UNIVERSITY OF CALIFORNIA, MERCED

Presynaptic Plasticity in Ethanol Tolerance Circuitry

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

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2022

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Dedication

I would like to dedicate this work to my family for their consistent and tireless support of me. I would not have been able to accomplish this without them.

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University of California, Merced CA **2022**
Ph.D. Candidate in Quantitative & Systems Biology
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California State University, Fresno CA **2016**
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Examining changes to presynaptic sites in the mushroom bodies after D. melanogaster is exposed to ethanol via immunohistochemistry. Confirming phenotypic changes via behavioral analysis of flies in tolerance development assays. Circuit mapping tolerance in the central brain.

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Designed experiments to exam the foraging and communication behavior of B. impatiens. Managed a team of undergraduate students to perform behavioral analysis of the bees' foraging habits in response to changes in their environment.

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Mager EM, Medeiros LR, Lange AP, McDonald MD. (2012). The toadfish serotonin 2A (5-HT2A) receptor: molecular characterization and its potential role in urea excretion. Comp Physio Biochem 163A: 319–326

CONFERENCE TALKS

Lange, AP, Artega, E, Bains, NK, Mohamed, A, Lent, DD. The Effect of Local Flower Distribution on the Foraging and Communication Behavior of the Common Eastern Bumblebee, Bombus impatiens. *Paper presented at the Central California Research Symposium, Fresno CA, USA 2016*

CONFERENCE ABSTRACTS

Lange, AP, Wolf, FW. Ethanol Induced Presynaptic Plasticity in a Tolerance Circuit. *Neurobiology of Drosophila 2021, Virtual, 2021*

Adhikari, P, Lange, AP, del Toro, A, Wolf, FW. Acute and Chronic Ethanol Induce Transcriptionally and Functionally Different Forms of Tolerance. *43rd Annual RSA Scientific Meeting, New Orleans, LA, USA 2020 (accepted, conference cancelled due to Covid-19)*

Adhikari, P, Lange, AP, Wolf, FW. Transcriptional Control Mechanisms for Ethanol Tolerance Development. *42nd Annual RSA Scientific Meeting, Minneapolis, MN, USA 2019*

Lange, AP, Yang, P, Mohamed, A, Artega, E, Lent, DD. Hives of the Common Eastern Bumblebee, Bombus impatiens, rapidly alter their foraging patterns based on sudden changes in local flower distribution. *Annual Meeting of the Society for Integrative and Comparative Biology, New Orleans LA, USA 2017*

Yang, P, Arteaga, E, Bains, NK, Mohamed, A, Morazan, C, Lange, AP, Lent, DD. The Effect of Local Flower Distribution on the Foraging and Communication Behavior of the Common Eastern Bumblebee, Bombus impatiens. *37th Annual Central California Research Symposium, Fresno CA, USA 2016*

Lange, AP, Artega, E, Bains, NK, Mohamed, A, Lent, DD. The Effect of Local Flower Distribution on the Foraging and Communication Behavior of the Common Eastern Bumblebee, Bombus impatiens. *Annual Meeting of the Society for Integrative and Comparative Biology, Portland OR, USA 2016*

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National Society of Collegiate Scholars

Abstract

Presynaptic Plasticity in Ethanol Tolerance Circuitry

By

Anthony P Lange

Doctor of Philosophy in Quantitative and Systems Biology

University of California, Merced

Professor Fred Wolf, Dissertation Advisor

The first exposure to ethanol alters neuron function that manifests behaviorally with subsequent exposures. Tolerance development, a simple stimulus-response paradigm for plasticity, is ideal for identifying functionally relevant cellular and molecular plasticity substrates. In Drosophila, the mushroom body circuitry promotes multiple forms of ethanol plasticity including tolerance.

Here, I show that ethanol physically changes presynapses in the mushroom bodies. Acute ethanol alters the expression of the presynaptic proteins Synapsin, Cdk5, and the $Ca_v2.1$ calcium channel (Cacophony) via the histone/protein deacetylase Sirt1 and the transcription factor Hr38. I found that acute ethanol increases presynapse size, scaling with ethanol dose. Increased synapse size is *Sirt1* and *Hr38*-dependent, suggesting that it is part of a unified signaling pathway for synaptic remodeling by ethanol.

Tolerance brain regions outside of the mushroom bodies are not known. I used a functional anatomical screen of sparsely expressed Gal4 strains driving Cacophony RNAi. I discovered three new sites for ethanol tolerance development, including the first demonstration of glutamatergic neurons in tolerance development. Further investigation of these glutamatergic tolerance neurons reveals that they are likely to be the DN1p clock neurons, demonstrating for the first time a role for the DN1p's in tolerance. Together these data imply that ethanol has a widespread impact on the presynapses of the brain and further research is necessary to determine if and how these presynapses are involved in other ethanol behaviors.

Introduction

The first exposure to ethanol an animal experiences alters neuron function to cause behavioral adaptation, which is manifest with subsequent exposures. Understanding these changes at the molecular level may provide a key to treating and preventing alcohol use disorder. Synapses are constantly adapting as new stimuli are encountered, making them a key area to study plasticity. The effect of ethanol on synapses at the molecular level may be key for how alcohol alters brain function.

Flies, like humans, show similar forms of behavioral plasticity when exposed to ethanol, including developing tolerance, preference, reward, withdrawal, and reinstatement after abstinence. Additionally, ethanol appears to affect the brains of different animals in remarkably similar ways at the molecular level (Pandey et al., 2008; Renthal et al., 2009; Sakharkar et al., 2012). Drosophila is amenable to genetic and cell-type specific manipulation, synaptic level anatomy, and experiments are rapid and inexpensive compared to rodents.

The mushroom bodies that encode learning and memory also promote behavioral plasticity induced by ethanol (Engel et al., 2016). Expression levels of presynaptic neurotransmitter proteins are regulated by ethanol (O'Brien et al., 2018). With this in mind, the presynaptic marker Elks/CAST ortholog Brp-short^{mcherry} (Christiansen et al., 2011) can be used to measure possible changes in synaptic release (Gupta et al., 2016).

Is the ethanol-induced presynaptic change regional within a specific neuron, or does it occur at all of its synapses? Is the plasticity dependent on circuit changes, or does ethanol, which readily distributes to the whole brain, have the same effect in all synapses and neurons? Are there other regions of the brain outside of the learning and memory centers that are necessary for tolerance? Using behavioral experimentation, transgenic techniques, and direct anatomical examination of the brain to study alcohol's effects on synapses I have endeavored to answer these questions.

Chapter 1 Role of Presynaptic Plasticity in Ethanol Tolerance in Drosophila

Summary

Here I demonstrate a role for presynaptic function and plasticity in ethanol tolerance. Presynaptic neurotransmission is necessary for tolerance development and the activity of the presynaptic protein Cdk5 is necessary for normal naïve and learned sedation response. Additionally, ethanol exposure induces physical changes to the presynaptic release sites as measured by Bruchpilot puncta size.

Role of Presynaptic Plasticity in Ethanol Tolerance in Drosophila

The impact of ethanol on presynaptic anatomy remains poorly understood. Tolerance and preference are two forms of plasticity that predict the development of alcohol use disorders in humans, and they are simple enough to be understood completely (Sakharkar et al., 2012). Flies and humans share many forms of behavioral plasticity when exposed to ethanol, including developing tolerance, preference, reward, withdrawal, and reinstatement after abstinence. Additionally, ethanol appears to affect the brain of different animals in remarkably similar ways at the molecular level (Ackermann et al., 2015; Lovinger & Kash, 2015; Lovinger & Roberto, 2011; McCool, 2011; Pandey et al., 2008; Sakharkar et al., 2012).

The mushroom bodies are key structures in learning and memory of flies. They are also important for behavioral plasticity induced by ethanol and other drugs of abuse (Adhikari et al., 2019; Engel et al., 2016; Renthal et al., 2009). Synapses are the communication points between neurons and between different areas of the brain. Insect synapses are remarkably similar to their mammalian counterparts at the molecular level (Ackermann et al., 2015). The machinery at the synapses of both mammals and insects that are involved in neurotransmitter release are both implicated in the actions of ethanol as well as other drugs of abuse, suggesting a conserved mechanism of action for ethanol in early plasticity development. Ethanol appears to hijack this communication, causing neurotransmitters to be released that create feedback loops that require increasing amounts of ethanol to be triggered again. In particular, ethanol decreases *Sirt1*, a histone deacetylase gene, in flies, which allows neurons to change the molecular makeup of their synapses (Engel et al., 2016). When these changes are prevented in the mushroom bodies, through genetic manipulation, ethanol tolerance does not develop (Engel et al., 2016).

The effect of ethanol on presynapses is an understudied area in the field and may be key for how ethanol alters brain function is crucial for gaining a complete understanding of how ethanol causes plasticity. At least 1 presynaptic protein, Unc13a, is known to effect ethanol behaviors and plasticity (Böhme et al., 2016; Reddy-Alla et al., 2017; Xu et al., 2018). It is also known that plasticity at presynaptic release sites can impact synaptic release probability (Christiansen et al., 2011; Delvendahl & Müller, 2019; Ehmann et al., 2018; Goel et al., 2019; Hige et al., 2015; Matkovic et al., 2013; Petzoldt et al., 2016; Reddy-Alla et al., 2017; Van Vactor & Sigrist, 2017). In particular, the protein Bruchpilot, which interacts with Unc13a, is key in regulating presynaptic plasticity (Matkovic et al., 2013; Petzoldt et al., 2016; Xu et al., 2018). It remains unknown what other presynaptic proteins are necessary for tolerance in the mushroom bodies and what the impact of ethanol on the structure of the presynapse.

To address this, presynaptic proteins known to be necessary for plasticity were investigated for their response to alcohol using confocal microscopy and behavioral experimentation. Presynaptic proteins were tagged with GFP/RFP reporters under control of the GAL4-UAS system in our wild-type white berlin flies and in mutant flies that are abnormal in tolerance development (*Sirt1, Hr38*). I began with proteins that indicate plasticity at the synaptic active zones (Bruchpilot). This allowed a model to be built for how alcohol changes presynapses at the molecular level. Flies expressing the marker Brp-short^{mstrawberry} (D3) in the beta lobes of the Mushroom Bodies (*17d-Gal4*) exposed to a ethanol show an increase in Brp puncta diameter, the magnitude of which increase scales with ethanol dose.

Results

Presynaptic Proteins are necessary for tolerance inside the Mushroom Bodies

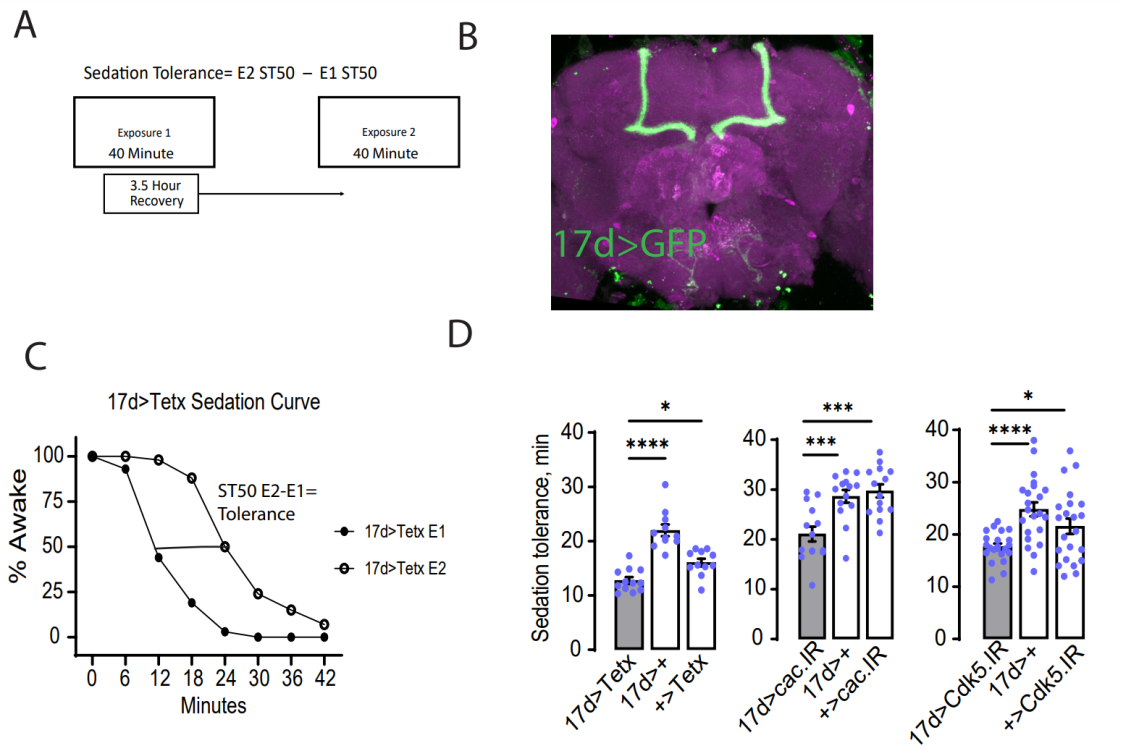


Figure 1- Presynaptic Proteins are necessary for tolerance in the Mushroom Bodies

A) EtOH exposure paradigm. Flies are exposed to EtOH vapor for 40 minutes and the ST50 is determined. Flies are rested for 3.5 hours and exposed a 2nd time. The difference between the 2nd ST50 and the 1st ST50 is the Sedation Tolerance time

B) 17d Gal4 driving UAS Myr::GFP

C) An representative examlple of sedation curves for 17d Gal4>UAS Tetx and controls. The difference between the curves is the Sedation Tolerance.

D) Silencing the kenyon cells of the mushroom bodies with UAS Tetx reduces tolerance. Presynaptic proteins Cdk5 and Cacophony are necessary in the mushroom bodies for tolerance.

It is known that the α/β lobes in the Mushroom Bodies are necessary for tolerance (Engel et al., 2016). It was also demonstrated that the presynaptic proteins Cacophony and Cdk5 may undergo plasticity during acute ethanol exposure under the control of the histone deacetylase *Sirt1* (Engel et al., 2016). In order to test the possible roles of Cacophony and Cdk5 I first confirmed that α/β lobe of the mushroom bodies signaling was necessary for tolerance development by expressing UAS Tetanus Toxin (*tetx*) using *17d-Gal4*. Flies were exposed to ethanol vapor using the boozemat (Wolf et al., 2002). In this assay flies were exposed to ethanol vapor for 40 minutes, during which time their righting reflex was measured every 10 minutes. Flies recovered for 4 hours and were re-exposed to ethanol (**Fig 1A**). The difference between the times to reach 50% sedation are calculated to

determine the sedation tolerance (**Fig 1C**). I found that while α/β lobe neurotransmission is necessary for tolerance development (**Fig 1D**), it is dispensable for the naïve ethanol response (**Sup Fig 1**). I next tested the role of different presynaptic proteins in tolerance development in the α/β lobes. These learning and memory centers are necessary for tolerance (Fig1D). Using Trip10 RNAi lines, I found that driving RNAi's against the presynaptic proteins Cdk5 (Nandi et al., 2017, p. 5) and Cacophony (Hidalgo et al., 2021; Hu et al., 2015) (the Ca^{2+} channel or cac) are necessary for tolerance (**Fig 1D**). Cdk5 is necessary for both naïve sensitivity (**Sup Fig 1**) and tolerance (**Fig 1D**), with RNAi against *Cdk5* (*Cdk5.IR*) expressing flies showing resistance to sedation during the flies' first exposure to ethanol and a reduced tolerance during the second exposure. *Cac.IR*, similar to the tetx results, only reduces tolerance development in these neurons (**Fig 1D**) and does not show an effect on naïve ethanol behavior (**Sup Fig 1**).

EtOH induces Plasticity in Presynapses

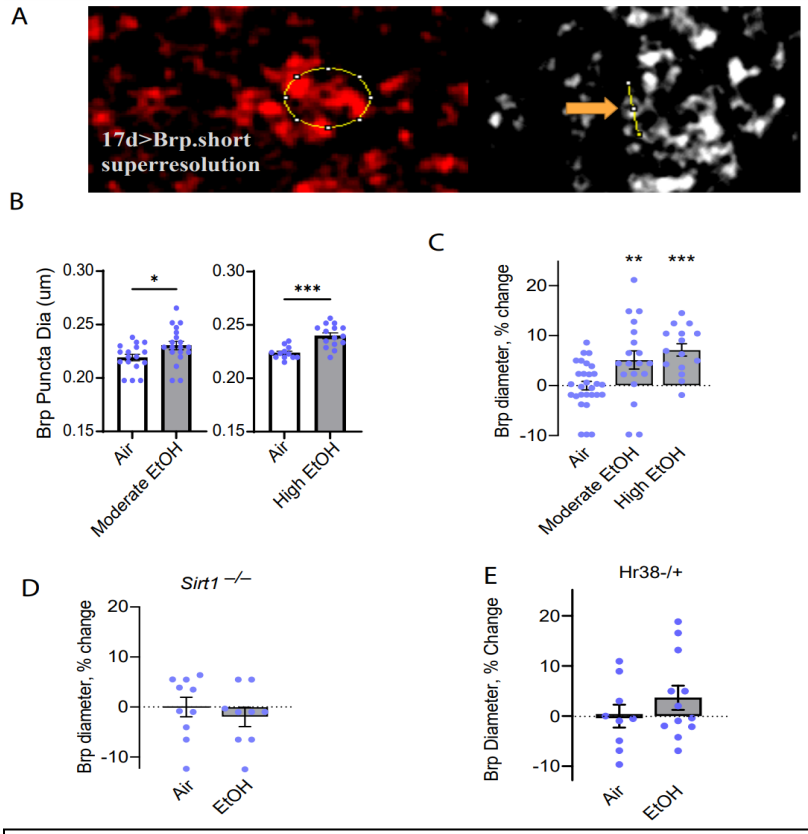


Figure 2 Ethanol Induces Plasticity in Brp Puncta

A) A superresolution image of a Brp ring in the 17d pattern with and without measuring in progress

B) Brp puncta increase in diameter 20 hours after ethanol exposure.

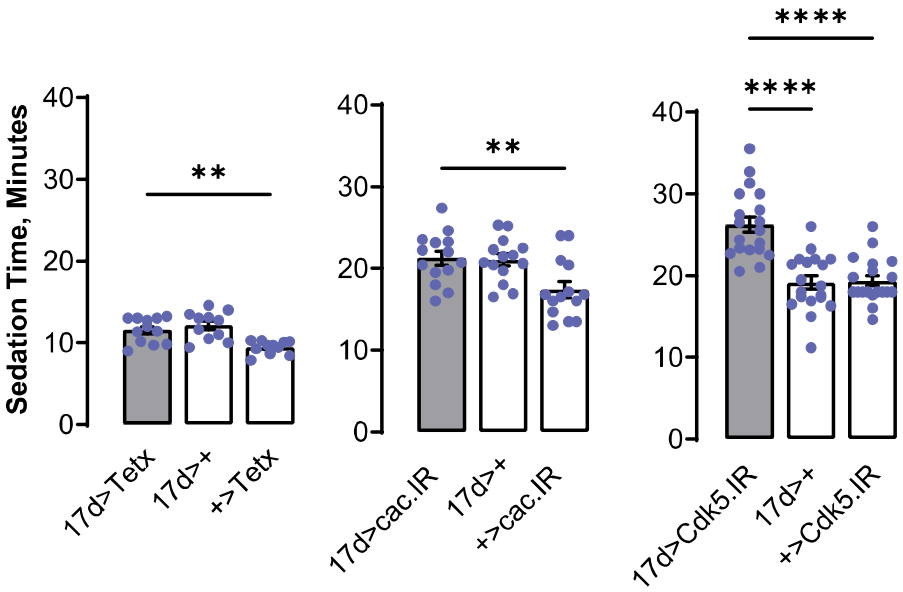
C) Brp puncta show a dose dependent increase in size 24 hours after ethanol exposure.

D) Sirt1 null mutants do not show Brp puncta size increases after ethanol exposure.

E) Hr38 dominant negative mutants show no changes in Brp puncta size after ethanol exposure.

Flies expressing the marker Brp-short^{mstrawberry}(D3) in the beta lobes of the Mushroom Bodies (*17d-Gal4*) exposed to a High Dose of 100/50 EtOH/Air show a change in Brp puncta diameter from 0.223µm (n=12) to 0.2394µm (n=15), an increase of 7.15% (**Fig 2B & 2C**). The magnitude of this increase scales with ethanol dose. A behaviorally relevant dose of 85/65 EtOH/Air increases puncta 5.15% (EtOH brains 0.2304µm, n=19 Control brains 0.2191µm n=17) (**Fig 2B & 2C**).

Importantly, ethanol's effect on puncta size is *Hr38* and *Sirt1*-dependent, suggesting that rapid tolerance is due in part to a unified signaling pathway for presynaptic remodeling. *Sirt1-* flies exposed to a High Dose (n=9) did not show any difference (-1.98%) from the controls (n=10) (**Fig 2D**). Flies heterozygous for the loss of function mutant of *Hr38* (y214) likewise exhibit reduced rapid tolerance (Adhikari et al., 2019) and no increase in puncta diameter (3.67% n=12) (**Fig 2E**). These data imply that ethanol-induced presynaptic plasticity is required for tolerance.



Supplementary Figure 1 Cdk5 is Necessary for Innate and Learned Tolerance in the Mushroom Bodies

17d>Tetx and 17d>cac.IR show no differences compared to controls during their first exposure to ethanol. 17d>Cdk5.IR shows a resistance compared to controls during the first ethanol exposure.

Discussion

In the mushroom bodies it is established that Kenyon cell activity is necessary for tolerance. However, the role of the presynapses in ethanol responses remains poorly defined. I have found that the presynaptic proteins Cacophony (Ca^{2+} channel) and Cdk5 are necessary for tolerance development in the mushroom bodies. Additionally, Cdk5 is necessary for the naïve response to ethanol, while the results of the *UAS cac.IR* and *UAS Tetx* expression reveal that synaptic activity may be dispensable for naïve ethanol response. This may indicate that the tolerance response in flies is driven by a plasticity in the presynapses.

Superresolution confocal microscopy imaging of the active zones in the Kenyon cells supports this conclusion. Within 20 hours of ethanol exposure Bruchpilot puncta (the fly homolog of mammalian ELKS/Cast) increase in size. This increase scales with dose in a nearly 1:1 ratio. This increase in puncta diameter indicates an increase in synaptic vesicle release probability (Christiansen et al., 2011; Delvendahl & Müller, 2019; Ehmann et al., 2018; Goel et al., 2019; Hige et al., 2015; Matkovic et al., 2013; Petzoldt et al., 2016; Reddy-Alla et al., 2017; Van Vactor & Sigrist, 2017). Brp is key in regulating presynaptic plasticity, and is likely responsible for the increase in Cacophony necessary for tolerance (Matkovic et al., 2013; Petzoldt et al., 2016; Xu et al., 2018), but it remains unclear what role Brp is playing in this ethanol-induced plasticity.

Additionally, in mutants shown to be deficient in tolerance (*Sirt1*- and *Hr38*-) the plasticity is ablated. This supports the idea that Sirt1 and Hr38 both function in ethanol tolerance by opening up access to the chromosome, allowing for increased protein translation (Adhikari et al., 2019; Engel et al., 2016). This, when taken with the behavioral data, strongly suggests that tolerance requires presynaptic activity in the Kenyon cells.

While phosphorylation of Brp is known to be necessary for plasticity (Driller et al., 2019), additional research is necessary to determine what role, if any, Cdk5 plays in the Brp plasticity seen in this study. Cdk5 has been implicated in multiple drugs of abuse (Allnutt et al., 2020, p. 5). Additionally, Cdk5 has been shown to be key to synaptic release and is responsible for regulating the reserve pool of synaptic vesicles (S. H. Kim & Ryan, 2010, p. 5).

Cdk5 has been shown to be potentiated by ethanol in cholinergic neurons in the nucleus accumbens in rats (Camp et al., 2006, p. 5) and appears to be a key regulator of neuroplasticity (Hernandez et al., 2016, p. 5; Liang et al., 2015, p. 5; Smith-Trunova et al., 2015, p. 5). Taken together these data indicate that Cdk5 mediated plasticity is necessary for ethanol tolerance, both naïve and learned.

Methods

Fly Lines Used

Flies were reared on food composed of molasses (9%), cornmeal (6.75%), yeast (1.7%), and agar (1.2%) food in Darwin Chamber incubators kept at 25°C and 60% humidity on a 16:8 hour light/dark cycle. All lines were outcrossed to the Berlin background genetic stock. 17d Gal4 (BDSC # 51631) was used to manipulate the α/β lobes of the mushroom bodies.

Reagent	*Source*	*Use*
17d Gal4	*Bloomington Drosophila Stock Center #51631*	
Brp-short^{mstraw}	*Gift from Sigrist? Mosca?*	
Unc13a::GFP	*Gift from Gregg Roman*	
UAS Tetx	*Bloomington Drosophila Stock Center*	
UAS Cdk5.IR	*Bloomington Drosophila Stock Center Trip Collection #27517*	
UAS cac.IR	*Bloomington Drosophila Stock Center Trip Collection #27244*	
Rabbit anti dsRed	*Clontech*	*1:500*
Chicken anti GFP	*Abcam*	*1:1000*
Discs Large	*DSHB*	*1:20*
NC82	*DSHB*	*1:20*
Goat anti-Rabbit 594	*Mol Probes*	*1:350*
Donkey anti Chicken FITC	*Pierce*	*1:350*
Goat anti mouse 488	*Mol Probes*	*1:350*
Goat anti mouse 594	*Mol Probes*	*1:350*

Exposure Assay

4-8 day old males were collected, stored in vials and allowed to recover from CO_2 for 24 hours. The next day flies were exposed to etoh vapor for 30 minutes and returned to food for 20-24 hours. Brains were dissected after the recovery and fixed overnight at 4° in 2% PFA. Brains were washed 5x in 0.5% PBT for 20 minutes each wash and blocked in high detergent block (HDB- 5% NGS, 0.5% BSA, 0.5% Triton-X) for 2 hours at room temperature. Brains were incubated in primary antibodies (in HDB) for 48-72 hours at room temperature. Brains were washed 4 times in PBT for 30 minutes each wash and 1 overnight wash at 4°. Brains were incubated in secondary antibodies (in HDB) for 24-48 hours at 4°. Brains were washed 4 times in PBT for 30 minutes each wash and 1 overnight wash at 4°. Brains were mounted in vectashield and imaged using a Zeiss 880 confocal microscope.

Presynaptic Release Site Measurement

Brp puncta were measured by imaging using a Zeiss 880 confocal microscope at 63x. Images were analyzed for complete puncta to be measured. Fiji was used to measure puncta diameter using the line tool. At least 6 puncta were measured per brain. The mean puncta diameter for each brain was calculated and used to calculate the mean puncta diameter per brain per condition. This was then normalized to the control brains that had not been exposed to ethanol to measure percent change.

Statistics

Statistics were generated on Prism 9.0 (Graphpad). Error Bars are SEM. Data is available upon request.

Chapter 2 Glutamatergic Neurons are necessary for tolerance outside of the mushroom bodies

Summary

In order to find tolerance neurons that lie outside the mushroom bodies I screened 107 Gal4 transgenic strains that express in the central brain to determine the effect of reducing presynaptic release on the development of rapid tolerance. Through this screen I identified a Gal4 line that expresses in glutamatergic tolerance neurons. This is the first time that a role for glutamatergic signaling, established fact in mammalian models, has be shown in ethanol plasticity in flies. Additional testing revealed these neurons are likely to be the DN1p glutamatergic neurons. These clock neurons are typically thought to be involved in morning and evening anticipatory behavior. This is the first time that clock neurons have been shown to reduce tolerance when knocked down and implies a role for anticipatory wakefulness in ethanol behavioral circuits.

Glutamatergic Neurons are necessary for tolerance outside of the mushroom bodies

Understanding the tolerance circuit will give insight into how ethanol alters presynaptic function and may indicate how other ethanol-induced behaviors are established at the synaptic level. While the mushroom bodies are well established as centers for ethanol behavior, little is known about what other regions of the brain are involved in tolerance. The ellipsoid body and fan shaped body have been implicated in other ethanol behaviors, but it remains unknown if these behaviors are encoded by the same neural circuit or different ones (Luan et al., 2006; Urizar et al., 2007).

To systematically discover the neural components responsible for the development of ethanol tolerance, I investigated what additional regions of the brain are necessary. 107 different Gal4 lines that express in the central brain were crossed to the RNAi strain *cac.IR*. This knocked down the calcium channel Cacophony, which was found to reduce tolerance when expressed in the mushroom body alpha/beta lobes using the *17d-Gal4*. Lines that showed comparable behavior to *17d-Gal4* were further screened against both *cac.IR* and Tetanus Toxin (*tetx*). After analysis 3 transgenic strains (50B06, 79H04, and 82F12) showed a significant difference from both controls, one of which (82F12) was found to have the tolerance phenotype recapitulated when driving *UAS dVGlut.IR*. To find the neurons in the 82F12 pattern that are responsible for the phenotype I tested all gal4 lines that are under the regulatory control of the *VGlut* locus against the dVGlut.ir transgenic strain. This ultimately led us to find that the DN1p clock neurons are necessary for ethanol tolerance, a finding that contradicts prior research on the role of clock neurons and ethanol tolerance (De Nobrega et al., 2017b, 2017a; De Nobrega & Lyons, 2016; Liao et al., 2016). This may mean that the DN1p tolerance phenotype is separate from these cells' typical role in maintaining morning/evening anticipatory behavior(Guo et al., 2017; Kunst et al., 2014; Lamaze et al., 2018) or it may mean that the DN1p's can only function in tolerance in an intact clock circuit.

Results

Presynaptic function is necessary for tolerance outside the Mushroom Bodies

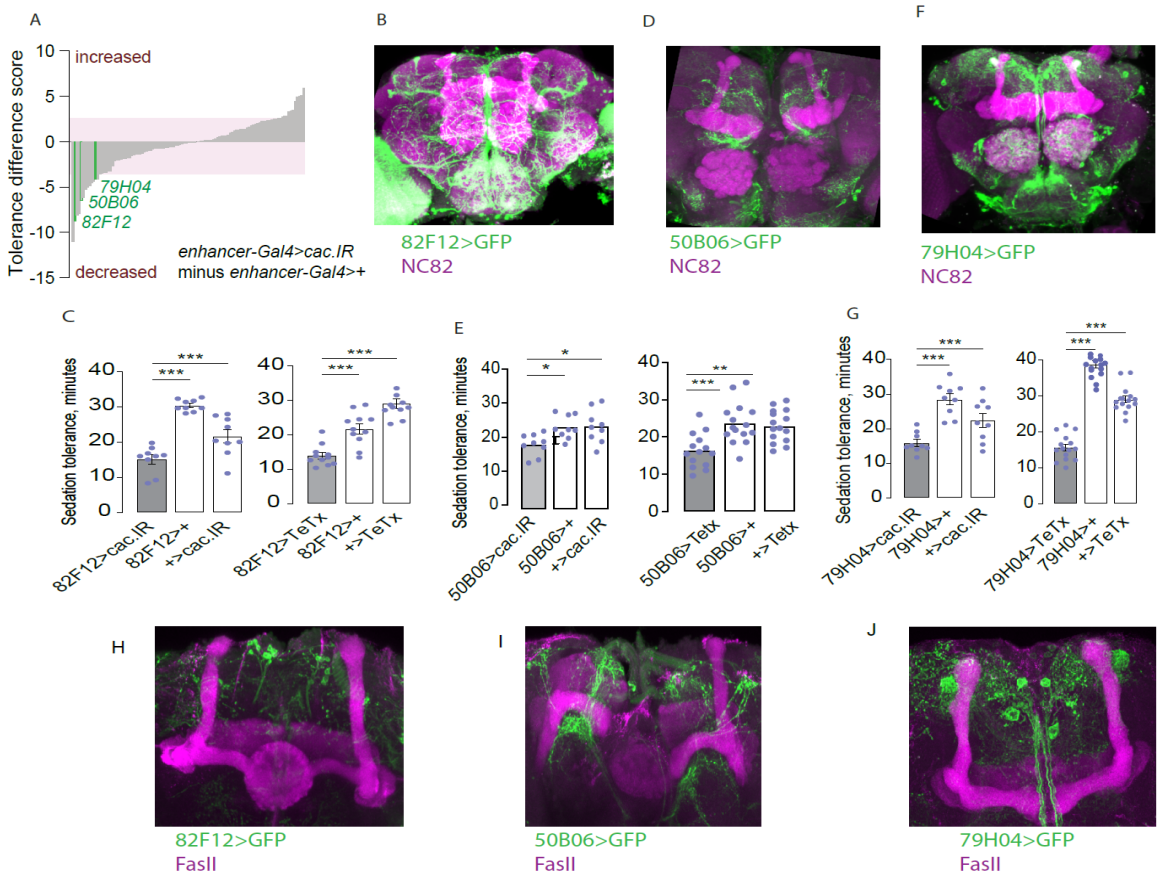


Figure 1- Presynaptic Function is Necessary for Tolerance Outside of the Mushroom Bodies

A) Unbiased screen of the central brain using UAS Cac.IR reavealed three lines, 82F12, 50B06, and 79H04 Gal4's that contain neurons necessary for tolerance.

B) 82F12 Gal4 driving UAS Myr::GFP

C) 82F12 Gal4 shows reduced tolerance when driving UAS Cac.IR and UAS Tetx

D) 50B06 Gal4 driving UAS Myr::GFP

E) 50B06 shows reduced tolerance when driving UAS Cac.IR and UAS Tetx

F) 79H04 gal4 driving UAS myr::GFP

G) 79H04 Gal4 shows reduced tolerance when driving UAS Cac.IR and UAS Tetx

To find tolerance neurons outside of the mushroom body I choose Gal4 transgenic strains that express in the central brain but not in the mushroom bodes and that already have splitgal4 pieces available. Lines were scored for sparseness and uniqueness and 107 were chosen (**Table 1**). These were crossed to UAS cac.IR to test for tolerance. Tolerance

change was measured by comparing Gal4>cac.IR to a negative control of either +>cac.IR or 17d>+. Lines were compared to the positive control of 17d Gal4>cac.IR. Any Gal4 line that developed a change in tolerance that fell within 1 standard deviation of the calculated tolerance difference shown by 17d>cac.IR (17d>cac.IR tolerance - +>cac.IR tolerance) was selected for further testing (**Fig 1A**).

This secondary screen consisted of both the experimental Gal4>+ (previously excluded) and +>cac.IR. Analysis revealed 5 transgenic strains (37G02, 50B06, 79H04, 82F12, and 92C03) had a significant decrease in tolerance. To confirm the phenotype these lines were tested a final time using UAS Tetanus toxin. Three lines (50B06, 79H04, 82F12) passed all three tests (**Fig 1A**).

The 3 strains were next imaged to determine what regions of the brain they expressed in by driving GFP. 82F12 expressed in a large number of neurons scattered across the brain, but with a particular concentration in the dorsal region (**Fig 1B**). 50B06 expressed in a small number of neurons, perhaps explaining its weaker phenotype (**Fig 1D**). 79H04 expressed strongly in both the dorsal region and the antenna lobes, and appeared to innervate the distal lobes of the α′ mushroom body (**Fig 1F**). To determine if any of the lines innervated the α/β neurons I imaged additional brains using the mushroom body specific stain FasII (**Figs 1H-J**). Only 79H04 showed any signal in the mushroom bodies, but no signal was detected in the α/β lobes (**Fig 1J**).

Screening for Neurotransmitters in Tolerance Strains

In comparing the three hits, 82F12 and 79H04 stood out as having the strongest phenotypes. Therefore only they were selected for further neurotransmitter screening. 82F12 was considered the strongest candidate because it has the most tools available (Gal4-AD, Gal4-dbd, and LexA).

Neurotransmitter screening of 82F12 quickly found tolerance phenotypes when knocking down the vesicular glutamate transporter VGlut (**Fig 2A**) and neuropeptide F(NPF) (**Fig 2A**). Tests using RNAi lines for NPF receptor (NPFR), vesicular GABA transporter (VGat), and Prohormone convertase required for neuropeptide processing gene Amontillado (Amon) revealed no phenotypes (**Fig 2A**). 79H04 showed no tolerance phenotype with any neurotransmitter specific RNAi (**Sup Fig 1**). The 82F12 VGlut phenotype was recapitulated using a short hairpin RNAi (**Fig 2A**), while the NPF phenotype was not. Therefore, VGlut was considered the stronger neurotransmitter candidate. Gal4 lines under regulatory control of both gene loci were obtained and screened for tolerance phenotypes using the respective neurotransmitter RNAi.

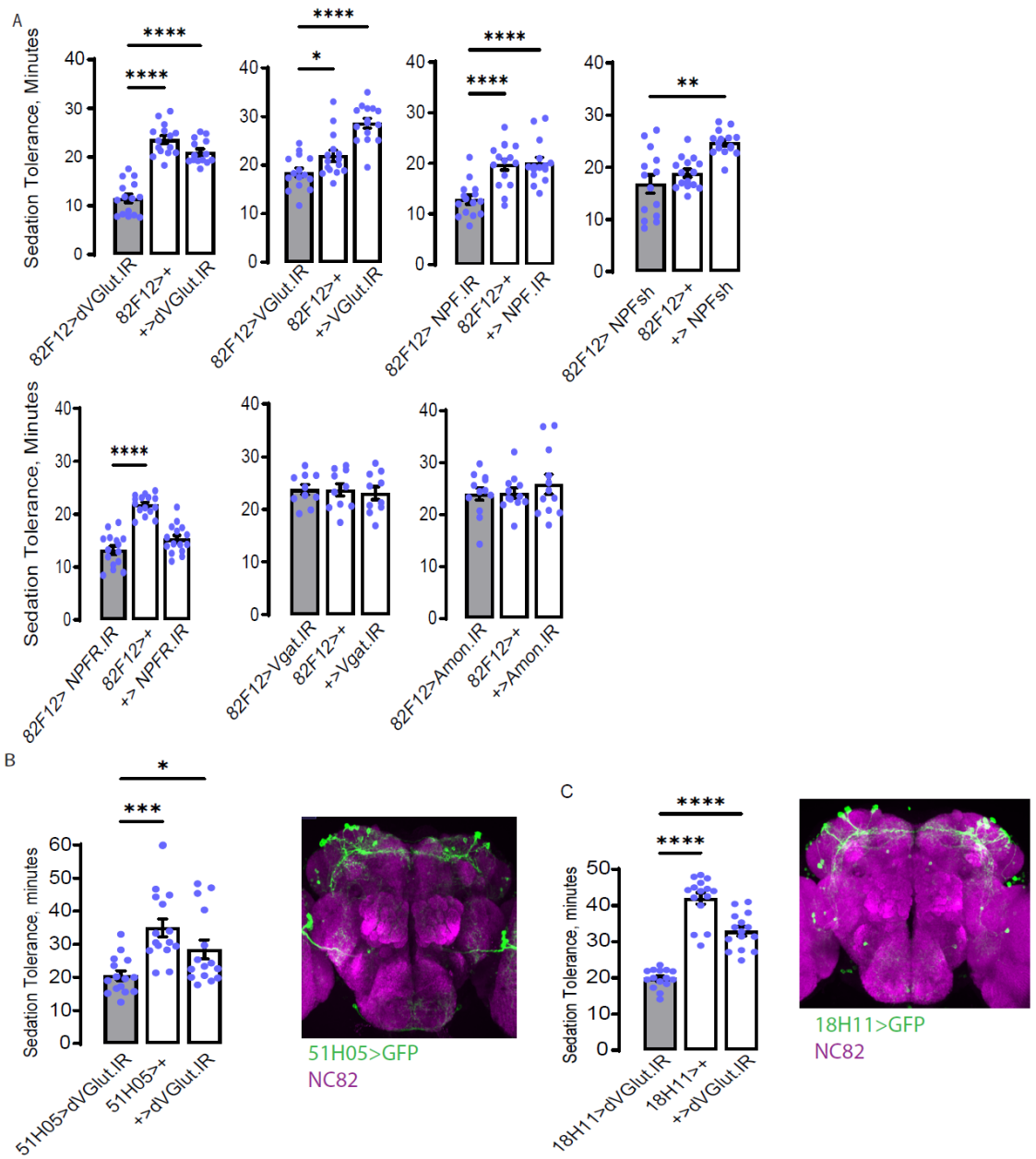


Figure 2- Glutamatergic Neurons Are Necessary for Tolerance in 82F12

A) A Neurotransmitter screen of 82F12 Gal4 reveals that glutamatergic neurons are necessary for the tolerance phenotype. NPF RNAi lines showed conflicting results for a tolerance phenotype. No other neurotransmitters were found to be necessary for the tolerance phenotype.

B) An investigation of Gal4 lines under regulatory control of vGlut showed that 51H05 Gal4 also contains glutamatergic neurons necessary for tolerance (image from Janelia).

C) 18H11 demonstrates reduced tolerance similar to 82F12 (image from Janelia).

Screening VGlut Gal4 Strains

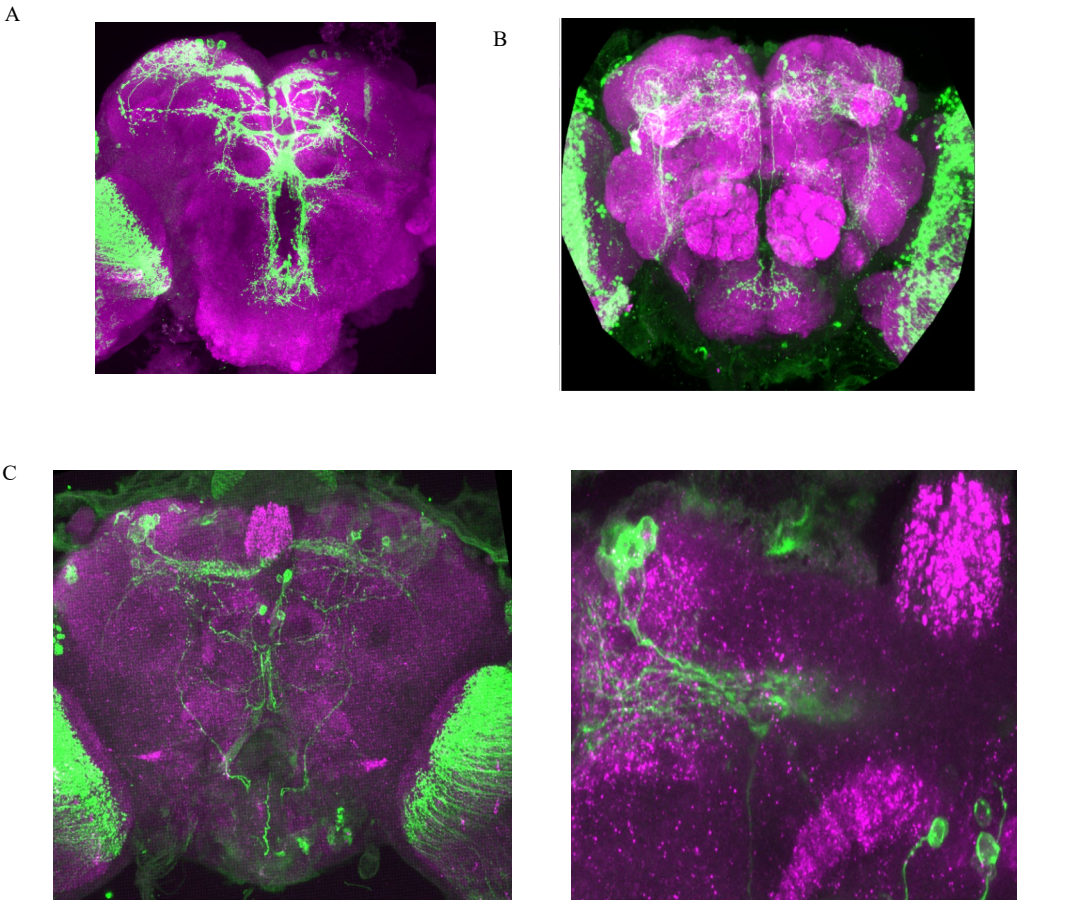


Figure 3- Investigating Shared Neurons between Glutamatergic Tolerance Lines

A) 82F12-AD∩51H05-dbd>GFP with NC82 counterstain

B) 82F12-AD∩18H11-dbd>GFP with NC82 counterstain

C) 82F12-AD∩51H05-dbd>GFP with vGlut counterstain with dorsal neurons showing overlap in GFP and vGlut (magenta) signal (white signal in cell body)

Because UAS dVGlut.IR (2689) showed a greater effect on tolerance in 82F12 Gal4, this line was chosen to look for additional Gal4 lines that may contain glutamatergic tolerance neurons. Upon investigating Gal4 lines under regulatory control of VGlut it was found that 51h05 Gal4 has both naïve resistance to and reduced tolerance when driving *dVGlut.IR* (**Fig 2B**). It has been used almost exclusively in circadian rhythm literature. It contains glutamatergic clock neurons (DN1p) (Andreani et al., 2021; Guo et al., 2017). It is unknown if other glutamatergic neurons on in the pattern. In order to determine if the 82F12 and 51H05 phenotypes are under control of the same neurons a split-Gal4 combination of 82F12-AD and 51H05-dbd was used to express myr::GFP. Approximately 12 neurons were found to be shared, including neurons in the region of the DN1p's (**Fig 3A**). Additional

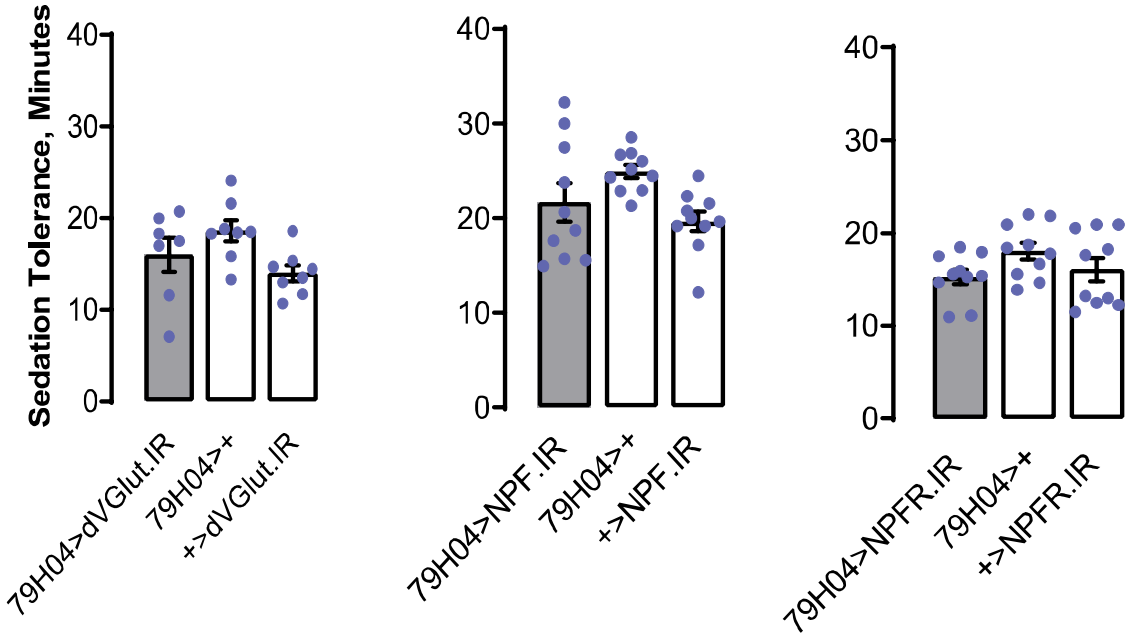
brains were imaged using a VGlut counterstain and the dorsal neurons in the 82F12-ADՈ51H05-dbd contained VGlut.

In order to investigate the possibility that DN1p neurons are responsible for the tolerance shown in 82F12 and 51H05 a second DN1p line was tested. 18H11 Gal4, which has been shown to contain glutamatergic DN1p neurons and to share DN1p neurons with 51H05 recapitulates the phenotypes seen in 82F12 (Andreani et al., 2021) (Fig 2C). Naïve 18H11 Gal4 flies expressing VGlut RNAi are resistant to ethanol's sedating effects. 18H11 flies are also deficient in tolerance development compared to 18H11 controls and RNAi controls. These data strongly support the role of DN1p neurons in ethanol sedation, including rapid tolerance development. 18H11 also shares neurons with 82F12, notably in the dorsal region of the brain (**Fig 3B**).

Table 1

49057	40338	48203	50290	48378	48333
40034	40607	39255	41227	49021	48087
39865	50210	39502	38728	48981	48108
50029	49998	39289	48312	47292	48916
40093	50377	49951	48395	38764	49074
40053	49539	40033	49067	48155	49087
39864	49613	38842	48982	39458	
39100	50044	41229	49059	49486	
40582	49766	39145	49101	50137	
48150	49447	38749	48079	49717	
49965	49967	41784	49125	49983	
49361	49660	41275	48127	49483	
49664	50047	48103	49172	38856	
50071	50317	40361	50385	40684	
49958	41289	38760	40409	49571	
50211	41267	48191	39522	49167	
49908	49008	49744	48971	49956	
39311	49863	49163	49072	41309	
48356	39939	50025	49394	39577	
40913	48099	49962	49120	49080	

Gal4 strains that were tested against *UAS cac.IR* for tolerance



Supplementary Figure 1 79H04 Gal4 Shows no VGlut or NPF Tolerance Phenotypes

79H04 Gal4 shows no tolerance phenotype when driving RNAi's against VGlut, NPF, or NPFR

Discussion

Here I have shown that glutamatergic neurons outside of the mushroom bodies are necessary for both naïve and learned behavior to ethanol. Three lines have shown a naïve resistance to the sedating effects of ethanol when *VGlut* is knocked down. These same lines have also expressed reduced tolerance development. Based on the known glutamatergic neurons in the lines 51H05 and 18H11, I strongly suspect that the DN1p neurons play a role in these phenotypes. Very little is known about the neurons expressed in the 82F12 Gal4 pattern. I can state that the line contains glutamatergic neurons and that the firing of these neurons is necessary for normal responses during ethanol exposure. 82F12 appears the express in the DN1p neurons, but the fact that 82F12 has a stronger phenotype than either 51H05 or 18H11 implies that 82F12 contains additional glutamatergic tolerance neurons outside of the DN1p's. If and how these newly discovered glutamatergic tolerance neurons communicate with the mushroom bodies remains unknown. Glutamatergic DN1p's are currently thought to communicate with the R2/R4 neurons of the ellipsoid body via the lateral bulb (Díaz et al., 2019). This may provide a connection to the altered coordination measured in our tolerance assay (loss of righting reflex), and the R2/R4 neurons have been implicated in tolerance before (Urizar et al., 2007).

These data may contradict prior research on the role of clock neurons and ethanol tolerance (De Nobrega et al., 2017b, 2017a; De Nobrega & Lyons, 2016; Liao et al., 2016). This may mean that the DN1p tolerance phenotype is separate from these cells' typical role in maintaining morning/evening anticipatory behavior (Guo et al., 2017; Kunst et al.,

2014; Lamaze et al., 2018) or it may mean that the DN1p's can only function in tolerance in an intact clock circuit. Future work will focus on determining if the neurons shared between these lines are indeed the glutamatergic tolerance neurons. I can also knock down the signaling molecule of the 18H11 DN1p neurons, DH31, (Kunst et al., 2014) to further test the DN1p neurons' role in tolerance.

Methods

Fly Lines Used

Flies were reared on food composed of molasses (9%), cornmeal (6.75%), yeast (1.7%), and agar (1.2%) food in Darwin Chamber incubators kept at 25°C and 60% humidity on a 16:8 hour light/dark cycle. All lines were outcrossed to the Berlin background genetic stock. *Cite uses of RNAi lines and make table of fly lines used. 17d Gal4 (BDSC # 51631) was used to manipulate the α/β lobes of the mushroom bodies.*

Lines Used

Reagent	*Source*	*Use*
17d Gal4	*Bloomington Drosophila Stock Center #51631*	
82F12 Gal4	*Bloomington Drosophila Stock Center #40338*	
79H04 Gal4	*Bloomington Drosophila Stock Center #40053*	
50B06 Gal4	*Bloomington Drosophila Stock Center #38728*	
Other Screen Gal4's	*Bloomington Drosophila Stock Center (see Table 1)*	
51H05 Gal4	*Bloomington Drosophila Stock Center #41275*	
52A01 Gal4	*Bloomington Drosophila Stock Center #47634*	
18H11 Gal4	*Bloomington Drosophila Stock Center #48832*	
UAS Tetx	*Bloomington Drosophila Stock Center*	
UAS cac.IR	*Bloomington Drosophila Stock Center Trip Collection #27244*	
UAS dVGlut.IR	*Bloomington Drosophila Stock Center Trip Collection #27538*	
UAS VGlut.IR	*Bloomington Drosophila Stock Center Trip Collection #40845*	
UAS VGAT.IR	*Vienna Drosophila Resource Center #45916*	
UAS Amon.IR	*Bloomington Drosophila Stock Center Trip Collection # 28583*	
UAS NPF.IR	*Bloomington Drosophila Stock Center Trip Collection #27237*	

UAS NPFsh	*Vienna Drosophila Resource Center #32069*	
UAS NPFR.IR	*Gift from Galit*	
Rabbit anti dsRed	*Clontech*	*1:500*
Chicken anti GFP	*Abcam*	*1:1000*
Discs Large	*DSHB*	*1:20*
NC82	*DSHB*	*1:20*
Goat anti-Rabbit 594	*Mol Probes*	*1:350*
Donkey anti Chicken FITC	*Pierce*	*1:350*
Goat anti mouse 488	*Mol Probes*	*1:350*
Goat anti mouse 594	*Mol Probes*	*1:350*

Behavioral Assay

4-6 day old males are collected, stored in vials (20-25 flies per vial) and returned to incubators at least 24 hours before etoh exposure. On the day of exposure flies are removed from incubators and placed in the boozemat tubes (Wolf et al., 2002). Flies are allowed to acclimate to the boozemat for 7 minutes while water vapor is run through the tubes. After the acclimation period the flow is switched to a 54% etoh/water vapor solution. The tubes are spun every 10 minutes to measure the flies' righting reflex for 40 minutes. The time to 50% sedation (ST50) is calculated for the 1st exposure (E1). The flies are allowed to rest in their vials while they metabolize the etoh. 4 hours after the beginning of the 1st exposure the flies are exposed a 2nd time. The difference between the 50% sedation times are calculated (E2-E1) to determine the sedation tolerance (Sed Tol).

Choosing Fly Lines

In order to find Gal4 lines that showed a tolerance phenotype a series of screens were performed. A comprehensive list of Gal4 lines that also had splitgal4 counterparts was compiled. The Janelia images of these lines were scored for presence in the central brain, sparseness, and uniqueness. The top (107) lines were tested in the primary screen. The primary screen consisted of the experimental (Gal4>cac.ir) lines and a positive (17d gal4>cac.ir) and negative (either 17d>+ or +>cac.ir). For each experiment, the sed tol of the negative control was subtracted from the sed tol of the positive control (so that a reduction in tolerance gave a negative number) to create a tolerance difference. The experimentals were treated likewise and their tolerance differences were compared to those of the control. If the experimental line had a tolerance difference that fell less than 1 SD away from the control it was chosen for further evaluation.

These lines (20) were tested in a secondary screen with full experiments and no positive control. To expedite testing 4 gal4 lines were tested during each experiment. An equal number of Gal4>cac.ir and Gal4>+ vials were collected and double that number were collected for the +>cac.ir negative control. When results were analyzed half of the +>cac.ir vials were eliminated to bring the n down to the Gal4>cac.ir using the random number generator feature of R. The 5 Gal4 lines that showed a tolerance phenotype were retested against UAS-Tetx as a confirmation. 3 lines (50B06, 79H04, and 82F12) still showed a tolerance phenotype. These 3 new tolerance gal4's were entered into a new screen to investigate what neurotransmitters were responsible for the phenotype.

Statistics

Statistics were generated on Prism 9.0 (Graphpad). Error Bars are SEM. Data is available upon request.

Chapter 3 Conclusions

Conclusions

My work has demonstrated that ethanol can induce plasticity in the active zones of synapses in the β lobes of the mushroom bodies, likely indicating a potentiation of some sort in these neurons. Further research is necessary to determine in these changes are regionally segregated to specific lobes or are more widespread. Additionally, the possibility of Cdk5 interacting with Brp should be investigated, as Cdk5 is necessary for both naïve and learned tolerance.

The addition of DN1p clock neurons to the mushroom bodies as neurons necessary for tolerance opens a range of possible questions. Do DN1p presynapses respond to ethanol in a similar manner to Kenyon cells? Is there any relationship between presynaptic response to ethanol and the identity of the postsynaptic partner? Are the DN1p neurons involved in other ethanol behaviors? More work is needed to answer these questions.

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