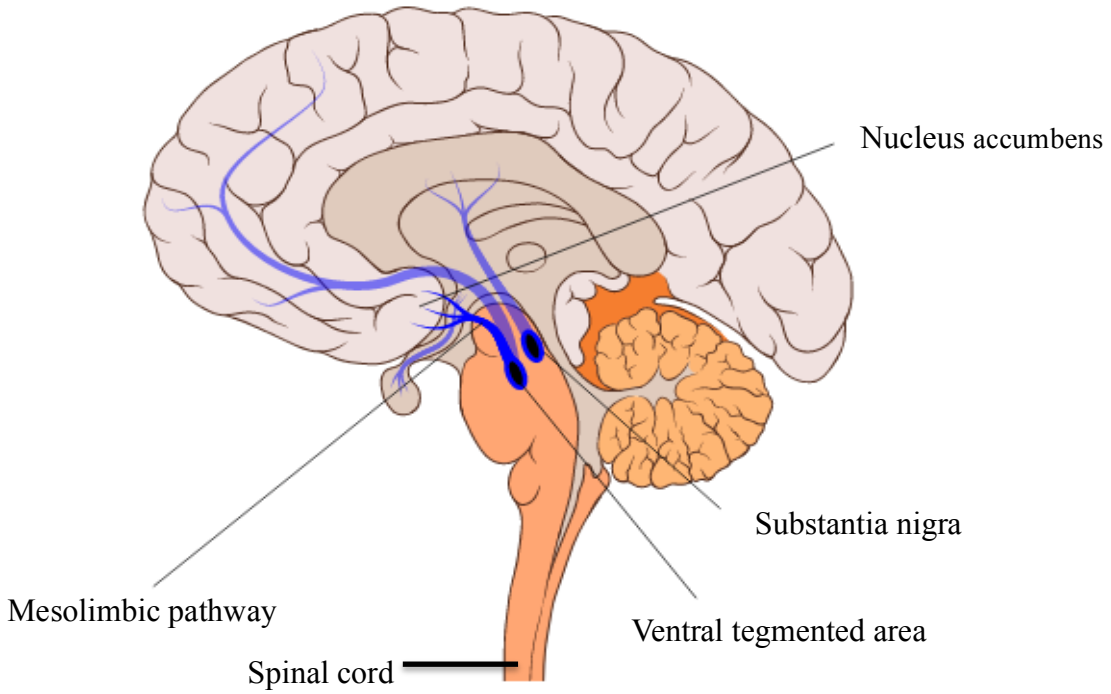


Figure 1. Mesolimbic Pathway Diagram. Smoking releases dopamine from the mesolimbic pathway, which regulates motivation and desire.

Nicotine and Nicotinic Acetylcholine Receptors (nAChRs). Tobacco plants are grown for their leaves, which are dried and fermented before being put in cigarettes (National Institute on Drug Abuse, 2017). When a person decides to smoke a cigarette, he or she is being exposed to smoke containing nicotine in small particles that are rapidly absorbed through the lungs and into the bloodstream. Finally, nicotine is lipid soluble, which allows it to freely cross the blood brain barrier (Riah et al., 1998).

Once inside the brain, nicotine binds to nicotinic acetylcholine receptors (nAChRs). The endogenous ligand for nAChRs is acetylcholine, a neurotransmitter involved in (among other functions) muscle movement, learning, memory, and heart rate (Kihara & Shimohama, 2004). Nicotine has a structure similar to that of acetylcholine and so will

bind and activate nAChRs (Xiu et al., 2009). When the body is bombarded with nicotine from smoking, these receptors are activated in the absence of acetylcholine, activating a variety of downstream signal transduction cascades. Because of the variety of nAChRs subtypes expressed in the brain, little is known about the specific downstream molecular and cellular events leading to the deleterious effects of developmental nicotine exposure.

Physiological Effects of Nicotine on Growth and Development

One adverse outcome of smoking during pregnancy is an increase in developmental mortality, which expresses itself in a variety of ways, including miscarriages, premature births which lead to a decrease in survival, and sudden infant death syndrome (SIDS). SIDS is defined as the sudden and unexplained death of an infant that is less than one year old. The children of women who smoked during pregnancy are at three times the normal risk of SIDS (Wisborg et al., 2000). Maternal smoking is a factor in up to 21% of all SIDS cases (Shah et al., 2006). Smoking or other tobacco use is a risk factor that contributes to adverse outcomes like premature births. One out of ten premature births in the United States in 2015 is due to maternal smoking (CDC, 2017). There is a strong dose response association with smoking and the increased risk of miscarriages (Mishra et al., 2000).

Nicotine Causes Reduced Brain Growth and Neurodevelopmental Abnormality.

Smoking during the second trimester of pregnancy affects the brain and is associated with reduced brain growth (Chatterton et al., 2017). In a previous in vivo study, it was found that nicotine exposed human fetal cortical plates contained fewer neurons than unexposed cortical plates, as defined by staining for Ki67, a marker for cellular

proliferation. This region normally has numerous cortical layers, but developmental nicotine exposure is associated with reductions in both numbers and neurons and cortical layers, but developmental nicotine exposure is associated with a reduction in neuronal content. Cigarettes contain thousands of chemicals, which makes it difficult to link compounds to specific phenotypes.

Further, it has been proposed that the exposure to nicotine due to smoking dysregulates the dopaminergic system and the nicotinic acetycholinergic circuits in the brain (Mcclernon & Kollins, 2008), an effect that appears to be connected to an increased incidence of attention deficit hyperactivity disorder (ADHD) (Mcclernon & Kollins, 2008). ADHD is characterized by inattention, hyperactivity, and impulsivity (Rosenthal et al., 2011). Smoking during pregnancy is one of the most significant risk factors for a diagnosis of ADHD (Rosenthal et al., 2011), and comorbidity of maternal prenatal smoking and ADHD is common and significantly more than predicted by chance, although the mechanisms by which prenatal smoking leads to ADHD are unknown (Mcclernon & Kollins, 2008).

Growth Deficiencies Associated with Smoking. Maternal smoking during pregnancy has been consistently linked to low birth weight in infants, especially when exposure occurs in the third trimester of pregnancy. Increased smoking during the third trimester has been shown to reduce birth weight (Bernstein et al., 2005). This aligns with many previous studies suggesting an increase in maternal smoking is associated with low birth weight (Pereira et al., 2017)

Craniofacial Abnormalities and Developmental Nicotine Exposure. In addition to the conditions described in previous sections, there is an association between smoking and craniofacial abnormalities. Cleft palate is an example; it is a condition in which there is an opening of the lip, which can extend into the nose (Figure 2). This craniofacial abnormality is one of the effects of prenatal smoking in women (Honein et al., 2017). There is a significant dose response trend that suggests maternal smoking and having a child with cleft palate are directly linked (Chung et al., 2000).

Figure 2. Cartoon of an infant with cleft lip/palate. Cleft palate is a craniofacial abnormality resultig from defects in midline closure during early development, and can be caused by developmental exposure to nicotine. (This image courtesy of Centers for Disease Control and Prevention, National Center on Birth Defects and Developmental Disabilities).

Nicotine and Ethanol are Often Comorbid

Maternal smoking alone carries health risks to the developing fetus, but when nicotine is co-abused with alcohol, the risk significantly increases. The combination of prenatal smoking and drinking is associated with an increased risk of mortality (Beeker et

al., 1992), SIDS (Fifer et al., 2009), and developmental delay (Polańska et al., 2015), but whether these increases are due to additive effects of the two drugs, or, alternatively, because the drugs have overlapping targets, is not known.

Drosophila Melanogaster as a Model for Development

D. melanogaster has been widely used in studies to model the effects of drugs of abuse, including alcohol, cocaine, and nicotine (Kaun et al., 2012), as well as the effects of developmental exposure to the same addictive drugs (Logan-Garbisch et al., 2014; McClure, French, & Hebelein, 2011). In addition, the molecular pathways of development are conserved between flies and humans.

Finally, *Drosophila* are ideal for developmental studies for a number of reasons. Flies have a short life cycle (approximately two weeks), and are inexpensive and easy to culture. For over 100 years, flies have been used to study basic cellular processes as well as complex behaviors, including but not limited to: basic developmental pathways (Arias, 2008), learning and memory processes (Gong et al., 1998), sleep (Shaw et al., 2000), and the physiological and behavioral response to drugs of abuse, including alcohol (Wolf & Heberlein, 2003).

This thesis will focus on the effects of nicotine on development in wild-type *D. melanogaster,* as well as interactions between nicotine and ethanol. We show that nicotine has a dose-responsive effect on both survival and development time, and that nicotine exposure may reduce adult fly body weight. In addition, we show that ethanol and nicotine have synergistic effects on developmental mortality, suggesting common

downstream targets within the cell. Finally, we show that developmental nicotine exposure does not alter larval brain size.

Materials & Methods

Fly Stocks

The strain used for all experiments was our standard lab background strain [w^{1118}; Wild Type Berlin (w;WTB). Fly stocks were maintained at 25 ^{o}C on a standard cornmeal/molasses medium unless otherwise stated.

Fly Food Recipes

- 0.01% Nicotine Food: 28.57 uL 35% w/v Nicotine were added to 99.91 mL of standard cornmeal/molasses medium.
- 0.02% Nicotine Food: 57.14 uL 35% w/v Nicotine were added to 99.94 mL of standard cornmeal/molasses medium.
- 0.03% Nicotine Food: 85.71 uL 35% w/v Nicotine were added to 99.91 mL of standard cornmeal/molasses medium.
- 5% EtOH Food: 2.5mL EtOH were added to 47.5 mL standard cornmeal/molasses medium.
- 0.01% Nicotine and 5% EtOH Food: 28.57 uL 35% w/v Nicotine and 5 mL EtOH were added to 94.97 mL of standard cornmeal/molasses medium.

Developmental Nicotine Exposure

Flies were transferred to egg laying bottles topped with petri dishes containing standard fly food. Egg collections were taken for 16-24 hours. A hundred eggs (N=4) were then transferred to vials containing either control food, nicotine-containing food (0.01%, 0.02%, or 0.03% nicotine final concentrations), 5% ethanol-containing food, or food containing both nicotine and ethanol, and, for ethanol exposure experiments, placed in a experimental condition of 5% ethanol bath (Figure 3). The ethanol bath ensures that developing animals are exposed to ethanol during their entire development, which continues for another 10 to 16 days. After eclosion, the newly hatched adult flies were counted daily at the same time for the duration of the experiment (between 9 and 20 additional days). The data were used to generate cumulative eclosion rate plots, a direct measurement of egg-to-adult survival, and the time to 50% of total eclosion. Time to 50% eclosion was calculated by linear interpolation.

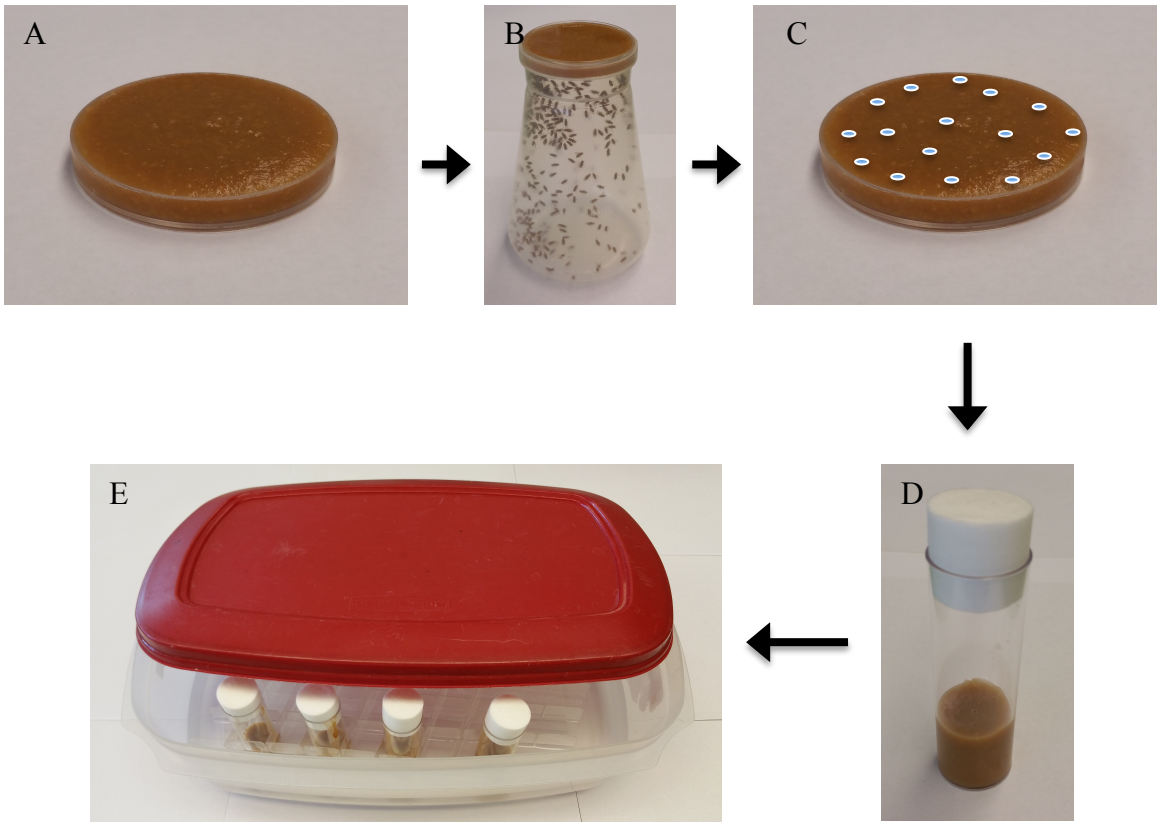


Figure 3. Developmental nicotine exposure assay diagram.
(A) Standard fly food is used in food plates. (B) Flies are placed in egg laying bottles to mate and lay eggs on food plate. (C) After 24 hours new food plates are used and 100 eggs are transferred into vials (D) containing either control or experimental food, and incubated in water or ethanol baths at 25°C for the duration of the experiment (E).

Weight

Flies were reared on control (0%) and experimental food (0.01%, 0.02%, or 0.03% nicotine). Upon eclosion, 100 female flies from each condition were collected and weighed. Average weight per fly was calculated by dividing the total weight for that condition (in mg) by 100.

Immunostaining and Imaging

Brains were dissected and fixed according to Wu and Luo (2006) with the following modifications: tissues were fixed for 1 hour, incubated with Normal goat serum (NGS) block for 24 hours. Brains were then incubated in NC82 primary antibody in block for 4 days followed by two days in secondary antibody. NC82 mouse anti-Brp was used at 1:50 (Developmental Studies Hybridoma Bank, AB 2314866). Secondary antibody Alexa Fluor 594 goat anti-mouse was used at 1:1000 (Jackson Immunoresearch, 705-586-147). Images were taken using a Zeiss LSM700 confocal microscope. A level of 0.05% was used in all experiments. Data are represented as mean ± s.e.m. Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's HSD *post hoc* test. No statistical tests were used to predetermine sample sizes, but our sample sizes are consistent with those reported in previous publications (McClure et al., 2010; Luu et al., 2016; Tran el al., 2014).

Results

Nicotine Exposure During Development Causes a Dose-Dependent Increase in Mortality and Development Time

Larvae were reared on a diet of standard fly food (see Materials and Methods) containing nicotine at a concentration of 0.01%, 0.02%, or 0.03% (experimental conditions) or food containing 0% nicotine (control conditions). Survival and development times were assayed starting 10 days after egg-laying by daily counts of eclosed adult flies. Nicotine at a concentration of 0.01% had no significant effect on survival, with a median survival on control food of 79.25% and 76.5% on food containing 0.01% nicotine (Figure 4). However, when we increased the nicotine concentration to 0.02%, median survival dropped to 41%, a significant reduction compared with controls ($p < 0.0001$, one-way ANOVA with Tukey post-hoc analysis, n=4). This trend continued when the nicotine concentration was increased to 0.03%. At this concentration, only 7% of flies survived to eclosion ($p < 0.0001$, one-way ANOVA with Tukey post-hoc analysis, n=4). These data demonstrate that nicotine exposure during development causes a dose-dependent decrease in survival in *Drosophila*.

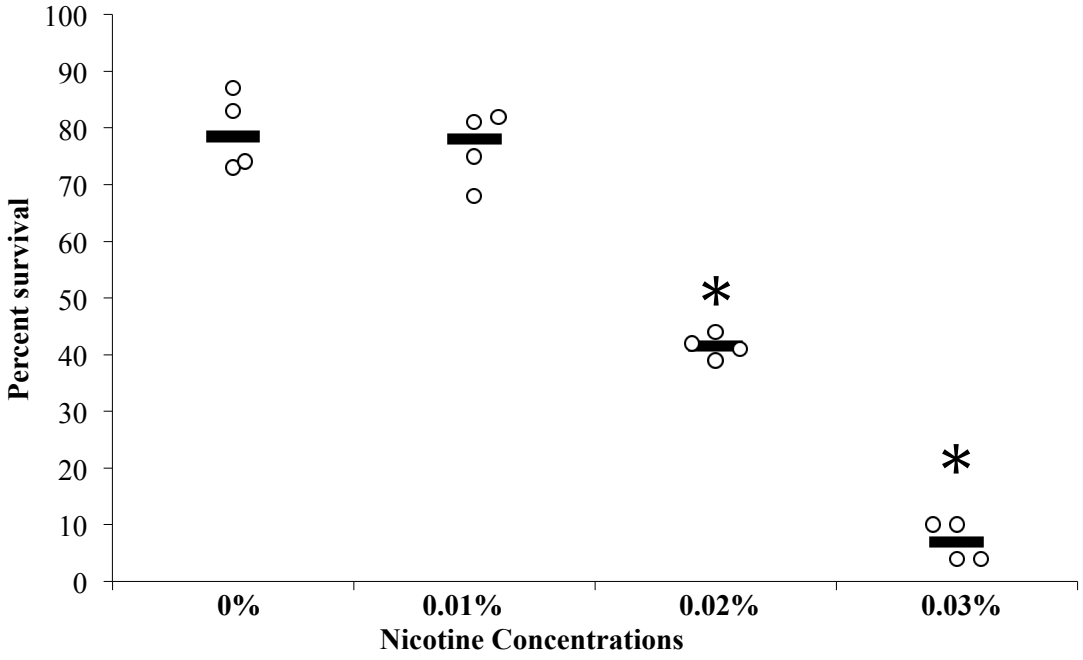


Figure 4. Nicotine causes a dose responsive reduction in survival? One hundred eggs were transferred to each vial containing 0% nicotine food as the control and 0.01%, 0.02%, and 0.03% nicotine food. The percent survival was assessed daily and data organized into a scatterplot. * denotes $p<0.0001$, differing from all other means. Horizontal bars represent mean. N=4 for all conditions, $p<0.0001$ one way ANOVA with Tukey's HSD post-hoc analysis.

In addition to its effects on survival, developmental nicotine exposure caused a developmental delay (Figure 5). In control animals, time to 50% eclosion was 10.46 days. While 0.01% nicotine had no effect on survival, it did result in a small but significant delay, with animals reared in 0.01% nicotine having a time to median eclosion of 11.29 days. As the concentration of nicotine was increased, time to median eclosion also increased: flies reared in 0.02% nicotine showed a 2.16 day delay ($p<0.0001$, one-way ANOVA with Tukey post-hoc analysis, n=4), while flies reared in 0.03% nicotine a 3.00

day delay when compared to control ($p<0.0001$, one way ANOVA with Tukey post-hoc analysis, n=4). These data are summarized in Table 1. These data demonstrate that nicotine exposure during development results in a significant developmental delay.

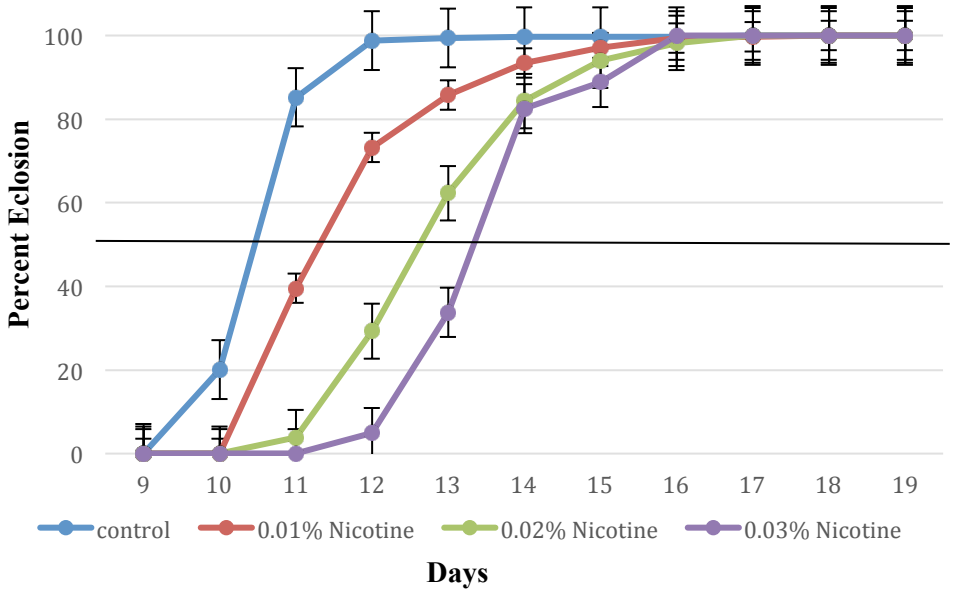


Figure 5. Developmental nicotine exposure causes developmental delay. 0% as the control and nicotine concentrations of 0.01%, 0.02%, and 0.03% were assessed. Data have been normalized to the total number of flies eclosed for each condition. Upon eclosion comparisons for time to 50% eclosion were assessed. ($p<0.05$, one way ANOVA with Tukey's HSD post-hoc analysis, n=4.

Table 1. *Nicotine Causes a Dose-Responsive Delay in Development Time*

Condition	Time to median eclosion (d)	Difference (d)
0%	10.46 ± 0.035	N/A
0.01% Nicotine	11.29 ± 0.14	0.83 (NS)
0.02% Nicotine	12.62 ± 0.14	2.16 (P<0.01)
0.03% Nicotine	13.46 ± 0.23	3.00 (P<0.01)

Note. Survival assay comparing developmental delay from nicotine-reared larvae with control larvae. Nicotine-reared wild-type larva showed a developmental delay as concentration increases.

Nicotine Exposure During Development May Decrease Adult Body Weight

For each experimental condition (0%, 0.01%, 0.02%, and 0.03% nicotine), we collected and weighed 100 adult female flies upon eclosion. The average weight of control flies was 1.23 mg. Flies reared on 0.01% nicotine weighed 1.16 mg on average, a 5% decrease in weight when compared with controls. Finally, 0.02% and 0.03% nicotine each resulted in a 13% decrease in body weight (average body weight 1.06 mg) (Figure 6). These data suggest that nicotine exposure during development may have an effect on adult body weight. As this experiment has not been repeated, we have not performed statistical analysis of our body weight data. We are continuing to examine the effects of nicotine on adult body weight.

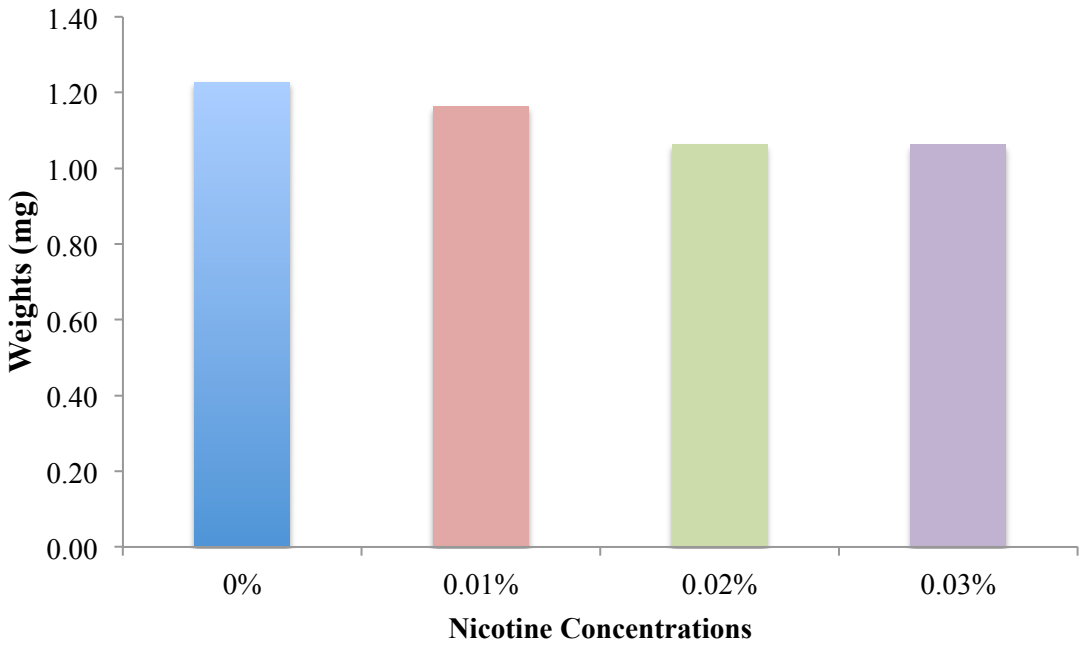


Figure 6. Developmental nicotine exposure may reduce adult body weight. Flies reared on 0.01% nicotine show a 5% reduction in adult weight, while both 0.02% & 0.03% nicotine result in a 13% reduction in adult weight.

Nicotine and Ethanol Have a Synergistic Effect on Survival

In cases of prenatal nicotine exposure in humans, simultaneous exposure to alcohol is common (McMurray et al., 2008). In addition, nicotine and ethanol cause similar effects on growth and development (McClure et al., 2011). We therefore exposed flies to a combination of nicotine and ethanol in order to ask whether the effects of the two drugs are synergistic, which would suggest overlapping molecular targets.

Larvae were reared on 0.01% nicotine, 5% ethanol, and a combination of 0.01% nicotine and 5% ethanol. These concentrations of nicotine (0.01%) and ethanol (5%) have minimal to no effect on growth and development, making it easier to detect possible synergistic effects. The results of these experiments are presented in Figure 7. Nicotine at

a concentration of 0.01% had no significant effect on survival, as seen previously (Figure 4). 5% ethanol had a significant negative effect on survival, but the combination of 0.01% nicotine and 5% ethanol resulted in a significant synergistic effect (Figure 7), which suggests that these drugs have common downstream targets.

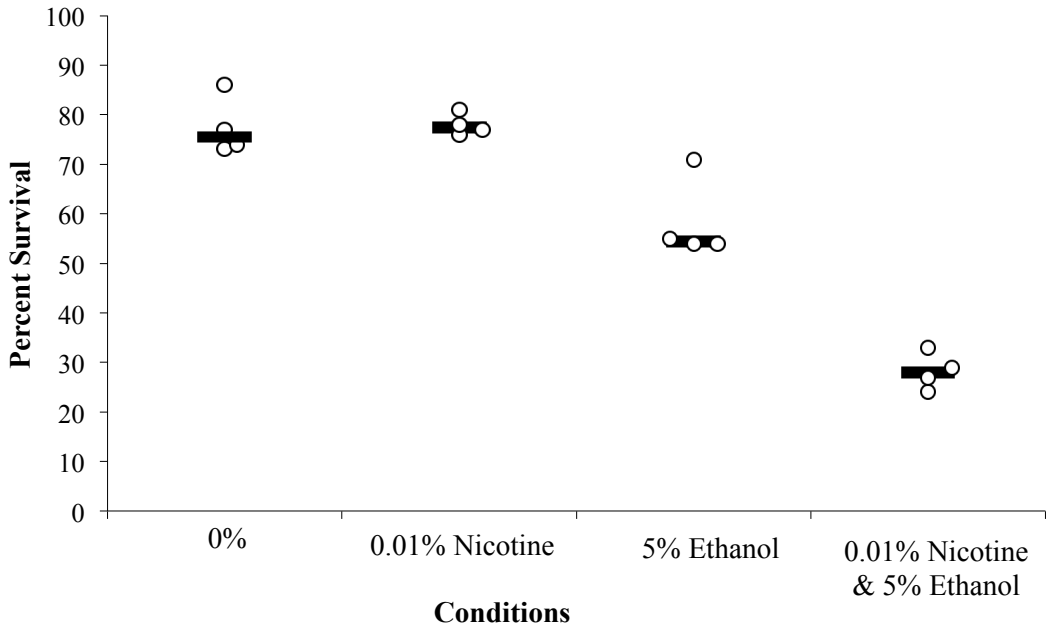


Figure 7. Nicotine and ethanol have synergistic effects on developmental mortality. One hundred eggs were transferred to each vial containing 0% nicotine food as the control, 0.01% nicotine food, 5% ethanol food, and a combination of 0.01% nicotine and 5% ethanol food. The percent survival assessed daily and data organized into a scatterplot. * = $p<0.0001$. Horizontal bars represent mean N=4 for all conditions, $p<0.0001$ two way ANOVA with Tukey's HSD post-hoc analysis.

Nicotine and Ethanol Exposure During Development Cause a Synergistic Increase in Development Time

Larvae were reared on 0.01% nicotine, 5% ethanol, and a combination of 0.01% nicotine and 5% ethanol as described in Section 3.1, and developmental delay was assayed by daily counts of the larva as they eclosed into adult flies as described in

Section 3.1. Larvae reared in 0.01% nicotine showed a 0.56 day delay compared with control flies, while rearing in 5% ethanol resulted in a 1.07 day delay when compared to control. When the two drugs were combined, the delay increased to 2.08 days as compared to control (Figure 8 and Table 2), and this effect is greater than the predicted effect of a simple combination of the two individual effects (p= 0.024, two-way ANOVA with Tukey post-hoc analysis, n=4). The combination of these two drugs, ethanol and nicotine, causes a synergistic increase in development time.

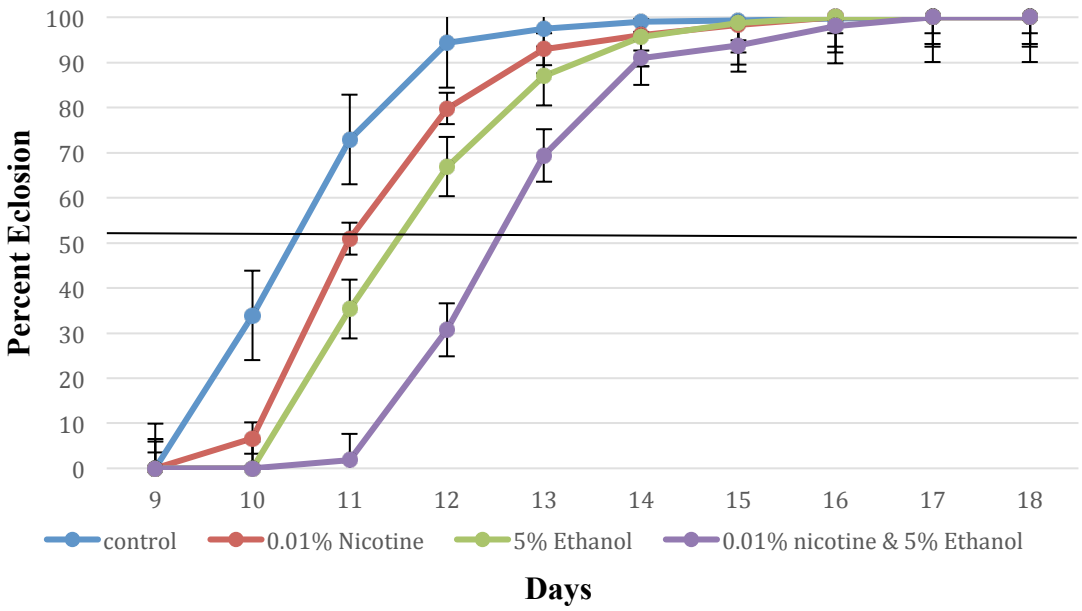


Figure 8. Nicotine and ethanol have synergistic effects on developmental mortality. Vials containing 0% nicotine food as the control, 0.01% nicotine food, 5% ethanol food, and a combination of 0.01% nicotine and 5% ethanol food were assessed. Data were normalized to the total number of flies eclosed for each condition. Upon eclosion comparisons for time to 50% eclosion were assessed. (p=0.024, two way ANOVA with Tukey's HSD post-hoc analysis, n=4).

Table 2. *Time to Median Eclosion For Flies Reared in Both Ethanol and Nicotine*

Condition	Time to median eclosion (d)	Difference (d)
0%	10.42 ± 0.11	N/A
0.01% Nicotine	10.98 ± 0.03	0.56 (P<0.0001)
5% Ethanol	11.49 ± 0.09	1.07 (P<0.0001)
0.01% Nicotine + 5% Ethanol	12.50 ± 0.10	2.08 (P=0.025)

Note. Survival assay comparing developmental delay from nicotine-reared larvae with control larvae. Nicotine-reared wild-type larva showed a developmental delay as concentration increases.

The Combination of Nicotine and Ethanol Exposure During Development Does Not Affect Adult Weight

Larvae were reared on 0.01% nicotine, 5% ethanol, and a combination of 0.01% nicotine and 5% ethanol. We collected and weighed 100 adult female flies upon eclosion, and found that the average weight for all conditions was similar (Figure 9; $p > 0.05$, two way ANOVA with Tukey post-hoc analysis, n=4). Flies reared on 0.01% nicotine, 5% ethanol, and a combination of both drugs show no difference in adult weights, indicating that, at these concentrations, there is no synergistic effect of ethanol and nicotine on reduction of adult body weight. Thus, low concentrations of nicotine and ethanol do not affect adult body size in *Drosophila*.

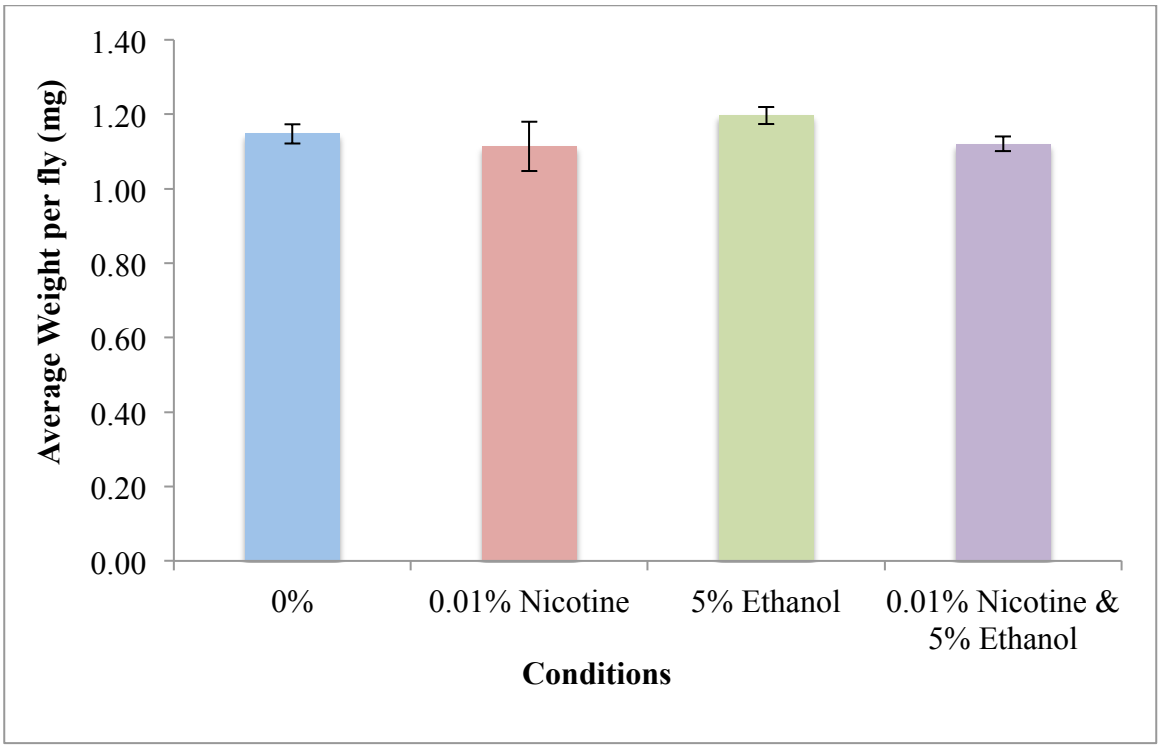
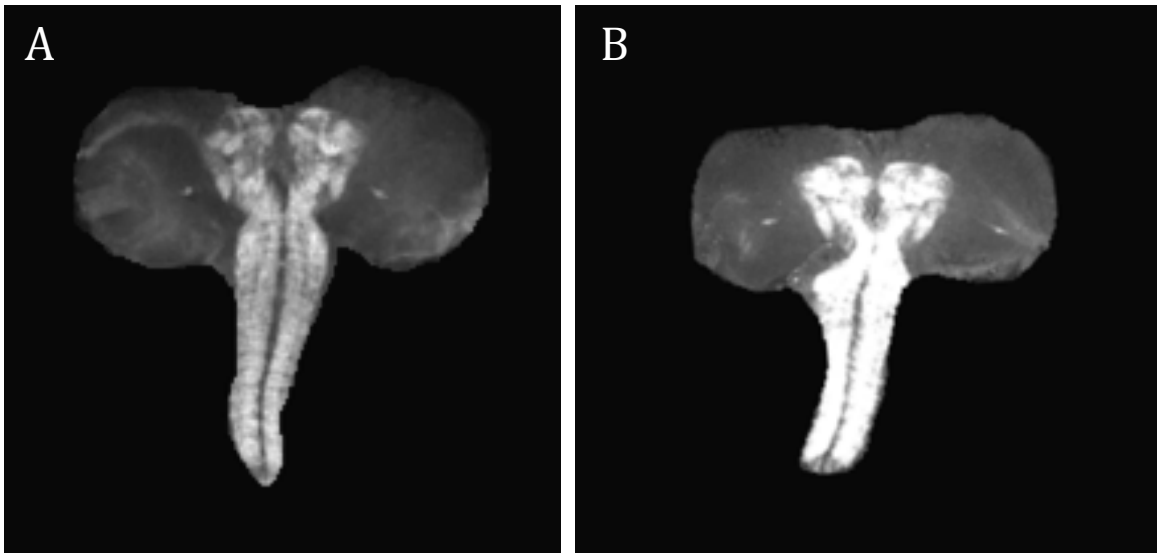


Figure 9. Nicotine and ethanol do not synergize to alter adult body weight. There is no synergistic effect of ethanol and nicotine on reduction of adult body weight ($p > 0.05$, two way ANOVA with Tukey's post-hoc analysis, n=4).

Nicotine Exposure During Development Does Not Affect Larval Brain Size

Developmental exposure to nicotine in animal models has indicated changes in neuronal cell replication and differentiation. When pregnant rats were exposed comparable nicotine levels to those experienced by human smokers there was a substantial reduction in cell number in the resulting pups, demonstrating that prenatal nicotine exposure compromised brain development (Roy et al., 2002). We therefore asked whether developmental nicotine exposure elicits reductions in brain size in *Drosophila*.

Larvae were reared on 0% (control conditions) or 0.02% nicotine (experimental conditions). Brains were dissected from wandering 3rd instar larva and stained with monoclonal antibody NC82. NC82 recognizes the protein Bruschilot, which labels the presynaptic active zone of all neurons and is the standard marker for labeling neuropil in fly brains (Figure 10). We used Image J to analyze confocal reconstructions of control (Figure 10A, n=14) and nicotine-exposed (Figure 10B, n=25) brains, measuring the inter-optic-lobe brain diameter. We found that brain sizes were similar in both conditions (see Figures 10C and D), indicating that exposure to 0.02% nicotine during larval development has no effect on brain size ($p > 0.05$, Student's T-test).



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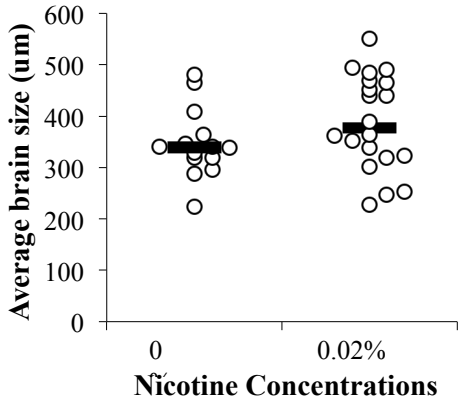

D

	Nicotine Concentrations	
	0%	0.02%
Average Brain Size	0.3470 ± 0.01807	0.3933 ± 0.01714

Figure 10. Developmental nicotine exposure does not alter larval brain size. A and B: Confocal micrographs of NC82-stained brains dissected from a 3rd instar control larva (A) and a 3rd instar larva after rearing in 0.02% nicotine (B). C. Brain size, as measured by inter-optic-lobe distance.

Discussion

Developmental nicotine exposure causes a wide range of adverse health risks including increased mortality, developmental delay, growth deficiencies, craniofacial abnormalities, and neurobehavioral deficits. Nicotine acts as an agonist for nAChRs, but the molecular events downstream of the nAChRs that lead to the deleterious developmental effects of nicotine are largely uncharacterized. We have established *Drosophila melanogaster* as a model for developmental nicotine exposure in order to begin identifying the molecular targets of nicotine exposure. We show here that larval nicotine exposure in *Drosophila* causes decreased survival, developmental delay, and reduced adult weight. In addition, we show a synergistic effect of ethanol and nicotine during development, suggesting that nicotine and ethanol have overlapping cellular targets.

Nicotine Exposure Causes Developmental Mortality and Developmental Delays

When exposed to nicotine during larval development, wild type flies displayed a dose-dependent increase in developmental mortality (Figure 4) and delayed time to median eclosion (Figure 5 and Table1). This result is similar to the effects of cigarette smoking on fetal development as seen in previous studies, which causes an increase in both prenatal and perinatal death: smoking during pregnancy is associated with an increased risk of miscarriage and stillbirth (Hofhuis, 2003). In addition, 21% of all cases of SIDS cases are associated with smoking during pregnancy (Shah et al., 2006). As shown with other studies a number of neurological delays are associated with prenatal nicotine exposure, including attention deficit hyperactivity disorder (ADHD) (Langley et

al., 2011). ADHD is a syndrome characterized by trouble paying attention, impulsive behaviors indicating insufficient executive function, and hyperactivity (National Institute of Mental Health, 2016).

Growth Deficiencies

Our study suggests that, at higher concentrations (0.02% or 0.03%) developmental nicotine exposure may lead to a decrease in adult weight. This is consistent with the literature on prenatal smoking. Prenatal nicotine exposure leads to growth deficiencies, and low birth weight is the primary fetal risk associated with smoking during pregnancy. As the amount of maternal smoking increases, there is a concomitant decrease in birth weight (Secker-Walker & Vacek, 2003).

Brain Size

This study did not show a significant effect on brain size due to developmental nicotine. This is not consistent with other studies that associate maternal smoking with reduced fetal brain growth. Maternal smoking impacts brain development and is associated with reduced fetal brain growth (Chatterton et al., 2017). It is possible that the dose of nicotine used here are not high enough to elicit large-scale effects on brain development; alternatively, this may be an area in which insect development differs from mammalian development. It is also possible that the effects of smoking on brain growth are not due to nicotine, but rather to another of the many harmful chemicals found in tobacco smoke. In future, we plan to address the question of the effects of nicotine on brain development in flies in two ways: first, by using higher doses of nicotine, and,

second, by examining the effects of nicotine on the development of specific brain structures, such as the mushroom bodies.

Future Directions

In the future, there are several questions that we would like to address. First, what is the critical period of development for the various phenotypes described in this thesis? This can be addressed by transferring developing larvae to nicotine-containing food at various developmental stages and assessing the effects on survival and growth. The importance of knowing the critical stage of development affected by developmental nicotine exposure will help to reduce the adverse health risks associated with maternal smoking.

In addition, as indicated above, we intend to pursue the question of nicotine's effect on brain development in two ways: by measuring brain sizes in animals exposed to higher concentrations of developmental nicotine, and by examining specific regions of the developing brain for changes in nicotine-exposed animals. Of particular interest are the mushroom bodies, the fly analog of the hippocampus, as the hippocampus is particularly sensitive to nicotine exposure in mammals (Gallinat et al., 2007). In addition, the hippocampus is affected in neurodegenerative disorders including Alzheimer Disease. A better understanding of the effects of nicotine on the development of these regions may result in greater understanding of the underlying mechanisms of Alzheimer Disease, and, ultimately, the development of therapies (Regensburger et al., 2014).

Finally, our data indicate a synergistic effect of nicotine and ethanol, suggesting that these drugs have overlapping downstream targets. We will begin identifying the common

targets by testing mutants that are known to be resistant or sensitive to the developmental effects of ethanol for similar alterations when reared in food containing nicotine.

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