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Ethanol Dependence in Drosophila Larvae

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Ethanol Dependence in Drosophila Larvae

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Ethanol Dependence in Drosophila Larvae

Brooks Gregory Robinson, Ph.D.

The University of Texas at Austin, 2013

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Addiction to alcohol is a disease of changed behavior that is uniquely human in it's complexity. Because of this, researchers have strived to develop animal models of individual endophenotypes of alcoholism in hopes that the larger picture will eventually come into focus. Recent studies in Drosophila have shown that many complex alcohol-related behaviors are conserved in this genetic model system. The series of projects presented in this dissertation outline the first account of physiological ethanol dependence in Drosophila. We first show that Drosophila larvae are able to form conditioned associations between an aversive heat stimulus and an attractive odor. We then show that an acute, low-dose ethanol exposure disrupts this learning ability. Finally, we present data

that demonstrate that larvae adapt to the presence of chronic ethanol to the point that they only perform normally in the learning assay when ethanol is present in the animal. We then propose that the major mechanism for this dependence involves ethanol regulating the acetylation level and therefore expression level of a large number of genes by inhibiting histone deacetylase enzymes. These experiments set the groundwork for the analysis of a network of genes, connected through interactions with histone deacetylase enzymes, that are involved in producing ethanol dependence.

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Chapter 1. General Introduction

IS ALCOHOLISM LEARNED? INSIGHTS FROM THE FRUIT FLY

The majority of text and the figures in this section of the introduction have been published as a review article in 2013 in the journal *Current Opinions in Neurobiology:*

Robinson, B. G., and Atkinson, N. S. (2013). Is alcoholism learned? Insights from the fruit fly. Curr Opin Neurobiol http://dx.doi.org/10.1016/j.conb.2013.01.016. [Epub ahead of print]

Co-author contributions: N.S. Atkinson is my P.I. and helped write this review.

Abstract

Alcohol addiction is a complex, uniquely human disease. Breaking addiction down into contributing endophenotypes enables it to be studied in a variety of model systems. In the past, the *Drosophila melanogaster* genetic model system has been used primarily to study alcohol sensitivity, tolerance, and physiological alcohol dependence. However, these endophenotypes cannot account for the near-permanent quality of the addicted state. It has been recently discussed that addictive drugs may hijack the learning-and-memory machinery to produce this persistent change in behavior. The learning and memory machinery is one facet of the emergent state that we call cognition. Because learning and memory is amenable to experimental study, it may provide us with a window into how alcohol affects higher-order mental functions. Changes in the higher-order cognitive functioning of the nervous system likely

directly contribute to compulsive drug use. The mechanisms of learning and memory show strong conservation between invertebrates and mammals. Here, we review the Drosophila literature, which links complex alcohol-related behaviors to learning and memory. Genetic analysis in Drosophila can provide a distinct view into how alcohol addicts.

Introduction

Alcoholism is a serious health concern worldwide. In the United States, almost 4% of the population meet the criteria for alcohol addiction, and alcohol-related problems are estimated to cost more than 223 billion dollars per year (Bouchery et al., 2011; Grant et al., 2004). Unfortunately, the success rate of treatment is dismal. During the first year of treatment, two-thirds of individuals have bouts of heavy drinking (Miller et al., 2001), while the best three year average shows ~25% rate of recidivism (Dawson et al., 2007). Rational treatment of alcoholism is dependent on a clear understanding of the mechanics of alcohol addiction.

Addiction to alcohol involves changes that are understandable at the single cell level and also changes that are clearly emergent properties of complex networks of many neurons. In the clinical diagnosis of alcohol dependence (a.k.a. alcohol addiction, alcoholism), an individual is expected to exhibit at least three of seven criteria (DSM-IV-TR, 2000). Two criteria, tolerance and withdrawal symptoms, are clearly rooted in cellular adaptations to ethanol. The five remaining diagnostic attributes include compulsive ethanol consumption, obsessive desire for alcohol, spending too much time pursuing alcohol, neglecting social, recreational, or occupational activities,

and continued alcohol use in spite of accumulating negative consequences. These latter five groups are clearly complex changes in behavior and are probably all emergent properties of a dysfunctional nervous system.

Behavioral responses to ethanol are highly conserved. In mammals and invertebrates, ethanol intoxication proceeds from stimulation to incoordination to sedation with increased dose. These can be followed by the appearance of functional-ethanol tolerance and physiological dependence. Ethanol tolerance is inducible ethanol resistance and in humans includes metabolic (pharmacokinetic) tolerance and functional (pharmacodynamic) tolerance. Functional tolerance of the nervous system is the earliest recognized neuronal plasticity change produced by ethanol. The cellular changes underlying functional tolerance have long been thought to overlap with the changes that produce withdrawal symptoms (Himmelsbach, 1941). Symptoms of withdrawal are indicative of physiological dependence (Koob and Le Moal, 2006). In Drosophila, a form of rapid ethanol tolerance and an ethanol withdrawal hyperexcitability phenotype have both been shown to share a common genetic basis - the involvement of the *slo* gene, which encodes the BK-type Ca^{2+}-activated K^{+} channel (Ghezzi et al., 2012).

The purpose of this section of the introduction is to recap recent developments that demonstrate that the Drosophila model system and mammals share some of the higher-order ethanol responses that are linked to alcohol addiction. In general, genetic analysis in Drosophila is more advanced than in mammals. However, the primary value of this model system lies in the fact that Drosophila studies are exponentially

cheaper and faster than genetic manipulation of mammals. Between Drosophila and mammals there is a strong and meaningful evolutionary concordance among the genes that underlie cellular activities of the nervous system. However, Drosophila and mammals show poor conservation of brain structures and neural circuitry. This suggests that the conservation of ethanol responses between Drosophila and humans arises because ethanol disrupts evolutionarily ancient attributes of neurons that are capable of adaptation.

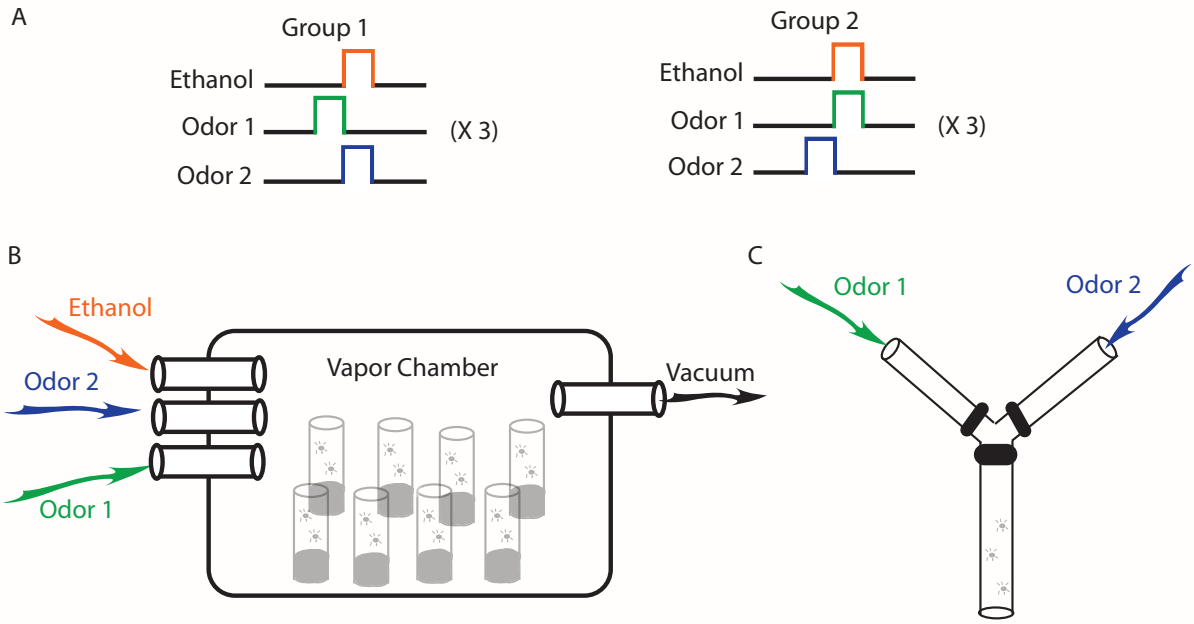
Alcohol Reward Learning in the Adult Fly

It has been proposed that addiction is a type of pathological associative memory that is produced by the over-activation of a reward pathway (Hyman et al., 2006). The capacity to learn and remember are functionally and mechanistically conserved phenomena in the Animalia. The learning-addiction link in Drosophila is supported by a recent study in adult flies on the rewarding properties of ethanol. In this study (Figure 1.1), flies were able to form associations between an odor (CS) and an intoxicating level of ethanol vapor (US, internal ethanol ~6 mM). Following training, flies preferentially moved into a compartment that included the conditioned odor. This assay mimicked aspects of the conditioned place preference (CPP) assay commonly used to study rewarding drugs in rodents. Additionally, the expression of conditioned odor preference in Drosophila was dependent on dopamine signaling in the fly brain (Kaun et al., 2011). The importance of dopamine signaling is a recurrent theme in addiction literature across many species. Here we also see another reccuring theme in the Drosophila alcohol literature—that the mushroom bodies, the brain structure most

tightly associated with learning and memory in flies, is of critical importance in forming the memory of the association between an ethanol "reward" and a specific odor. Blocking mushroom-body signaling blocks the retrieval of this memory.

The rewarding properties of ethanol were further examined by evaluating how ethanol reward relates to, and whether it is represented internally in the same way as, another natural reward--sex (Shohat-Ophir et al., 2012). In this study, reward appears to be encoded as elevated neuropeptide F (NPF) signaling. NPF is the fly homolog of mammalian neuropeptide Y, which has been linked to reward and ethanol behaviors in mammals (Josselyn and Beninger, 1993; Thiele et al., 1998). In flies, increased NPF signaling (produced by sexual satiation or transgenic overexpression) decreases the salience of an ethanol reward, while a deficit in NPF signaling (produced by sexual rejection or transgenic RNAi expression) increases the drive for other rewards, such as ethanol.

Figure 1.1: Adult Drosophila can associate the rewarding aspects of ethanol intoxication with an odor. A) In group 1, flies are exposed to two odors, one of which is delivered in the presence of an intoxicating dose of ethanol vapor. In group 2, the odor that is paired with ethanol is switched. B) Simultaneous training of many vials of flies can be performed in a single vapor chamber. C) Twenty four hours following three training sessions, when placed at the base of a T- or Y-maze, flies chose the odor paired with the ethanol over the unpaired odor (Kaun et al., 2011).



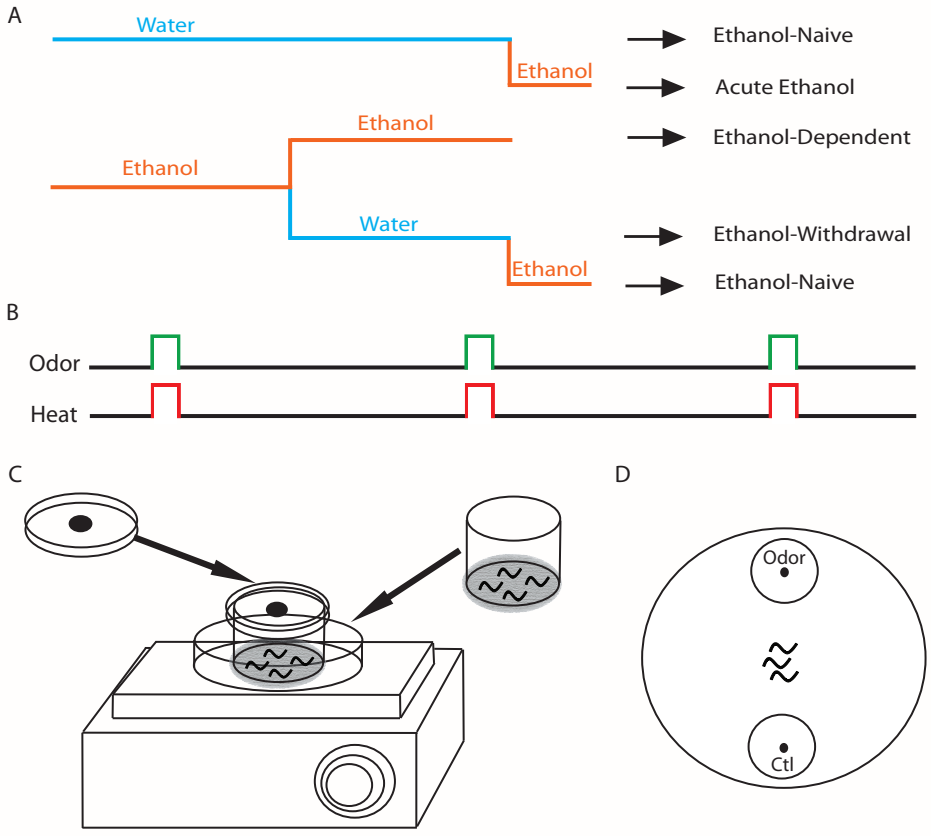
Alcohol-Adapted Larvae show Cognitive Dependence

In a recent study (Robinson et al., 2012b), we established the third instar larva of *Drosophila melanogaster* as an animal model for exploring the relationship between small doses of ethanol and associative learning. In this assay, an otherwise attractive odor (CS) is paired with a heat pulse (US) in three training trials over a 30 minute period. Untrained larvae will crawl to a spot of odorant in a petri dish. However, trained animals have learned to be repulsed by the odor and most of them avoid the odorant. It was shown that an internal ethanol concentration of ~7 mM ethanol, which did not affect heat sensitivity, odor sensitivity, or locomotion, would nevertheless disrupt this type of learning. These results are consistent with the idea that higher-order neural activites that are dependent on extensive neuronal inter-connectivity, such as learning and memory, are more sensitive to the effects of ethanol than are the simpler neural functions underlying sensory input and motor activity.

Because we could isolate the effect of ethanol on learning and memory, we used this system to model cognitive ethanol tolerance and dependence (Robinson et al., 2012a). Drosophila larvae eat continuously, and they treat ethanol-laced food as palat-able. As expected, when larvae consume 5% ethanol food for 1 hour, their capacity to learn plummets. However, chronically consuming ethanol food over a five-day period causes them to adapt to the point that the magnitude of learning is equivalent to that of ethanol-naive animals. This adaptation is chronic tolerance. In the ethanol-adapted larvae, it is the withholding of ethanol that impairs learning, while the capacity to learn is restored by ethanol reinstatement. Thus, chronic ethanol consumption has

made the animals functionally dependent on ethanol for normal cognition. These effects occurred in larvae with internal ethanol concentrations equivalent to 0.05 to 0.08 BAC (10-17 mM). In a human, this level would be mildly intoxicating. Alcohol addiction is a disease of complex changes in behavior. The adaptations that affect larval learning might, in a human, contribute to cognitive changes that promote uncontrollable drinking. Thus, a mechanistic description of the changes produced by chronic ethanol in larvae is an important goal.

Figure 1.2: Larval Drosophila that chronically feed on food containing ethanol become functionally dependent on the drug. A) The larval ethanol and control treatment schedule results in 5 separate groups. The control group is naive to ethanol. An acute ethanol group receives a 1 hour ethanol treatment. A chronically treated ethanol group receives ethanol continuously for 6 days. A withdrawal group receives a chronic ethanol treatment followed by a 6-hour ethanol abstention. The ethanol reinstatement group receives the withdrawal treatment followed by a subsequent 1-hour ethanol treatment. B) All of these groups were then trained with three rounds of 42°C heat shock-odor pairing to induce associative conditioning. C) Larvae are placed in a transfer chamber with a mesh bottom for training. The chamber is placed on a heated petri dish and covered with a plastic cap spotted with odor for an associative conditioning trial. D) Following the training, larvae were placed in the middle of an agar plate with the odor on one side and a control on the other to measure the level of attraction to the odor. Animals that have learned to associate the odor with the unpleasant heat treatment will avoid the odor zone, while animals that fail to learn will move into the odor zone (Khurana et al., 2012).



Mutations in Learning Genes Disrupt Alcohol-Related Behaviors

Mutation studies have provided further evidence for a linkage between learning and memory genes and addiction. A long list of memory genes have been associated with alcohol sensitivity, functional tolerance, conditioned place preference, and drinking in flies (Table 1). One striking recent addition is a mutant allele of the Drosophila *discs large 1* (*dlg1*) gene. The *dlg1* gene encodes two proteins—DlgA and DlgS97. The human homolog of DlgA is the PSD-95 synaptic scaffolding protein and the DlgS97 product most closely resembles human SAP97. Maiya *et al*. (Maiya et al., 2012) identified a new mutant allele of *dlg1,* generated by P element mutagenesis, based on a reduced capacity of the mutant to display rapid ethanol tolerance. This allele, called $dlg1^{intol}$, eliminates expression of the DlgS97 splice variant. The SAP97, NMDAR, and CASK proteins have all been shown to interact and to have roles in learning and memory, long-term potentiation (LTP), or long-term depression (LTD) (Hodge et al., 2006; Xu, 2011). DlgS97 protein was shown to co-immunoprecipitate with the fly NMDA receptors and is also thought to bind the Caki/Camguk protein (homolog of human CASK). In flies, mutations in any one of these genes impede the production of ethanol rapid tolerance. This study also showed that mutant analysis of the role of DlgS97 in an ethanol response was predictive of the consequence of a reduction in SAP97 expression in mice. As for DlgS97 in flies, a loss of SAP97 expression in mice caused the mice to be unable to acquire rapid ethanol tolerance in a loss-of-righting-reflex assay.

Table 1.1: Drosophila genes and their proteins that have been implicated in both alcohol-related behaviors and learning and memory. The abbreviations in the fourth column indicate whether the mutation alters ethanol sensitivity (S), tolerance, (T), conditioned place preference-like responses (CPP), and/or ethanol drinking (D).

Gene	Protein	Function	Phenotype
amn^{cheapdate}(Moore et al., 1998)	PACAP	cAMP Pathway	S
aru(Eddison et al., 2011)	Eps8	EGFR Pathway	S
dco(Rodan et al., 2002)	PKA	Kinase	S
dlg1(Maiya et al., 2012)	PSD-95/SAP97	Synaptic Scaffolding	T
DopR(Kong et al., 2010b)	DA D1 Receptor	Dopamine Signaling	S
Egfr(Corl et al., 2009)	EGFR	EGFR Pathway	S
exba(Berger et al., 2008; Devineni and Heberlein, 2009)	Initiation Factor 5C	Translation Regulation	S, T, D
fas2(Cheng et al., 2001)	Fasciclin 2	Cell Adhesion	S
homer(Urizar et al., 2007)	Homer	Scaffolding	S, T
KCNQ(Cavaliere et al., 2012)	KCNQ	Synaptic Transmission	S, T
klg^{ruslan}(Berger et al., 2008)	Klg	Axon Guidance	T
Nmdar1(Kaun et al., 2011)	dNR1	Synaptic Transmission	DPP
npf(Shohat-Ophir et al., 2012; Wen et al., 2005)	NPF	Neuropeptide Signaling	S, CPP
pum$^{milord-1}$(Berger et al., 2008)	Pum	Translation Regulation	T
pxb^{baika}(Berger et al., 2008)	Pxb	Axon Guidance	T
RhoGAP18B(Rothenfluh et al., 2006)	RhoGAP18B	Rho GTPase Regulation	S
rho^{iks}(Berger et al., 2008)	Rho	Developmental	T
rut(Moore et al., 1998)	Adenylate Cyclase	cAMP Pathway	S
sca(Shohat-Ophir et al., 2012)	Notch Pathway	Notch Pathway	CPP
scb, mys(Bhandari et al., 2009)	α, β Integrin	Cell Adhesion	T
Sir2(Kong et al., 2010a)	Sir2	HDAC Activity	S, T
Syn(Godenschwege et al., 2004)	Synapsin	Presynaptic Scaffolding	T
Tbh(Scholz et al., 2000)	Octopamine	Oct. Signaling	T
TH(Bainton et al., 2000; Kaun et al., 2011)	Dopamine	Dopamine Signaling	S, CPP

Thoughts and Conclusions

One particularly vexing aspect of addiction is the persistent nature of the disease. The addicted state persists beyond the period of functional tolerance, physiological dependence, and the manifestation of withdrawal symptoms that are precipitated by abstention. The idea that addictive drugs co-opt the learning-and-memory machinery to produce the long-lasting addictive state is attractive. Addiction has been proposed to represent maladaptive associative learning, in which the drug hyperactivates brain reward pathways and results in overlearning that rapidly transitions from mild associative conditioning to habit (Hyman et al., 2006). However, there exists a generally recognized contradiction. The negative effect of ethanol on learning has been well documented in both hippocampal and cortical LTP studies and behavioral assays (Matthews and Silvers, 2004; McCool, 2011). Therefore, how can ethanol result in overlearning to the point of pathology if acute or chronic ethanol intoxication results in a depression in the capacity for learning and memory?

Recently, Bernier *et al*. (Bernier et al., 2011) addressed this question in a study of the effects of ethanol on LTP in the mouse ventral tegmental area (VTA), a mammalian structure that is strongly implicated in drug addiction. This group used an LTP protocol that closely replicates the stimulation experienced during reward-based learning. They observed that chronic intermittent ethanol facilitates the inducibility of activity-dependent plasticity in the VTA. This type of change is considered an example of metaplasticity, which is a higher order modulation of the capacity for LTP. This novel response to ethanol might be a reflection of the novel LTP induction protocol employed, or it might mean that cellular learning in the VTA responds to

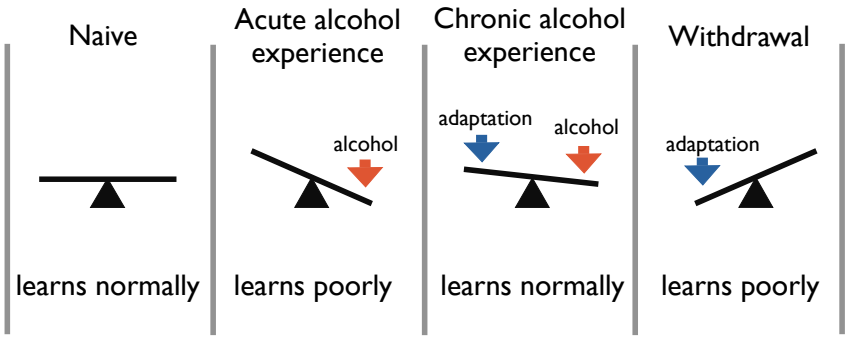
ethanol in an manner opposite to other parts of the brain. The latter interpretation is supported by behavioral experiments showing that ethanol experience inhibits most forms of rodent associative learning but enhances cocaine CPP, which involves the VTA (Bernier et al., 2011; Hunt and Castillo, 2012).

We propose that the reason ethanol responses are so tightly conserved between mammals and invertebrates is because the list of functionally relevant ethanol targets include some evolutionarily ancient cellular mechanisms. A recent addition to this list are the enzymes that modulate histone acetylation—a target that is linked to both functional tolerance and withdrawal in mammals (Starkman et al., 2012) and one that could be an additional point of unification for ethanol responses and the learning-and-memory machinery. Over the last 10 years, the learning and memory field has accumulated substantial evidence that histone acetylation (and other epigenetic modifications) contribute to the formation of long-term memory (Day and Sweatt, 2011). It has been shown that different types of learning can produce different patterns of histone acetylation (Peixoto and Abel, 2013). This is remarkable for a modification that was relatively recently considered generic and uninteresting. Ethanol exposure, in turn, has been shown to potently increase histone acetylation in the brain. While there is some disagreement concerning how the increase is produced, there is evidence that a metabolite of ethanol (probably acetate) is responsible (Choudhury and Shukla, 2008; Pandey et al., 2008; Soliman and Rosenberger, 2011).

Much of the recent alcoholism research in Drosophila has focused on the development and characterization of fly behavioral assays that are already well established

in mammalian model systems. This is necessary because Drosophila has only more recently become an alcoholism model system. The conservation of behavioral responses to ethanol has to this point been impressively high. Not only are the adaptive responses of tolerance and dependence conserved but there is evidence of similar interactions between ethanol and the reward-and-learning mechanisms in flies and mammals. The novel genetic tools available in Drosophila will allow questions to be addressed in ways that are not possible or perhaps not practical with a mammalian model system. The diminutive fruit fly is becoming invaluable in the discovery of the mechanisms leading to alcohol addiction.

Figure 1.3: Interpretation of the alcohol-induced homeostatic adaptation of learning demonstrated with Drosophila larvae. Not only is learning an important process in the development of addiction, but the ability to learn adapts concomitantly with the progression of dependence. An acute alcohol exposure initially results in an impaired learning capability. With continued ethanol exposure however, homeostatic adaptations countering the intoxicating effects of alcohol result in a seemingly normal learning ability. The adaptations remain however and are no longer balanced when the drug is removed creating a withdrawal state in which learning is again impaired (Robinson et al., 2012a).



DROSOPHILA LARVAE AS A MODEL TO STUDY PHYSIOLOGICAL ALCOHOL DEPENDENCE

The text and figures in this section of the introduction have been published as a review article in 2013 in the journal *Communicative and Integrative Biology*:

Robinson, B. G., Khurana, S., and Atkinson, N. S. (2013). Drosophila larvae as a model to study physiological alcohol dependence. Commun Integr Biol *6*, e23501.

Co-author contributions: S. Khurana, a post-doctoral collaborator and N.S. Atkinson, my P.I. helped write this review.

Abstract

Alcohol addiction is a disease that includes a diverse set of phenotypes. Functional alcohol tolerance is an adaptation to the effects of alcohol that restores neuronal homeostatic balance while the drug is present. When the drug is suddenly withheld, these adaptations unbalance the nervous system and are thought to be the origin of some withdrawal symptoms. Withdrawal symptoms, which can be a motivating factor for alcoholics to relapse, are taken as evidence of physiological ethanol dependence. Both tolerance and withdrawal symptoms are diagnostic criteria for alcoholism. Recent studies have demonstrated that the larvae of Drosophila show conserved alcohol tolerance and withdrawal phenotypes indicating that Drosophila genetics can now be used in studying this endophenotype of alcohol addiction. This section of the introduction reviews the prevailing theories we believe best describe the processes that lead to physiological ethanol dependence.

Homeostatic Theory of Drug Dependence

In humans, alcoholism can be thought of as having two distinct stages. Physiological tolerance and dependence make up the first stage and the second stage encompasses an unknown number of psychological events that promote the transition to uncontrolled and compulsive alcohol consumption (Ripley and Stephens, 2011). While these phenomena have proven difficult to reproduce in animals, the physiological responses of functional alcohol tolerance and dependence have lent themselves to study in animal model systems. Understanding these processes is important because they arise from neural changes that occur during the early stages of alcohol addiction. These changes contribute to the psychological dysregulation observed in alcoholics, producing continued drinking despite serious family, health, or legal problems.

Physiological alcohol dependence is a core endophenotype of alcoholism. According to Koob and LeMoal (Koob and Le Moal, 2006), dependence is defined by manifestation of withdrawal symptoms that originate from the physiological adaptations that occur in response to the drug. This definition is rooted in the counter-adaptive theory of drug addiction (Martin, 1968), which postulates that dependence arises from the same neuroadaptive mechanisms that produce drug tolerance. These adaptations oppose the pharmacological effects of the drug, but once the drug is cleared, their persistence is counter-adaptive and produces symptoms of withdrawal. Thus, withdrawal symptoms serve as an indicator of physiological drug dependence. The underlying counter-adaptive changes are believed to directly contribute to the motivational aspects of drug addiction. A framework for the psychological interrelationship between tolerance and dependence was outlined in the opponent process theory

(Solomon and Corbit, 1974). Here, both tolerance to the positive affective state and the negative consequences of withdrawal lead to the motivational changes that escalate drug use (Koob and Le Moal, 1997).

Alcohol tolerance, withdrawal-induced seizure, and the rewarding effects of alcohol have all been modeled in the fruit fly *Drosophila melanogaster* (Cowmeadow et al., 2006; Ghezzi et al., 2012; Kaun et al., 2011; Scholz et al., 2000). These studies show not only a between-species conservation in the fly and mammalian behavioral responses to alcohol but also a conservation of the role of genes in these responses. While the neural circuitry of the fly and the mammalian brain do not resemble one another, there is substantial conservation of genes and signaling pathways in neurons. This conservation is sufficiently high that fly genes that modulate neural excitability or that regulate animal behavior have been used to identify their mammalian counterparts where they perform similar functions (Atkinson, 2009; Brodie et al., 2007; Ghezzi and Atkinson, 2011; Hardin, 2011; Mackay and Anholt, 2006; Salkoff et al., 1992; Treistman and Martin, 2009). Thus, it is likely that alcohol responses that are mechanistically conserved from flies to mammals arise from the conserved cellular effects of alcohol. Behavioral outputs of flies can serve to magnify the effects of small changes in neural function.

One variant of alcohol dependence, which has been little studied in animal models, is the adaption that allows high-functioning alcoholics to appear behaviorally normal and to be productive members of society for much of their lives (Benton, 2010; Moss et al., 2007). During alcohol abstinence, their addiction becomes more

noticeable because of alcohol-withdrawal symptoms. In extreme cases, symptoms can include alcohol-withdrawal seizures, but abstinence can also produce an inability to concentrate, remember, or learn (Parsons and Nixon, 1993; Stavro et al., 2012).

We have recently shown that acute alcohol treatment impairs the performance of Drosophila larvae in a simple associative learning and memory assay (Robinson et al., 2012b). In our learning assay (Khurana et al., 2009), we leveraged the capacity of larvae to associate a noxious heat stimulus with an otherwise attractive odor. Once the association is made, memory retention can be tested by observing how the larvae respond to the odorant. Avoidance of the previously attractive odor is indicative of memory. Larvae are ideal for this purpose because large numbers of animals can be simultaneously tested in a single petri dish. Similar learning and memory paradigms are becoming popular in larvae due to the additional model system advantages of speed and economy of both behavioral assays and genetic manipulations.

In addition to acute alcohol effects, we have also seen striking effects of chronic alcohol exposure on larval behavior. Using the same learning and memory paradigm, we have shown that when larvae chronically feed on alcohol food, they adapt to it (acquire tolerance) and then can learn as well as animals that have never been exposed to alcohol. Concurrent with the development of this chronic tolerance, physiological alcohol dependence was apparent as evidenced by withdrawal symptoms. Specifically, larvae chronically treated with alcohol that underwent a subsequent abstention showed a learning deficit. Alcohol reinstatement restored normal learning in withdrawn larvae, further verifying the presence of dependence. Larvae in with-

drawal also had an increased sensitivity to the convulsant drug picrotoxin indicating an underlying nervous system hyperexcitability (Robinson et al., 2012a).

In our study, the larvae become dependent on alcohol after maintaining internal alcohol concentrations around 10 mM for 6 days. In a human, this would correspond to a blood alcohol concentration below the legal limit for driving in the United States. It would be unusual to observe a similar consumption pattern in humans, however in mice, alcohol withdrawal hyperexcitability has been seen following persistent low blood-alcohol levels over a period of days (Goldstein and Pal, 1971).

Our findings indicate that the larvae of Drosophila can adapt to alcohol and display similar tolerance and withdrawal phenotypes as mammals. This conservation advances Drosophila larvae as an additional instrument to study the adaptations that lead to physiological alcohol tolerance and dependence. Alcohol addiction is a multi-faceted disease that has yet to be comprehensively modeled in a non-human system. Thus, many models systems are needed because the experimental advantages and disadvantages of each allow focus to fall on a specific set of questions. The distinct toolset of the Drosophila model system may provide insight into aspects of alcohol-related behaviors that are difficult to study in mammals.

Chapter 2. Olfactory Conditioning in Drosophila Larvae

OLFACTORY CONDITIONING IN THE THIRD INSTAR LARVAE OF DROSOPHILA MELANOGASTER USING HEAT SHOCK REINFORCEMENT

The majority of text, data, and figures presented in this chapter have been published in a 2012 manuscript in the journal *Behavior Genetics*:

Khurana, S., Robinson, B. G., Wang, Z., Shropshire, W. C., Zhong, A. C., Garcia, L. E., Corpuz, J., Chow, J., Hatch, M. M., Precise, E. F., Cady, A., Godinez, R. M., Pulpanyawong, T., Nguyen, A. T., Li, W. K., Seiter, M., Jahanian, K., Sun, J. C., Shah, R., Rajani, S., Chen, W. Y., Ray, S., Ryazanova, N. V., Wakou, D., Prabhu, R. K., and Atkinson, N. S. (2012). Olfactory conditioning in the third instar larvae of Drosophila melanogaster using heat shock reinforcement. Behav Genet *42*, 151-161.

Co-author contributions: S. Khurana and myself are co-first authors on this published study and. Z. Wang, W.C. Shropshire, A. Zhong, L.E. Garcia, J. Corpuz, J. Chow, M.M. Hatch, E.F. Precise, A. Cady, R.M. Godinez, T Pulpanyawong, A.T. Nguyen, M. Seiter, K Jahanian, J.C. Sun, R. Shah, S. Jajani, W.Y. Chen, S. Ray, N. V. Ryazanova, D. Wakou, and R.K. Prabhu were undergraduate researches that I trained and assisted with experiment preparation and completion. N.S. Atkinson is my P.I. and funded the study and assisted with writing.

Abstract

Adult Drosophila melanogaster has long been a popular model for learning and memory studies. Now the larval stage of the fruit fly is also being used in an increasing number of classical conditioning studies. In this study, we employed heat shock as a novel negative reinforcement for larvae and obtained high learning scores following just one training trial. We demonstrated heat-shock conditioning in both reciprocal and non-reciprocal paradigms and observed that the time window of association for the odor and heat shock reinforcement is on the order of a few minutes. This is slightly wider than the time window for electroshock conditioning reported in previous studies, possibly due to lingering effects of the high temperature. To test the utility of this simplified assay for the identification of new mutations that disrupt learning, we examined flies carrying mutations in the dnc gene. While the sensitivity to heat shock, as tested by writhing, was similar for wild type and dnc homozygotes, dnc mutations strongly diminished learning. We confirmed that the learning defect in dnc flies was indeed due to mutation in the dnc gene using non-complementation analysis. Given that heat shock has not been employed as a reinforcement for larvae in the past, we explored learning as a function of heat shock intensity and found that optimal learning occurred around 41˚C, with higher and lower temperatures both resulting in lower learning scores. In summary, we have developed a very simple, robust paradigm of learning in fruit fly larvae using heat shock reinforcement.

Introduction

A comprehensive understanding of the brain requires a clear understanding of both the hardwired and plastic aspects of the nervous system. Drosophila melanogaster is a widely used model system for the study of sensory behavior and neuronal plasticity. The low cost of the D. melanogaster system makes it ideal for high-throughput behavioral assays and the rich molecular and genetic tools facilitate the genetic dissection of behavior. Drosophila exhibits a vast array of bona fide associative learning responses including olfactory associative conditioning (Quinn et al., 1974) and mating suppression learning (Gailey et al., 1984).

The larval stage of Drosophila melanogaster has the added attraction of fewer functional neurons than the adult fruit fly. Current estimates point to ~2000 functional neurons in the larval central nervous system as opposed to ~100,000 in the adult fly (Iyengar et al., 2006), though further studies are needed to establish the exact numbers. Much is known about the neural structures of the larval brain that underlie olfactory learning. In larvae, there are only 21 olfactory receptor neurons that transmit information to the larval antennal lobe (Fishilevich et al., 2005). From the antennal lobe, 25 neurons pass information to higher olfactory centers: the lateral horn and the mushroom body (Masuda-Nakagawa et al., 2009; Ramaekers et al., 2005). The mushroom body has been shown to be involved in olfactory associative conditioning in both larvae and adults and to be involved in olfactory appetitive conditioning in the larval stage (Pauls et al., 2010). The mushroom body neurons (~300) involved in olfactory appetitive conditioning in larvae are of embryonic, not larval, origin. Because the behavioral responses of the third instar larvae are stable for an entire

experimental day (Khurana, 2003a), there is ample opportunity to explore mechanisms of learning, as well as memory decay (Khurana et al., 2009).

There exist a variety of larval associative learning assays, employing different positive (Dukas, 1999; Honjo and Furukubo-Tokunaga, 2009; Schipanski et al., 2008) or negative reinforcers (Aceves-Pina and Quinn, 1979; Dukas, 1999; Khurana et al., 2009; Selcho et al., 2009). In a non-reciprocal design, for an attractive stimulus the use of negative reinforcements is better able to resolve learning, while for a repulsive stimulus the same is true of positive reinforcements. Large array of experimental paradigms in larvae is useful for characterizing differences in circuitry, physiology, and signaling between distinct types of learning. Many differences between aversive and attractive reinforcers are known in mammalian literature but now even in larvae, Honjo and Furukubo-Takunaga reported different memory decay rates and differences in circuitry for conditioning using attractive versus aversive reinforcements (Honjo and Furukubo-Tokunaga, 2009). Electroshock has been the most commonly used negative reinforcer for olfactory conditioning (Aceves-Pina and Quinn, 1979) and has been successfully used to explore different phases of memory decay (Khurana et al., 2009). Unfortunately, electroshock training requires significant effort to set up and multiple training trials to generate high learning scores, both of which limit the scalability of this assay and thus its application to mutant screening.

We have developed a novel olfactory learning assay in which heat shock reinforcement replaces electroshock reinforcement as the unconditional stimulus. Interestingly, this change results in high associative learning scores after single heat shock reinforcement. This change significantly reduces the time and equipment

needed to experimentally measure the capability of larvae to learn and to remember.

Methods and Materials

Stocks and Cultures

The Canton S strain of Drosophila melanogaster and the learning mutants dnc^{1} and dnc^{ML} were reared on cornmeal, molasses, and yeast agar medium at 25 ± 1˚C on a 12/12 hour light–dark cycle (9 a.m. – 9 p.m.). CS and dnc^{ML} were obtained from Bloomington stock center (stock numbers 1 and 9407) and dnc1 was a kind gift from Dr. Gregg W. Roman.

Larval Rearing and Harvesting

To obtain third instar larvae, 100 flies were allowed to lay eggs on well-yeasted medium for 24 hours and mid-third instar larvae were obtained after 5 days (± 8 hrs). The top media containing the larvae was gently dispersed in water and fine debris was removed with a sieve. The larvae were separated from the coarse media through a density separation with a 30% solution of 1500 molecular weight polyethylene glycol (Acros Organics, AC19226-0051), with the larvae floating on the top and the debris settling at the bottom (Khurana et al., 2009; Khurana, 2003b). After the density separation, larvae were rinsed in water using a sieve that does not retain the smaller second instar larvae and also removes traces of PEG. Larvae were then rested in 9 cm Petri dishes containing 0.5 ml of Ringer's solution until the onset of the experiment. The Ringer's solution contained 128 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM Na_2HPO_4, and 0.37 mM KH_2PO_4.

Odor and Heat Delivery

All conditioning experiments consisted of two steps: training and testing. Independent of the training and testing designs, all testing with larvae relied exclusively on their olfactory responses. The training and testing steps were different for the reciprocal and the non-reciprocal design, but the methods for delivering the heat shock and odor were the same and are described below.

For all experiments, we used heat as the unconditioned stimulus (US). To administer the heat shock, 9 cm glass Petri dishes (Pyrex, 08-747A) were filled with 30 ml of 0.5% agar (Neogen Corporation Bacteriological Agar 7178A) and placed on heat blocks with inverted metal plates to give a flat surface (Analog Dry Block Heaters VWR 12621). To obtain the desired temperature on the agar surface, we had to maintain the heat block 2˚C above the desired temperature and keep the Petri dish on it for 8 minutes prior to administering the heat shock. Apart from the experiment designed to find the optimal heat shock temperature for learning (Figure 2.4), a temperature of 41˚C on the agar surface was used for all experiments. A 9 cm glass Petri dish with 30 ml of 0.5% agar maintained at 22˚C acted as a rest plate that the larvae were placed on in between heat shock presentations.

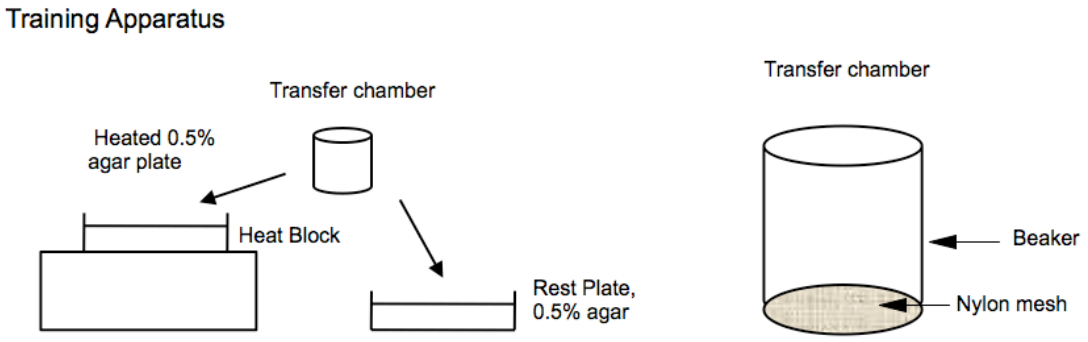
For the odor presentation, Petri dishes similar to the heat shock plates were made, but just before agar solidification, pure odorant was added to the molten agar and vigorously mixed. The odorants used, ethyl acetate (EA) and n-Butanol, are not soluble in water. The odorants form small micelles that, for the purpose of odor presentation, can be uniformly spread by vigorously shaking the molten-agar odor

mix before pouring it in the plates. When heated, these odor plates allow a simultaneous exposure to the CS and the US.

We constructed a transfer chamber that allowed us to rapidly move larvae between Petri dishes and therefore to precisely control the timing of the heat treatments and odor exposures (Figure 2.1). The transfer chamber was constructed by replacing the bottom of a plastic beaker (50 ml Tri-pour beaker no. 50-996-322, Fischer Scientific) with nylon organza mesh (obtained from a local fabric store). Precautions were taken to ensure that all of the larvae were on the mesh, thus being uniformly exposed to the agar for the experimental manipulation. The mesh prevented larvae from coming in direct contact with the agar and odorant. In addition, larvae were gently rinsed in water before and after exposure to any surface. Rinsing kept the larvae moist and removed any traces of odor that may have been present on the larvae or transfer chamber from the CS presentation.

To verify whether heat shock was a good reinforcement for olfactory conditioning, we used an alternative method of odor presentation. This method consisted of covering the transfer chamber with a Petri dish top that had four 50 µl drops of 10^{-4} EA evenly spaced on it, similar to a design employed for electroshock conditioning in a previous study (Khurana et al., 2009).

Figure 2.1: Experimental Design and Apparatus. Larvae were moved between heated and rest plates using a transfer chamber. We made the transfer chamber by replacing the bottom of the plastic beaker with a nylon mesh. This allowed larvae to be exposed to the odor in the agar plates as well as the heat.



Larval Discomfort

Larval discomfort was qualitatively assessed by observing jerky sideways movements during heat shocks. Several investigators, blind to the trial number, rated larval writhing on a scale of 0 to 5, with 5 being the highest level of writhing.

Non-reciprocal Conditioning

Major difference between reciprocal and non-reciprocal conditioning design is that in the reciprocal design animals are trained to prefer one CS over another CS while in non-reciprocal design animals are trained to change their response to one CS after pairing with a US. The reciprocal design is often tested as preference between two CS and hence can also be called preference conditioning, while the non-reciprocal design tests for response to the CS in different control and conditioned groups.

In non-reciprocal conditioning, a CS is paired with the presentation of a US to elicit the conditioned response. We used simultaneous conditioning, in which the CS and US are presented concurrently (Figure 2.2A). Approximately 300 larvae were placed in a transfer chamber and rested on a room temperature 0.5% agar Petri dish without odorant. To administer the heat shock, larvae were placed on a heated odor plate (10^{-4} EA dilution (v/v)) for 30 seconds and then returned to the rest plate for an 8-minute inter-trial interval. This was repeated for the desired number of trials. Within 5 minutes of the final trial, larvae were tested for aversion to the odorant acquired as a result of the CS-US pairing. For control groups in this protocol, larvae were exposed to either heat-only (odorless heated agar plate) or odor-only (room

temperature odorant-containing agar plate) conditions in lieu of the CS-US simultaneous presentation. Apart from the CS-only and the US-only groups, larvae naïve to both the CS and US were also used as controls.

We trained larvae with an odorant concentration that was 10 fold higher (10^{-4}EA) than the concentration used during testing (10^{-5} EA) because we were concerned that the mesh between the larvae and agar surface would prevent larvae from experiencing the full concentration of EA on the training plate. Testing was done using 15 cm plates (BD Falcon, 08-757-148) containing 25 ml of 2% solidified agar. An odor zone of 2 cm radius was demarcated with the center of the zone 2.5 cm from the edge of the plate (Figure 2.2B). In the odor zone, 20 µl of 10^{-5} EA dilution in liquid paraffin (v/v) was placed onto a 0.4 cm diameter filter paper disc. On the diametrically opposite end, liquid paraffin was placed on a filter disc at the same distance from the edge. 30 larvae from the pool of 300 trained larvae were then placed in a 1 cm center zone and the plate was covered. At the end of 3 minutes, we noted the number of larvae in the odor zone and the total number of larvae on the plate, excluding the central 1 cm plate (nonparticipants, usually 0 or 1 larva).

For the single odor avoidance, a response index was calculated as the fraction of larvae on the plate that were in the odor zone (Figure 2.2A). Learning indices were calculated using the response indices of the trained and the control groups (Figure 2.2C). The learning index represents the fractional amount of response that is reduced because of the association of the odorant with the heat shock. We report the response indices, either in the figures or in the text, so that the absolute difference can easily be determined in addition to the fractional change.

Odorants for testing were prepared by diluting high purity stock chemicals (99+%) in odorless liquid paraffin. All odorants used in this study are soluble in hydrophobic heavy carbon chain solvents like liquid paraffin. Ethyl acetate (Fisher Scientific E145-1) and n-Butanol (Fisher Scientific A399-1) were obtained from Fisher Scientific while liquid paraffin (AC17140-0010) from Acros Organics.

Reciprocal Conditioning

In reciprocal conditioning, two CS are presented to the larvae. Only one CS however, is paired with US and the shift in preference between the odors after conditioning demonstrates the associative nature of the conditioning. The benefit of this type of training is that the two reciprocally trained groups act as controls for each other. In this protocol, one group of larvae received 3 CS-US pairings using the EA as the odorant with 8-minute inter-trial intervals similar to the simultaneous conditioning described above. Four minutes after the presentation of EA however, larvae were placed on a non-heated odor plate containing 10^{-2} n-butanol dilution (v/v) for 30 seconds. Another group of larvae was trained reciprocally, with n-butanol in the heated plate and EA in the non-heated plate. Figure 2.6A illustrates the training paradigm for preference conditioning. After the conditioning, larvae were tested for olfactory preference (as described below and in figure 2.6B). The n-butanol concentration of 10^{-2} was used because larvae were similarly attracted to this concentration compared with 10^{-5} EA.

Following the reciprocal training paradigm, larvae were tested for a preference between the two odorants. Testing was done as described for non-reciprocal

conditioning, but 10^{-2} n-butanol (v/v) was spotted opposite the 10^{-5} EA rather than liquid paraffin (Figure 2.6B). At the end of the 3-minute testing period, we recorded the number of larvae in both odor zones as well as the total number of larvae.

A preference index (PI) was calculated for the reciprocal training paradigm (Figure 2.6C). The PI is the difference in response between the two odorants (on opposites sides of the testing plate) divided by the sum of the two responses. A PI can be calculated for both odors and then averaged to give an averaged preference index for the experiment.

Testing dunce

dnc^1 homozygous larvae were trained in the non-reciprocal conditioning mode explained above. To test for non-complementation with a different allele of dunce, we crossed virgin female dnc^1 flies with male dnc^{ML} flies. Because the dnc locus is X-linked, only the female progeny carried both mutant alleles of dunce. Immediately prior to training these larvae, we separated out the males and only used the female larvae for learning experimentation. Male and female larvae can easily be distinguished by the size of the gonadal imaginal disc. This transparent organ resides around the fifth abdominal segment (~2/3 down the length of the larvae) and is much larger and more visible in males and resembles a spherical hole in the opaque larvae fat body. Under 10x magnification, many larvae can be sexed in this manner in a short amount of time. To control for the additional handling and any gender differences, female homozygous dnc^1 and wild type CS larvae were trained and tested alongside the dnc^1/dnc^{ML} strain.

Statistical Analysis

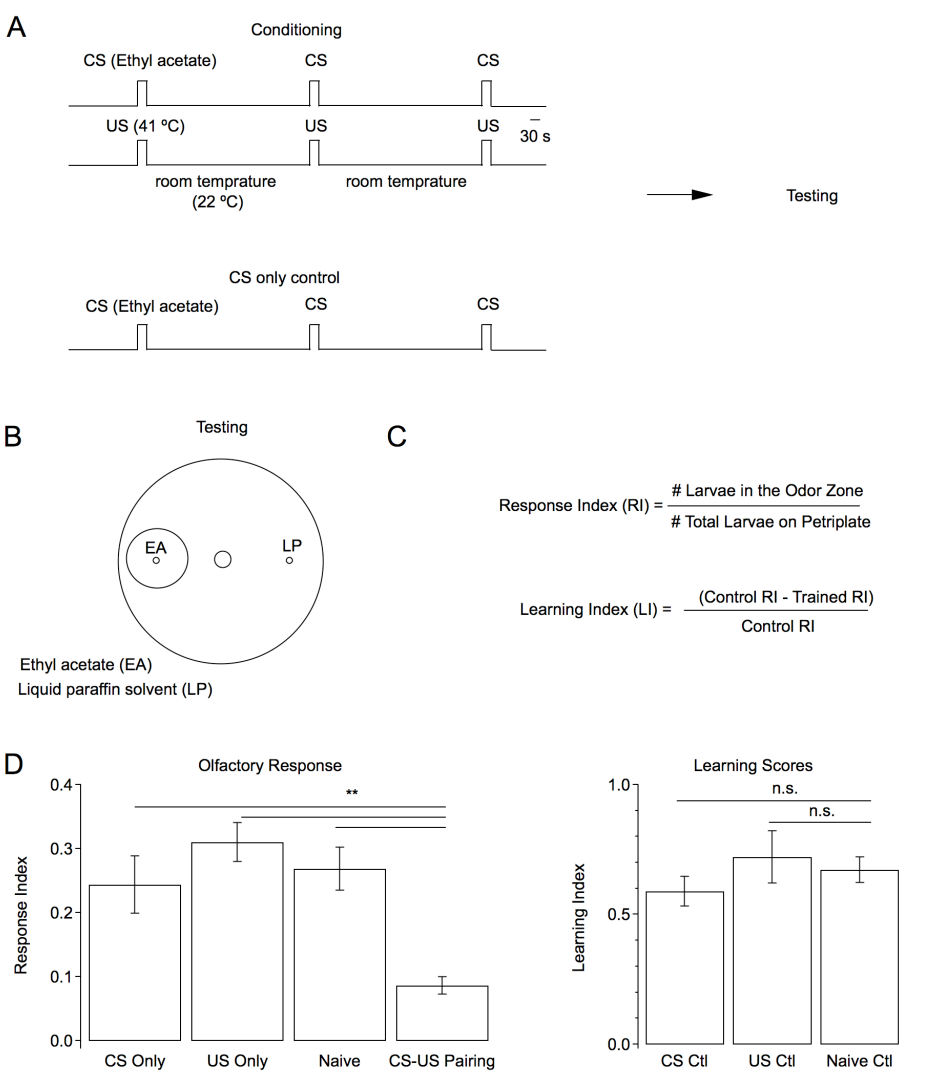
Animals that were trained together were considered to be the same sample (N=1), due to a lack of independence, despite the fact that multiple testing plates were used to evaluate learning. For example, 300 animals trained simultaneously might be tested for learning on 10 testing plates with each testing plate containing 30 animals. Nevertheless, these would be averaged and considered to be a single sample trial (N=1). The learning index for each sample was the average of a minimum of three testing plates of 30 animals each. Error bars presented throughout the study are the standard error of the mean (SEM). The number of independent experiments for each mean is specified in the text, figure or figure legend. For paired difference tests, we chose the Wilcoxan signed rank test, a non-parametric statistical hypothesis test, because it does not assume the population to be normally distributed. Less than 20 independent experiments, as used by most studies including ours, are not amenable to testing for conformity to a normal distribution. Therefore, we have chosen to use a more conservative Wilcoxan signed-rank test but any significance reported in our text is also significant if a t-test is performed on the data. The significance value of <0.05 is indicated with "*" and <0.01 with "**", while significance values above >0.05 are indicated with "n.s.". No multiple comparisons were made in this study.

Results

Non-reciprocal Olfactory Conditioning

Using the method described in Figure 2.1, as well as in the methods section, we performed training trials (Figure 2.2A) in which two stimuli were paired simultaneously (US-CS pairing) and trials in which each stimulus was delivered alone (CS-only, US-only). After training (Figure 2.2A), animals were tested in groups of 30 (at least 3 repetitions for each experiment) for their response to ethyl acetate (Figure 2.2B). The response index (Figure 2.2C) after conditioning underwent a 3-fold reduction from naïve, CS-, or US-only groups (Figure 2.2D). Figure 2.2E shows that high learning indices are obtained regardless of whether we compared the CS-US paired animals to the CS-only control, to the US-only control, or to the naïve larvae control. Only the simultaneous presentation of the CS and US, and not the individual presentation of either CS or US, diminished the larval attraction to the 10^{-5} dilution (v/v) of EA (ethyl acetate) indicating that the larvae form an association between the two stimuli.

Figure 2.2: Non-reciprocal conditioning. A Conditioning protocol: Above, following an 8-minute plate-warming period, larvae are given 30-second heat shocks with 8-minute inter-trial interval. Below, we present a schematic of the CS-only control, but similar US-only and naïve controls were also run. B Testing: The 14 cm diameter single-odor avoidance testing plate consisted of an odor spot placed 2.5 cm from the edge of the plate. We demarcated the odor zone as a 2 cm radius ring around the odor spot. On the diametrically opposite end of plate the solvent, liquid paraffin was placed as a control. C Response indices were calculated as the fraction of larvae on the plate that were in the odor zone. Using response indices, learning indices were calculated as fractional decrease in response index due to the conditioning. D Left: Pairing the CS (odor) with the US (heat shock) resulted in a significant drop in the number of larvae attracted to 10^{-5} EA (v/v) compared with either stimulus presented alone or naïve groups as obvious in response index measurements (n=6). Right: Learning indices calculated from the three control conditions. All control conditions gave similarly high learning indices.

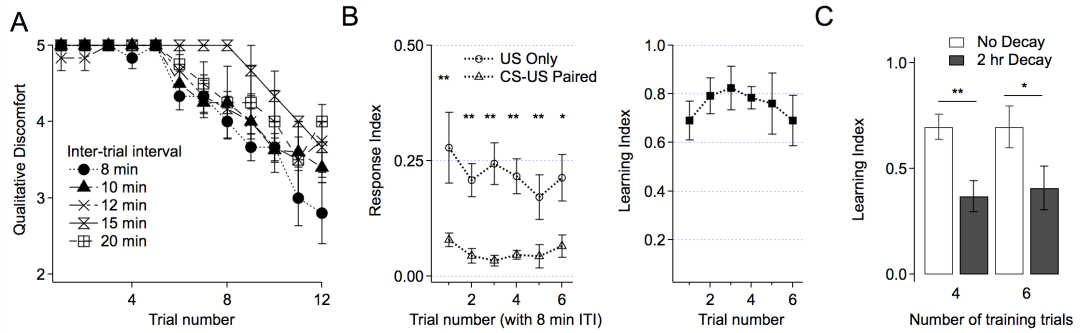


Limitations and Advantages of Heat Shock Reinforcement

We were concerned about possible desensitization of larvae to the heat shock that would cause the US-CS pairing to become equivalent to a CS presentation only, and which could produce extinction of the conditioned responses. To determine when desensitization occurs, we qualitatively analyzed larval writhing in 12-trial training sessions using a variety of inter-trial intervals. Four investigators, blind to the trial number, observed larvae for quick and jerky movements that were ranked on a 5-point scale. Independent of inter-trial intervals of the heat shock, the larvae exhibited less writhing beginning at trial number 6 (Figure 2.3A). Thus from trial 6 and onwards, desensitization to heat shock results in suboptimal negative reinforcement. In agreement with the larval writhing assay, less learning was observed from trial 6 onwards (data not shown). In conclusion, the limitation of our assay is that it must be employed for fewer than 6 trials in a single training session.

In an assay with gradual learning acquisition, desensitization to the US would be very problematic. In our heat shock paradigm however, larvae learn to avoid the olfactory CS following just a single CS-US pairing as demonstrated by the immediate separation of odor response indices between the control and trained larvae (Figure 2.3B). A fractional learning index of nearly 0.7 is achieved after the first CS-US pairing and this rises to a peak of 0.8 following just three training trials (Figure 2.3C).

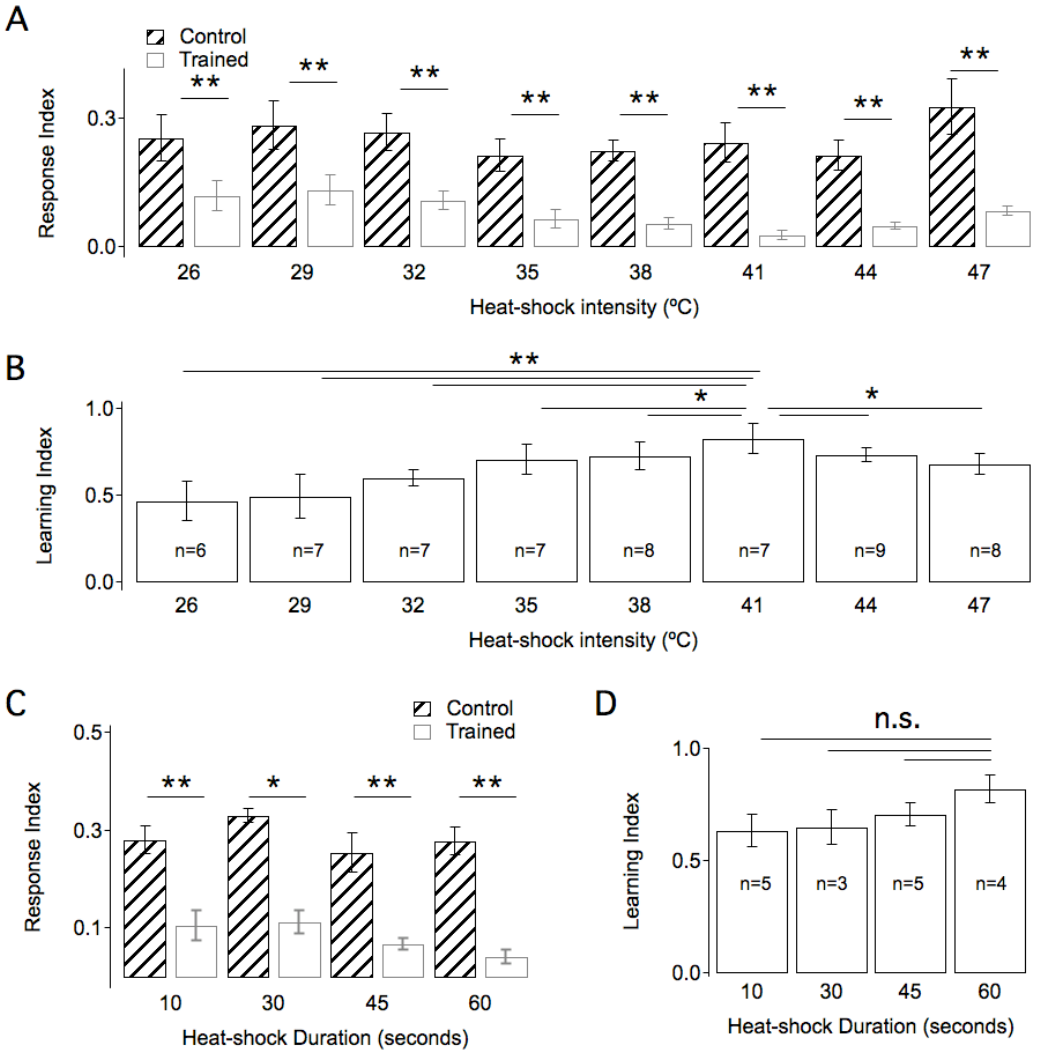
Figure 2.3: Acquisition and retention of learning. A Qualitative analysis of larval discomfort during heat shocks (n=6). Larvae were observed for visible signs of discomfort during each of 12 shocks with different inter-trial intervals and ranked on a scale of 0 to 5. Maximal discomfort persists through six shocks but then desensitization occurs regardless of the inter-trial interval (ITI). B. Larvae immediately acquire learned avoidance of the odor. Learning remains high and significant through 6 repetitions ($p<0.01$) and following desensitization, learning decreases and becomes highly variable. On the left are the response indices and on the right are the learning indices (n=7) C. 4-trial and 6-trial learning persists through a 2-hour memory decay period (n= 6).



Optimization of Heat Shock

We found that the temperature of the heat shock affects learning. Response indices for trained and US-only control (Figure 2.4A) are shown for a range of heat shock temperatures. For all of the temperatures tested, there was a significant difference between the control and the conditioned response indices. The learning indices (Figure 2.4B) increased from 26-41˚C, but a further increase in temperature resulted in a decrease in learning. We also tested conditioning for four different durations of heat shock (Figure 2.4C). For the narrow range that we explored, we did not find any statistically significant difference in learning scores for the different durations (Figure 42.D). Our choice of temperature for the heat shock for all subsequent experiments was 41°C because it produced the optimal learning score. Heat-shock duration of 30 seconds was chosen based on handling convenience.

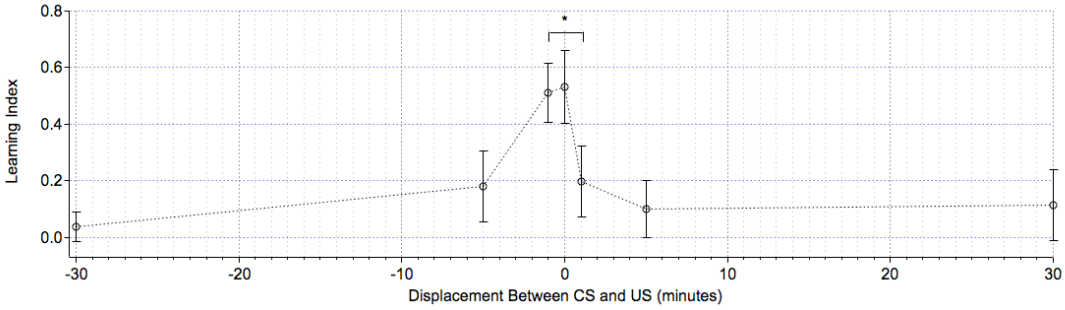
Figure 2.4: Learning as a function of heat-shock intensity. Larvae were trained at different temperatures or with different heat shock durations. A. The response indices of all the conditioned groups were significantly less than the respective control groups. B. Learning increases with increasing temperature in the range of 26˚C–41˚C. Above this temperature learning decreases, creating an inverted U shaped curve for learning as a function of the US intensity. C. Response indices of all groups, whether given heat shock for 10 seconds or a minute were significantly different than the control groups. D. The learning indices for different durations of heat shock were statistically indistinguishable from each other. The numbers of experiments are indicated in the figure itself.



Coincidence Window for Odor and Heat Shock Pairing

For many forms of associative conditioning, a narrow window of association between the CS and US is known to exist. In the case of electroshock, the CS closely following the aversive US acts as a signal of relief-from-punishment and can result in added attraction to the CS (Khurana et al., 2009; Tanimoto et al., 2004). While in some cases like taste-aversion conditioning the coincidence window of learning can be much wider and result in backward conditioning (Domjan and Gregg, 1977). To assess the nature of heat shock as a punishing reinforcement, we varied the time between the presentation of the odor and the heat shock (Figure 2.5A for schematic). Interestingly, we find that the coincidence window for heat shock is fairly narrow (less than 30 minutes), but broader than electroshock conditioning in larvae (Khurana et al., 2009). Akin to electroshock, it is asymmetrical around the simultaneous presentation. The presentation of the CS first elicits stronger learning than when the CS follows the US (-1 minutes vs. 1 minutes, $p<0.05$). Nonetheless, unlike electroshock we do not see a relief from punishment and the presentation of the heat shock before the odorant results in a mild conditioned response (with a 50% reduction in learning).

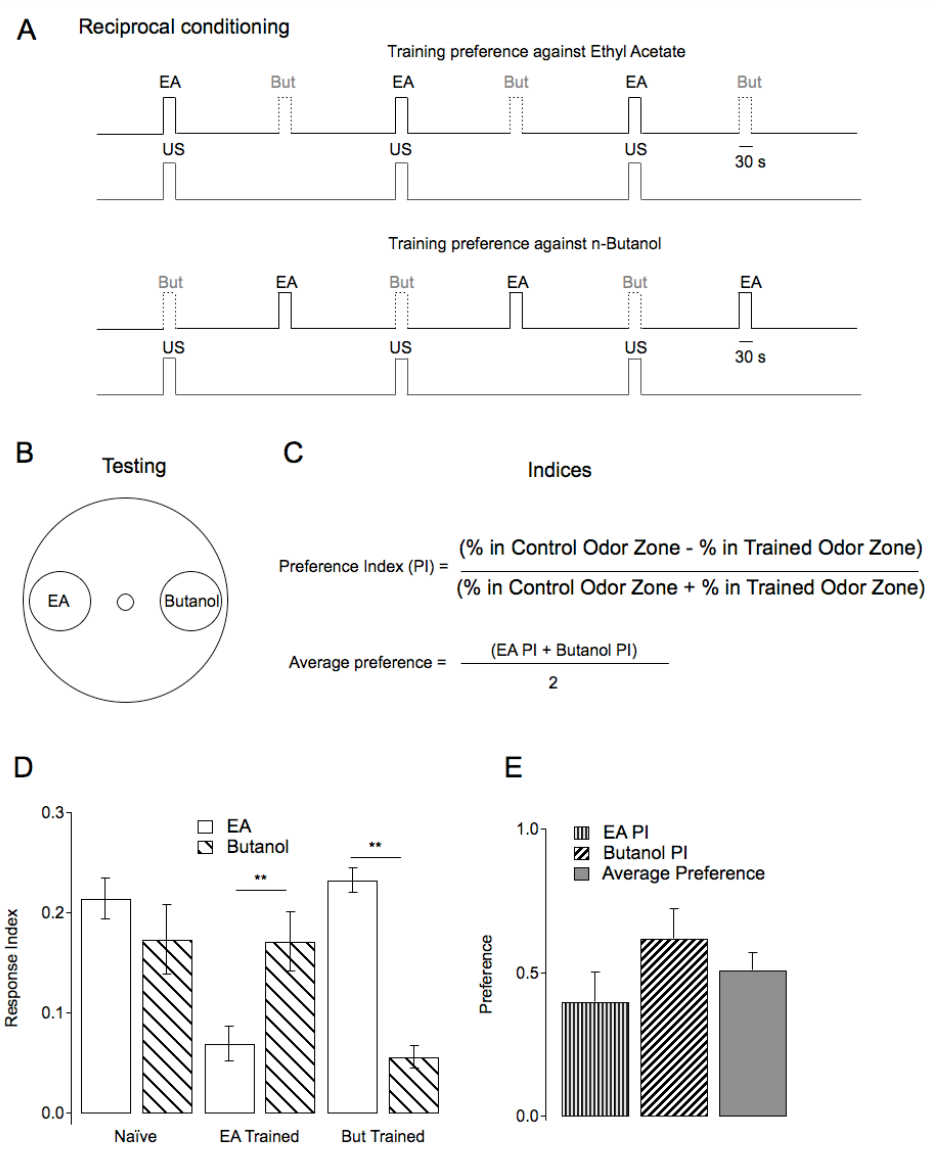
Figure 2.5: Window of association for odor and heat shock. Top: schematic, bottom: group data. The relative timing between a single presentation of odor and heat shock was varied from -30 minutes to +30 minutes. Significantly higher learning was obtained if odorant preceded the heat shock than the other way around.



Reciprocal Conditioning

A reciprocal conditioning paradigm, in which larvae were exposed to two odors with only one being paired with the US, has been previously employed in fruit fly research (Khurana et al., 2009; Tully et al., 1994). Given that the window of coincidence for our learning paradigm showed steep time dependence, we presented one odorant at the time of punishment and another 4 minutes after the heat shock. Two groups were used and a different odor was paired with the heat shock in each group (Figure 2.6A and methods). Figure 2.6 shows the response index (Figure 2.6D), preference, and average preference (Figure 2.6E) following this reciprocal conditioning. Larvae learn to prefer one of two odors when the other is paired with a negatively reinforcing heat shock, demonstrating the associative nature of heat-shock conditioning.

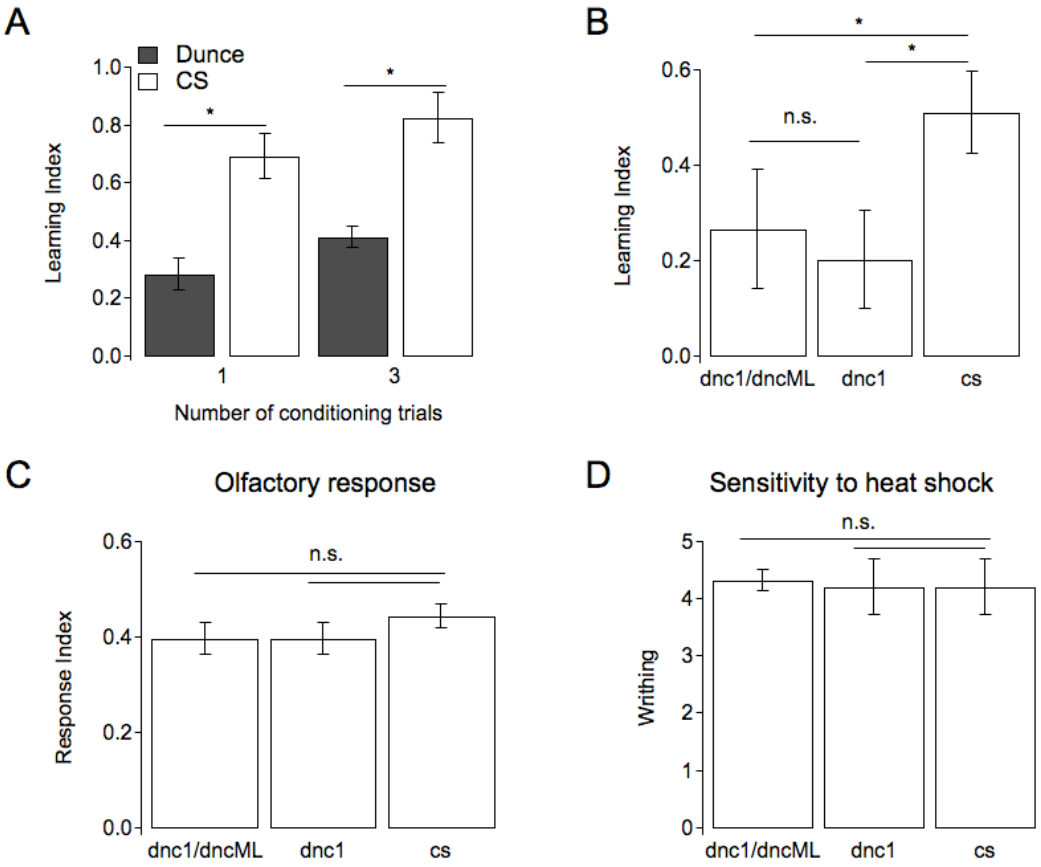
Figure 2.6: Reciprocal preference conditioning. A. Larvae were trained on a reciprocal two-odor preference paradigm where they were exposed to two odors but only trained to avoid one. B. For preference testing, the plate was set up in a similar manner as figure 2.2, but 10^{-2} n-butanol was spotted opposite the 10^{-5} EA rather than liquid paraffin. C. Preference for each odor was quantified by subtracting the responses to the two odors and dividing by the total response. Preference learning was defined as the average of the preferences for the two odors. D. Response indices to two different odors on the same plate for the reciprocally trained larvae groups. One group was given EA along with the US heat shock, while the other group was exposed to n-butanol simultaneously with the heat shock (n=5). E. Preference indices for EA and n-butanol trained groups and preference learning displaying a transfer of partiality from odor to odor (n=5).



Analysis of a Learning Mutant

To determine if we could use heat shock associative conditioning to identify learning mutants, we tested *dunce* mutants in a non-reciprocal training paradigm. We found that larvae carrying mutations in the *dunce* gene learned very poorly. After 1 and 3 training trials the learning indices were significantly reduced compared to wild type larvae (Figure 2.7). To establish that the learning defect was indeed due to a defect in the *dnc* locus, we performed non-complementation analysis on female larvae that carry two different *dnc* alleles (X-linked) for olfactory responses, writhing, and learning scores.

Figure 2.7: Learning in dunce. A. Using non-reciprocal heat shock learning, larvae with a mutation in the dunce[1] gene acquire learning, but at a significantly lower level than wild type larvae (n= 5). B. The female larvae of dnc1 and dnc1/dnc^{ML} exhibited significant learning defect but their learning scores were indistinguishable from each other (n=6). C. The olfactory responses were not affected by the dnc mutation (n=14). D. The response to heat shock was also unaltered due to mutation in the dnc locus (n=9).



Alternative Design for Non-reciprocal Conditioning

Even though we have measured olfactory response and preference in the testing phase of the conditioning assay, one may argue that the presentation of odorant from below the mesh of the transfer chamber might also permit gustatory contact with the odor molecules. To ascertain that gustation is not important in this learning assay we compared the learning indices obtained when the odorant was presented in the agar to the learning indices obtained when the diluted odorant was added to the inside of a petri dish lid. Both methods were performed at the same time. Presentation of odorant either in the agar or from the Petri dish lid resulted in statistically indistinguishable learning scores (0.38 ± 0.08 vs. 0.37 ± 0.06 for odor presented from bottom vs. odor presented from top; $p = 0.48$; $n=7$).

Discussion

In this study, we describe an olfactory conditioning paradigm that induces high levels of learning in *Drosophila melanogaster* larvae, that is very simple to setup, and that requires only a single training trial. This last point is important because it reduces the time for each experiment making this assay well suited for mutant screening.

Electroshock is the most commonly used negative reinforcer. Electroshock reinforcement produces gradual conditioning over multiple trials (Khurana et al., 2009); however, with heat shock as the negative reinforcer we obtained significant learning scores much faster. Heat-shock reinforcement also differs from electroshock reinforcement in two other characteristics: desensitization and window of

coincidence. Larvae desensitize more rapidly to heat shock than to electroshock. As a result, heat-shock entrainment results in diminishing returns at lower trial numbers than that reported for electroshock entrainment (Figure 2.3A and B). Another key difference is that heat shock has a wider window of association than electroshock (Figure 2.5), which we speculate is due to lingering effects of the heat shock. It would be interesting to see how heat shock reinforcement compares with other negative reinforcers such as gustatory negative reinforcement.

We chose to use a 41°C heat pulse as a negative reinforcer. It should be noted that, in the wild, larvae encounter this temperature but would be motivated to avoid it because of its negative effect on viability. Field studies have shown Drosophila larvae to be present in sunlit fruit with an internal temperature up to 44˚C. However, fruit temperatures above 40˚C result in a substantial increase in mortality (Feder and Krebs, 1997). Larvae sense heat using a well-described nociception pathway that is reliant on the transient receptor potential (TRP) cation channels encoded by the *painless* gene (Sokabe et al., 2008; Tracey et al., 2003). Although learning can occur using evolutionarily irrelevant stimuli, it must occur because the stimulus co-opts a learning pathway that was selected to respond to a different stimulus. The use of a stimulus normally found in the environment may produce more robust learning because there exists a sensory system evolutionarily optimized for its detection.

We also explored the role of US intensity in this study. For many model organisms, there is a positive correlation between the intensity of the reinforcement and the learning scores up to a point at which an overly intense reinforcer interferes with the capacity to learn. Beyond this point, there is a zero or negative correlation,

resulting in a sigmoid or inverted U shaped relation between the intensity of the reinforcer and the learning score (Baldi et al., 2004). In adult Drosophila a sigmoidal relationship has been observed using electroshock as the negative reinforcer (Diegelmann et al., 2006). Not surprisingly, heat shock reinforcement has an inverted U-shaped relationship between reinforecer intensity and magnitude of learning, probably because after a very high intensity the reinforcer becomes disruptive to learning.

In recent years, larval olfactory associative conditioning and the neurobiology of larval olfaction have witnessed a resurgence of interest (Khurana et al., 2009; Khurana et al., 2010; Pauls et al., 2010; Schipanski et al., 2008; Tully et al., 1994). Larval conditioning assays have been used to explore three major themes: (1) how the odor is represented in the nervous system, (2) the cellular mechanisms of learning and memory and recently, (3) the neurons and circuitry involved in learning.

The neural representation of sensory input can only be fully understood from in vivo analysis of cellular and circuit physiology during odor information processing. In the interim, conditioning experiments offer promising glimpses into the nature of olfactory processing (Chen et al., 2011; Mishra et al., 2010). For such an exploration, a robust conditioning assay is required. It also remains to be addressed if and how conditioning itself alters the olfactory representation in larvae.

To date, all mutants found to have deficits in adult conditioning have also been seen to have a similar deficit in larval conditioning paradigms. These mutants have proven useful for the exploration of the separate phases of memory decay in larvae (Khurana et al., 2009). Genes involved in learning and memory in flies have

also been shown to be important for learning and memory in mammals (Waddell, 2010; Wang and Zhong, 2004). Because Drosophila has a genetic toolkit that differs from that of mammalian model systems, Drosophila studies can complement mammalian work. However, an unbiased Drosophila genetic screen requires a survey of a great number of independent mutants. For the identification of learning mutants, a single-trial learning paradigm would be advantageous.

Efforts towards brain mapping in Drosophila are on the rise (Jefferis et al., 2007; Turner et al., 2008) and optophysiological tools are growing in popularity for the *in vivo* analysis of neural circuits (Knopfel et al., 2010). The application of these techniques to the study of learning and memory holds great promise. Drosophila larvae are particularly well suited for optogenetic studies because of their translucent body and have been used in recent years to explore questions of olfactory coding hitherto beyond the reach of previous techniques, using optogenetic tools (Bellmann et al., 2010; Schroll et al., 2006).

Every novel learning paradigm offers distinct advantages that tailor the assay towards a specific end. A single-trial learning assay in a model organism with powerful genetics should prove advantageous for the description of the cellular mechanics of learning and memory.

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Chapter 3. Acute Ethanol Impairs Learning in Drosophila Larvae

A LOW CONCENTRATION OF ETHANOL IMPAIRS LEARNING BUT NOT MOTOR AND SENSORY BEHAVIOR IN DROSOPHILA LARVAE

The majority of text, data, and figures in this chapter have been published in a 2012 manuscript in the journal *PLoS One*:

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Co-author contributions: S. Khurana, J.B. Pohl, W. Li, and A Ghezzi were lab mates and assisted with various aspects of planning and executing experiments. A. Cady, K. Najjar, M.M. Hatch, R.R. Shah, A. Bhat, O. Hariri, K.B. Haroun, M.C. Young, K. Fife, J. Hooten, T. Tran, D. Goan, F. Desai, F. Husain, R.M. Godinez, J.C. Sun, J. Corpuz, J. Moran, A.C. Zhong, and W.Y. Chen were undergraduate researches that I trained and assisted with experiment preparation and completion. N.S. Atkinson is my P.I. and funded the study and assisted with writing.

Abstract

Drosophila melanogaster has proven to be a useful model system for the genetic analysis of ethanol-associated behaviors. However, past studies have focused on the response of the adult fly to large, and often sedating, doses of ethanol. The pharmacological effects of low and moderate quantities of ethanol have remained understudied. In this study, we tested the acute effects of low doses of ethanol (~7 mM internal concentration) on Drosophila larvae. While ethanol did not affect locomotion or the response to an odorant, we observed that ethanol impaired associative olfactory learning when the heat shock unconditioned stimulus (US) intensity was low but not when the heat shock US intensity was high. We determined that the reduction in learning at low US intensity was not a result of ethanol anesthesia since ethanol-treated larvae responded to the heat shock in the same manner as untreated animals. Instead, low doses of ethanol likely impair the neuronal plasticity that underlies olfactory associative learning. This impairment in learning was reversible indicating that exposure to low doses of ethanol does not leave any long lasting behavioral or physiological effects.

Introduction

Ethanol consumption is known to affect sensory and motor abilities and to compromise more complex cognitive functions, such as attention, learning, and memory. These effects are dose dependent and sensitive to heterogeneity in ethanol metabolism, body weight, gender, genetic background, and prior experience with ethanol. Cognitive tests have established that low amounts of ethanol disrupt

attention-requiring tasks including learning and memory, while higher amounts of ethanol disrupt sensory and motor responses (Koelega, 1995; Richter and Hobi, 1975; Schweizer and Vogel-Sprott, 2008). Many studies in animal models have focused on obviously intoxicating levels of ethanol that produce motor defects (Atkinson, 2009; Bell et al., 2006; McBride and Li, 1998; Wolf and Heberlein, 2003). The effects of low doses of ethanol are understudied in animal models because the behavioral consequences are subtle. However, society incurs significant cost from accidents while operating machinery or driving at the levels of ethanol that affect judgment and attention in the absence of obvious effects on motor coordination (Camino Lopez et al., 2011; Phillips and Brewer, 2011).

Ethanol has previously been shown to have many effects on learning and memory. In humans, ethanol disrupts performance on a variety of short-term memory tasks, from verbal list learning (Acheson et al., 1998; Lister et al., 1991; Miller et al., 1978) to spatial memory (Matthews and Silvers, 2004). Model systems have also been used to gain an understanding of the mechanisms behind ethanol-associated behaviors (Atkinson, 2009; Barr and Goldman, 2006; Koob, 2000; McIntire, 2010; Wolf and Heberlein, 2003). In rodents, acute doses of ethanol have been shown to impair many learning tasks, including spatial memory(Gibson, 1985; Givens, 1995; Melchior et al., 1993; White et al., 1997), nonspatial working memory (Givens, 1996; Givens and McMahon, 1997), and spatial reference tasks (Markwiese et al., 1998). In honeybees, consumption of a 5% or higher ethanol solution disrupts Pavlovian conditioning (Abramson et al., 2000; Mustard et al., 2008). Adult *Drosophila melanogaster* have been a particularly useful model organism for the genetic analysis of ethanol

responses because they show many of the same responses to ethanol as do humans and, in addition, have the most experimentally malleable genome of any metazoan (Atkinson, 2009; Rodan and Rothenfluh, 2010). As observed in mammals, flies become hyperactive when initially exposed to ethanol but suffer incoordination and sedation as their internal ethanol concentration rises (Moore et al., 1998). Furthermore, like mammals, flies acquire functional tolerance to ethanol intoxication (Cowmeadow et al., 2005; Heberlein, 2000; Scholz et al., 2000). To date, the analyses of the effects of ethanol in Drosophila have been mostly restricted to the study of locomotor impairment. There is a need for a genetic model system to dissect how small amounts of ethanol affect emergent properties of the nervous system.

Adult flies have long been used to study behavioral plasticity, but over the last few years, larval Drosophila have become valuable as a genetic model for the study of learning and memory (Gerber and Stocker, 2007; Honjo and Furukubo-Tokunaga, 2009; Khurana et al., 2009; Khurana et al., 2012; Pauls et al., 2010). With a powerful genetic toolbox and a simple nervous system that generates a variety of behaviors, larvae are an excellent choice for genetic analysis of neural plasticity. Most ethanol-related studies in larvae have focused on ethanol preference and metabolism (Depiereux et al., 1985; Fry and Saweikis, 2006; Parsons, 1980; Pecsenye et al., 1997; van der Zel et al., 1991; Vigue et al., 1982). One recent study examined the effects of larval ethanol exposure on adult development and ethanol responses (McClure et al., 2011). The natural habitat of larvae includes fermenting fruits that contain significant ethanol content (McKenzie and McKechnie, 1979), and it is likely that ethanol has significant impact on the physiology and behavior of larvae. Nevertheless, there has

not been an in-depth study of the acute effects of ethanol on larval behavior.

In this study, we establish early third instar larvae of *Drosophila melanogaster* as an animal model to probe how small doses of ethanol affect learning, a higher order emergent property of the nervous system, while leaving the sensory and motor functions intact. We use heat-shock conditioning, a robust larval associative learning paradigm (Khurana et al., 2012), to explore the effect of low pharmacologically relevant doses of ethanol on learning in *Drosophila melanogaster* larvae.

Methods

Morphologically, larvae have three instars or stages, but the third and most advanced instar is functionally subdivided into two substages; an early third instar and a wandering late third instar (Rajamohan and Sinclair, 2008). The olfactory paradigm and conditioning protocol used in this study (Khurana et al., 2012) were established for early third instar larvae.

Fly Husbandry and Harvesting of Larvae

Wild type flies of the Canton S strain (Bloomington stock # 1) were raised on standard cornmeal/yeast/molasses media on a 12/12 light/dark schedule at 24°C. To produce age-matched early 3rd instar larvae, adult flies were allowed to lay eggs on the media for 24 hours and then removed. Five days (+/- 8 hours) later, early 3rd instar larvae were collected by dissolving the larvae-containing media in water and rinsing away softer media through a sieve that does not retain the smaller second instar larvae. Larvae were then placed in a 1500 molecular weight polyethylene glycol

(PEG, Acros Organics AC19226-0051) solution that acts as a density separator wherein the larvae float and dense food particles sink. After the density separation, larvae were rinsed in water to remove traces of PEG. Larvae were then rested in 9 cm Petri dishes containing 0.5 ml of Ringer's solution until the onset of the experiment. The Ringer's solution contained 128 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM Na_2HPO_4, and 0.37 mM KH_2PO_4.

Ethanol Treatment

Larvae were treated with ethanol to determine if the ethanol affected their odor response or their associative learning capabilities. One hundred larvae were placed in a transfer chamber that was resting in a 5 cm diameter Petri dish containing 3 ml of 20% ethanol v/v diluted in water. The transfer chamber was a plastic beaker with the bottom replaced with a nylon mesh. Up to six groups of 100 larvae were treated simultaneously (see Larval Training and Testing below). Larvae were treated with ethanol for 20 minutes, rinsed with water and then Ringer's solution. Additionally, a group of larvae was treated with water only to serve as a control for the ethanol-treated group. Both ethanol- and water-treated animals underwent the same duration (20 minutes) of ethanol or water treatment and the same duration (45 seconds) of rinsing (See Figure 3.1B). After the ethanol or water treatment and subsequent rinsing, larvae were taken through one of the behavioral tasks: olfactory conditioning, olfaction, locomotion or heat avoidance.

Associative Conditioning Apparatus and Set-up

The experimental set up was similar to a previous study (Khurana et al., 2012). In associative conditioning, a conditioned stimulus (CS) is paired with an unconditioned stimulus (US) to produce an altered or conditioned behavioral response to the CS. In our heat shock learning paradigm, 10^{-4} dilution of the attractive odor ethyl acetate (EA, Fisher Scientific E145-1) was used as the CS and heat was used as the aversive US. The behavioral response following the pairing of these two stimuli is seen as a decrease in attraction to the EA. The presentation of the CS and US was done using agar-filled 9 cm glass Petri dishes. Specifically, 30 ml of 0.5% agar was poured into the Petri dishes. For heat presentation, the Petri dish was rested atop a heat block (Analog Dry Block Heaters VWR 12621) for 8 minutes prior to use. At equilibrium, the temperature of the top agar is 2°C lower than the temperature of the heat block. The temperature of agar surface was monitored in all experiments. Agar surface temperatures of 41˚C and 35˚C were used for training. For odor presentation, pure EA was mixed into the 0.5% melted agar just prior to solidification and vigorously shaken. Heating a Petri dish with odor mixed in allowed the simultaneous presentation of odor and heat. During the training, larvae were kept in a transfer chamber that was made by replacing the bottom of a plastic beaker (50 ml Tri-pour beaker no. 50-996-322, Fischer Scientific) with fine nylon mesh (obtained from a local fabric store). The transfer chamber allowed the experimenter to quickly move the larvae to different experimental conditions.

Figure 3.1: Experimental design. A. Schematic of the flow of the experiment. B. Schematic of the ethanol/water treatment protocol.

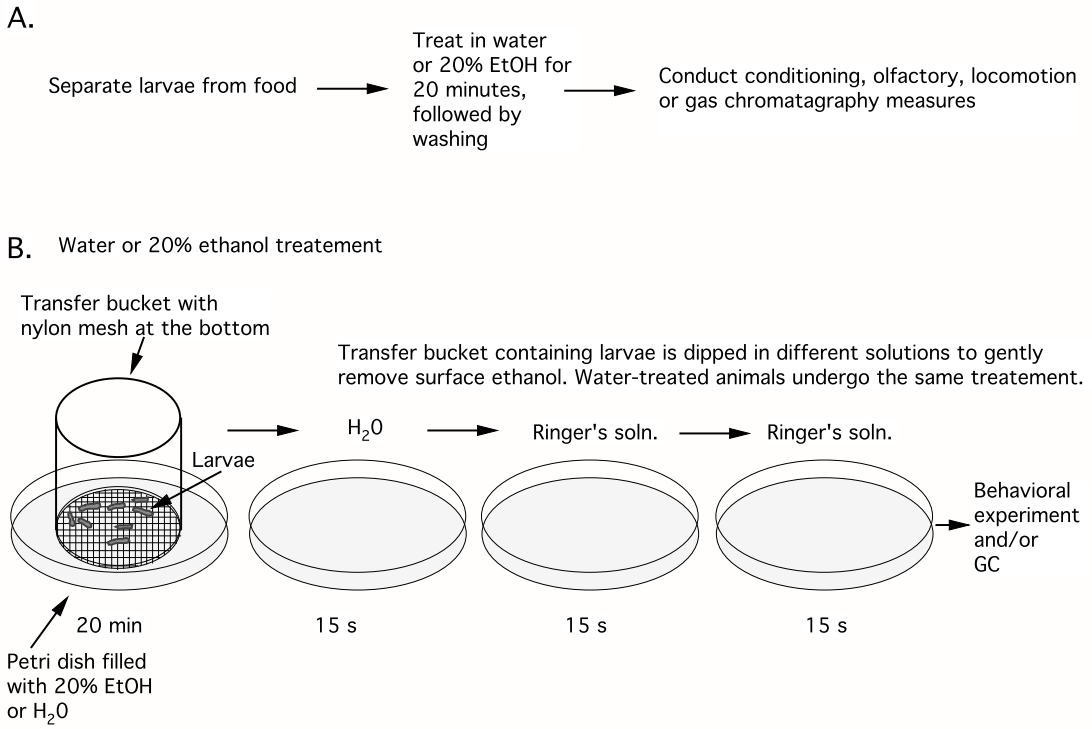


Figure1

Larval Training Procedure

Larvae were put in the transfer chamber and placed on a Petri dish filled with 0.5% agar at room temperature. To administer a heat shock, the transfer chamber was dipped in Ringer's solution, and then placed on a heated agar Petri dish. After a 30 second shock, the chamber was again dipped in Ringer's and placed back on the rest dish. An interval of 8 minutes was used between heat shocks. The training consisted of 3 shocks and the larvae were tested for their olfactory attraction within 5 minutes of the final shock. We confirmed that, regardless of the ethanol or water treatment, larvae exposed to the CS or the US alone had the same olfactory responses as sham-conditioned animals (Figure 3.4A). Because of this, we used one control group for the remainder of the study, the heat only stimulus (US), in addition to the trained group that received the simultaneous odor-heat (CS-US) pairing.

Larval Olfactory Testing

Following the training procedure, larvae were tested for their olfactory attraction to EA (Khurana et al., 2012). Olfactory testing was done on 15 cm Petri dishes containing ~15 ml of 2% agar. 30 larvae were placed in the center of the plate in a zone of 1 cm radius. On one side of the plate, 20 µl of EA diluted in pure liquid paraffin to a concentration of 10^{-4} v/v was spotted onto a paper disc. The odor was spotted 3 cm from the edge of the plate. Diametrically opposite to the odor, liquid paraffin was spotted similarly. Larvae were allowed to roam the plate freely for 3 minutes, at which point the number of larvae in a 2 cm radius zone around the odor was noted as well as the total number of larvae on the plate. We also noted the

number of larvae in a 2 cm radius zone opposite the odor zone. No attraction to the solvent alone was observed. Larvae that remained within the 1 cm drop zone were not counted because their lack of movement could be due to poor health. The number of these non-participants did not exceed 5% in any experiment. For each test plate, a response index (RI) was calculated as the fraction of total participating larvae on the plate that were found in the odor zone at the end of 3 minutes. For each experiment repetition, a minimum of 3 test plates were performed and averaged to give a single response index. For learning experiments, the response indices of the control group and trained group were compared to give a learning index. The learning index was defined as $(RI_{control} - RI_{conditioned})/RI_{control}$ and it represents the decrease in response to the odor caused by the training.

Tracking Larval Olfactory Response

Larval tracking was done in a manner similar to a previous study (Khurana et al., 2010). Twenty-four hours prior to the start of the experiment black food dye was added to the larvae-containing food. The dyed food inside the larvae is easily visible due to the transparent larval body wall. Using a standard olfactory testing procedure (described above), a camera was placed above the Petri dish and captured a frame of the plate each second. The particle counting algorithm applied a binary threshold to each frame, so only pixels darker than the threshold were counted. These pixels were then grouped together into objects, and each object larger than three pixels was counted as a larva. We manually verified that the 3-pixel threshold was sufficient to capture over 99% fully separated larvae without capturing erroneous noise. The

algorithm then calculated the coordinates of the centers of each larva. Using these coordinates, the algorithm then calculated the distance of each larval object to the odor center, which was predetermined. A Larval object that was less than 2 cm from the odor was counted as in the odor zone, and otherwise outside the odor zone. A correction factor had to be applied to each frame to account for the fact that individual larvae cannot be resolved in the very beginning of the test, when they are all aggregated in the center of the plate, and near the end of the test when the larvae are aggregated near the odor. The correction factor was as follows: for each frame of the movie, the number of larvae tracked was reported. The frame number that had the highest larval count was noted (and manually verified). For each frame prior to the highest larval count frame, the difference between the highest larval count and the count at that frame was assumed to be larvae in the center of the plate, i.e., outside the odor zone. For each frame after the highest larval count frame, the difference was assumed to be larvae inside the odor zone. After the correction factor was applied, the algorithm then calculated the number of larvae inside the odor zone and outside the odor zone. The response index was then calculated as described above. These steps were applied to each frame in the movie, so a response index vs. time plot could be generated.

Gas Chromatography

Approximately 100 larvae were placed in 750 µl of pure toluene in a micro centrifuge tube immediately following the ethanol treatment and rinse. The weight of larvae was determined by weighing the centrifuge tube before and after adding the

larvae. We determined the water content of larvae to be 81.4% of their weight by weighing larvae before and after desiccation in a 65˚C oven. The larvae were crushed with a small pestle and the supernatant was removed after spinning the tube at 15K rpm for 2 minutes. An auto sampler injected 3 µl of the extract into an SRI-310C Gas Chromatograph (SRI Instruments, Torrance, CA). The temperature protocol was: 50˚C for 1 minute, ramp for 10 minutes to 150˚C, and hold for 10 minutes. An ethanol peak is observed at approximately 2.2 minutes and toluene at approximately 10 minutes. All data were analyzed using PeakSimple (SRI Instruments, Torrance, CA). The area of the ethanol peak was determined using the integration tool with a threshold area size of 100. The ethanol content of the larvae was determined by a comparison to a known standard curve of ethanol. The concentration of ethanol in the larvae was determined by calculating the total number of mmoles of ethanol extracted from the larvae and dividing this by the total water content of the larval sample.

Heat Avoidance Assay

Larvae were tested for their sensitivity to heat or cold, in a manner adapted from Rosenzweig *et al.* (Rosenzweig et al., 2005), to ensure that effects on learning were caused by psychopharmacological properties of ethanol rather than anesthetic properties. To test heat sensitivity, a 9 cm glass Petri dish filled with 2% agar was situated on a heat block so that half of the plate was on the heat block and half of the plate was not. To test the cold sensitivity the agar plate was situated half on a heat block that had been cooled by ice to 18˚C. In all, we tested the two temperatures used for training, 41˚C and 35˚C, as well as 31˚C, 28˚C, 26˚C and 18˚C to explore the

sensitivity of the assay. Thirty larvae were placed in the center of the plate and at every 1-minute interval, for a total of 6 minutes, the number of larvae on each half of the plate was noted. An avoidance index was calculated by subtracting the number of larvae on the heated or cooled side from the number of larvae on the room temperature side and dividing the difference by the total number of larvae on the plate. In this index a negative score indicates attraction.

Statistics

Animals that were trained together were considered to be the same sample (N = 1) due to a lack of independence despite the fact that multiple testing plates were used to evaluate learning. The learning index for each sample was the average of a minimum of three testing plates of 30 animals each. Error bars presented throughout the study are the standard error of the mean (SEM). The significance score was calculated using Students *t*-test for pair-wise two-tailed comparison. The number of experiments and p-values are stated in the results section and figure legends. In the figures we use "*" to indicate significance level <0.05 and >0.01, and "**" for p-values <0.01. For all single comparisons we present the exact p value and for multiple pair-wise comparisons we present p values as lower or greater than 0.05.

Results

We wished to determine whether low-level ethanol intoxication affects the capacity for learning in *Drosophila melanogaster* larvae. We define a low dose as a dose that does not produce obvious changes in locomotor activity nor blocks sensation. Larvae were separated from the media, treated with ethanol or water, and

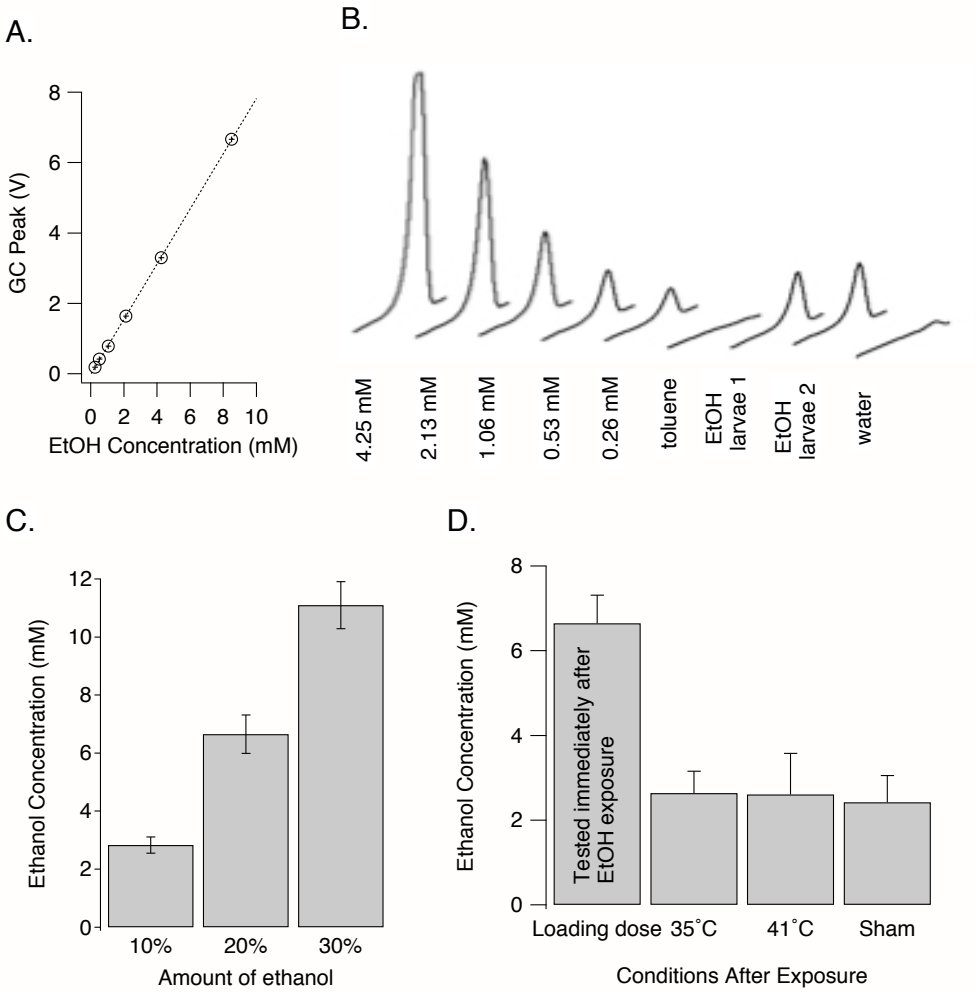
then taken through a behavioral test (Figure 3.1A). After the water or ethanol treatment we measured larval locomotion, olfaction, olfactory conditioning, and heat avoidance. Given that we wanted to correlate the behavior with the amount of ethanol in larvae, we also assessed larval ethanol content using gas chromatography. Additionally, we evaluated if the changes in animal behavior produced by ethanol are temporary by testing whether larvae recover a normal conditioning response after the ethanol has been metabolized.

Larvae Readily Absorb Low Concentrations of Ethanol and Retain it for the Duration of the Learning Assay

We used gas chromatography to measure internal ethanol concentration in the larvae. Larvae were crushed into toluene, and compared to a known standard curve of ethanol in toluene. We used a large volume of toluene (750 µl) in which to crush approximately 100 larvae to ensure that the metabolic processes of the larvae were completely and abruptly stopped and to ensure that the ethanol in the larvae directly enters the solvent. Ethanol was clearly detectable in the larvae and within our standard curve (Figure 3.2A-B). Figure 3.2B shows an example trace from two larval groups that were exposed to 20% ethanol for 20 minutes, one water-exposed larval group, and a standard curve. We used the weight of the larvae to determine the internal ethanol concentration (larvae are ~81% water by weight, see methods for details). We found that larvae absorb ethanol in a dose dependent manner (Figure 3.2C). Immediately after ethanol exposure, the internal ethanol concentration of larvae treated for 10 minutes with 10%, 20%, and 30% ethanol v/v diluted in water was 2.8 ± 0.3 mM (n = 8), 6.6 ± 0.7 mM (n = 13), and 11.1 ± 0.8 mM (n = 8),

respectively. No ethanol was detected in water-treated larvae (detection threshold ~0.5 mM, data not shown). We also observed that significant ethanol is retained through the entire conditioning experiment (Figure 3.2D). Given the volatile nature of ethanol and that conditioning involves heat exposure, we measured ethanol decay after taking larvae through the exact conditioning protocol. We showed that, regardless of heat-shock temperature, 3 heat shocks of 30 s did not cause a significant change in the clearance of ethanol content compared to larvae not receiving heat shocks (n= 13 for “Loading Dose”; n= 5 for all others; $p = 0.98$, 0.79 and 0.87 for 41˚C vs. 35˚C, 41˚C vs. sham and 35˚C vs. sham).

Figure 3.2: Perdurance of internal ethanol. A. Example standard curve for ethanol gas chromatography. All of the measurements noted in this document fall within the linear range of the gas chromatograph standard curve. B. Chromatographs of ethanol from larvae. Standard responses for known concentrations of ethanol (4.25 mM, 2.13 mM, 1.06 mM, 0.53 mM, and 0.26 mM) diluted in toluene as well as pure toluene are shown. Representative traces from larvae treated for 20 minutes with 20% ethanol (EtOH larvae 1 and 2) or water (control larvae) are also shown. C. The amount of ethanol absorbed by larvae depended on the amount of ethanol in the treatment solution. D. The brief heat shocks (41˚C and 35˚C) that were used in the conditioning experiments did not reduce internal ethanol below that measured in sham-treated larvae. Animals were treated for 20 minutes with 20% ethanol (Loading Dose) and then taken through the heat shock protocol at 35°C or 41°C as used in conditioning experiments. The loading dose is the same data shown in the panel C 20% bar graph and is repeated for comparison purposes. Sham-treated animals were taken through same protocol except that they did not receive the heat shocks but instead were moved to room temperature (24˚C) plates.



Low Internal Ethanol (~7 mM) does not Affect Olfaction of Locomotion

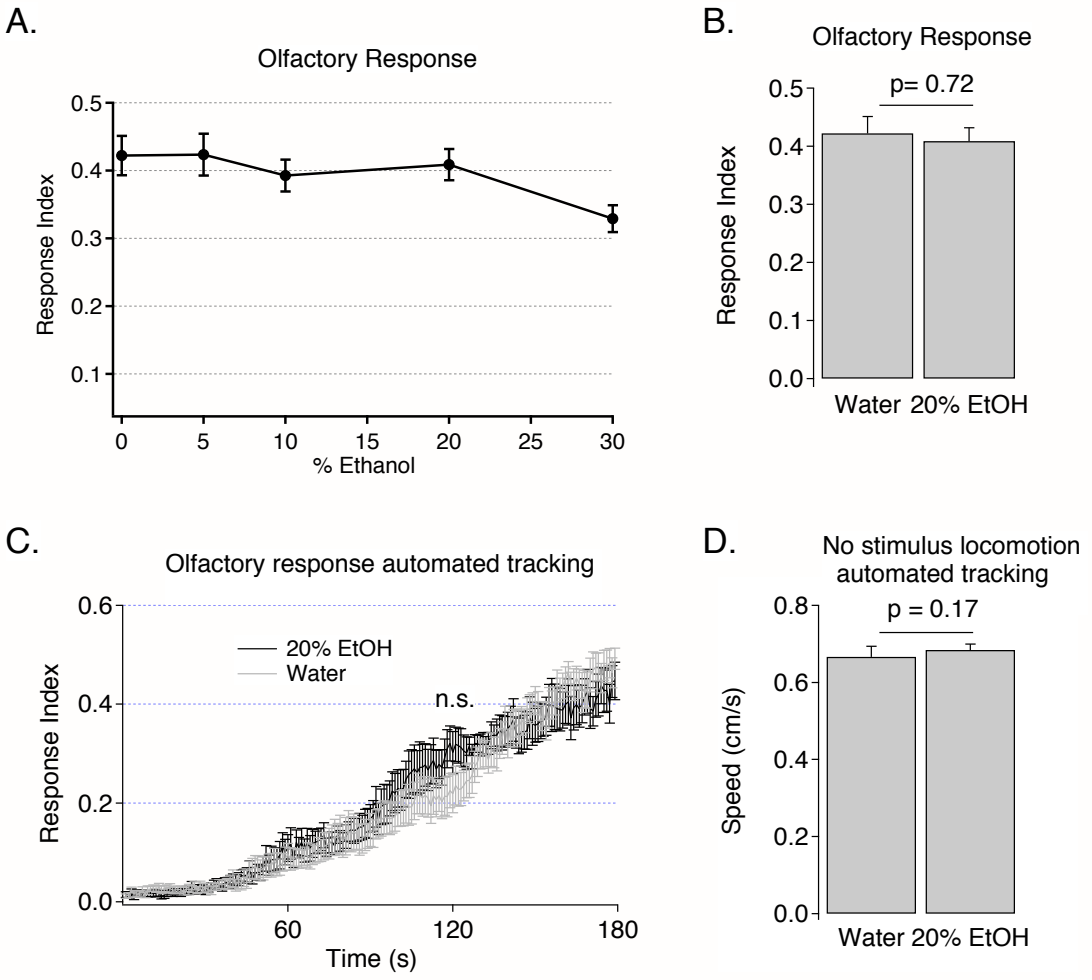
Ethanol might cause reduced learning because it specifically disrupts learning itself or because it alters sensory perception of either the conditioned stimulus (CS) or of the unconditioned stimulus (US). Given that we used an odor as the CS in our learning assay, we tested larvae for their olfactory response to 10^{-4} ethyl acetate (EA) following 20-minute treatments of 0% (water-only), 10%, 20%, or 30% ethanol. The response index to the odorant was determined by manually counting the number of larvae in proximity of odorant, defined as the odor zone (see methods) and the rest of Petri dish at the end of 3 minutes. Only the 30% ethanol-treated group showed a reduction in the olfactory response index, (30% vs. water, 5%, 10%, and 20% ethanol had $p = 0.013$, 0.022, 0.052, and 0.018 respectively; $n = 16$, 9, 9, 22 and 13 for water, 5%, 10%, 20% and 30% ethanol respectively), although, statistical significance was lost when a Bonferroni correction was applied. However, because we were interested in studying the consequences of an ethanol dose that perturbs higher-order functions without disturbing motor and sensory functions, we chose the 20-minute treatment with 20% ethanol in all further experiments. The 20% ethanol-treated larvae and the water-only group responded equally to 10^{-4} EA (Figure 3.3B, $n = 22$, $p = 0.72$).

Manual end point response measurements can hide differences in the rate of entry to the odor zone that are likely to be more sensitive measures of subtle differences in olfactory responses. Thus we used automated tracking to look at rate of entry of control and ethanol-treated larvae (Khurana et al., 2010). Using the tracks generated by the software, we were able to analyze the larvae's response indices (similar to manual counting) at any given time. Figure 3.3C shows the odor response

curve of ethanol-treated and water-treated larvae over a 3-minute period. The two groups of larvae showed statistically indistinguishable response indices throughout the test ($n = 8$, $p > 0.05$ for all individual frames). Using automated tracking, we also quantified speed of larval movement in the absence of odor to see if there are any gross defects in larval locomotion due to ethanol exposure. No significant difference in speed was observed due to ethanol exposure ($n = 7$, $p = 0.17$).

The similarity in response indices and speed indicated that animals retain the ability to respond to a conditioning experience even when exposed to ethanol. Therefore, it is reasonable to assume that any defects in learning reflect the impact of low-level ethanol on higher order information processing.

Figure 3.3: Olfactory response and locomotion are unaffected by ethanol. (A-C) Response indices are shown for larvae when placed in the middle of an agar dish with ethyl acetate on one side and liquid paraffin on the other. The number of larvae in each odor zone was counted after 3 minutes. A. The Olfactory response shows a mild reduction with 30% ethanol treatment but not with 20% ethanol treatment. B. Larvae had been previously submerged for 20 minutes in either pure water or 20% EtOH. No significant difference was seen. C. Automated tracking. Left: Response index over a three-minute period when larvae are being tracked. Larvae had been previously submerged for 20 minutes in either pure water or 20% EtOH. D. Average speed in the absence of a stimulus is shown for larvae during the three minute tracking period. 20% EtOH did not cause a significant reduction in either locomotion speed or olfaction.



The Effect of Ethanol on Learning is Dependent on the Intensity of the US

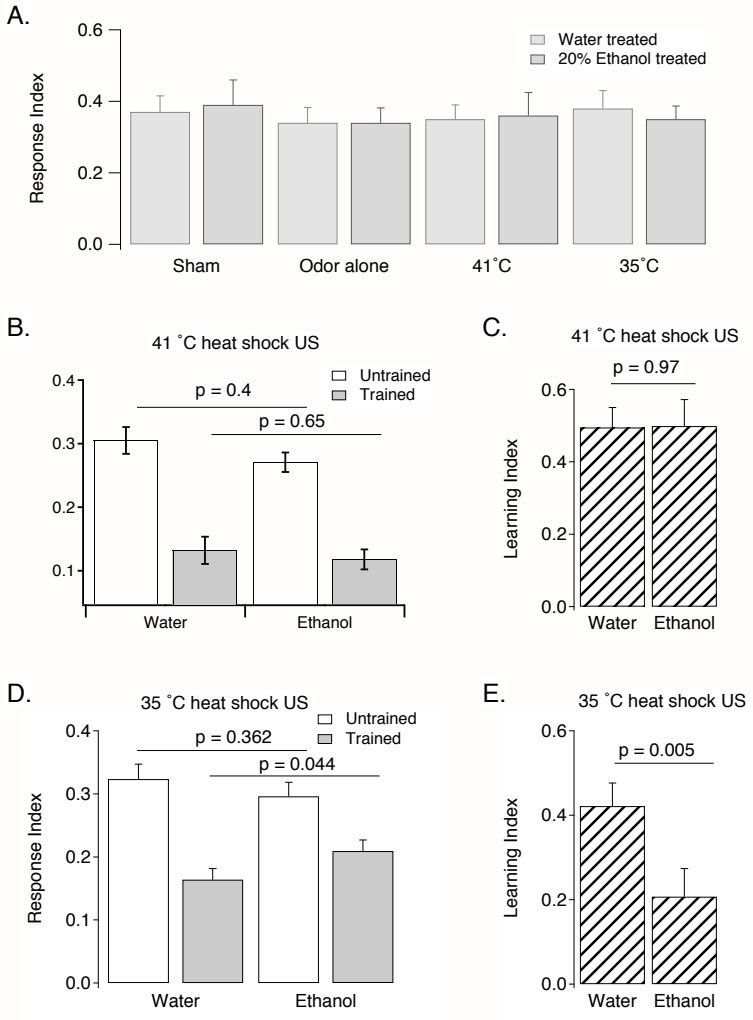
To explore the effect of ethanol on learning, we paired an odor with a heat shock and compared olfactory responses of this trained group with an untrained group. The learning index was calculated as the fractional decrease in olfactory response from the control group following conditioning [(Response $Index_{(control)}$ − Response $Index_{(conditioned)}$)/Response $Index_{(control)}$]. We confirmed that, regardless of the ethanol or water treatment, larvae exposed to the CS or the US alone had the same olfactory responses as sham-conditioned animals (Figure 3.4A). Because of this, we used one control group for the remainder of the study, the heat only stimulus (US), in addition to the trained group that received the simultaneous odor-heat (CS-US) pairing. The ethanol treatment did not alter the odor response of the untrained larvae (immediately after ethanol exposure; Figure 3.3) when the internal ethanol concentration was ~7 mM nor when the internal ethanol concentration was ~2.5 mM (at the time of the training session; Figure 3.4A).

The optimal punishment temperature for this learning paradigm is 41˚C (Khurana et al., 2012). Here we treated larvae with 20% ethanol and then trained the larvae in our heat shock paradigm by pairing 10^{-4} EA with a 41˚C heat shock. After 3 training trials, we found that larvae treated with ethanol learned similarly to larvae that received a water-only treatment ($n = 17$, $p = 0.97$). For both treatment groups, the trained larvae responded to the odor significantly less than the control larvae, indicating that learning has occurred. Trained and control response indices were similar for the water- and ethanol-treated larvae ($n = 17$, $p = 0.40$ for control and $n = 17$, $p = 0.65$ for trained response indices; Figure 3.4B) and learning indices for the

two groups were nearly identical (n = 17, p = 0.97; Figure 3.4C).

These results suggest that an internal ethanol concentration of ~7 mM has no effect on larval learning. However, other learning and memory studies have shown that the effects of ethanol on learning become apparent when suboptimal conditioning parameters are used (Hunt et al., 2009; Jacobson et al., 2011). To test the hypothesis that different learning conditions will reveal effects of ethanol on learning, we tested ethanol-treated larvae in the same paradigm, using a lower heat shock temperature. We found that ethanol-treated larvae had a significantly lower learning index than water treated larvae when trained with a 35˚C heat shock (Figure 3.4D and 3.4E). Figure 3.4D shows the trained and control response indices for the ethanol and water treated groups. While the control response indices of the ethanol- and water-treated groups were similarly high (n = 32, p = 0.362) the trained response index of the ethanol group was higher than that of the water group (n = 32, p = 0.044). This resulted in the ethanol-treated larvae having a significantly lower learning index than water-treated larvae (Figure 3.4E; n = 32, p = 0.005).

Figure 3.4: Ethanol treatment affects olfactory learning when the heat shock unconditioned stimulus is below the temperature optima. A. Either heat alone or odor alone presentations resulted in the same response index (RI = #Larvae in odor zone/#Larvae total) as sham-treated larvae ($p>0.05$ for any comparison). B. Response indices for untrained (control) and trained larvae are shown for animals that either received water or 20% EtOH. All larvae were trained to associate the odor with a 41˚C heat-shock. The response indices were similar for water-treated and ethanol-treated groups when comparisons were made for similar conditions such as the untrained group or the trained group. C. Learning indices ($LI = (RI_{control} - RI_{conditioned})/RI_{control}$) calculated from the data in Panel B. D. Response indices for untrained (control) and trained larvae are shown for animals that either received water or 20% EtOH. All larvae were trained to associate the odor with a 35˚C heat-shock. The conditioned response indices are significantly different in the ethanol treated groups ($n = 32$; $p = 0.044$). E. Learning indices calculated from the data in Panel D. Ethanol induced a significant reduction in learning.

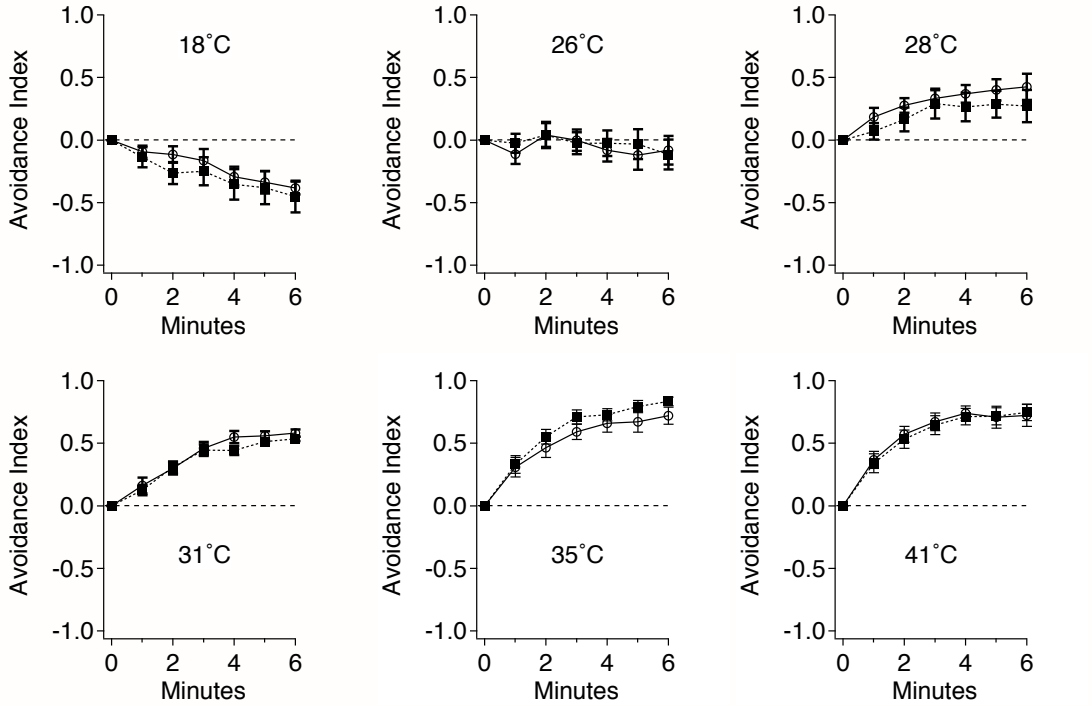


Decreased Learning is not Caused by Anesthetic Properties of Ethanol

One possible explanation for a decrease in learning at 35˚C is that ethanol anesthetizes the larvae to reduce the aversive properties of the heat pulse. This would make the heat shock a less effective unconditioned stimulus. We therefore compared the aversion of larvae to heat before and after ethanol exposure in a manner adapted from Rosenzweig *et al*. (Rosenzweig et al., 2005).

We compared the capacity of untreated and ethanol-treated larvae to sense a wide range of temperatures (18°C to 41°C). One half of a Petri dish was cooled or heated and larvae were placed in the center of the dish. Larvae quickly sense the temperature gradient and move towards the side that is closer to their preferred temperature. We calculated the fraction of larvae on the room temperature side of the plate during a six-minute assay. With heat blocks set to produce agar temperatures of 41˚C, 35˚C, 31˚C, 28˚C, 26˚C or using a cooled block to produce 18˚C agar, we observed that the untreated and ethanol treated larvae partitioned similarly between the temperature extremes. The capacity to sense and avoid temperature extremes was not affected by the ethanol treatment (Figure 3.5, $n = 20$, $p > 0.05$).

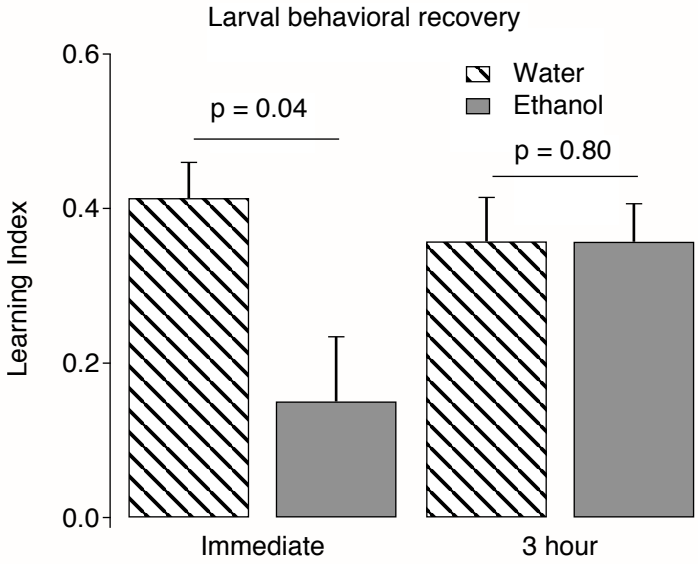
Figure 3.5: The learning deficit is not caused by ethanol anesthesia. The ethanol treatment did not reduce the sensitivity of larvae to the heat treatment (US). Larvae were placed onto an agar dish in which half of the dish is 24˚C and the other half is held at a different temperature. An avoidance index was calculated based on how many larvae avoided the artificially heated or cooled half of the plate. Shown are plots indicating the avoidance index at every minute for the total duration of heat-avoidance assay. The dotted lines with filled squares are ethanol treated group and solid lines with empty circles are the water treated group. The ethanol and water treated groups are not different for any temperature tested ($p>0.05$ for all points).



The Effects of Ethanol Exposure are Transient

We wanted to know if the acute effects of ethanol were permanent or transient. To test this, we conditioned larvae three hours after ethanol exposure. We found that the learning indices of larvae measured three hours following a 20 minute 20% ethanol exposure were statistically indistinguishable from larvae tested three hours following a water exposure (Figure 3.6). The complete reversibility of the learning deficit indicates that this ethanol treatment does not compromise learning because it induces permanent damage.

Figure 3.6: Effects of ethanol are temporary. Behavioral recovery of learning ability after ethanol exposure was tested by dividing both the ethanol-exposed and water-exposed groups into two subgroups, one conditioned immediately after treatment and one conditioned three hours later. No significant difference was observed in the conditioning scores of alcohol and water treated groups at the end of three hours in contrast to the immediately-conditioned group ($p = 0.8$, $n = 5$).



Discussion

In this paper, we show that ethanol negatively affects heat shock induced olfactory associative learning in Drosophila larvae. The deficit in learning is not caused by a deficit in locomotion or olfaction because the drug does not affect odor response indices or speed. Neither is the learning deficit a product of the anesthetic effect of ethanol, since this ethanol treatment did not reduce the response of the larvae in the heat avoidance assay. Thus, the deficit in learning must reflect a subtle perturbation of the learning process by a low dose of ethanol.

This is one of the first Drosophila behavioral assays to capture an effect of low doses of ethanol on learning and memory. Low doses of ethanol, which are commonly thought to be harmless, frequently cause occupation-based injuries or deaths (Camino Lopez et al., 2011; Phillips and Brewer, 2011). For the study of the effects of low doses of ethanol, fruit fly larvae are advantageous because they sport all of the genetic tools of Drosophila and have a simple nervous system (~2000 functional neurons as opposed to ~100,000 in the adult fly) (Iyengar et al., 2006) that lends itself to genetic dissection. Finally, subtle changes in behavior can be quantified because the behavior of large populations of larvae can be quantified using simple and inexpensive methods.

An ethanol-dependent learning deficit is observed when the heat-shock reinforcing temperature is below the optimal temperature for learning. We find a deficit in learning when larvae are heat-shocked at 35˚C, but not at 41˚C. We suspect that the effects of ethanol are not strong enough to suppress associative conditioning to a strong 41˚C heat shock, but can influence a less salient 35˚C heat shock. We

believe that the use of a sub-optimal US shifts the assay to a region of the stimulus-dependent learning curve that is better suited to reveal the subtle effects of ethanol (Khurana et al., 2012). In rats, low-level ethanol has been shown to reduce the capacity for attention (Givens and McMahon, 1997). In larvae, a diminished capacity for attention could reduce learning by further lowering the effectiveness of the suboptimal US. A lack of visible reduction in learning at 41˚C could also be the result of overtraining caused by an ethanol-induced increase in the valence of the punishment at this temperature.

We quantified the internal amount of ethanol in this study using gas chromatography. It is interesting to note that when placed in 20% (3.425 M) ethanol for 20 minutes, the concentration of ethanol within the larvae rises to only ~7 mM. In humans 7 mM ethanol, which corresponds to a blood alcohol concentration (BAC) of ~0.03, is considered to be a rather low dose of ethanol. This is a level at which it is currently legal to drive throughout the United States. In larvae, the slow metabolism of ethanol cannot account for the differential between external and internal ethanol concentration (Figure 3.2D) indicating that the absorption of ethanol by larvae is somehow severely restricted. This is unsurprising, as the natural habitat of larvae includes fermenting fruits that can contain over 7% ethanol (McKenzie and McKechnie, 1979). If there were no system to control ethanol absorption then the ethanol that larvae encounter in their natural life cycle would likely be fatal. This capacity to limit ethanol absorption is shared by another invertebrate inhabitant of fermenting fruit (Davies et al., 2003; Felix and Braendle, 2010). *C. elegans* also show a remarkable ability to limit ethanol absorption when placed in a high ethanol

environment. Each model organism provides a unique perspective on a biological process. This paper establishes the use of the genetically malleable fruit fly as a model to study ethanol-induced effects on higher order behaviors like learning.

Acknowledgements

SK, BGR, JBP, WL, and NSA designed the study and wrote the paper. BGR, SK, JBP and other co-authors conducted the experiments and analyzed the data. This work was funded by NIH R01 AA018037 to NSA and NSF grant IOS-0641370 to NSA supported the undergraduate contribution. BGR and JBP were supported by the NIH Ruth L. Kirschstein National Research Service Award Institutional Research Training Grant # 5T32AA007471. AC and RG received The University of Texas Undergraduate Research Fellowship. We thank George Pollack for allowing use of his lab space.

Chapter 4. Chronic Ethanol Exposure leads to Physiological Dependence

NEURAL ADAPTATION LEADS TO COGNITIVE ETHANOL DEPENDENCE

The majority of the text, data, and figures in this chapter have been published in a 2012 manuscript in the journal *Current Biology*:

Robinson, B. G., Khurana, S., Kuperman, A., and Atkinson, N. S. (2012a). Neural Adaptation Leads to Cognitive Ethanol Dependence. Curr Biol 22(24), 2338-41.

Co-author contributions: S. Khurana is a postdoctoral collaborator that helped design the study. A. Kuperman is an undergraduate research assistant that helped fill in experiments involving larval hyperexcitability. N.S. Atkinson is my P.I. and funded the study and helped with writing.

Summary

Physiological alcohol dependence is a key adaptation to chronic ethanol consumption that underlies withdrawal symptoms, is thought to directly contribute to alcohol addiction behaviors, and is associated with cognitive problems such as deficits in learning and memory (Koob and Le Moal, 2006; Parsons and Nixon, 1993; Stavro et al., 2012). Based on the idea that an ethanol-adapted (dependent) animal will perform better in a learning assay than an animal experiencing ethanol withdrawal will, we have used a learning paradigm to detect physiological ethanol dependence in Drosophila. Moderate ethanol consumption initially degrades the capacity of larvae to

learn, but they eventually adapt and are able to learn as well as ethanol-naive animals. However, withholding ethanol from ethanol-adapted larvae impairs learning. Ethanol reinstatement restores the capacity to learn, thus demonstrating cognitive dependence on ethanol. The larval nervous system also shows ethanol withdrawal hyperexcitability. Larvae reach ethanol concentrations equivalent to 0.05 to 0.08 BAC—levels that would be mildly intoxicating in humans. These ethanol-induced changes in learning are not the product of sensory deficits or state-dependent learning. This is the first demonstration of cognitive ethanol dependence in an invertebrate genetic model system.

Methods

Detailed methods can be found in Supplemental Experimental Procedures.

Ethanol Treatment. Standard fly media was supplemented to 5% (v/v) ethanol.1

Learning Assay. Larvae were trained in three trials in which an aversive heat shock was paired with an attractive odor. Training trials were separated by 8 minute inter-trial intervals. Following the conditioning, larvae were tested for their attraction to the odor. Approximately 30 larvae were placed on an agar plate that had the odor spotted on one side. At the end of a 3-minute period the fraction of larvae in a 1 cm zone around the odor were recorded. As a control, the same protocol was performed with larvae that received a heat shock without the paired odor.

Picrotoxin Treatment and Measuring Excitability. In an assay adapted from (Stilwell et al., 2006) larvae were removed from their food and incubated in two mL of 10 mM picrotoxin solution for six hours.,Then the number of peristaltic

contractions over a 30 second period were visually recorded.

Statistics. Student's *t* test was used to compare two groups. Multiple-point comparisons were performed by one-way ANOVA. Multiple-condition comparisons were performed by two-way ANOVA. Bonferroni posttests were performed when significant

Supplementary Experimental Procedures

Fly Food, Fly Stocks, and Harvest of Larvae

Ethanol-supplemented fly food was made from melted standard fly food that was cooled to ~45°C (a temperature immediately before agar re-solidification) and then supplemented with absolute ethanol (Decon Laboratories Inc., King of Prussia, PA) to achieve a 5% v/v concentration. Non-ethanol control fly food (water-supplemented) was processed in the same manner except that water was added in place of ethanol. The food was held at room temperature for 24 hours prior to use. Each day of the experiment, 0.3 ml of a 10% ethanol solution was pipetted on top of the larvae-containing food, and 1 ml of 10% ethanol was pipetted onto the cotton plug that seals the fly bottle. For non-ethanol controls the same volumes of water were added in place of the ethanol solution.

Wild-type Canton S strain of *Drosophila melanogaster* (Bloomington stock #1) were used for all experiments and were raised on standard cornmeal/agar/molasses medium at 25°C on a 12/12 light/dark schedule. Larvae were collected as originally described in Khurana *et al*., (Khurana et al., 2009). Briefly, adult flies were allowed to

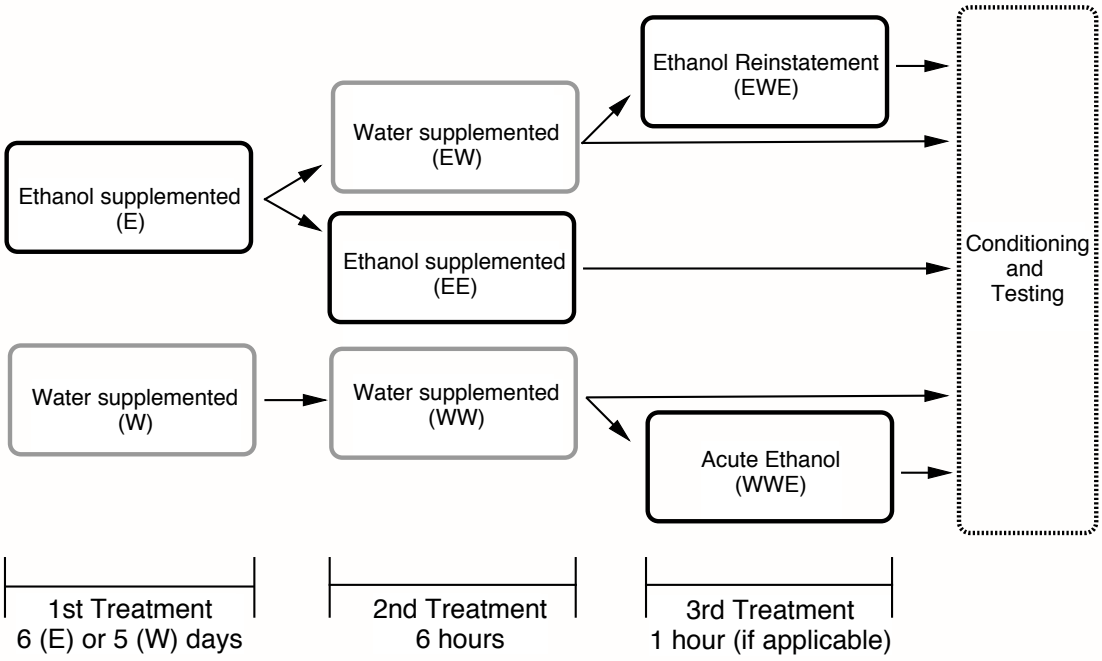
lay eggs in bottles for 24 hours. Larvae in the middle of the 3rd instar (5-6 days after egg laying) were used in all experiments. Once larvae were the proper age, the larvae-rich top layer of food was dissolved in water, sieved, and rinsed with water. Small larvae (1st and 2nd instar larvae) and small particles of food pass through the sieve. The remaining larvae were separated from food particles by resuspension in 50 ml of a 30% polyethylene glycol (PEG mwt 1500, Acros Organics AC19226-0051) solution. In this solution, larvae float while the food sinks. Larvae were then rinsed in water and placed in Ringer's solution until the start of the experiment (less than 2 minutes). Ringer's solution was 128 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM Na_2HPO_4, and 0.37 mM KH_2PO_4. The entire procedure allows the isolation of approximately 200 larvae in 5 minutes.

Acute and Chronic Ethanol Treatment

To determine the effects of chronic ethanol exposure and ethanol withdrawal, the larvae were raised on ethanol food or non-ethanol food (control) until they reached the third larval instar. Third instar larvae perform well in the learning and memory assay (Khurana et al., 2012). As shown previously, ethanol slightly slows development (McClure et al., 2011) so that ethanol-treated larvae reach third instar on day 6 while water-treated larvae reach third instar on day 5. The ethanol-treated larvae were extracted from their food and divided into two groups. One group was placed in a fresh bottle of ethanol-supplemented food (so-called EE group; Figure 4.1) for 6 hours. The second group was placed in non-ethanol, water-supplemented food (so-called EW group; Figure 4.1) for 6 hours. Animals were raised concurrently on

water-supplemented food (so-called WW group; Figure 4.1). While the WW group was never exposed to ethanol it was otherwise handled identically to the EE group. After these treatments, larvae from all groups were tested for their ability to learn. In addition, half of the EW group was placed into another fresh ethanol-supplemented bottle for a 1-hour ethanol reinstatement (so-called EWE group; Figure 4.1) and then assayed for the ability to learn. Acute ethanol treatment and ethanol reinstatement (WWE and EWE, respectively; see Figure 4.1) were performed by placing larvae in ethanol food for 1 hour before behavioral testing.

Figure 4.1: Experimental design. Larvae were raised in ethanol (E)- or water-supplemented food (W, non-ethanol). To test for an ethanol-withdrawal effect on learning, two thirds of the E larvae were placed on water-supplemented food (EW; withdrawal group) for six hours. The remaining one third of the E larvae were placed on ethanol-supplemented food (EE) for six hours. To determine whether ethanol withdrawal compromised learning, learning was compared in the EW and EE groups. To determine if ethanol reinstatement could reverse withdrawal effects, half of the EW group was moved back to ethanol food for one hour (EWE), and the capacity to learn was measured. Larvae raised on non-ethanol food were also prepared (W; the water supplemented group) purified and placed back on food without ethanol (WW). After six hours WW was split in half. One half was assayed for the ability to learn. The other half was placed on ethanol food for one hour (WWE) and then assayed for the capacity to learn.



Acute and Chronic Sucrose Treatment

To ensure that the ethanol withdrawal symptoms were not merely symptoms of changing calories (calorie withdrawal) we performed the same treatment as described above using sucrose instead of ethanol. Ethanol provides 7 kcal/g whereas sucrose provides 4 kcal/g. To make a sucrose-supplemented media that is isocaloric with the 5% ethanol media we added 7 grams of sucrose for every 4 grams of ethanol that were added in the ethanol-supplemented food. This resulted in 7% or 200 mM sucrose food. The treatment schedule was the same as for the ethanol-treated larvae (see Figure 4.1). We tested for any acute effects of calorie-rich food by giving larvae a 1-hour treatment of sucrose-supplemented food (WS) and compared this group to sucrose-naive larvae (WW). We also tested the effects of a chronic sucrose treatment (SS) and a chronic sucrose treatment followed by a 6-hour withdrawal from sucrose (SW). These controls were performed as described in the previous section for ethanol except that sucrose was substituted for ethanol.

Heat-Shock Olfactory Conditioning

Heat-shock olfactory conditioning was carried out as described in Khurana *et al*., (Khurana et al., 2012). Heat shock treatment was the unconditioned stimulus (US). Briefly, approximately 100 larvae were placed in a transfer chamber that was constructed from a 1 cm high section of a 50 ml falcon tube that was glued to nylon mesh on one end. The transfer chamber allows the larvae to be easily moved to and from the training plate. Heat shock was delivered by placing the larvae, in the transfer chamber, on a pre-warmed glass Petri dish containing 20 ml of 0.5% agar. Pre-

warming was performed by placing the dish on a heat block set to 43°C for 8 minutes after which the surface of the agar had equilibrated to 41°C. Prior to and in between each conditioning trial, the transfer chamber and larvae were moved to a room-temperature Petri dish that contained 20 ml of 0.5% agar.

To pair the heat unconditioned stimulus (US) with the odor conditioned stimulus (CS) both were presented to the larvae at the same time. To achieve this, 2 µl of pure ethyl acetate odorant (EA) was added to 20 ml of 0.5% molten agar just prior to solidification and poured into a Petri dish. After the agar fully solidified, this Petri dish was placed on a 43°C heat block as described above. To administer a conditioning trial, the larvae (in a transfer chamber) were placed on the heated odor plate for 30 seconds, rinsed in Ringer's saline and returned to a room temperature plate. After an 8-minute inter-trial interval, another heat shock was administered. The training was complete after 3 heat-shock trials. It has been previously shown that neither the heat shock alone nor the odor exposure alone cause a change in the olfactory response (which is used for quantifying learning; see below) (Khurana et al., 2012). In this study, we ran a group of larvae that received only heat shocks (no odor pairing) alongside every trained group as a control. Additionally, we confirmed all of our learning results using a training procedure in which the odor CS was presented to the larvae from above the transfer chamber rather than from in the agar (Khurana et al., 2012). The two protocols produced qualitatively identical results (data not shown).

Behavioral Quantification of Learning

To determine how well the trained larvae learned, we performed an olfactory response test. Thirty larvae were placed in the middle of a 15 cm plastic Petri dish filled with ~15 ml of solidified 2% agar. On one side of the plate, 20 µl of a 10^{-4} dilution of EA in liquid paraffin was spotted onto a disc of filter paper 2 cm from the edge of the plate. Diametrically opposite, 20 µl of pure liquid paraffin was spotted onto a filter paper disc. Larvae were allowed to roam the plate for 3 minutes, at which point the number of larvae in a 2 cm radius odor zone around the odor and the total number of larvae on the plate were noted. Untrained larvae congregate closely around the odor whereas a much smaller fraction of larvae are found around the odor following training. For each trained and control group of larvae, we calculated an odor response index (RI):

RI = # Larvae in odor zone / # Larvae total

We calculated a learning index (LI) by comparing the response indices of the control and trained groups:

$$LI = (RI_{control} - RI_{trained}) / RI_{control}$$

Heat Avoidance Assay

In order to determine the extent of the larval aversion to the heat-shock US, we placed 30 larvae in the center of a 9 cm Petri dish that was situated so that half of the plate was being heated by a heat block while the other was not being heated (Rosenzweig et al., 2005). We measured how many larvae were on each half of the plate every minute for six minutes. A heat-avoidance index was calculated as:

HA = (# Larvae on non-heated side – # Larvae on heated side) / # Larvae total

Gas Chromatography to Determine Ethanol Content

To test the internal ethanol levels of larvae in the ethanol food, we picked larvae individually from the food, rinsed them briefly in Ringer's solution and put them in toluene. Approximately 50 larvae were combined in a microcentrifuge tube containing 500 µl of toluene. We also tested the ethanol concentration in the larvae following the *en masse* PEG separation from their food (described above). Immediately after the PEG extraction, approximately 50 larvae were placed in 500 µl of toluene in a microcentrifuge tube. The weight of larvae was determined by weighing the centrifuge tube before and after adding the larvae. The water content of third-instar larvae is 81.4% of their weight (Robinson et al., 2012b). The larvae were crushed with a small pestle, and the supernatant was removed after centrifugation. An auto sampler injected 3 µl of the supernatant into an SRI-310C Gas Chromatograph (SRI Instruments, Torrance, CA). The temperature protocol was as follows: 50°C for 1 minute, 10 minute ramp to 150°C, and hold for 10 minutes. The ethanol peak elutes at approximately 2.2 minutes and the toluene peak elutes at approximately 10 minutes. All data were analyzed using PeakSimple (SRI Instruments, Torrance, CA). The area of the ethanol peak was determined using the integration tool. The ethanol content of the larvae was determined by a comparison to a known standard curve of ethanol.

Larval Picrotoxin Treatment and Seizure Assessment

The effects of picrotoxin (PTX) on larval locomotion were tested in a manner adapted from Stilwell et al. (Stilwell et al., 2006). To treat larvae with PTX, larvae were extracted from their food bottles and placed on 3% agar plates. PTX was dissolved in TE buffer with 20% grape juice to achieve a concentration of 10 mM PTX. Two mL of this PTX solution was put on the agar plate with the larvae. After 5 hours, larvae in the PTX solution were observed under a microscope and the number of full peristaltic contractions in 30-second bins were counted. The peristalsis rate of ethanol food raised larvae was compared to the peristalsis rate of ethanol naive larvae in both PTX-free and PTX-treated conditions. Additionally, after 6 hours of PTX treatment, the larvae were rinsed, split into 2 groups, and placed on fresh plates for a 1 hour recovery. One of the groups was given 2 mL of 5% ethanol during recovery. After the recovery, larvae were again observed for the number of peristaltic contractions performed over 30 seconds.

Adult Fly Ethanol Sensitivity Assay

In this experiment, flies raised on non-ethanol food were compared to those raised on ethanol food. We wished to analyze only the effects of larval ethanol exposure. Therefore, for the ethanol-raised flies, once the larvae had begun to pupate on the side of the food bottles, the ethanol-supplemented food was replaced with ethanol-free food. This was achieved by cutting off the bottom of the food bottle and replacing it with a new food bottle bottom and sealing it with Parafilm.

Adult female flies were collected 3-5 days following eclosion. Ten flies were placed in plastic vials and 6 vials (60 total flies) were used for each group. Pure ethanol vapor was blown through the vials until all flies in all groups were sedated (10-12 minutes). At this point the ethanol vapor was replaced by an air flow. Every two minutes the number of flies that had regained postural control following the sedation was recorded until all of the flies were recovered or determined to be dead.

Adult Fly Seizure Induction

In this experiment, flies raised on non-ethanol food were compared to those raised on ethanol food. Individual female flies were immobilized in a clay molding by pressing their legs and wings into the clay. Detailed methods for the electrophysiological assay can be found in previous studies performed by Dr. Alfredo Ghezzi (Ghezzi et al., 2010; Ghezzi et al., 2012). Two stimulating electrodes were inserted into the fly brain at the junction of the eye and forehead on each side of the head. A recording electrode was placed the the flight muscle in the thorax of the fly. Every eight minutes the flies were given a 1.5 second long, high-frequency electroconvulsive shock consisting of 1 ms pulses at 200 Hz. Each successive shock was at an increasing voltage until the fly reliably displayed a seizure. A seizure was defined as an immediate high-frequency discharge, followed by a prolonged period where a single pulse given to the fly brain failed to evoke a response.

Results and Discussion

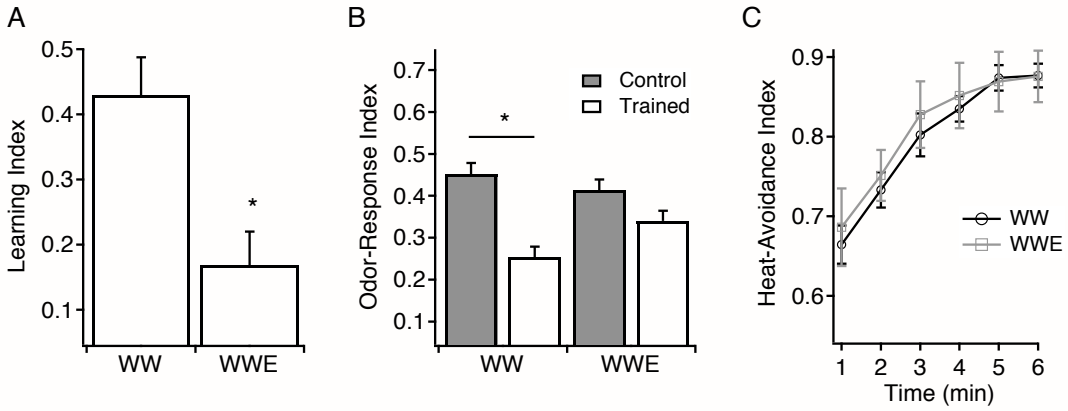
To test for chronic ethanol adaptation and to determine whether abstinence precipitates a withdrawal syndrome, we used an olfactory heat-shock conditioning assay (Khurana et al., 2012) in which larvae associate a heat pulse (US) with an otherwise attractive odor (CS). Associative learning reduces attraction to the odor. The paradigm in Figure 4.1 allows comparisons of the effects of acute ethanol, chronic ethanol, ethanol abstention, and ethanol reinstatement. An abbreviated nomenclature for each group is described in Figure 4.1 (WWE, EE, EW, EWE). In this paradigm, larvae are exposed to ethanol as a 5% supplement to their food. This "ethanol food" is at the high end of the range of ethanol concentrations encountered by larvae in the wild (Gibson et al., 1981).

Acute Ethanol Impairs Learning

Larvae fed ethanol food for 1 hour (WWE in Figure 4.1) learn poorly compared to larvae that had not consumed ethanol food (Figure 4.2A). The odor-response index of untrained animals maintained on ethanol food for 1 hour (WWE) or on non-ethanol food (WW) did not differ (Figure 4.2B), demonstrating that the ethanol-induced depression of learning was not caused by a reduced ability to sense the odor. The effect of ethanol was apparent only in trained animals (Figure 4.2B). Because the WWE and WW larvae sensed heat equally well (Figure 4.2C), the reduction in learning was not caused by anesthesia. Moreover, none of the additional ethanol treatments shown in Figure 4.1 reduced the capacity of larvae to sense either the odorant used as the CS (*cf*. Control groups; Figure 4.3B) or the heat used as the US

(Figure 4.3C).

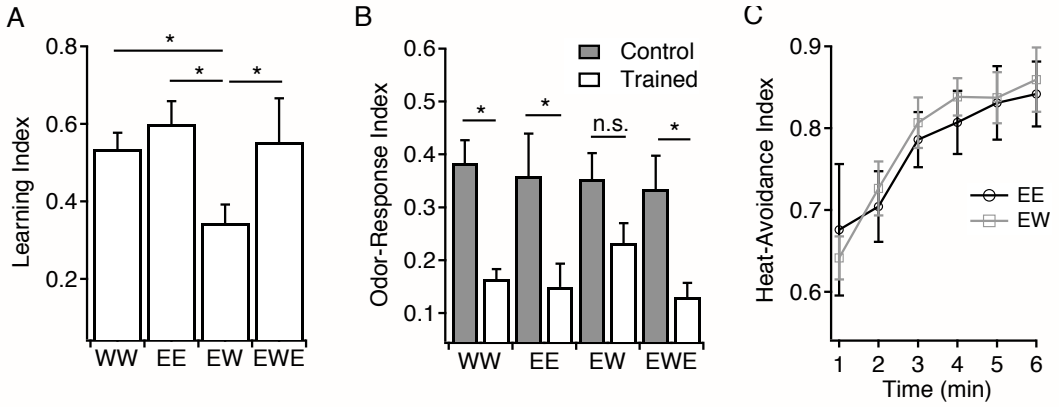
Figure 4.2: Acute ethanol treatment impairs learning. A) Larvae fed ethanol food for 1 hour (WWE) showed reduced learning when compared to ethanol-naive larvae (WW). (* p=0.006, N=7). B) Shown are odor-response indices for the water-treated (WW) and acutely treated ethanol group (WWE). Gray bars are mock-trained and open bars are trained larvae. There was an overall significant effect of training (* p<0.001), however posttests indicated that trained and control responses differed only within WW group but not within WWE group (* p<0.001 for WW p>0.05 for WWE, N=8). C) The ethanol treatment did not alter larval sensitivity to heat as both groups avoided the heated section of a dish at the same rate. There was a significant effect of time during the test (* p<0.0001), but not of treatment (p=0.7514).



Dependence and Withdrawal

To test for ethanol dependence, larvae fed ethanol food for 6 days were divided into two groups. Group EE was placed on ethanol food for an additional 6 hours and the EW group was subjected to a 6-hour ethanol withdrawal period. After the 6-hour period, half of the EW group received a 1-hour ethanol reinstatement to generate group EWE. Chronically exposed (EE) and ethanol-naive (WW) animals learned equally well (Figure 4.3A). However, the EW withdrawal group showed reduced learning in comparision to the WW or EE groups. This withdrawal phenotype (reduced learning) can be attributed to ethanol dependence because a one-hour ethanol reinstatement (EWE) restored learning to normal, non-withdrawal levels (Figure 4.3A).

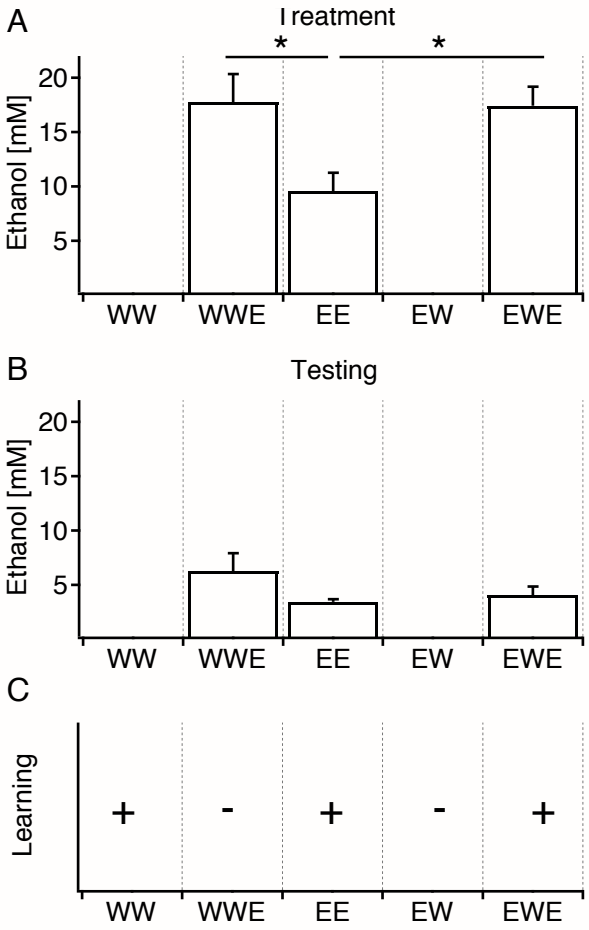
Figure 4.3: Chronic ethanol consumption induces ethanol dependence. A) Larvae continuously treated with ethanol (EE) learn as well as ethanol naive larvae (WW). A six-hour withdrawal (EW) decreases learning. A one-hour ethanol reinstatement reverses this deficit (EWE). ANOVA indicated a between-group difference ($p = 0.0025$), and post-hoc analyses indicated that EW differed significantly from all other groups (Bonferroni correction, * $p<0.05$; N=9). B) Odor-response indices show a significant overall effect of training (* $p<0.0001$, N=9) across all groups. A pairwise posttest indicated that within the EW treatment group the trained and control conditions did not differ significantly ($p>0.05$) meaning that learning was absent or weak in this group. C) The differences in associative learning are not caused by anesthesia since the EE and EW larvae responded equally in the heat-avoidance assay ($p>0.4$, N=6). Additionally, the coloric value of ethanol could not account for the changes in learning ability because an isocaloric supplement of sucrose (to 5% ethanol) to the larval food did not mimic the dependence and withdrawal phenotype (Figure 4.5).



Internal Ethanol Concentration

We measured the internal ethanol concentration for each treatment group (Figure 4.1). Larvae were plucked from the food at the end of the ethanol or water treatment, dipped in saline to remove adhering food, crushed in toluene, and the extract analyzed by gas chromatography. The EE group (6 days of ethanol food) had an internal ethanol concentration of ~10 mM (Figure 4.4A). No ethanol was found in groups that had been housed on non-ethanol food for 1 or more hours (WW and EW; detection threshold of ~0.5 mM). Finally, the acute ethanol group (WWE) and the ethanol reinstatement group (EWE) reached an internal concentration of approximately 17 mM (Figure 4.4A).

Figure 4.4: Quantification of internal ethanol concentration. A) Larvae were individually picked out of the food, rinsed to remove clinging food, and analyzed for ethanol content. The means were found to be significantly different (*p=0.005) and posttests indicate that both WWE and EWE larvae had higher internal ethanol concentration than EE larvae (* p<0.05 ; N=13). WW and EW larvae did not contain measurable ethanol (detection threshold of ~0.5 mM). B) The en masse PEG larvae purification reduces internal ethanol but WWE, EE, and EWE larvae did not have statistically different levels of ethanol following PEG purification (p=0.1543; N=8) C) Summarized learning capacity of each category of larvae as determined in Figures 4.2 and 4.3. Plus signifies normal and minus signifies a poor capacity to learn. The poor capacity to learn in the EW group also correlates with larval nervous system hyperexcitability (see Figure 4.6).



When the larvae are isolated *en masse* for the learning and memory assay, they are separated from their food by floating them on a 30% PEG solution. We were concerned that this two-minute rinse may reduce the internal ethanol concentration. Indeed, after mass isolation of larvae, the internal ethanol for groups EE, WWE, and EWE dropped almost 50% into the 3-6 mM range. This change in internal ethanol cannot account for the observed withdrawal and reinstatement behaviors since there is no systematic or statistically significant difference in ethanol concentration between the relevant groups (Figure 4.4B). We recognize that the PEG exposure is changing the animals, but all of the animals are exposed to the PEG solution.

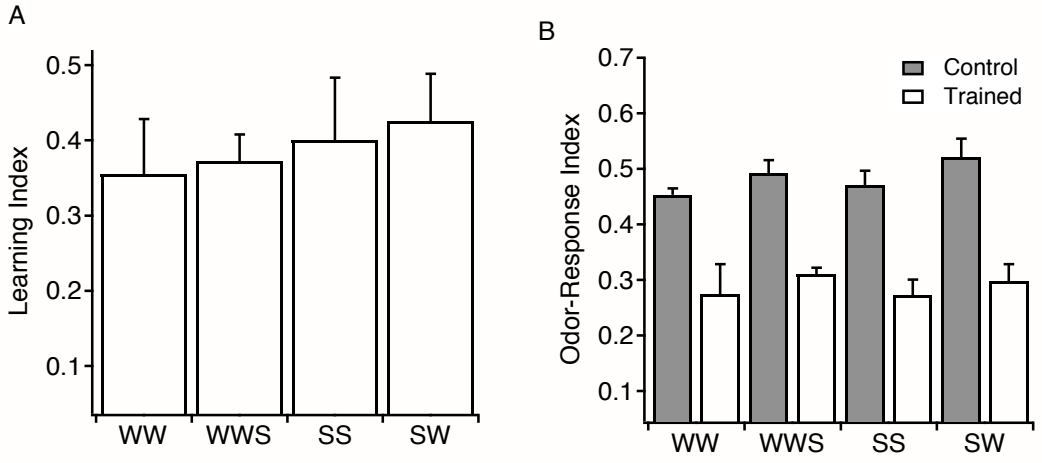
The maintained internal ethanol concentration (10 mM to 17 mM) is equivalent to a blood-alcohol concentration (BAC) of 0.05 to 0.08 g/100 ml. In a human, this would be near the legal limit for driving in the United States. While maintaining this level of internal ethanol for many days would be unusual for a human, persistent low blood-alcohol levels have been maintained in mice and shown to trigger ethanol withdrawal responses (Goldstein and Pal, 1971). This concentration is pharmacologically relevant to the larvae because it impedes learning (*cf.* WWE animals to WW animals in Figure 4.2). However, when larvae chronically consume ethanol, they adapt and learn as well as animals that have never been exposed to ethanol. Functional tolerance is obvious in a comparison of the EWE and WWE treatment groups. The larvae raised on ethanol food for 6 days (EWE, Figure 4.3A) learned at a normal level while those raised on non-ethanol food and exposed to ethanol for one hour (WWE. Figure 4.2) displayed impaired learning (summarized in Figure 4.4C). Metabolic adaptation cannot account for the learning deficit because the

ethanol reinstatement (EWE) animals and the acute ethanol animals (WWE) have almost identical levels of internal ethanol (Figure 4.4) but only the acutely exposed animals learn poorly.

Ethanol dependence was evidenced in the comparison between the EE group and the EW group (withdrawal) showing that acute abstention hindered learning. Furthermore, ethanol reinstatement (EWE) restores normal learning despite producing a higher internal ethanol concentration than in chronically exposed (EE) animals (Figure 4.4). A limitation of the third instar larval model system is that the window for assaying learning is so short (~1 day) that we cannot examine the decay of dependence. By the following day, some animals have settled into a stage of immobility that precedes pupation making the assay impossible.

Ethanol withdrawal or reinstatement behavior cannot be attributed to state-dependent learning because the ~20 minute training and testing assay for all treatment groups occurs on non-ethanol plates. Furthermore, the ethanol-naive (WW) and ethanol-withheld larvae (EW) contained no detectable ethanol. Nevertheless, the EW group underperformed in the learning assay. Finally, ethanol is a calorically-rich food supplement. Therefore it is concievable that the withdrawal response is a response to the change in food calories. However, sucrose supplementation, calculated to contain the same number of calories as the ethanol supplement, did not affect learning during supplementation or when the supplement was withheld (Figure 4.5).

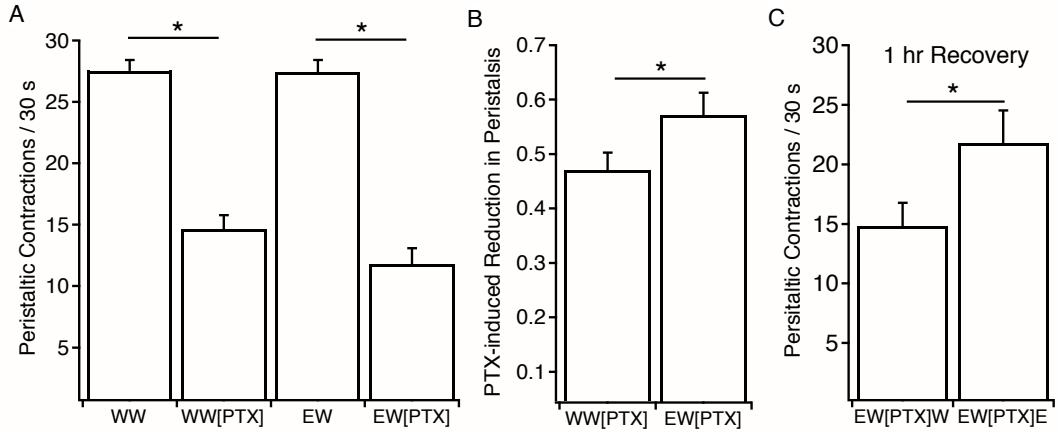
Figure 4.5: The effect of ethanol is not a response to a change in calories. The ethanol-withdrawal learning deficit is not caused by calorie withdrawal. This experiment replicates key parts of the experimental paradigm used to test for ethanol dependence as described in Figure 4.1. Here however, in place of ethanol, the food was supplemented to 200 mM sucrose which was calculated to have the same caloric value as 5% ethanol. A) Switching the larvae to and from sucrose-supplemented food did not affect the capacity to learn. No difference in learning was found between larvae continuously fed unsupplemented food (WW), larvae switched from unsupplemented to sucrose-supplemented food (WWS), larvae continuously fed sucrose-supplemented food (SS), or larvae that experienced a 6 hour withdrawal from sucrose-supplemented (SW) ($p=0.880$; $N=6$). B) When comparing the trained and control response indices for all of the treatments there was a significant effect of training (* $p<0.0001$) and within all of the treatments the trained animals had lower response indices than the control animals (* $p<0.05$; $N=6$).



Withdrawal Hyperexcitability

Nervous system hyperexcitability is a well-documented alcohol withdrawal response that could compromise learning. To test for this response we asked whether the EW larvae are more susceptible to picrotoxin-induced seizures (Stilwell et al., 2006). PTX blocks the Drosophila counterpart of the $GABA_A$ receptor enhancing neural excitability promoting seizures that cause body wall muscle bunching which reduces peristalsis. When treated with PTX, ethanol withdrawn (EW[PTX]) larvae displayed a greater reduction in peristaltic contractions than ethanol-naive (WW[PTX]) larvae (Figure 4.6A & B). Increased sensitivity to PTX indicates that withdrawal enhances nervous system excitability. Finally, a 1 hour 5% ethanol reinstatement partially reverses the increased PTX sensitivity of the withdrawal group (Figure 4.6C). The PTX sensitivity assay confirms that during withdrawal that neuronal signaling is abnormal. The fact that both the withdrawal-induced learning deficit and the neuronal hyperexcitability response are reversed by ethanol reinstatement (EW[PTX]E) suggests that they have related origins and that withdrawal learning may suffer because the nervous system is overly excitable.

Figure 4.6: Withdrawal-induced Hyperexcitability. Picrotoxin (PTX)-sensitivity was used to evaluate the excitability of the larval nervous system during ethanol withdrawal. PTX-induced seizures reduce the frequency of body-wall peristalsis. Increased basal neural hyperexcitability causes increased PTX-sensitivity (Stilwell et al., 2006). Larvae were treated as described in Figure 4.1 except that they were also treated with PTX. Larvae were raised in ethanol-(E) or water-supplemented food (W, non-ethanol) for six days and then divided into two groups. For six hours, one group was housed on ethanol-free agar plates (groups WW and EW) while the other group was housed on ethanol-free plates overlaid with 2 ml of 10 mM picrotoxin (groups WW[PTX] and EW[PTX]). As described in Figure 4.1, EW represents the group experiencing ethanol withdrawal. Seizure activity was assessed as a reduction in body wall peristaltic contractions over a 30 second interval. A) When not treated with PTX (WW and EW), the number of peristaltic contractions did not differ between the two groups. When treated with PTX, both groups had significantly reduced rates of peristaltic contractions (* $p<0.0001$ for effect of PTX, N=7). B) The percent reduction of peristaltic contractions was significantly higher in larvae raised on ethanol-supplemented food compared with larvae raised on water-supplemented food (EW[PTX] vs. WW[PTX]. * $p=0.025$). C) Giving larvae 5% ethanol during the first hour after PTX treatment (EW[PTX]E) causes a significant recovery of peristaltic contraction rate compared to a group not given ethanol during recovery (EW[PTX]W) (* $p=0.0079$ for effect of PTX).



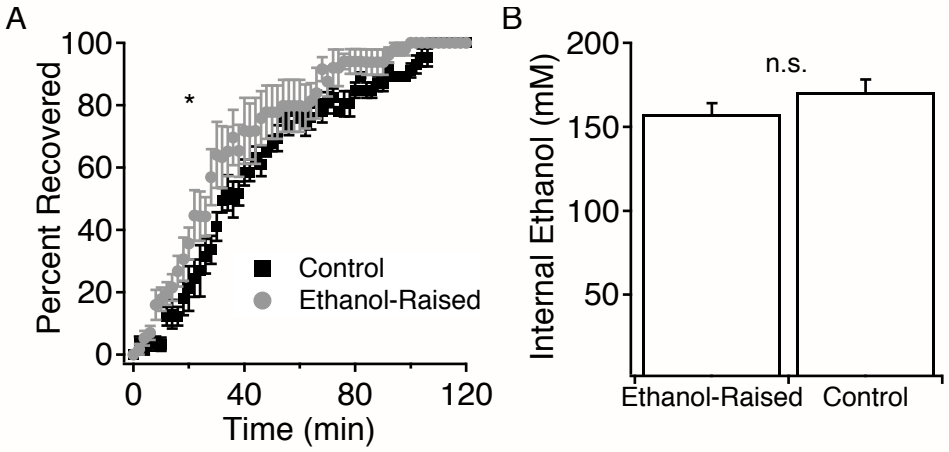
In humans, alcohol dependence is often associated with learning and memory deficits that last up to a year after abstinence (Stavro et al., 2012). Here, we show that similar changes can be observed in Drosophila larvae. Thus, genetic analysis in the Drosophila model system is now poised to contribute to the understanding of the cognitive consequences of ethanol dependence.

Ethanol-related Behaviors Survive Metamorphosis

One of the limitations of using third instar Drosophila larvae as a model is the relatively short duration in which experimentation can occur. Within one to two days after reaching this larval stage, the larvae enter a "wandering" stage in which they leave their food media in search of an area to pupate and begin metamorphosis. For a better characterization of our model of alcohol dependence, we wished to determine the level of transience associated with our phenotypes. Therefore, in adult flies that were treated with ethanol only as larvae, but during the entirety of the larval stages, we looked at two alcohol-related phenotypes: sensitivity and hyperexcitability. We found that, compared to flies that were completely naive to ethanol, flies treated with ethanol during the larval stages were significantly more resistant to the sedative effects of vaporized ethanol (Figure 4.7A). Flies were sedated with 100% ethanol vapor and the amount of time it took to recover postural control after the removal of ethanol was quantified. Flies treated as larvae recovered significantly faster than the control group. This decrease in recovery time in the ethanol-treated flies was not caused by increased ethanol metabolism (absorbance or clearance) as halfway through the

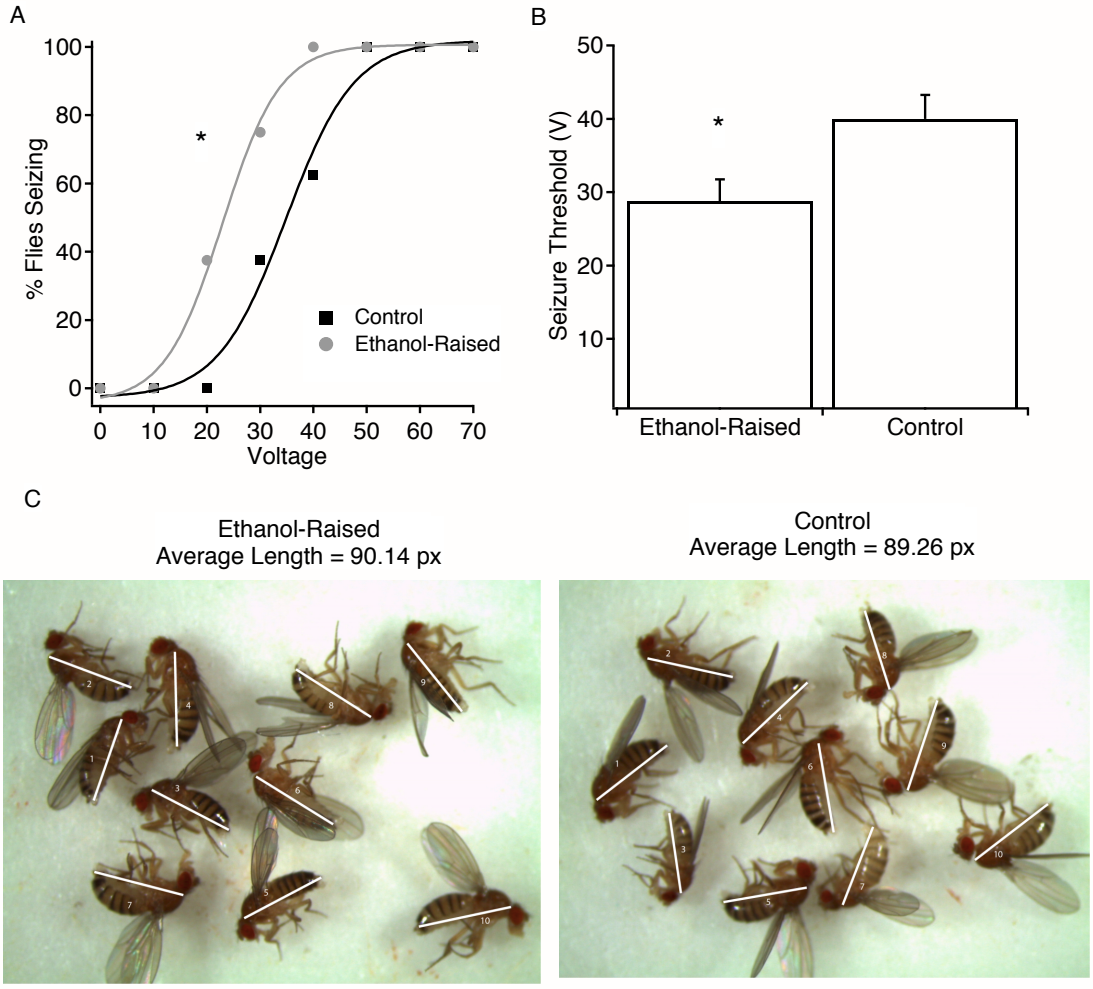
recovery period, the two groups of flies had statistically indistinguishable levels of internal ethanol (Figure 4.7B).

Figure 4.7: Adult flies treated with ethanol as larvae are resistant to the drug's sedatory effects. A) Adult flies were sedated with 100% ethanol vapor. Once all flies were knocked out, the ethanol vapor was replaced by air. The amount of time it took the flies to regain postural control following the sedation is plotted. Compared to flies naive to ethanol (black squares), flies that were treated with ethanol during the larval stages (gray circles) recovered more quickly (* $p<0.05$; N=6). B) The internal ethanol concentration of the ethanol-raised and control flies was measured 30 minutes into the recovery period using gas chromatography. The two groups of flies did not differ significantly in internal ethanol concentration indicating that their ethanol metabolism rates were similar ($p>0.05$; N=4).



Additionally, our lab has previously shown that adult flies treated with a single sedating dose of ethanol or benzyl alcohol show nervous system hyperexcitability 24 hours following the drug exposure (Ghezzi et al., 2010; Ghezzi et al., 2012). This hyperexcitability is measured by electrophsyiologically inducing seizures in alive but immobilized adult drosophila. The adult fly brain is stimulated with successively higher voltage trains until the fly has a seizure as recorded by activity of the flight muscle - a direct measurement of the activity of the giant fiber pathway. The minimum voltage that evokes a seizure in each fly is noted. We found that adult flies that had been treated with ethanol only during the larval stages, had a significantly lower induced-seizure threshold compared with ethanol naive flies (Figure 4.8A & B). In other words, the larval ethanol treatment causes adult flies to have hyperexcitable nervous systems. This corresponds with the hyperexcitable phenotype we observe in alcohol withdrawn larvae. One cause of a reduced inducible seizure phenotype in adult flies might be a reduced body size due to developmental effects of larval ethanol exposure. Smaller flies would be expected to require a lower voltage to induce a seizure. We therefore measured the body sizes of flies from the control and ethanol treated groups and found no differences (Figure 4.8C).

Figure 4.8: Adult flies treated with ethanol as larvae have a reduced seizure threshold. A) Seizures were induced in adult flies by stimulating the brain with pulses of successively higher voltage. The percentage of flies seizing at each voltage is plotted. The ethanol-raised group (gray circles) begin seizing at lower voltages than the control (black squares) group. B) The voltage at which 50% of flies seize is significantly lower when the flies were fed ethanol food as larvae compared with ethanol naive control flies ($p<0.05$; N=7). C) The average body length of ethanol-raised and control flies were identical. Measurements were performed by drawing a straight line from the bottom of the abdomen to the place where the thorax meets the head and determining the length of the line ($p>0.05$; N=10).



Interpretations of changes that survive metamorphosis necessarily need to be viewed with caution. During metamorphosis, a large-scale re-wiring of neurons occurs in the nervous system. Included in this process is the loss of many neurons that made up the larval brain (through apoptosis) and the incorporation of many more neurons that, in the larvae, were present in the imaginal discs, but not the larval brain. At this point, we do not know the mechanism for how certain phenotypes might survive metamorphosis. We hypothesize that changes can occur in individual neurons that cause alcohol-related phenotypes such as tolerance and withdrawal. Many of the neurons that will make up the adult brain are present in the larvae although not in the larval brain. Therefore, these neurons are still getting the ethanol treatment and likely undergoing ethanol-induced changes. One possible mechanism is that ethanol is causing epigenetic alterations to the genome. These alterations, in the form of histone tail acetylation and methylation, cause changes in gene expression. Previous studies in our lab have shown that ethanol causes changes in histone acetylation across the genome. It is possible that chronic ethanol exposure causes changes that are capable of surviving metamorphosis and are still present in the adult animal.

It cannot be ruled out that developmental deficits play a role in the behaviors observed in the post-metamorphic Drosophila. However, the reduction in seizure threshold we observe in the adult flies is not consistent with predicted developmental deficits produced by ethanol. Ethanol exposure during development has been shown to decrease cell division in Drosophila (McClure et al., 2011) as well as to decrease dendritic arborization and dendritic spine density in mammals (Granato et al., 2003; Hamilton et al., 2010; Whitcher and Klintsova, 2008). All of these studies indicate a

decrease in overall connectivity in the nervous system. Seizures however, are positively correlated with excitability and therefore also connectivity and mutations in excitability genes (causing a decrease in excitability) infer robust protection against seizures (Song and Tanouye, 2008).

Acknowledgements

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Chapter 5. Discussion and Future Directions

I have strived to further Drosophila larvae as a useful model for studying learning and memory and establish this system as a valuable tool for studying alcohol-related behaviors.

Learning and Memory in Drosophila Larvae

Initially, I developed a novel paradigm for inducing associative conditioning in Drosophila larvae. This assay uses an aversive heat stimulus as the unconditioned stimulus (US). An attractive odor (conditioned stimulus, CS) is temporally paired with this US resulting in a learned association between these two stimuli. Behaviorally, this manifests as a reduction in the fraction of larvae that closely approach the odor in a testing arena. The importance of this new assay mainly lies in the ease and speed of which it can be performed. The preparation is safe and simple and just involves passively heating an agar plate. Previous aversive conditioning involved electroshock as the US. This requires tight control of the plate conductance and rigorous safety measures to prevent electrocution. My heat shock assay has been successfully taught to over 50 undergraduate assistants and high school students. Additionally, the typical heat shock conditioning assay involves three repetitions of the heat-odor pairing spaced by eight minutes each. Including the behavioral testing following the last trial, this assay is complete in ~20 minutes. In contrast, electroshock conditioning, which is currently the most popular punishment to use, can take up to six or eight training trials causing just the training aspect of the assay to last

over an hour. This simple learning and memory assay has become the primary tool used in the remainder of my research.

Acute Ethanol Exposure in Drosophila Larvae

My main interest lies in understanding the action of ethanol on the nervous system and the resulting neuroadaptions that counteract ethanol's actions. My first ethanol-related project was to test the effects of acute ethanol on learning and memory. I have shown that low doses (20% ethanol bath for 20 minutes resulting in ~7 mM internal concentration) of ethanol affect learning negatively. However, this effect is subtle. At the optimal temperature for heat shock negative reinforcement (43°C) learning was unperturbed by this low dose of ethanol. However, we repeated the experiment using a suboptimal heat shock temperature of 37°C. In this instance, acute ethanol exposure cause the larvae to learn significantly worse than untreated larvae (figure 3.4). Importantly, this learning deficit occured without overt disturbances in locomotor or olfactory behaviors. This indicates that some higher order cognitive functions are more sensitive to low doses of ethanol than sensory and motor outputs.

Additionally, we wished to be able to test whether the ethanol treatment was having an anesthetic effect on the larvae. If the ethanol treatment were numbing the larvae, they would naturally not learn as well when a heat-shock punishment was used. Therefore, we developed a heat avoidance assay that was adapted from a previous study (Rosenzweig et al., 2005). We showed that larvae sense and avoid uncomfortable temperatures (high and low) equally regardless of whether they were

treated with 20% ethanol or mock treated with 0% ethanol indicating that the ethanol was not having an anesthetic effect on the larvae (figure 3.5).

This study is notable for several reasons. First, it confirms that ethanol has cognitive disrupting effects in Drosophila as has been shown in numerous other species. Therefore the diverse and unique genetic tools available for use in Drosophila can be employed to look at mechanisms involved. Secondly, the results of this study provide further evidence that, at low doses, ethanol can disrupt higher order nervous system processing (such as attention and learning) while not obviously affecting sensory and motor processing. If a mutant screen were conducted to determine the targets of ethanol that cause a learning deficit, some of the earliest and most sensitive ethanol targets would likely be implicated. Thirdly, we gained valuable knowledge regarding treating larvae with ethanol. In this study on the effects of acute ethanol exposure on larvae, we treated the larvae with ethanol by "soaking" them in a Petri dish of ethanol solution. This proved minimally effective in getting large amounts of ethanol in the larvae. Indeed, it seems as though the larvae have a mechanism for keeping ethanol out of their bodies or removing it very quickly (apart from metabolism). Finally, this study is of import because it paves the way for an investigation into alcohol dependence. According to the homeostatic theories of addiction, an initial drug exposure pushes an organism out of homeostasis and causes some set of phenotypes. However adaptations occur to counteract the effects of the drug, which result in the drug having a diminished effect upon subsequent exposures. This is known as functional tolerance. However, the mechanisms that produce tolerance remain after drug clearance and result in drug withdrawal phenotypes.

Withdrawal is the primary method for determining the presence of drug dependence. With regard to my acute ethanol phenotype, we would predict that with enough of an ethanol exposure, the larvae will develop tolerance to ethanol and dependence which would allow the larvae to learn normally in the presence of the drug. This was the hypothesis leading to the seminal study of my dissertation.

Chronic Ethanol Exposure in Drosophila Larvae

For the final aspect of my dissertations, I have shown that Drosophila larvae can become physiologically dependent on ethanol. I raised larvae on 5% ethanol food. They feed on this ethanol media for 6 days-until they reach the third instar stage. This is the stage just before the larvae leave their food and pupate and start the metamorphosis process. It is also the stage at which all learning and memory studies are done in larvae. After the larvae have fed on ethanol food their entire lives, I remove them from the media and split them into 2 groups. One group is kept on ethanol food and the other is kept on media that contains no ethanol. This treatment lasts 6 hours. At this point, I test both groups for their ability to learn. As part of this study, I also tested the learning ability of control, ethanol-naive larvae and larvae treated with a 1 hour acute dose of ethanol (see figure 4.1 for complete treatment schedule). I found that the acute dose of ethanol causes a dramatic decrease in learning ability compared with ethanol-naive larvae (figure 4.2). The larvae kept continuously on ethanol food however, were able to learn at the same level as larvae completely naïve to ethanol. On the other hand, larvae raised on ethanol food and given a 6 hour withdrawal from ethanol learned at a much lower level compared to

ethanol-naïve and continuous ethanol treated larvae. Importantly if the ethanol withdrawn larvae are given an ethanol reinstatement, their learning jumps back up to a normal level (figure 4.3).

This assay tests all of the conditions to ensure that the larvae are indeed becoming dependent on ethanol. In summary, acute ethanol treatment causes a decrease in learning ability. Upon chronic ethanol administration extensive adaptations occur in the larval nervous system that successfully counteract the effects of the drug and produce a state in which larvae only learn normally in the presence of the drug. This state is physiological ethanol dependence. Once larvae are in this state, a sudden withdrawal from ethanol results in a lowered capacity to learn. None of the learning effects we observed could be attributed to sensory or locomotion differences between the groups. The effects were also not caused by the increased calories added to the food with the addition of ethanol and subsequent withdrawal of these calories (figure 4.5). The effects could not be attributed to metabolic tolerance because the larvae getting the acute ethanol treatment learn poorly and have the same internal ethanol concentration as dependent larvae receiving a reinstatement treatment that learn at the normal level (figure 4.4). Finally, state-dependent learning is not an issue in this assay because all of the groups are in the same intoxication state during the training as during the testing aspect of the protocol.

We wished to further characterize the dependent state. We were able to detect nervous system hyperexcitability in the larvae that were in ethanol withdrawal. These larvae displayed increased sensitivity to the proconvulsant drug picrotoxin which is an inhibitor of the Drosophila homologue of the GABAa channel. Specifically, larvae

fed picrotoxin show abnormal peristaltic contractions. Their body wall muscles all contract and they show a bunching phenotype. Larvae in ethanol withdrawal showed a more severe phenotype than larvae not in withdrawal (figure 4.6).

Finally, in establishing this model, we wished to determine whether the chronic larval ethanol treatment resulted in long-lasting effects. This line of experimentation highlights a main disadvantage of the larval model in that the third instar stage lasts for only 2 days before the animal pupates. For this reason, we treated larvae with ethanol and allowed them to go through metamorphosis and then looked at the adults for altered ethanol related behaviors. We found that adult flies treated with ethanol as larvae were more resistant to the sedatory effects of ethanol compared to ethanol naïve flies (figure 4.7). We also observed that the nervous system of the adult flies treated with ethanol as larvae remained hyperexcitable. This was seen as a reduction in the threshold for electrophysiologically induced seizures (figure 4.8). These phenotypes were not due to the adults being developmentally stalled as the two groups of flies had the same average body sizes. We have not been able to rule out developmental issues in the brain only. However, our phenotypes are not indicative of a reduction in neuron number or connectivity. In general, decreases in neural connectivity result in a decrease in excitability and conversely we observe increased excitability.

This study represents the first reported case of cognitive dependence in Drosophila.

General Discussion and Future Directions

The model organism *Drosophila melanogaster* is primarily well-regarded as a genetic model system. The genes and neurons in the Drosophila brain are well conserved up to mammals. The low cost and speed of rearing make this system effective for dissecting the genetic basis of behavioral and other phenotypes. My string of projects has deviated from traditional projects in Drosophila because I have mainly performed behavioral assays to characterize the effect of ethanol on Drosophila larvae. However, when genetically dissecting behaviors it is first necessary to have a robust behavioral assay. Considering that alcoholism is a uniquely human disease, it behooves the field of Drosophila alcohol researchers to identify every human alcohol response that is also shared by fruit flies. In this pursuit, Drosophila will continue to serve as a useful mechanistic tool.

The culmination of my research has resulted in a novel model that closely resembles a human high-functioning alcoholic (Benton, 2010). While this term is not widely referred to in academic literature, it describes an individual who has become physiologically dependent on alcohol and performs everyday tasks normally *only* with alcohol in their systems. Such individuals live among the general population and are often only discovered once access to alcohol is restricted in some way and they undergo noticeable withdrawal symptoms. In my Drosophila larvae model, the larvae chronically consume alcohol and reach a point where they learn, smell, and locomote normally with significant levels of alcohol on board. However when alcohol is withheld, learning decreases and evidence of nervous system hyperexcitability is uncovered. This, along with other recent studies, represents a significant increase in

the complexity of alcohol-related behavior that can be modeled in Drosophila. Specifically, we are now able to determine the effects of alcohol exposure and alcohol withdrawal on higher order functioning. The completion of this model now means that the power of Drosophila genetics can be used to dissect another important aspect of addiction.

An important future direction of this work will likely involve pharmacological manipulation. The alcohol-related phenotypes of sensitivity and tolerance are not commonly thought of as "treatable". With the exception that the FDA approved drug Disulfiram causes extreme nausea when alcohol is consumed – essentially making individuals ultra sensitive to alcohol, most efforts at producing a drugs to treat alcoholism target the craving and rewarding aspects of alcohol consumption or aim to relieve the state of negative affect during withdrawal. With the recent advancements of behavioral models of addiction in Drosophila, it is now possible to conduct drug screens on exactly those "treatable" addiction phenotypes-reward and withdrawal. My larval model of physiological alcohol dependence is particularly amenable to drug screens. The larvae of Drosophila feed continuously. Any number of substances can be mixed into their food (as alcohol was for rendering the larvae dependent) and the larvae will consume it. While not widely used for drug screens, the larvae of Drosophila have been successfully established as a model for testing anti-epileptic drugs (Stilwell et al., 2006).

Our next step in this project, which is ongoing, is to leverage the fact that ethanol and other addicting drugs cause epigenetic changes at specific genes sites that result in altered transcription (Feng and Nestler, 2013). We and others have shown

that ethanol is an epigenetically active drug. We have seen that upon a single ethanol exposure, specific DNA elements within the promoter of the *slowpoke* gene display dynamic changes in histone acetylation. This correlates tightly with changes in transcription as measure by mRNA (unpublished Data from (Li et al., 2012)). Additionally, others have shown that ethanol (or metabolites (Choudhury and Shukla, 2008; Soliman and Rosenberger, 2011)) is a potent inhibitor of HDAC activity (Pandey et al., 2008). This also correlates with changes in transcription levels of specific genes. In this study, animals also showed adaptation to this HDAC suppressing action displayed by ethanol. Specifically, chronic inhibition of HDAC activity resulted in a homeostatic adaptation that increases endogenous HDAC activity that was exposed upon drug removal when HDAC activity becomes overactive. Amazingly, alcohol withdrawal-induced anxiety could be alleviated in rats by treatment with the non-specific HDAC inhibitor TSA. These results indicate that the general acetylation level in the genome is potentially important in alcohol dependence and withdrawal. A related alternative to this idea is that alcoholism is caused by the actions of the drug on one or more "master regulator". The alteration in the function of the master regulators has widespread and profound consequences for the organism. It is possible that a master regulator in alcoholism is an epigenetically active enzyme that drastically changes the epigentic landscape upon chronic ethanol exposure, alters gene expression, and causes behavioral manifestation of alcohol-related phenotypes. This master regulator might be altering gene expression of an essential network of genes involved in ethanol dependence.

Additionally, previous research in the Atkinson lab has demonstrated that

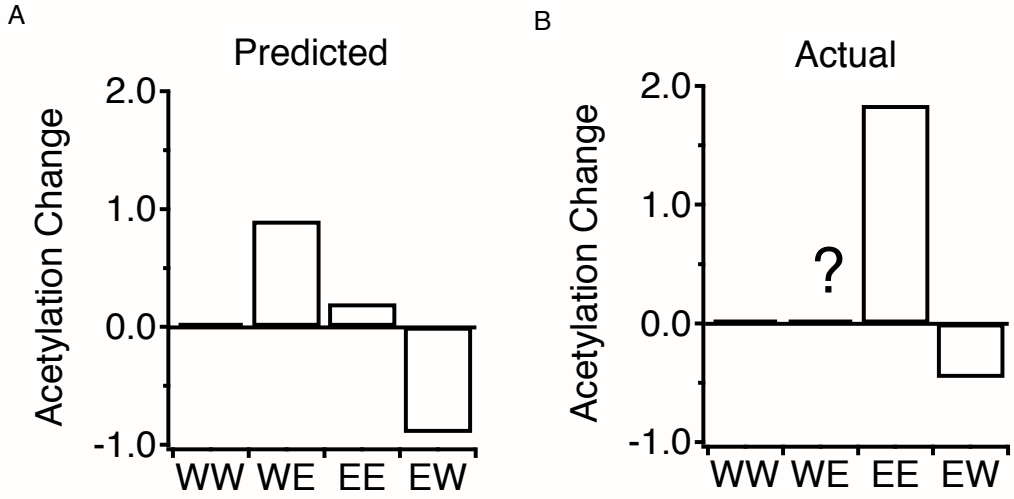
consumption of the HDAC inhibitor sodium butyrate (SB) causes behavioral resistance to benzyl alcohol in adult Drosophila. In essence, the SB treatment mimics a drug sedation that causes subsequent drug exposures to have less of an effect (Wang et al., 2007). While benzyl alcohol is not the same as ethanol, the two drugs cause mutual cross-tolerance to each other and both drugs cause histone acetylation changes (and presumably expression changes) at a common set of genes that are necessary for drug tolerance (Ghezzi et al., 2013).

We propose that our larval dependence phenotype involves the dynamic regulation of histone acetylation. In addition to the above evidence, aspects of ethanol dependence survive metamorphosis from the larval to the adult stage, and one possible explanation is that there are lasting epigenetic modifications that allow ethanol phenotypes to survive a massive neural re-organization. With regard to our findings, an acute dose of ethanol would increase histone acetylation. However, with chronic ethanol administration and the resulting chronic inhibition of HDACs, a natural homeostatic adaptation increases HDAC activity and balances the HDAC inhibition from ethanol. During ethanol withdrawal, the adaptation persists but no longer is countering any effects of the drug and thus HDAC activity would be overactive. This would be seen as a decrease in acetylation. Insidiously, increased HDAC activity may prevent or hinder nervous system plasticity and potentially the adaptation back to a completely normal state.

To address this hypothesis, we are currently collecting larval brains in all states the ethanol withdrawal phenotype from ethanol naïve to dependent to withdrawn (c.f. figure 4.1) to see if there is an overall indication of gross acetylation

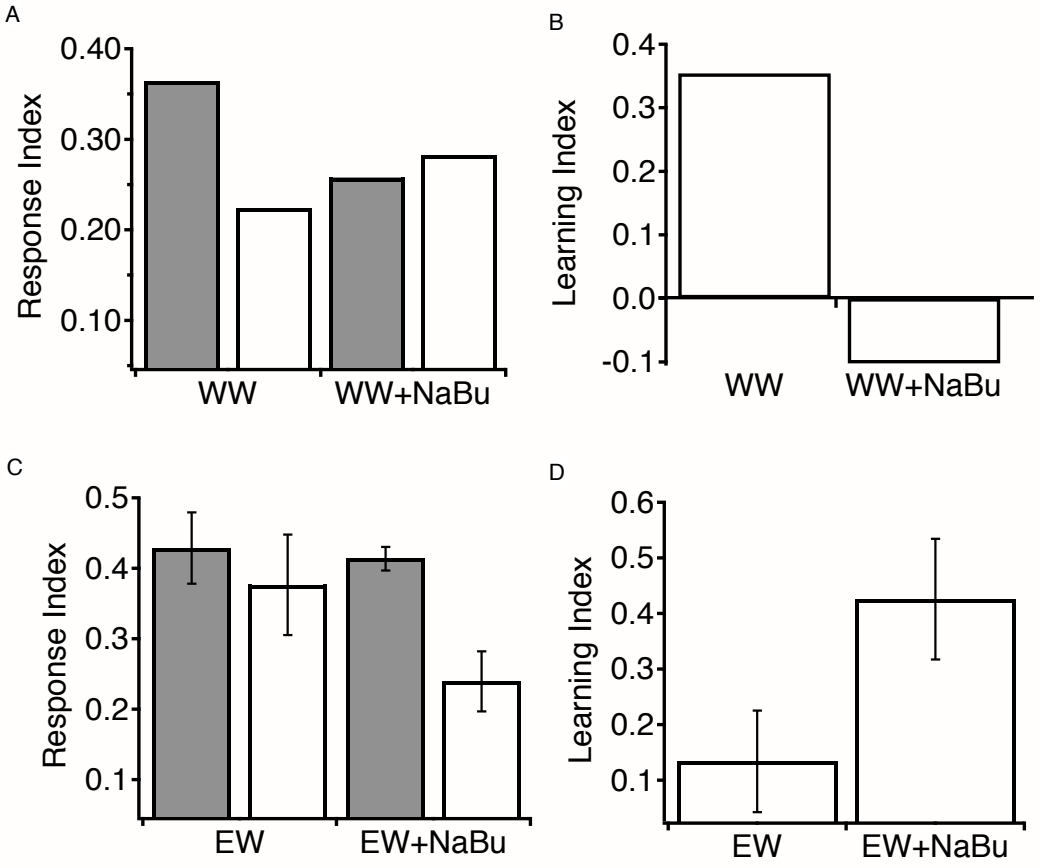
regulation and adaptation. Nuclear extracts from the larval brains will be immunoprecipitated with an antibody that recognizes only acetylated histones. We can then compare the different groups in the ratio of acetylated histones to total protein. Preliminary results have shown that, somewhat unexpectedly, larvae chronically treated with ethanol have vastly elevated acetylation levels compared to ethanol-naive larvae. Larvae chronically treated with ethanol followed by a six hour withdrawal have acetylation levels slightly lower than ethanol-naive larvae (figure 5.1). It is possible that, in larvae, rather than homeostatically balancing acetylation levels, adaptations occur that reset the baseline in dependent animals to the new high acetylation level. It is also possible that this higher acetylation in the EE group is a significant adaptation to what an acute dose of ethanol might cause. This will be tested by measuring the acetylation level of a WE group (question mark in figure 5.1).

Figure 5.1: Preliminary results on global brain acetylation levels during the development of physiological ethanol dependence and withdrawal. A) We would predict that dependent larvae (EE group) would adapt to the HDAC inhibitor effects of ethanol (WE group) by increasing HDAC activity. This would result in the EE group having a similar level of global acetylation. However, when the ethanol is removed (EW group) the adaptations would remain and result in a decrease in acetylation. B) In the initial experiments, we have seen that the acetylation level in dependent animals (EE group) is massively elevated compared to ethanol-naive larvae. The animals in withdrawal have a similar, but slightly lower level of acetylation compared to the ethanol-naive larvae.



In addition to measuring the histone acetylation levels associated with each state in the progression to withdrawal, we are investigating how HDAC inhibitor drugs affect ethanol dependence. Previous research (Pandey et al., 2008) has shown that administering an HDAC inhibitor administered during ethanol withdrawal alleviates anxiety associated with that state. We are interested to see whether an HDAC inhibitor also alleviates the reduction in learning ability during ethanol withdrawal in Drosophila larvae. We therefore have been treating larvae with 100 mM sodium butyrate (SB) during the 6-hour ethanol abstention period that causes withdrawal in chronically ethanol treated larvae. Preliminary results show that SB significantly increases the larvae's ability to learn during withdrawal (figure 5.2). Additionally, preliminary results show that SB administration does not have a general effect on learning because the drug does not increase learning in ethanol-naive larvae, but actually decreases learning. It is interesting that SB decreases learning ability in ethanol-naive larvae because, to our knowledge, SB has never been shown to have this effect and is usually shown to have a memory enhancing effect. However, we hypothesize that this six-hour SB treatment is drastically increasing acetylation levels in a relatively non-specific manner. According to homeostatic theory of drug exposures and the resulting adaptations that occur and produce tolerance and dependence, a large increase in acetylation would be an initial effect of a drug that would disrupt homeostasis. It is possible that this disruption in homeostasis is large enough to disrupt the learning capacity of larvae.

Figure 5.2: Preliminary results for effect of sodium butyrate on ethanol withdrawal. A and B) In ethanol-naive larvae a six hour treatment with 100 mM sodium butyrate causes a reduction in response index and a huge reduction in learning ability. C and D) However, when larvae are treated with 100 mM sodium butyrate after the development of dependence and during an ethanol withdrawal period, the sodium butyrate causes the larvae to regain their learning ability. Statistics not included because of insufficient number of experiments.



A nice complementary set of experiments to the ones described in the previous paragraph would be to test if adaptations occur to SB itself, that is; do larvae become functionally dependent on a non-intoxicating HDAC inhibitor. In this instance larvae would be fed SB chronically, then given a SB withdrawal period to determine if some aspects of SB withdrawal mimics aspects of ethanol withdrawal (replace ethanol with SB in figure 4.1). In this case, we would be testing if adaptations occur in larvae to an HDAC inhibitor alone. If larvae adapt to SB and experience withdrawal when it is withheld from them, it is possible that administering ethanol during SB withdrawal would alleviate the SB withdrawal because of the HDAC activity of ethanol. This would indicate that ethanol and SB are targeting a similar network of genes that produce drug dependence. The two drugs could be said to cause mutual cross dependence.

The experiments proposed above are exploring our hypothesis that the regulation of histone acetylation of a network of genes plays a central role in ethanol dependence. Our preliminary results and previous studies from other labs indicate that ethanol initially causes an increase in histone acetylation, but in response to chronic ethanol adaptations occur acting to decrease acetylation which counteracts ethanol's actions. When ethanol is removed, the adaptations remain and decrease acetylation contributing to some withdrawal symptoms. That global HDAC inhibitor drugs (a) cause ethanol resistance in Drosophila and (b) successfully treat larval ethanol withdrawal symptoms indicates that the HDAC activity of ethanol is targeting at least some of the same genes as the global HDAC inhibitors TSA and SB. In mammals SB has been shown to inhibit class I and class IIa HDACs, while TSA inhibits those plus

class IIb HDACs e.g.(Chuang et al., 2009). Classes I, IIa, and IIb include ten proteins in humans e.g. (Carey and La Thangue, 2006), however in Drosophila there are only four HDAC genes in classes I and II (Cho et al., 2005). Therefore we propose to perform mutant analysis with these 4 HDAC genes to determine which is/are necessary for the effects of ethanol that are attributable to HDAC regulation. The speed of genetics and ability to regulate gene activity both up and down in Drosophila will be of great value in identifying the HDACs relevant in ethanol-related behaviors. This identification would go a long way in determining the mechanism by which ethanol causes such widespread changes throughout the nervous system.

We also plan on performing a genome-wide analysis of the genes that are involved in ethanol dependence. This would be done using chromatin immunoprecipitation using an antibody for acetylated histone following a chronic ethanol treatment and subsequent withdrawal. However, in genome-wide analyses it is difficult to separate the relevant changes from non-specific changes (Mulligan et al., 2006). There are various methods for improving the "hit rate" on relevant genes. One method we have employed successfully in our lab is by comparing the genome-wide changes produced by two different drugs that produce mutual cross-tolerance (ethanol and benzyl alcohol; Ghezzi et al., unpublished). The genes that are changed by both of these drugs have a high probability of being involved in tolerance. We believe the same concept could be applied to ethanol-induced changes and SB-induced changes. Exposure to either drug causes a decreased subsequent effect of ethanol and SB effectively treats ethanol withdrawal and we hypothesize that larvae become dependent on SB and a SB withdrawal could be treated with ethanol.

Therefore genes regulated by both of these substances are relatively likely to be involved in dependence.

The results of the projects in this dissertation have set the foundation for a potentially very useful model of physiological ethanol dependence. We have shown that Drosophila larvae adapt to the presence of chronic ethanol to the point that they only function normally in a learning and memory assay in the presence of the drug. I propose that the HDAC inhibiting effects of ethanol play a crucial role in the development of this dependence and outline some preliminary data that support this hypothesis. Eventually, we hope to identify a network of genes regulated by ethanol that are central in the development of dependence.

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