Disorders of the Nervous System

Ethanol Regulates Presynaptic Activity and Sedation through Presynaptic Unc13 Proteins in *Drosophila*

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Abstract

Ethanol has robust effects on presynaptic activity in many neurons, however, it is not yet clear how this drug acts within this compartment to change neural activity, nor the significance of this change on behavior and physiology *in vivo*. One possible presynaptic effector for ethanol is the Munc13-1 protein. Herein, we show that ethanol binding to the rat Munc13-1 C1 domain, at concentrations consistent with binge exposure, reduces diacylglycerol (DAG) binding. The inhibition of DAG binding is predicted to reduce the activity of Munc13-1 and presynaptic release. In *Drosophila*, we show that sedating concentrations of ethanol significantly reduce synaptic vesicle release in olfactory sensory neurons (OSNs), while having no significant impact on membrane depolarization and Ca^{2+} influx into the presynaptic compartment. These data indicate that ethanol targets the active zone in reducing synaptic vesicle exocytosis. *Drosophila*, haploinsufficent for the Munc13-1 ortholog *Dunc13*, are more resistant to the effect of ethanol on presynaptic inhibition. Genetically reducing the activity of *Dunc13* through mutation or expression of RNAi transgenes also leads to a significant resistance to the sedative effects of ethanol. The neuronal expression of *Munc13-1* in heterozygotes for a *Dunc13* loss-of-function mutation can largely rescue the ethanol sedation resistance phenotype, indicating a conservation of function between *Munc13-1* and *Dunc13* in ethanol sedation. Hence, reducing *Dunc13* activity leads to naïve physiological and behavioral resistance to sedating concentrations of ethanol. We propose that reducing *Dunc13* activity, genetically or pharmacologically by ethanol binding to the C1 domain of Munc13-1/Dunc13, promotes a homeostatic response that leads to ethanol tolerance.

Key words: Drosophila; ethanol; Munc13-1; presynaptic; resistance; tolerance

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Significance Statement

At relatively low concentrations, ethanol inhibits the activity of many presynaptic termini (Liu and Hunt, 1999). Homeostatic changes in presynaptic activity are proposed to underlie the formation of functional tolerance (Koob and Bloom, 1988; Ghezzi and Atkinson, 2011). We do not currently know where ethanol binds to bring about changes in presynaptic activity and homeostasis. We now show that ethanol will bind to the C1 domain of the Munc13-1 protein at intoxicating concentrations and inhibit the binding of diacylglycerol (DAG). Reducing the activity of the *Drosophila* Dunc13 ortholog leads to a homeostatic change that promotes behavioral and physiologic resistance to intoxicating levels of ethanol. Hence, Unc13 proteins are likely sites for ethanol's action within the presynaptic compartment that bring about tolerance.

Introduction

Addiction to alcohol remains one of the most significant mental health problems throughout of the world. A major challenge is to understand how ethanol changes behavior and the brain during the descent into addiction. A promising approach is to examine endophenotypes for this disease in model systems (Dick et al., 2006; Salvatore et al., 2015). One major endophenotype for the development of alcoholism is the naïve behavioral sensitivity to ethanol, wherein the sons of alcoholics there is a fourfold increase in the likelihood of alcoholism among those with a reduced naïve sensitivity to the intoxicating effects of alcohol (Schuckit, 1980, 1994; Schuckit et al., 1996). The naïve resistance to ethanol intoxication may be mechanistically related to the formation of tolerance that follows exposure to high concentrations of ethanol (Harris et al., 2008; Mayfield et al., 2008).

During binge alcohol exposure, ethanol creates widespread and long-lasting changes in neural activity, altering both presynaptic and postsynaptic activity. An effect of ethanol on presynaptic release is seen at concentrations below 100 mM, where this drug typically produces its sedative effects (Diamond and Gordon, 1997). A single amino acid polymorphism in the Munc-18 active zone protein and its homolog unc-18 were found to generate strong resistance to ethanol sedation in mouse and *Caenorhabditis elegans*, respectively (Fehr et al., 2005; Graham et al., 2009). This mutation in Unc18 also decreased the frequency of synaptic vesicle release (Graham et al., 2009). The loss of synaptic vesicle proteins Rab-3A and its homolog Rab-3 also results in the resistance to ethanol sedation in both *C. elegans* and mouse (Kapfhamer et al., 2008). Ethanol inhibits presynaptic vesicle release in rat hippocampal slices induced by extracellular K^+ triggered depolarization and dependent on voltage-gated calcium channel activity (Maldve et al., 2004). Since Munc-18, Rab-3A, and voltage-gated channels are essential for presynaptic exocytosis, ethanol may alter presynaptic activity by changing the probability of synaptic vesicle fusion. The mechanism by which ethanol effects reduction in presynaptic activity is unknown, but one candidate is

the *slowpoke* large conductance BK channel. The activity of the *slowpoke* BK channel has an important role in ethanol sedation and tolerance in both *Drosophila* and *C. elegans* (Davies et al., 2003; Cowmeadow et al., 2005, 2006). Ethanol can directly bind to BK channels and the binding is essential for ethanol sedation in *C. elegans* (Bukiya et al., 2014; Davis et al., 2014). However, BK channel is expressed both presynaptically and postsynaptically, and it is not yet clear how much of the change in presynaptic activity is attributed to ethanol binding to presynaptic BK channels (Sailer et al., 2006).

Munc13-1 is also an alcohol binding protein and a strong candidate for producing an effect of alcohol in the presynaptic compartment (Das et al., 2013). Munc13-1 is an active-zone protein essential for synaptic vesicle fusion (Betz et al., 1997, 1998; Rhee et al., 2002). Munc13-1 directly interacts with and coordinates several other members of vesicle fusion machinery at presynaptic action zones in the mammalian brain, including syntaxin, RIM, Munc18, and voltage-gated calcium channels (VGCCs), (Rizo and Xu, 2015). The C1 domain of Munc13-1 binds diacylglycerol (DAG), which promotes membrane localization of this protein and lowers the energy barrier for vesicle fusion, facilitating synaptic vesicle release (Basu et al., 2007). Ethanol binds to the Munc13-1 C1 domain *in vitro* at concentrations below 100 mM (Das et al., 2013). Reduction in the level of *Dunc13*, the *Drosophila* ortholog of *Munc13-1*, results in flies that self-administer ethanol at significantly higher levels than wild-type controls (Das et al., 2013). Herein, we demonstrate that ethanol inhibits DAG binding to the Munc13-1 C1 domain at concentration as low as 25 mM. Furthermore, we show in *Drosophila* that ethanol impairs synaptic vesicle release in excitatory neurons downstream of Ca^{2+} influx into the active zone and that the reduction in *Dunc13* produces a behavioral and physiologic resistance to sedative effects of ethanol.

Materials and Methods

Fly strains

All flies were raised on standard cornmeal food at 25°C on a 12/12 h light/dark cycle. All the stocks were outcrossed into the Canton-S background for a minimum of six generations before behavioral analysis. $Dunc13^{P84200}/ci^D$ (FBst0300878) was generously provided by the Kyoto Stock Center. $Dunc13^{KK101383}$ RNAi line (RRID: FlyBase_FBst0479208) was provided by Vienna Drosophila Resource Center. The *elav*-Gal4 (FBst0008760, RRID: BDSC_8760), UAS-*Arclight* (RRID: BDSC 51056, FBst0051056), UAS-*GCaMP5* (RRID: FlyBase_FBst0042037) and $Dunc13^{JF02440}$ RNAi line (RRID: BDSC_29548) were provided by Bloomington Drosophila Stock Center. The *n-syb*-Gal4 was generously provided by Herman Dierick (Baylor College of Medicine). The *Or42b*-Gal4 was kindly provided by Scott Pletcher (University of Michigan). The *tublin*-Gal80^ts2^, ry^{506}, and UAS-*pHluorin* were generously provided by Ronald L. Davis (Scripps, FL).

The Munc13.1-EGFP cDNA was digested from the pBRETU vector (Roman et al., 1999; Das et al., 2013), and cloned into pUAST-attB vector (Bischof et al., 2007). The Munc13.1-pUAST-attB vector was recombined into attP40

in second chromosome, and the position and orientation were confirmed by PCR.

For the ethanol sedation assay, the *Dunc13*[P84200]/+ genotype was generated by crossing virgin females of *ry*[506], with *w*[+]; +; *ry*[506]; *Dunc13*[P84200]/*ci*[D] males. For the functional complementation of *Dunc13*[P84200], the experimental genotype was generated by crossing virgin females of *w*[+]; *tublin*-Gal80[ts]; *ry*[506]; *Dunc13*/*ci*[D], with *w*[+]; UAS-*Munc13-1* (or UAS-*dunc13A*); *elav*-Gal4;+ males. For optical imaging, the genotype Or42b-Gal4/+; UAS- pHluorin (or *Arclight*, *GCaMP5*)/+ was generated by crossing virgin females of Or42b-Gal4, with UAS-*pHluorin* (or *Arclight*, *GCaMP5*) males, and the genotype Or42b-Gal4/+; UAS- pHluorin/+; *Dunc13*[P84200]/+ was generated by crossing virgin females of Or42b-Gal4;; *Dunc13*[P84200]/*ci*[D], with UAS-*pHluorin* males.

Ethanol sedation

The ethanol loss of righting reflex (LOR) assay was performed as previously described (van der Linde et al., 2014). Female flies were collected and placed in vials containing fresh food (1 *n* = 30 flies per vial) for 24 h before behavioral testing. They were then transferred to the empty plastic vials of the test apparatus and exposed to a stream of ethanol vapor (50% unless noted) at a flow of 250 ml/min for 1 h to test for ethanol sensitivity. The ethanol vapor was produced by bubbling fresh air through 100% ethanol and pure water and mixing the two kinds of vapors. The % ethanol refers to the percentage of the final air/ethanol stream made up of the air bubbled through ethanol. The percentage of flies sedated in each vial was recorded at 5-min intervals. A fly was counted as being sedated if it had fallen onto its back or side and could not right itself. The time to 50% LOR was calculated for each exposure tube by linear interpolation of the two time points around the median and then averaged over the number of tubes (Ojelade et al., 2015).

Ethanol metabolism and absorption

The ethanol metabolism and absorption assay has been described before (Urizar et al., 2007). To study ethanol absorption, 30 female flies were exposed to 50% ethanol vapor at a 250 ml/min flow rate for 0, 20, 40, or 60 min. Immediately after exposure, flies were frozen in liquid nitrogen and homogenized in 200 μl of 50 mM Tris-HCl, pH 7.5. The homogenate was then centrifuged at 15,000 × *g* at 4°C for 20 min, and the supernatant was collected. The ethanol concentration in the supernatant was measured using the Ethanol Assay kit (catalog #MAK076, Sigma-Aldrich). To determine whether equivalent amounts of flies were assayed for each genotype, the protein concentration in the fly extracts was measured using the DC protein assay (Bio-Rad Laboratories). Ethanol metabolism was examined by exposing flies to 50% ethanol vapor for 60 min and allowing flies to recover for a period of 0, 30, 60, or 120 min. The ethanol concentration in fly extracts was measured at the end of each recovery period.

Optical imaging and ethanol injection

Optical imaging was performed on live flies as previously described with modifications (Zhang and Roman, 2013). Each female fly was fixed on a pipette tip and its head was secured to the tip opening with wax. The cuticle on the top of head was removed and covered with a piece of plastic wrap.

Before injection, each dissected female fly was checked through gently touching the tip of its foreleg with forceps. If the fly's femur did not show any movement, it would be discarded since it might be dead or close to dying. To inject ethanol, a small piece of pipette tip which contained fixed fly was removed to expose its scutellum. An insect pin (catalog #26002-10, Fine Science Tools) was inserted between scutum and scutellum to make a hole. A glass pipette (top diameter ~20 μm), was inserted into the hole. Then an injector (MINJ-PD, Tritech Research) linked with the glass pipette was turned on to inject ~0.1 μl ethanol solution into fly. The injected solution was Ringer's solution plus 15% ethanol.

After 10 min, the injected fly was imaged under an upright confocal microscope with a 20× objective (SP8, Leica). Ethyl acetate was diluted in mineral oil to 1% and delivered with an air flow at a rate of 400 ml/min bubbled through the mineral oil. The delivery of odorants was accomplished with a CS55 stimulus controller (Syntech), which added the odor to an airstream. Flies were exposed to 1-min air and then 3-s odor; there were no mechanical disturbances during the transition from air to odor.

After the imaging, each fly was taken out of the pipette tip to check for viability. Most of the flies exhibited leg movements. However, if a fly's legs showed no movement, it was considered dead, and the data were discarded.

Biochemistry

The Munc13-1 C1 domain in pGEX-KG vector was kindly provided by Dr. Josep Rizo (University of Texas Southwestern Medical Center at Dallas, TX). Site-specific mutations in the Munc 13-1 C1 domain were introduced with PCR by presenting the mutation in oligonucleotide primers using the he QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies). The Munc 13-1 C1 domain wild-type or the mutant fused with glutathione *S*-transferase (GST) were expressed in BL21 DE3 gold *Escherichia coli* (Agilent Technologies). Munc13.1C1 and its E582A mutant were purified following the methods described earlier (Das et al., 2013). Fluorescence resonance energy transfer (FRET) between Munc13.1 C1 and dansyl-DAG were determined using a PTI fluorimeter (Photon Technology Instruments; Pany and Das, 2015). Samples containing Munc13.1C1 (1 μM) and dansyl-DAG (1 μM) in buffer (50 mM Tris, 100 mM NaCl, and 5 mM ZnSO$_4$; pH 8) were incubated for 30 min at 25°C. For measuring the effect of ethanol, mixtures were incubated with ethanol (25 and 50 mM) for 30 min at 25°C. Samples were then excited at 280 nm, and emission spectra were recorded from 300 to 550 nm. Emission maxima of Munc13.1 C1 and dansyl-DAG were found to be at 337 and 500 nm, respectively. Relative FRET indices were calculated from fluorescence intensity at 500 nm using formula: [(Fi + Munc13.1C1) − (Fi, −Munc13.1C1)] − [(F0 + Munc13.1C1) − (F0− Munc13.1C1)], where Fi + Munc13.1C1 and Fi − Munc13.1C1 are the intensities of Dansyl-DAG in the presence and absence of Munc13.1C1, respectively,
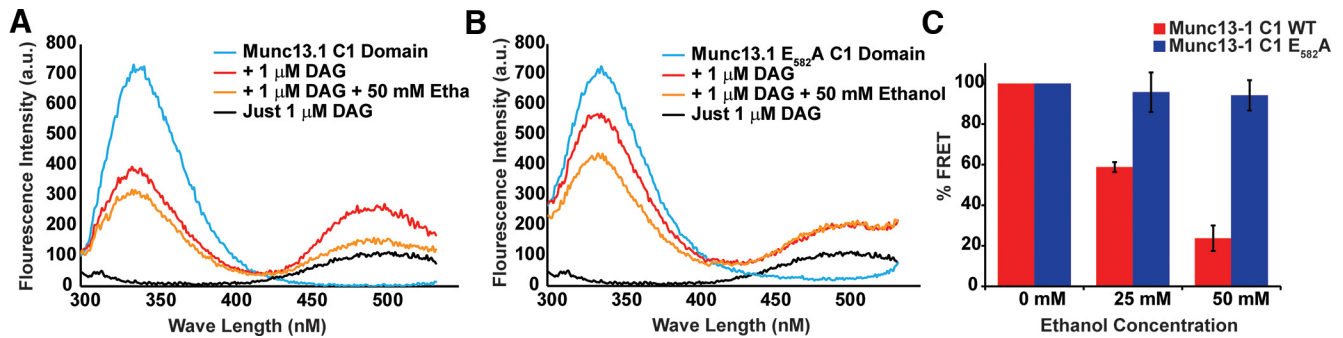
Figure 1. Ethanol binding to the C1 domain inhibits DAG binding. **A**, Spectral emission after excitation at 290 nm reveals FRET between the Munc13-1 C1 domain and dansyl-DAG (red trace). The C1 domain emission peaks at ∼335 nm (cyan trace), while the dansyl-DAG emission peak is found at 500 nm (black trace). This FRET is disrupted by 50 mM ethanol (green trace). **B**, Spectral emission of the Munc13-1 E_{582}A mutation after excitation by 290-nm light is shown. **C**, The binding of dansyl-DAG as revealed by FRET emission at 500 nm is reduced at both 25 and 50 mM ethanol concentrations. The E_{582}A C1 domain mutation fails to bind ethanol (Das et al., 2013). Ethanol does not inhibit dansyl-DAG binding to the E_{582}A Munc13-1 C1 domain ($n = 3$ each; *$p < 0.05$, ***$p < 0.001$). Error bars are standard error of the mean (SEM).

and F0+ Munc13.1C1 and F0− Munc13.1C1 are fluorescence intensities of the buffer solution in the presence or absence of Munc13.1C1, respectively. The change in fluorescence intensities for each concentration of alcohol was normalized using the equation: $(1-F/F0) \times 100$, where F and F0 are intensities of dansyl-DAG plus Munc13.1C1 in the presence or in the absence of alcohol.

Experimental design and statistical analysis

The differences between two groups were tested with two-tailed, unpaired Student's t tests; the differences among multi-group data were tested with two-way ANOVA (treatment × genotype), followed by Bonferroni-Dunn *post hoc* tests. Planned within genotype comparisons within these data were analyzed by Student's t tests. For each fly in the optical imaging experiments, the maximum fluorescent intensity change ratio before and after stimulation were calculated first. Then the ratio of vehicle-injected flies and ethanol-injected flies was analyzed by two-tailed, unpaired Student's t tests. For t test and ANOVA, $\alpha = 0.05$.

Results

Ethanol binding to the C1 domain inhibits DAG binding

Ethanol may affect sedation by binding to Munc13-1/Dunc13 and directly changing the activity of these regulators of vesicle priming. Previously, ethanol was found to bind the E_{582} residue in the C1 DAG binding domain of Munc13-1 (Das et al., 2013). Since the distance between the E_{582} ethanol binding residue and the DAG binding site, His_{567}, is only 8.8 A, ethanol binding to the C1 domain may impact DAG binding (Das et al., 2013). To investigate this question, we measured the effect of ethanol on the ability of the Munc13-1 C1 domain to bind DAG *in vivo*. FRET between the C1 domain and dansyl-DAG was used to quantify the relative amounts of DAG bound to the C1 domain *in vitro*. C1 domain fluorescence at ∼340 nm excited the dansyl-DAG and the dansyl-DAG emission was quantified at 500 nm (Fig. 1A,B). Overall, changing ethanol concentration and genotype of the C1 domain

had a significant effect dansyl-DAG binding ($F_{(3,14)} = 10.321$, $p < 0.001$; genotype, $t = 4.058$, $p = 0.001$). Ethanol concentrations of 25 and 50 mM significantly reduced FRET between the wild-type Munc13-1 C1 domain and dansyl-DAG ($t = 16.97$ and $t = 12.121$, respectively, $p < 0.001$ for both). However, FRET between the Munc13-1E_{582}A and dansyl DAG was not significantly altered by 25 and 50 mM ethanol ($t = 0.439$, $p = 0.684$ and $t = 0.781$, $p = 0.479$, respectively). This resistance to ethanol meant that the mutant E_{582}A C1 domain was significantly less sensitive to the effects of ethanol at both 25 and 50 mM concentrations as compared to the wild-type C1 domain ($t = 3.68$, $p = 0.021$ and $t = 7.21$, $p = 0.002$; Fig. 1C). Hence, ethanol binding to the C1 domain reduces the affinity for DAG, presumably leading to reduced Munc13-1 activity. This inhibition relies on ethanol's interaction with the C1 domain at E_{582}.

Ethanol inhibits presynaptic vesicle release in *Drosophila*

If the ethanol-induced inhibition of Munc13-1 activity is an important mechanism for ethanol effects on presynaptic activity, then ethanol should impact synaptic vesicle release *in vivo*, without impacting preceding presynaptic activation events. To examine this prediction, we measured the effect of ethanol on presynaptic activity in *Drosophila* of the OR42b olfactory sensory neurons (OSNs) using optical physiology techniques. The OR42b neurons are excitatory cholinergic neurons that are strongly activated by ethyl acetate (Hallem and Carlson, 2006). The OR42b OSN axons converge in the DM1 glomeruli (Gao et al., 2000; Couto et al., 2005). The *Or42b*-Gal4 line was used to drive three different genetically encoded sensors: ArcLight to measure changes in membrane depolarization (Cao et al., 2013); G-CaMP5 to measure Ca²⁺ influx into the presynaptic compartment (Akerboom et al., 2012); and synapto-pHluorin to measure synaptic vesicle fusion (Yuste et al., 2000; Reiff et al., 2005). These changes in fluorescence were measured in the DM1 glomeruli of the antennal lobe after a 3-s presentation of ethyl acetate. To measure the effect of ethanol on the physiologic changes

in the OR42b OSN, we injected either 0.1 μl vehicle or 15% ethanol before imaging each fly. The ethanol-injected flies stopped normal leg movements, and appeared completely sedated for a period of ~20 min. The flies were all imaged during this period of sedation. Vehicle-injected flies continued normal leg movements throughout the imaging period. Flies that did not recover leg movements after 20 min were excluded from the analysis.

Ethanol sedated flies did not have any detectable changes in ethyl acetate-induced membrane depolarizations within the presynaptic compartments of OR42b OSNs compared to vehicle-injected flies ($t = 0.198$, $p = 0.845$; Fig 2A,B). Similarly, we failed to detect a significant change in ethyl acetate induced Ca^{2+} influx into the OR42b OSN presynaptic compartment after ethanol sedation ($t = 0.744$, $p = 0.464$; Fig. 2C,D). In contrast, a significant decrease in the ethyl acetate elicited synaptic vesicle fusion was detected in the ethanol sedated flies ($t = 4.129$, $p = 0.0002$; Fig 2E,F). Together, these data that indicate that intoxicating levels of ethanol inhibits synaptic vesicle fusion largely independent of the incoming action potentials and resulting Ca^{2+} influx into the presynaptic compartment. These data are consistent with a role for ethanol-induced inhibition of Dunc13 activity, leading to a reduction in presynaptic release. It remains possible that ethanol may still impact Ca^{+2} influx within the presynaptic compartment in other time scales, such as minutes, but that we were unable to detect this effect. Munc13-1 enhances the function of presynaptic VGCC through direct interactions between these channels and the Munc13 C2B domain (Calloway et al., 2015). Hence, it remains possible that ethanol may subtly inhibit VGCC and presynaptic activity by inhibiting the binding between Munc13-1 and DAG.

Lastly, we asked if the inhibition of presynaptic vesicle fusion in response to ethanol was altered in the behaviorally resistant Dunc13^{P84200}/+ flies. The Dunc13^{P84200} allele is a loss-of-function mutant with a P-element insertion into the Dunc13 locus (Aravamudan et al., 1999). Homozygotes for this allele fail to display neurotransmission in the embryonic neuromuscular junction and die late in embryogenesis (Aravamudan et al., 1999). The heterozygotes have ~50% wild-type levels of Dunc13 mRNA (Das et al., 2013). In this experiment, there was a significant difference between groups ($F_{(2,81)} = 21.474$, $p < 0.0001$). As previously shown, injection of ethanol decreased the vesicle fusion in the OR42b OSNs elicited by ethyl acetate, however, the Dunc13^{P84200}/+ flies were less sensitive to this presynaptic inhibition as compared to wild-type controls ($t = 2.224$, $p = 0.031$; Fig 2G,H). Naïve Dunc13^{P84200}/+ heterozygotes are physiologically more resistant to ethanol's inhibition of presynaptic activity.

Dunc13 activity modulates ethanol sedation

To examine whether the Dunc13-ethanol interaction impacts behavioral responses to this drug, we further examined the effect of reducing the activity of the Dunc13 on behavioral sensitivity to ethanol. Dunc13^{P84200}/+ flies were previously shown to display a significantly higher level of ethanol self-administration (Das et al., 2013).

Since increases in ethanol self-administration in Drosophila are likely a response to the hedonic properties of this drug (Devineni and Heberlein, 2009; Kaun et al., 2011; Xu et al., 2012), it is possible that the increased self-administration in the Dunc13^{P84200}/+ heterozygotes is due to a naïve difference in the sensitivity to the neural effects of ethanol. To determine whether Dunc13 activity is involved in ethanol intoxication, we examined heterozygotes for the Dunc13^{P84200} mutation in a LOR assay (van der Linde et al., 2014). In the LOR assay, flies are exposed to ethanol vapor, which passively enters through their respiratory systems, increasing their internal alcohol concentrations (van der Linde et al., 2014). This leads to progressively more flies losing their righting-reflex with time. We use the $T_{1/2}$, which is time needed for 50% of the flies to lose their righting reflex, to compare differences in the rate of intoxication (van der Linde et al., 2014).

Genetically reducing Dunc13 activity results in a reduced sensitivity to the sedative effects of ethanol. The Dunc13^{P84200}/+ heterozygotes are significantly more resistant to the sedative effects of ethanol than wild-type controls ($t = 7.246$, $p < 0.001$; Fig. 3A). This increased resistance to ethanol vapor found in the Dunc13^{P84200}/+ heterozygotes was not due to differences in the absorption of ethanol ($F_{(4,41)} = 9.19$, genotype difference $t = 0.583$, $p = 0.563$; Fig. 3B), or in the rate at which the heterozygotes metabolize the ethanol ($F_{(4,43)} = 11.16$, genotype difference $t = 0.037$, $p = 0.971$; Fig. 3C). Moreover, driving the expression of the Dunc13^{KK101383} RNAi transgene throughout the nervous system using the n-Syb-Gal4 driver, also dramatically reduced the ethanol sedation sensitivity ($F_{(2,33)} = 72.73$, $p < 0.0001$; Fig. 3D). The $T_{1/2}$ LOR for the experimental genotype, with Dunc13 RNAi being driven throughout the nervous system, was significantly longer than both the Gal4 driver control ($t = 7.36$, $p < 0.0001$) and the Dunc13 RNAi control genotypes ($t = 19.18$, $p < 0.0001$). The flies were viable, which suggested a partial knockdown of Dunc13 in neural tissue. Lastly, we induced the expression of the Dunc13^{JF02440} RNAi transgene postdevelopmentally in neurons with the elav-Gal4 and Gal80^{ts20} transgenes (McGuire et al., 2003). There were significant differences between groups in this experiment ($F_{(3,31)} = 5.191$, $p < 0.005$). A 24-h 30°C induction of Dunc13 RNAi expression also leads to a significant decrease in ethanol sedation sensitivity compared to the within genotype control ($t = 2.95$, $p = 0.011$; Fig. 3E). Hence, reducing Dunc13 activity through mutation or by using two nonoverlapping RNAi lines expressed in the nervous system by two independent neuronal drivers, all result in flies that are more resistant to the sedative effects of ethanol. The independence of these drivers and RNAi lines provide a strong indication that reducing Dunc13 activity, similar to the Dunc13^{P84200}/+ haploinsufficient flies, produces a resistance to the sedative effects of ethanol. The ability of the induced Dunc13^{JF02440} RNAi expression to decrease ethanol sedation sensitivity further suggests that this change results from a physiologic rather than a developmental response to reduced Dunc13 activity, however, the RNAi transgene was expressed for
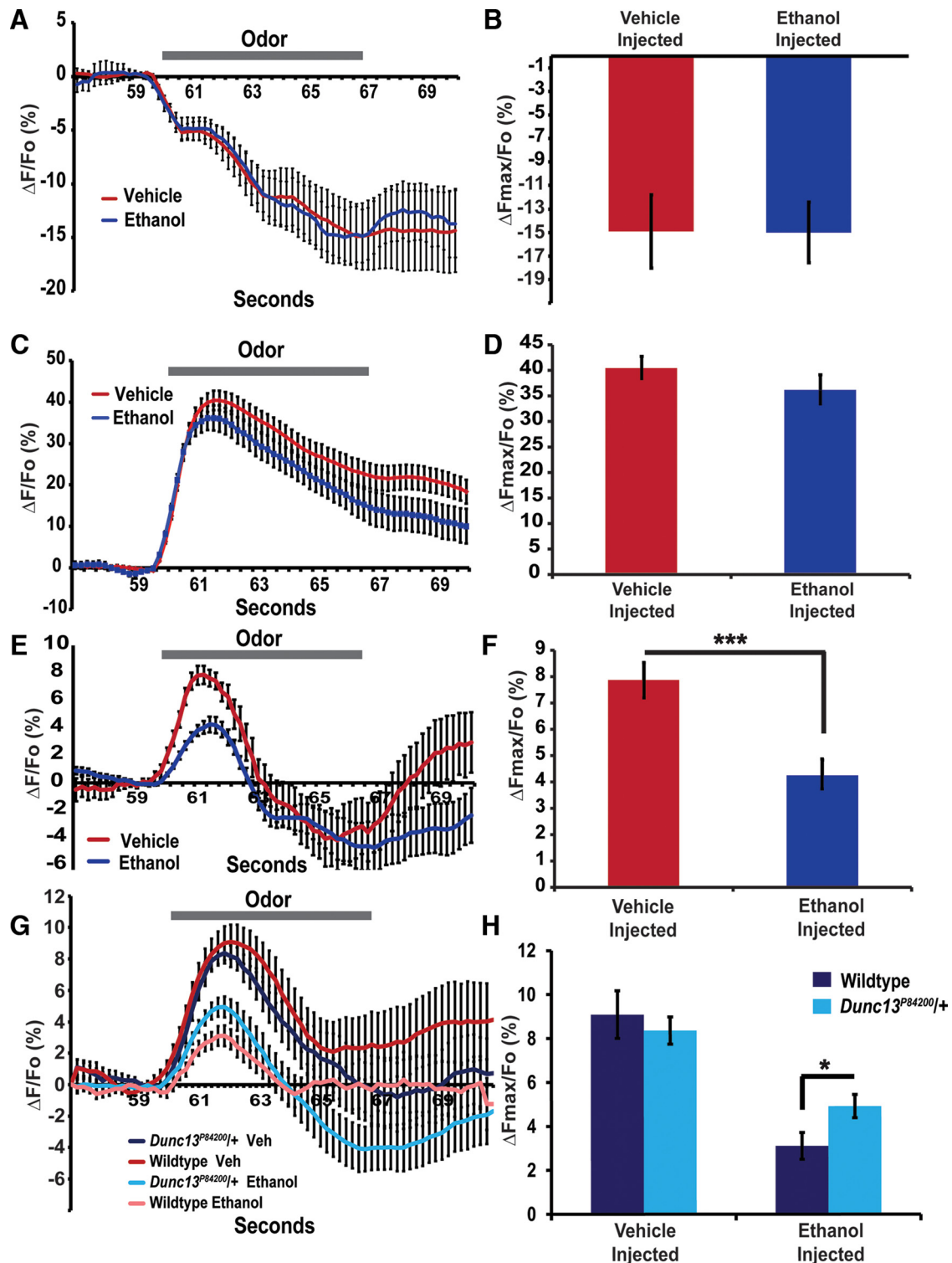
Figure 2. Ethanol specifically inhibits presynaptic vesicle release at excitatory synapses. *A*, *B*, Presynaptic activity was elicited by ethyl acetate delivered to the antennae and imaged within the DM3 glomeruli. Flies were imaged with ArcLight to measure membrane depolarization, which leads to a decrease in the maximal fluorescence ($\Delta Fmax$). Intoxication due to ethanol injection did not impact measured depolarization as measured by % $\Delta Fmax/Fo$ ($p > 0.05$, $n = 17$). *C*, *D*, Flies were also imaged with G-CaMP5 to indicate the intracellular Ca^{2+} concentration. Increasing Ca^{2+} leads to an increase in fluorescence. Intoxication due to ethanol injection did not impact measured Ca^{2+} influx as measured by % $\Delta Fmax/Fo$ ($p > 0.05$, $n = 15$). *E*, *F*, Flies were imaged with pHluorin to exhibit the presynaptic vesicle release. Intoxication due to ethanol injection significantly reduced synaptic vesicle fusion as measured by % $\Delta Fmax/Fo$ (***$p < 0.001$, $n = 17$). *G*, *H*, In vehicle-injected flies, $Dunc13^{P84200}/+$ heterozygotes do not show a significant reduction in synaptic vesicle release ($p > 0.05$, $n = 19$); however, they showed

continued

less ethanol-induced depression of synaptic release (∗$p < 0.05$, $n = 25$), after 15% ethanol was injected. All error bars are SEMs.

2 d in this experiment, which would permit homeostatic response to the reduced *Dunc13* activity.

We further verified the requirement for wild-type levels of *Dunc13* for normal sedation sensitivity by rescuing the haploinsufficiency of the *Dunc13P84200*/+ heterozygotes. Previously, we had shown that a rat *Munc13-1::EGFP* cDNA was capable of rescuing the *Dunc13P84200*/+ increased ethanol self-administration phenotype when expression was induced throughout the nervous system (Das et al., 2013). Herein, we examined whether *Munc13-1::EGFP* expression could also complement the *Dunc13P84200* haploinsufficiency in ethanol sedation ($F_{(3,56)} = 9.875$, $p < 0.0001$). The induced expression of this *Munc13-1::EGFP* transgene (48 h at 30°C) reduced the ethanol sedation sensitivity phenotype of *Dunc13P84200*/+ females ($t = 3.90$, $p = 0.001$; Fig. 3G). The activity of Munc13-1 can therefore functionally complement the *Dunc13* activity required for normal ethanol sedation sensitivity. These data further indicate that ethanol sedation sensitivity is modulated by Dunc13/Munc13-1 activity.

We next examined whether *Munc13-1::EGFP* could also rescue the lethality of *Dunc13P84200* homozygotes. Two different UAS-*Munc13-1::EGFP* transgenes failed to rescue the late embryonic lethality of *Dunc13P84200* when driven by *Tubp*-Gal4, *elav*-Gal4, or *n-Syb*-Gal4, suggesting that *Munc13-1* may not be able to fully complement *Dunc13* neural functions (over 300 progeny of each genotype, with no viable homozygous *Dunc13P84200* adult progeny). One possible reason for the lack of full complementation could be due to problems in trafficking the relatively large Munc13-1::EFGP protein to the presynaptic compartment. To see whether the Munc13-1::GFP protein was localized to the presynaptic compartment in *Drosophila*, we examined the neuromuscular junction of 3rd instar larvae with the genotype: UAS-*Munc13-1::GFP*/+; *nsyb*-Gal4/+. Munc13-1::EGFP was located within the presynaptic compartment in puncta. Bruchpilot, a protein localized to presynaptic active zones (Wagh et al., 2006), was found to be largely colocalized with Munc13-1::EGFP, although there exists some Munc13-1::EGFP puncta that are nonoverlapping with Bruchpilot signals and vice versa (Fig. 3H). Hence, the Munc13-1 was trafficked to presynaptic compartments.

Discussion

The work that we present here identifies a new presynaptic mechanism for modulating ethanol sedation sensitivity. Ethanol binds to the C1 domain of Munc13-1 (Das et al., 2013), which inhibits DAG binding *in vitro*. This inhibition occurs within the physiologically intoxicating range of 25–50 mM ethanol (Majchrowicz, 1975). Inhibiting DAG binding will decrease the activity of Unc13 proteins by reducing the membrane localization of this protein, leading to an increased energy barrier for vesicle fusion and a reduction in synaptic vesicle release (Lackner et al., 1999; Basu et al., 2007). We found using *in vivo*

optical physiologic approaches in *Drosophila* that intoxicating levels of ethanol did not significantly affect either membrane depolarization or Ca^{2+} influx within the presynaptic compartment of activated OR42b neurons, but synaptic vesicle fusion was dramatically inhibited in the neurons by these intoxicating levels of ethanol. Although our results do not show a significant effect on presynaptic Ca^{2+} levels after alcohol, we cannot rule out that the localization of Ca^{2+} microdomains was not impacted by ethanol. Nevertheless, our imaging results indicate that ethanol acts to inhibit synaptic vesicle release downstream of voltage-gated Ca^{2+} influx, which is consistent with ethanol inhibition of presynaptic activity through the reduction of DAG binding to Dunc13.

To verify a role for *Dunc13* in the physiologic and behavioral sensitivity to ethanol, we examined flies that had reduced levels of *Dunc13* activity. A simple prediction would be that these genetically sensitized flies, having already lost some *Dunc13* activity would be more sensitive to the effects of ethanol. However, the genetically sensitized flies were physiologically and behaviorally resistant to sedating concentrations of ethanol. The *Dunc13P84200*/+ heterozygotes displayed reduced sensitivity to the acute effects of ethanol on synaptic vesicle fusion in the OR42b OSNs, and these flies also displayed reduced behavioral sensitivity to ethanol sedation. The reduced behavioral sensitivity to ethanol in the *Dunc13P84200*/+ heterozygotes was partially reversed by neural expression of either *Dunc13* or *Munc13-1* cDNAs, indicating the phenotype was produced by a haploinsufficiency of *Dunc13*. Moreover, two independent and nonoverlapping *Dunc13* RNAi lines also displayed reduced sedation sensitivity. Together these data indicate that reducing *Dunc13* levels reduces the naïve responsiveness to the sedative effects of ethanol.

The functional complementation of *Munc13-1* for *Dunc13* haploinsufficiency in the LOR assay but not for the viability of the *Dunc13P84200* homozygotes indicates at least a partially conserved function between these orthologs in *Drosophila*. There can be several reasons for the inability of a *Munc13-1* cDNA to rescue the lethality of the homozygous *Dunc13P84200* flies, including differences in functional activity, expression levels, and protein localization. Munc13-1 contains several identified functional domains: C1 domain for DAG binding (Betz et al., 1998), CBS domain for binding calmodulin (Dimova et al., 2009), C2A domain for heterodimerization with RIM and regulating later steps in vesicle docking and priming (Betz et al., 2001; Camacho et al., 2017), C2B domain for binding phospholipid and VGCCs (Shin et al., 2010; Calloway et al., 2015), C2C domain, which may bind the plasma membrane (Rizo and Südhof, 1998), and MUN domain for binding syntaxin (Li et al., 2011). Although the C1, CBS, C2B, C2C and MUN domains of mammalian unc-13 and *Drosophila* unc-13 are highly conserved, Dunc13 lacks a conserved C2A domain (Aravamudan et al., 1999). It is
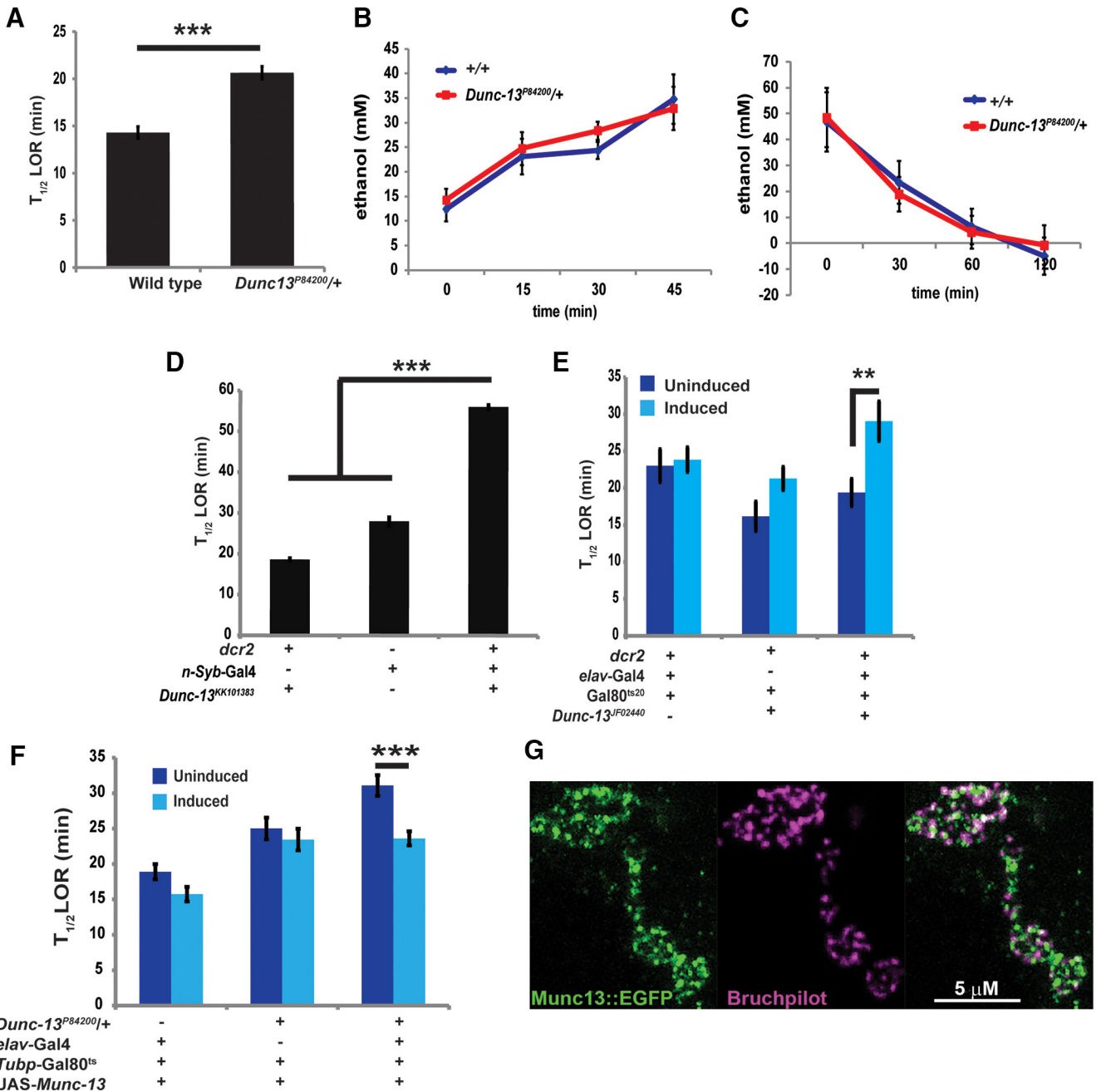
Figure 3. A reduction in Dunc13 activity leads to an increased resistance to ethanol sedation. **A**, The $Dunc13^{P84200}/+$ heterozygotes require a greater time to reach 50% LOR (T1/2 LOR) reflex levels (***$p < 0.001$, $n = 17$). **B**, The concentration of ethanol was determined in $Dunc13^{P84200}/+$ and control flies exposed to 50% ethanol vapor for 0, 15, 30, or 45 min; no significant differences were found ($p > 0.05$, $n = 6$). **C**, The ability of $Dunc13^{P84200}/+$ and control flies to metabolize ethanol was determined by first exposing flies to ethanol vapor for 45 min, and then by measuring the ethanol remaining in the flies 0, 30, 60, and 120 min after the exposure. No significant differences in ethanol metabolism were detected at each time point ($t = 0.037$, $p > 0.05$, $n = 6$). **D**, The neural expression of the $Dunc13^{KK101383}$ RNAi transgene led to significantly slower T1/2 LOR compared to the genotype controls (***$p < 0.05$, $n = 10$). **E**, The induced neural expression of the $Dunc13^{JF02440}$ RNAi transgenes also led to a significantly slower T1/2 LOR as compared to the within genotype control ($p < 0.01$, $n = 8$). Induction was accomplished with a 24 h, 30°C heat treatment, followed by a 3-h recovery period at room temperature. **F**, Inducing the expression of a wild-type Munc13-1 cDNA for 48 h led to a significant decrease in LOR for the $Dunc13^{P84200}/+$ flies (***$p = 0.001$, $N = 9$). Induction was accomplished with a 48 h, 30°C heat treatment, followed by a 3-h recovery period at room temperature. **G**, Munc13-1::EGFP is colocalized with Bruchpilot, a protein localized to presynaptic active zones, in the presynaptic compartment of the larval neural muscular junction. All error bars are SEMs.

currently unclear if the Dunc13 protein physically interacts with RIM in docked vesicles, but since this is a central interaction for vesicle priming it is likely this interaction is conserved in Drosophila. Interestingly, the C2A domain and the neighboring N-terminal sequences are necessary for the *C. elegans* unc-13 isoform L to be correctly located at the active zone (Hu et al., 2013; Zhou et al., 2013). We found that the Munc13-1::EGFP was localized to puncta in the larval neural muscular junction, although not completely overlapping with the active zone Bruchpilot protein.

How does a reduction of *Dunc13* activity lead to a resistance to the sedative effects of ethanol?

The $Dunc13^{P84200}/+$ haploinsufficiency and the neurally-expressed *Dunc13* RNAi lines likely mimic the initial effects of intoxicating concentrations of ethanol on this protein by genetically reducing its activity. The genetic or pharmacological reduction in Dunc13 activity would be expected to generate a widespread reduction in the size of the readily releasable pool (RRP; Augustin et al., 1999). This reduction in the RRP would produce synaptic depression, triggering a homeostatic response in the affected synapses (Davis and Goodman, 1998; Frank, 2014). Work from the *Drosophila* neural muscular junction has demonstrated two convergent mechanisms for homeostatic increases in presynaptic activity: potentiating Ca^{2+} influx through VGCCs and by increasing the RRP (Frank et al., 2009; Müller et al., 2012). In vertebrate central synapses, similar presynaptic homeostatic mechanisms have been discovered in which inhibition of activity leads to increases in both presynaptic Ca^{2+} influx and in synaptic vesicle release (Zhao et al., 2011). The presynaptic portion of this process may be largely driven by changes in cyclin-dependent kinase 5 (CDK5) activity (Kim and Ryan, 2010). Suppression of synaptic activity decreases CDK5 activity, which in turn promotes presynaptic Ca^{2+} influx and increases the resting vesicle pool available for promotion into the RRP (Kim and Ryan, 2010).

The homeostatic changes in synaptic efficacy brought about by ethanol reducing Dunc13 activity may mimic tolerance. Ethanol resistance and tolerance share a reduced response to this drug but differ in their ontogeny. Tolerance is a reduced responsiveness to alcohol brought about by previous exposure to this drug, whereas resistance refers to an innate difference in sensitivity to alcohol (Ghezzi and Atkinson, 2011; Rothenfluh et al., 2014). *Drosophila melanogaster* undergoes functional tolerance when exposed to high levels of alcohol, but does not develop metabolic tolerance (Scholz et al., 2000). This functional tolerance in *Drosophila* can be long lasting and dependent on epigenetic changes in gene expression and new protein synthesis (Berger et al., 2004; Cowmeadow et al., 2006; Engel et al., 2016).

There has been increasing evidence from *Drosophila* indicating that the development of functional tolerance is triggered by changes in presynaptic activity. Conditional alleles in the dynamin gene *shibire* (*shi*) can completely block the formation of rapid functional tolerance (Krishnan et al., 2012). At nonpermissive temperatures, the dominant negative *shi^{ts}* alleles block synaptic vesicle recycling resulting in a depletion of the vesicle pool and a loss of presynaptic activity as well as other endocytosis-dependent events, such as receptor internalization (Kosaka and Ikeda, 1983). When this block in vesicle recycling was induced during sedation, there was a failure in the production of tolerance, while if it occurred after the initial binge exposure, there was no impact on tolerance, indicating a role for *shi* in the induction of tolerance (Krishnan et al., 2012). Interestingly, blocking neural activity during sedation using a *paralytic^{ts}* conditional allele in a voltage-gated sodium channel or temperature-sensitive alleles of the *comatose* NSF protein did not block the formation of functional tolerance. These data suggest that dynamics in the presynaptic vesicle pool during ethanol sedation, and not changes in presynaptic activity per se, are required for tolerance formation (Krishnan et al., 2012).

Alcohol exposures that induce functional tolerance also bring about long-lasting changes in the levels of several presynaptic proteins that are required for the induction of functional tolerance (Ghezzi et al., 2013). Some of these transcriptional changes necessary for chronic tolerance require the activity of the Sir2 histone deacetylase (Engel et al., 2016). These Sir2-dependent epigenetic changes regulate the activity of presynaptic proteins including Synapsin and also likely the *cacophony* voltage-gated Ca^{2+} channel and the *cdk5* kinase, which are known to have important roles in presynaptic homeostatic responses to reduced synaptic activity (Davis, 2013; Frank, 2014). Mutants in Synapsin are defective in tolerance formation, but not in naïve sedation (Godenschwege et al., 2004; Engel et al., 2016). Moreover, increases in the level of the slowpoke BK channel are also required for the development of functional tolerance (Cowmeadow et al., 2005; Cowmeadow et al., 2006; Li et al., 2013). Increases in *slowpoke* increase the ability for hire frequency firing (Ghezzi et al., 2010). This increase in high-frequency firing may reverse synaptic depression by facilitating the ability of Ca^{+2} to increase the rate at which the RRP is replenished (Wang and Kaczmarek, 1998).

In sum, there is strong evidence for specific homeostatic changes in presynaptic activity underlying ethanol tolerance, and an initial trigger for these changes may be ethanol inhibiting the binding of DAG to the C1 domain of Dunc13. A testable prediction from this hypothesis for the inhibition of Dunc13 as an initial event in the formation of tolerance is that compounds or mutations, such as the Munc13-1 $E_{582}A$, that inhibit ethanol binding to the C1 domain will reduce tolerance formation (Das et al., 2013). However, for these reagents to be useful in elucidating the role of Dunc13 inhibition in tolerance formation, they need to have a limited effect on Dunc13/Munc13-1 activity in the absence of ethanol. Otherwise they may lead to the same homeostatic changes in naive animals found after exposure to intoxicating levels of ethanol.

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