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Authors

Engel, Gregory L
Marella, Sunanda
Kaun, Karla R
et al.

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Sir2/Sirt1 Links Acute Inebriation to Presynaptic Changes and the Development of Alcohol Tolerance, Preference, and Reward

Gregory L. Engel,[1] Sunanda Marella,[2] Karla R. Kaun,[3] Julia Wu,[2] Pratik Adhikari,[1] Eric C. Kong,[2] and Fred W. Wolf[1,2]

[1]School of Natural Sciences, University of California, Merced, Merced, California 95343, [2]Ernest Gallo Clinic and Research Center, University of California San Francisco, Emeryville, California 94608, and [3]Department of Neuroscience, Brown University, Providence, Rhode Island 02912

Acute ethanol inebriation causes neuroadaptive changes in behavior that favor increased intake. Ethanol-induced alterations in gene expression, through epigenetic and other means, are likely to change cellular and neural circuit function. Ethanol markedly changes histone acetylation, and the sirtuin Sir2/SIRT1 that deacetylates histones and transcription factors is essential for the rewarding effects of long-term drug use. The molecular transformations leading from short-term to long-term ethanol responses mostly remain to be discovered. We find that Sir2 in the mushroom bodies of the fruit fly *Drosophila* promotes short-term ethanol-induced behavioral plasticity by allowing changes in the expression of presynaptic molecules. Acute inebriation strongly reduces Sir2 levels and increases histone H3 acetylation in the brain. Flies lacking Sir2 globally, in the adult nervous system, or specifically in the mushroom body α/β-lobes show reduced ethanol sensitivity and tolerance. Sir2-dependent ethanol reward is also localized to the mushroom bodies, and Sir2 mutants prefer ethanol even without a priming ethanol pre-exposure. Transcriptomic analysis reveals that specific presynaptic molecules, including the synaptic vesicle pool regulator Synapsin, depend on Sir2 to be regulated by ethanol. Synapsin is required for ethanol sensitivity and tolerance. We propose that the regulation of Sir2/SIRT1 by acute inebriation forms part of a transcriptional program in mushroom body neurons to alter presynaptic properties and neural responses to favor the development of ethanol tolerance, preference, and reward.

Key words: addiction; behavior; *Drosophila*; ethanol; transcriptional regulation

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Significance Statement

We identify a mechanism by which acute ethanol inebriation leads to changes in nervous system function that may be an important basis for increasing ethanol intake and addiction liability. The findings are significant because they identify ethanol-driven transcriptional events that target presynaptic properties and direct behavioral plasticity. They also demonstrate that multiple forms of ethanol behavioral plasticity that are relevant to alcoholism are initiated by a shared mechanism. Finally, they link these events to the *Drosophila* brain region that associates context with innate approach and avoidance responses to code for reward and other higher-order behavior, similar in aspects to the role of the vertebrate mesolimbic system.

Introduction

Alcoholism is the most prevalent form of drug addiction, and it has broad socioeconomic impacts, including lost productivity,

interpersonal discord, and high medical costs. The pharmacological effects of ethanol lead to long-term changes in neurophysiology and behavior. Some of these changes are maladaptive and underlie the shifts in behavior that are characteristic of alcohol abuse and dependence. However, the molecular mechanisms underlying these maladaptive changes are not well understood. Recently, gene expression regulation in the nervous system, facilitated by changes in chromatin conformation, have been shown to contribute to the development of ethanol tolerance, preference, and reinstatement (Mahadev and Vemuri, 1998; Kerns et al., 2005). Covalent modification of core histones is a major mechanism for dynamically regulating chromatin conformation and gene expression. Ethanol and other drugs of abuse cause marked changes in histone acetylation and methylation

(Maze and Nestler, 2011). Specifically, short-term ethanol exposure increases the acetylation of histones H3 and H4 in the amygdala, and induces histone modifications in other brain regions (Pandey et al., 2008; Finegersh and Homanics, 2014).

Ethanol exerts some of its long-term effects through altering the activity of the enzymes that modify histones. This includes the protein lysine deacetylase/histone deacetylases (HDACs) that remove acetyl groups to generally decrease transcription from nearby genes. Short-term ethanol exposure in rats decreases HDAC activity, and withdrawal from long-term ethanol use increases HDAC activity, changes that are predicted to increase and decrease gene transcription, respectively (Pandey et al., 2008). Further, pharmacological reduction of HDAC activity in ethanol withdrawal reduces anxiety-like behavior and tolerance to the anxiolytic effects of ethanol (Sakharkar et al., 2012). Similarly, long-term cocaine use increases HDAC activity, and pharmacological manipulation of the sirtuin HDAC Sirt1 alters cocaine reward (Renthal et al., 2009). These data suggest that changes in chromatin structure through the regulation of HDACs and other chromatin-modifying enzymes contribute to drug-induced behavioral plasticity, possibly through transcriptional regulatory mechanisms.

Drosophila become inebriated by ethanol produced by fermenting fruit, which is a major source of nutrition, a site for reproduction, and also a source of protection from predators (Dudley, 2000; Kacsoh et al., 2013). *Drosophila* develop a preference for ethanol intake and tolerance to its inebriating and sedating effects after repeated or prolonged exposure (Scholz et al., 2000; Devineni and Heberlein, 2009). Furthermore, flies find ethanol rewarding, and they show signs of withdrawal after a period of abstinence (Kaun et al., 2011; Shohat-Ophir et al., 2012; Ghezzi et al., 2014). Molecularly, ethanol broadly alters gene expression and also histone modifications in the *Drosophila* nervous system, including acetylation of histones H3 and H4 at specific sites across the genome (Morozova et al., 2006; Urizar et al., 2007; Kong et al., 2010; Ghezzi et al., 2013). The expression of RNA for the *Drosophila* Sirt1 homolog *Sir2* is markedly decreased following short-term exposure to ethanol, and both locomotor and postural control responses to ethanol are affected in flies with decreased Sir2 expression levels (Morozova et al., 2006; Kong et al., 2010). It remains unclear, however, how short-term ethanol exposure causes changes in gene expression and what characteristics of the nervous system are affected. This is an important question to answer because gene expression can lead to lasting changes in nervous system function that are plausibly the basis for increased addiction liability. Here, we use *Drosophila* to define the role of Sir2 in ethanol-induced behavioral plasticity, and show that ethanol works through Sir2 to change the expression of key presynaptic molecules.

Materials and Methods

Drosophila genetics and culturing conditions. All strains were outcrossed for at least five generations to the Berlin genetic background carrying the w^{1118} genetic marker mutation. The genetic background strain was used as an experimental control, indicated as "control" in the figures. Flies were cultured on standard cornmeal/molasses/yeast medium at 25°C and 70% relative humidity with an ~16/8 h light/dark schedule, unless otherwise indicated. Strains used in this study include $Sir2^{2A-7-11}$ (catalog #8838, Bloomington Drosophila Stock Center [BDSC]), *UAS-UAS-Sir2.IR* 32481 (catalog #32481, BDSC), *elav(c155)-GAL4* (catalog #458, BDSC), *tub-GAL80^{ts}* (catalog #7019, BDSC), *17d-GAL4* (catalog #51631, BDSC), *UAS-Sir2* (a gift from Alexei Tulin, Fox Chase Cancer Center, Philadelphia, PA), *MB-GAL80* (a gift from Scott Waddell, University of Oxford, Oxford, UK; Krashes et al., 2007), *UAS-TeTx* (a gift from Sean

Sweeney, University of York, York, UK), *UAS-Sir2.IR* 105502 (catalog #105502, Vienna Drosophila RNAi Center), $Sir2^{NP1145}$ (catalog #112534, Kyoto Drosophila Genetics Resource Center), and Syn^{97} (a gift from Giovanni Bosco, Dartmouth College, Hanover, NH).

Ethanol tolerance. Crosses were allowed to lay eggs for 2 d, and groups of ~20 2- to 4-d-old adult male progeny were collected into standard food vials without yeast. These flies were allowed to recover from CO_2 anesthesia for 2 d and were then tested behaviorally.

Behavioral tests were conducted in the booz-o-mat, as previously described (Wolf et al., 2002). Each strain was tested on at least 2 separate days, to control for day-to-day variation. *n* indicates the total number of groups tested. Flies were allowed to acclimate to the testing chamber for 7–9 min in a stream of humidified air and were then switched to a stream of ethanol vapor (85/65 ethanol vapor/air). At 6 min intervals, the number of flies unable to right themselves was counted. Following a 4 h interexposure interval, flies were given a second identical exposure. Sedation sensitivity was measured as the time for 50% of the flies to become sedated during the first exposure (ST50), except where otherwise indicated. Sedation tolerance was measured as the difference between the time to 50% sedation in the first and second exposures.

Ethanol preference. The capillary feeding assay (CAFE) was used to determine ethanol preference, as previously described (Ja et al., 2007; Devineni and Heberlein, 2009). Groups of eight adult males were collected 3–4 d after eclosion and were allowed to recover for 1 d. They were then exposed to either 85/65 ethanol vapor/air mixture or humidified with air alone for 20 min. After 16 h of recovery, flies were placed into the CAFE chamber, which consists of empty *Drosophila* culture vials with capillary tubes containing liquid food, with or without 15% ethanol, embedded in the vial plug. The preference index was measured as the volume of food consumed over 1 night from the ethanol capillaries minus that consumed from the no-ethanol capillaries over the total volume consumed, corrected for evaporation by measuring the volume lost in tubes with no flies. Bitter taste avoidance was measured by presenting flies with a choice of 1.25% agarose containing either 50 mM sucrose (S) or 100 mM sucrose and 1 mM quinine (SQ). Approximately 20 male flies were food deprived on water for 14 h, placed in a $40 \times 90 \times 10$ mm clear acrylic arena, and 150 μl of S and SQ dots were then placed in apposition at the center of the arena. The number of flies on each dot was counted at 120 min. Avoidance was calculated as $(SQ - S)/(SQ + S)$, such that complete avoidance of bitter taste gives a value of -1.

Ethanol reward. The conditioned olfactory preference assay was performed as previously described (Kaun et al., 2011). Groups of 30 male flies were trained to associate an odor with ethanol in three 10 min repetitions of odor/ethanol pairing. Memory was tested 24 h later in a Y maze with ethanol-paired and unpaired odors streaming from opposite arms. A preference index was calculated as follows: (number of flies selecting paired odor − number of flies selecting unpaired odor)/total number of flies.

Ethanol absorption and metabolism. Flies were frozen in liquid nitrogen and homogenized in 50 mM Tris-HCl, pH 7.5. Ethanol concentrations were measured in fly homogenates using the Ethanol Assay Kit from Diagnostic Chemicals Ltd. (catalog #229-29). To calculate the ethanol concentration in flies, the volume of one fly was estimated to be 1 μl (Moore et al., 1998).

Western blot. Protein Western blot analysis was performed using standard methods. Whole male fly heads were lysed in RIPA buffer (catalog #20-188, Millipore); and protein was run on polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies, including the following: rabbit α-histone H3 (Active Motif), rabbit α-Histone H3 K9 acetylated (Active Motif), rabbit α-Sir2 818 (a gift from James Kadonaga, University of California, San Diego, La Jolla, CA), mouse α-tubulin (catalog #T6074, Sigma-Aldrich), mouse α-SynORF1 (catalog #3C11, Developmental Studies Hybridoma Bank), and rabbit α-Brp-D2 (a gift from Stephan Sigrist, Free University of Berlin, Berlin, Germany). Blots were secondarily stained with α-rabbit HRP (catalog #NA934V, GE Healthcare Life Sciences) and imaged using the enhanced chemiluminescence method (Mruk and Cheng, 2011), or secondarily stained with goat α-Rabbit IRDye 800CW and goat α-Mouse IRDye 680RD (LI-COR) and imaged on a LI-COR Odyssey infrared scanner.
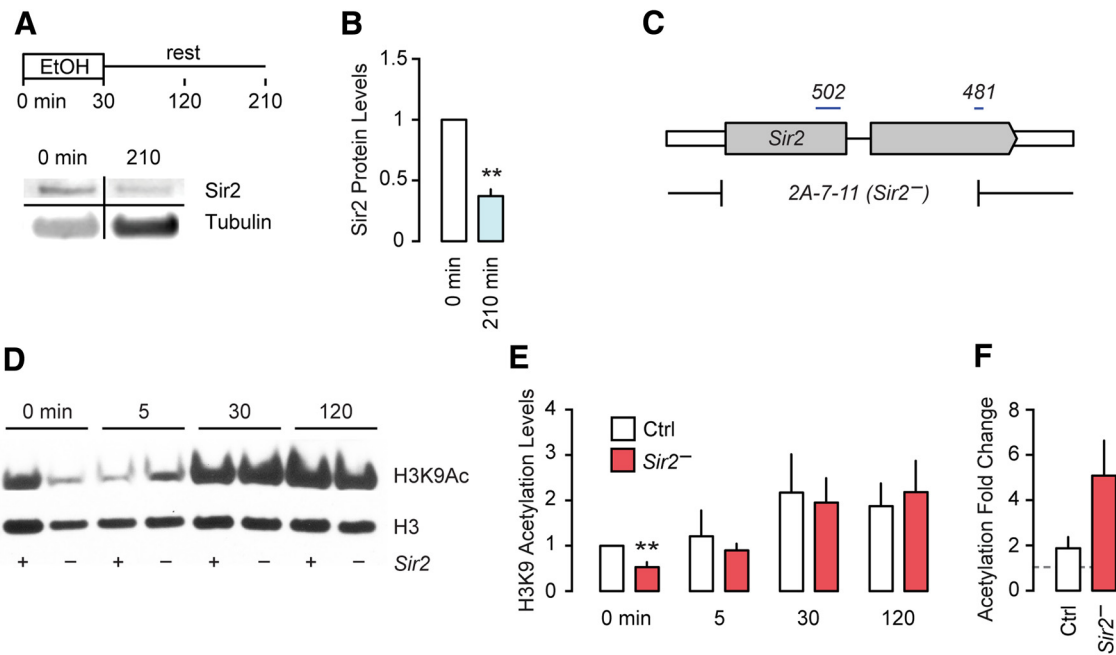
Figure 1. Levels of Sir2 and histone acetylation in fly heads following short-term exposure to ethanol. ***A***, Schematic diagram of experimental procedure, and Western blot detecting Sir2 and tubulin proteins (bands rearranged from a single blot). ***B***, Quantification of Sir2 expression normalized to tubulin, relative to unexposed flies ($n = 3$). ***C***, Schematic diagram of *Sir2* coding region (gray) depicting boundaries of the *2A-7–11* deletion (*Sir2$^-$*) and regions used to generate the *502* and *481* RNAi lines. ***D***, Western blot of control ($+$) and *Sir2$^-$* ($-$) heads for acetylated and total H3K9 for the indicated time points during/after ethanol exposure. ***E***, Quantification of ***D*** showing H3K9Ac levels normalized to total H3, relative to untreated controls ($n = 5$). ***F***, Total fold change in H3K9 acetylation in control and *Sir2$^-$* flies between unexposed and ethanol-exposed flies ($n = 5$). Error bars indicate the SE. **$p < 0.01$.

Immunohistochemistry. Immunohistochemistry was performed based upon standard protocols (Wu and Luo, 2006). Adult brains expressing CD2-mCherry were dissected and stained using rabbit α-dsRed (catalog #632496, Clontech), mouse α-Brp (catalog #nc82, Developmental Studies Hybridoma Bank), goat α-rabbit 594 (ThermoFisher Scientific), and goat α-mouse 488 (ThermoFisher Scientific). Imaging was performed using a Nikon Eclipse Ti C1 Confocal System, and z-stacks were compressed using FIJI, and corrected for sample orientation and brightness/contrast with Adobe Photoshop.

RNA measurement. Groups of 600 male flies were exposed to an 85/65 ethanol vapor/air mixture or a matched flow of humidified air alone for 30 min and allowed to recover for 3.5 h. Flies were then frozen in liquid nitrogen, and heads were collected. Head RNA was purified using TRIzol reagent (Invitrogen). Poly-A pulldown and cDNA library generation were performed using the manufacturer protocols with a TruSeq Prep Kit (Illumina), then sequenced to a depth of 25 million paired-end reads (Expression Analysis, Inc). Data were analyzed using Galaxy main (https://usegalaxy.org) using the Tuxedo suite of tools (Bowtie, Tophat, and Cufflinks) to align reads to release five of the *Drosophila melanogaster* annotated genome (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010; Trapnell et al., 2012). The resulting gene expression data were filtered for genes with known functions in the nervous system that showed a onefold or higher change in expression between groups and had a minimum expression of 0.1 fragment per kilobase per million. Genes meeting these criteria were further compared with those enriched in the mushroom body (MB; Henry et al., 2012). The putative effector genes identified in this way were further assessed by quantitative PCR (qPCR) across multiple biological and technical replicates.

Statistics. All statistical tests were performed using GraphPad Prism software. Tests used include unpaired *t* test, one sample *t* test, and one-way ANOVA with Tukey's *post hoc* test. All tests performed were two tailed. Significance indicators on the figures indicate the results of *t* tests or Tukey's tests. Error bars represent the SEM.

Results

Sir2 is downregulated by acute ethanol exposure

Strongly reduced *Sir2* transcript levels soon after acute ethanol exposure led us to ask whether Sir2 regulation initiates an effector program to alter gene expression and behavior (Kong et al., 2010). First, we asked whether Sir2 protein and one of the targets of Sir2 deacetylase activity are affected by acute ethanol exposure. Western blot analysis demonstrated that Sir2 protein is also strongly decreased (unpaired *t* test, $t_{(4)} = 6.82$, $p = 0.0024$; Fig. 1*A*,*B*). A dramatic ethanol-induced increase in acetylation of histone H3 at lysine 9 (H3K9ac), a Sir2 target for deacetylation, in whole head extracts persisted for at least 90 min after ethanol exposure had ended (Fig. 1*D–F*). In *Sir2* mutant flies (*Sir2$^-$*) that carry a deletion of the *Sir2* gene (Fig. 1*C*), we observed an almost doubled increase in H3K9ac by ethanol that did not reach statistical significance (unpaired *t* test, $t_{(8)} = 1.98$, $p = 0.0828$; Fig. 1*D–F*). Maximum levels of H3K9ac did not appear different across genotypes. However, untreated *Sir2$^-$* flies had decreased levels of H3K9Ac (one-sample *t* test, $t_{(4)} = 5.18$, $p = 0.0035$; Fig. 1*E*), suggesting either the existence of compensatory epigenetic mechanisms in the absence of Sir2 HDAC activity or a complexity in the actions of Sir2 on histones. These data indicated that ethanol causes marked changes in histone acetylation that depends, in part, on the regulation of the HDAC Sir2 by ethanol in flies.

Sir2 acts in the brain to promote ethanol tolerance

Since ethanol treatment increases protein acetylation, and this is dependent on Sir2, we hypothesized that changes in protein acetylation by Sir2 contributes to the behavioral responses of flies to ethanol. We measured ethanol sedation sensitivity and the rapid functional tolerance that develops soon after the ethanol accumulated during the first exposure has been metabolized. Naive *Sir2$^-$* flies were strikingly less sensitive to the sedating effects of acute ethanol exposure, taking nearly twice as long to reach 50% sedation (unpaired *t* test, $t_{(10)} = 23.83$, $p = 0.0001$; Fig. 2*A–C*). *Sir2$^-$* flies also showed a marked decrease in sedation tolerance (unpaired *t* test, $t_{(10)} = 5.08$, $p = 0.0005$; Fig. 2*D*). These strong behavioral effects were not due to alterations in either ethanol absorption or metabolism (Fig. 2*E*). Therefore, Sir2 is required to
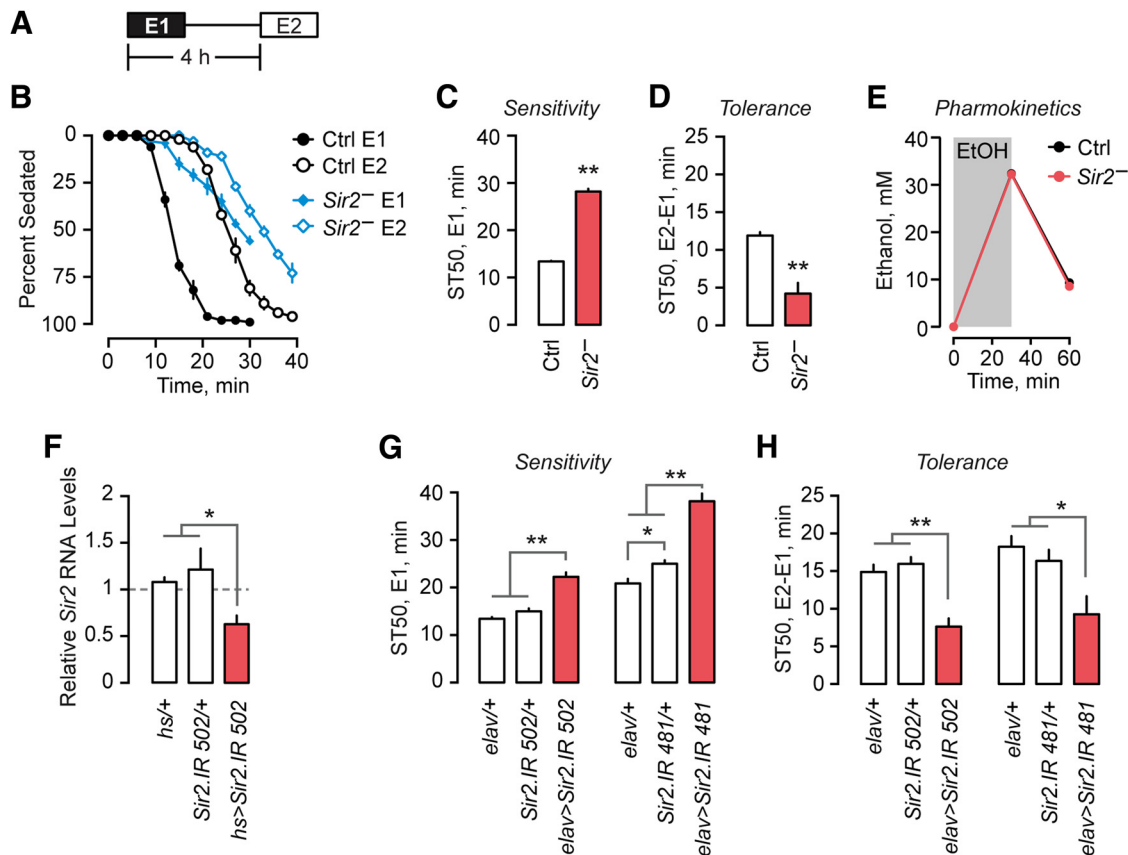
Figure 2. Ethanol sedation sensitivity and tolerance in flies with reduced *Sir2* expression. ***A***, Exposure scheme for measuring ethanol sedation sensitivity (E1) and tolerance (E2-E1). ***B***, Time course of sedation for E1 (closed symbols) and E2 (open symbols) in control (black) and *Sir2⁻* (blue) flies (n = 6). ***C***, Sedation sensitivity (time to 50% sedation e1) in *Sir2⁻* and control flies (n = 6). ***D***, Sedation tolerance (time to 50% sedation e2-e1) in *Sir2⁻* and control flies (n = 6). ***E***, Ethanol absorption and metabolism in *Sir2⁻* and control flies. ***F***, *Sir2* gene expression following RNAi-mediated knockdown using the *502* RNAi line, relative to control flies (n = 3). ***G***, ***H***, Sedation sensitivity (***G***) and sedation tolerance (***H***) in flies expressing *Sir2* RNAi transgenes in the nervous system (n = 9). Error bars indicate the SE. *$p < 0.05$, **$p < 0.01$.

promote ethanol sensitivity and facilitate the development of ethanol tolerance.

To determine the tissues where *Sir2* is required for ethanol behaviors, we used the *GAL4/UAS* transgenic system to express *Sir2* inverted repeat (*Sir2.IR*) dsRNA to generate *Sir2* RNAi. We determined first that ubiquitous expression of *Sir2.IR* driven by a heat shock-inducible GAL4 (*hs*) decreased *Sir2* RNA abundance (one-way ANOVA, $F_{(2,12)} = 8.68$, $p = 0.0047$; Fig. 2F). Decreasing *Sir2* in all neurons using the *elav-GAL4* driver (*elav*) reduced both sedation sensitivity (one-way ANOVA, $F_{(2,34)} = 17.09$, $p < 0.0001$; Fig. 2G) and sedation tolerance (one-way ANOVA, $F_{(2,34)} = 66.70$, $p < 0.0001$; Fig. 2H). To test for off-target effects, we decreased Sir2 in neurons using a UAS-RNAi that targeted a different region of *Sir2* (*Sir2.IR 481*) and showed that it decreased sedation sensitivity (one-way ANOVA, $F_{(2,36)} = 59.07$, $p < 0.0001$; Fig. 2G) and sedation tolerance (one-way ANOVA, $F_{(2,36)} = 6.61$, $p = 0.0036$; Fig. 2H). *Sir2.IR 481* also decreased *Sir2* RNA abundance (one-way ANOVA, $F_{(2,6)} = 10.56$, $p = 0.0108$; data not shown). The expression of *Sir2.IR* in other tissues, including glia and the fat body adipose tissue, did not change ethanol sedation sensitivity or tolerance (data not shown). These results demonstrate that *Sir2* is required in neurons to promote ethanol sensitivity and tolerance.

To determine whether the expression of *Sir2* in the nervous system was sufficient to allow normal ethanol sensitivity and tolerance, and thus could account for the role of *Sir2* in etha-

nol behaviors, we performed a genetic rescue experiment, expressing *Sir2* in all neurons of flies otherwise lacking functional *Sir2* using a *UAS-Sir2* transgene. The expression of *Sir2* solely in neurons did not rescue the decreased ethanol sedation sensitivity of *Sir2⁻* mutants (one-way ANOVA, $F_{(3,26)} = 0.78$, not significant; Fig. 3A). However, it did rescue the decreased sedation tolerance phenotype of *Sir2⁻* mutants (one-way ANOVA, $F_{(3,26)} = 10.32$, $p < 0.0001$; Fig. 3B). Importantly, these results indicate that the decreased sedation sensitivity and decreased sedation tolerance *Sir2⁻* phenotypes can be separated: *Sir2*-dependent tolerance is not due solely to alterations in ethanol sensitivity. The role of *Sir2* in ethanol sensitivity may also require its activity in non-neuronal tissues. Alternatively, our genetic rescue scheme may reinstate *Sir2* in either an inappropriate expression pattern or level in neurons for the role of *Sir2* in sensitivity. However, *Sir2* is both necessary and sufficient in the nervous system for the development of ethanol tolerance.

Sir2 is required in adults for ethanol tolerance
Neuronal Sir2 may contribute to nervous system development, maintenance, physiological function, or a combination thereof to affect ethanol behavioral responses. We performed two complementary experiments to decrease Sir2 function during specific periods of the fly lifespan. First, nicotinamide is a potent end-product inhibitor of Sir2 deacetylase activity that is active against Sir2 *in vivo* (Das et al., 2009). Treatment of adult flies with nico-
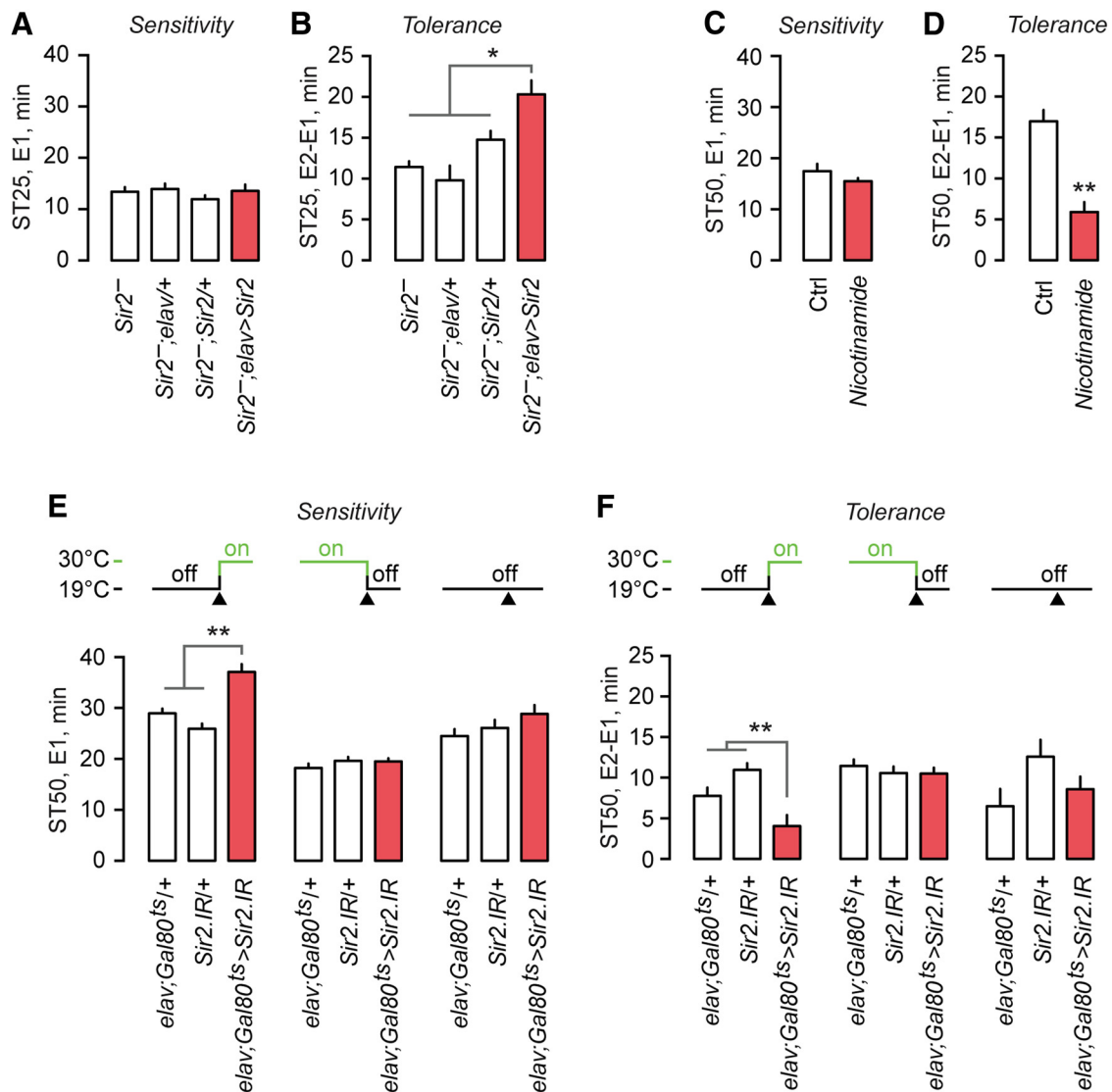
Figure 3. Sir2 activity is necessary in the adult brain for normal ethanol response behaviors. ***A***, ***B***, Sedation sensitivity (***A***) and sedation tolerance (***B***) in $Sir2^-$ flies expressing wild-type Sir2 in the brain. Measurements are presented as the time to 25% sedation due to the marked increase in sedation resistance during exposure 2 in $Sir2^-$;$elav > Sir2$ ($n = 6 – 8$). ***C***, ***D***, Sedation sensitivity (***C***) and sedation tolerance (***D***) in flies that have consumed the Sir2 inhibitor nicotinamide ($n = 8$). ***E***, ***F***, Sedation sensitivity (***E***) and sedation tolerance (***F***) in flies expressing Sir2 RNAi under temporal control, limited to adulthood ($n = 18$), development ($n = 7 – 8$), or inhibited through the entire lifespan ($n = 6$). Error bars indicate the SE. $*p < 0.05$, $**p < 0.01$, triangle on temperature shift schematics indicates beginning of adult stage.

tinamide did not affect ethanol sedation sensitivity (unpaired t test, $t_{(14)} = 1.27$, $p = 0.2251$; Fig. 3C) but strongly decreased ethanol sedation tolerance (unpaired t test, $t_{(14)} = 6.03$, $p < 0.0001$; Fig. 3D). Second, we took advantage of a temperature sensitive GAL80 to limit $Sir2.IR$ reduction of Sir2 expression levels to either development or adulthood. Ubiquitously expressed GAL80^{ts} inhibits GAL4 activity at 19°C and allows GAL4 activity at 30°C. When neuronal decreases in Sir2 were limited to adulthood, sedation sensitivity was decreased (one-way ANOVA, $F_{(2,51)} = 23.57$, $p < 0.0001$; Fig. 3E), and sedation tolerance was also decreased (one-way ANOVA, $F_{(2,51)} = 10.23$, $p = 0.0002$; Fig. 3F), recapitulating the $Sir2^-$ behavioral phenotypes. When neuronal decreases in $Sir2$ were limited to development, no difference in behavior was observed (one-way ANOVA, $F_{(2,20)} = 0.46$, difference was not significant; Fig. 3E,F). Therefore, Sir2 activity is required in the adult nervous system for the development of ethanol tolerance.

Sir2 is required in the mushroom bodies for ethanol tolerance

The mushroom bodies are a bilateral brain neuropil implicated in learning and memory, and also in ethanol preference and reward (Dubnau and Tully, 2001; Roman and Davis, 2001; Fiala, 2007; Busto et al., 2010; Kaun et al., 2011; Ojelade et al., 2015). In particular, the α/β-lobes of the mushroom body are implicated in ethanol responses (Kaun et al., 2011; King et al., 2011; Ojelade et al., 2015). We investigated the role of the mushroom bodies in ethanol tolerance. To test whether mushroom body neurotransmission is important, we expressed the tetanus toxin light chain ($TeTx$) in the mushroom body α/β-lobes using the $17D$-GAL4 driver line (Fig. 4A), preventing presynaptic vesicular release. While we observed variable effects for sedation sensitivity, there was a marked decrease in sedation tolerance (one-way ANOVA, $F_{(3,56)} = 14.94$, $p < 0.0001$; Fig. 4B,C). Addition of MB-GAL80, which prevents GAL4 activity specifically in the α/β-lobes, attenuated, but did not completely ameliorate, the decreased tolerance
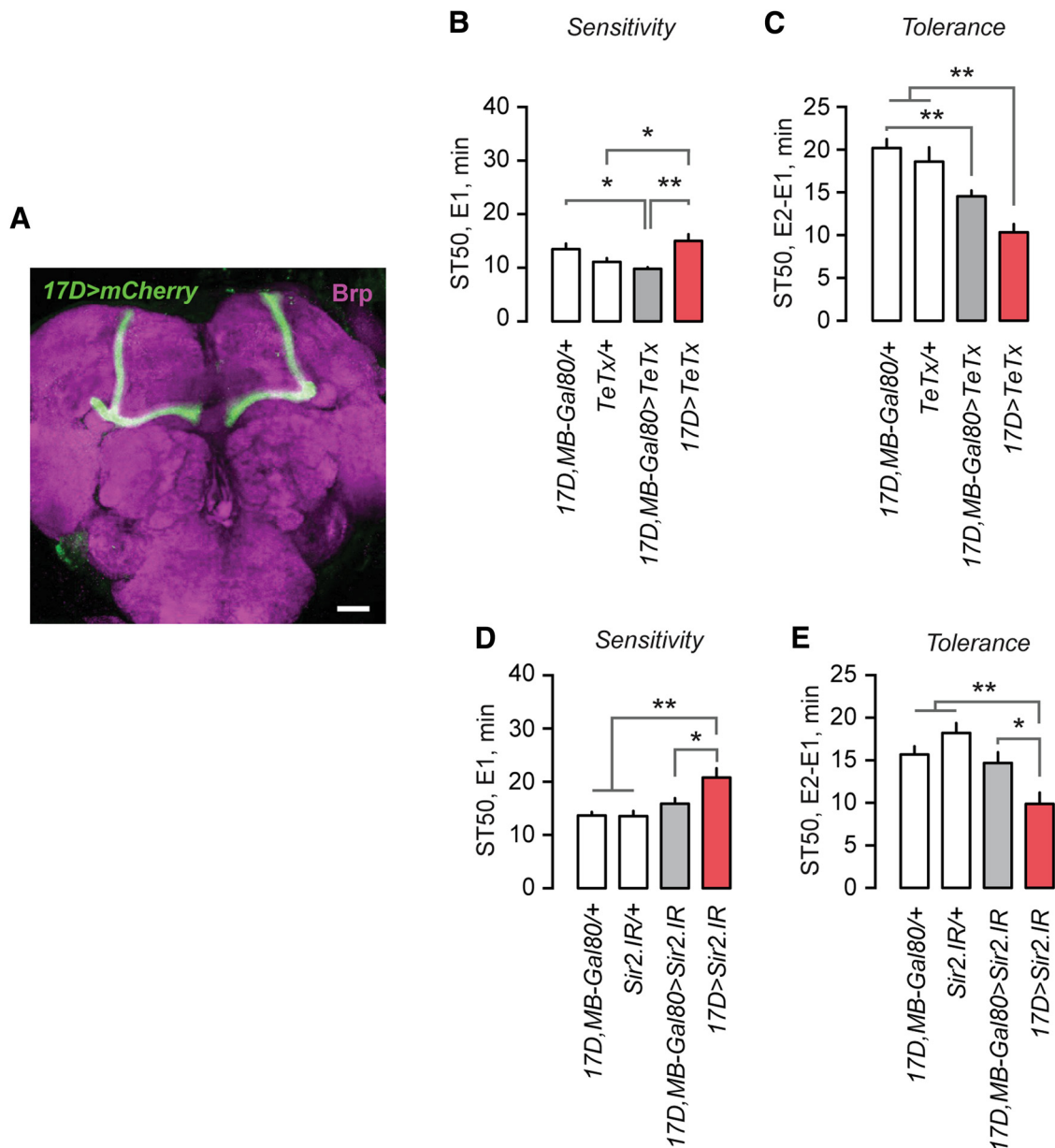
Figure 4. Sir2 acts in the mushroom body α/β-neurons to promote ethanol tolerance. ***A***, Compressed z-stack image of an adult brain immunostained for mCherry (driven in the *17D-GAL4* pattern, green) and the presynaptic protein Bruchpilot (Brp; magenta). ***B***, ***C***, Sedation sensitivity (***B***) and sedation tolerance (***C***) of flies expressing the tetanus toxin light chain (TeTx) in the *17D* pattern with or without *MB-GAL80* to block mushroom body expression (n = 8). ***D***, ***E***, Sedation sensitivity (***D***) and sedation tolerance (***E***) of flies expressing *Sir2* RNAi in the *17D* pattern in the mushroom bodies, with and without *MB-GAL80* (n = 15). Error bars indicate the SE. *$p < 0.05$, **$p < 0.01$.

phenotype (Tukey's test, $q = 3.69$, $p = 0.0653$; Fig. 4C). This experiment suggested that neuronal activity in the mushroom body α/β-lobes promotes ethanol tolerance development.

We next asked whether *Sir2* was required specifically in the mushroom body α/β-lobe neurons. Mushroom body-specific *Sir2.IR* resulted in decreased sedation sensitivity (one-way ANOVA, $F_{(3,28)} = 9.08$, $p < 0.0001$; Fig. 4D) and decreased sedation tolerance (one-way ANOVA, $F_{(3,28)} = 8.70$, $p < 0.0001$; Fig. 4E). This recapitulation of the *Sir2* loss-of-function phenotypes was due to reduced *Sir2* expression in the α/β-lobes: adding MB-GAL80 blocked the *Sir2.IR* ethanol behavioral phenotypes (sensitivity: Tukey's test, $q = 4.07$, $p = 0.0283$; tolerance: Tukey's test, $q = 4.37$, $p = 0.0159$; Fig. 4D,E). Thus, *Sir2* is required in the mushroom bodies to promote ethanol sensitivity and the development of ethanol tolerance.

Sir2 is required for ethanol preference and reward

The mushroom bodies are required for ethanol preference and reward (Kaun et al., 2011; Xu et al., 2012; Ojelade et al., 2015). We asked whether the role of Sir2 may extend to these other forms of ethanol behavioral plasticity. Flies given a choice of liquid food with and without added ethanol will develop a preference for the ethanol food in the CAFE assay (Ja et al., 2007; Devineni and Heberlein, 2009). Flies that have developed ethanol preference will overcome adversity to gain the ethanol and show preference rebound following a period of abstinence. Additionally, flies pre-exposed to ethanol vapor develop ethanol preference much more rapidly (Peru y Colón de Portugal et al., 2014; Fig. 5A). Whereas ethanol pre-exposed control flies developed robust preference, $Sir2^-$ flies did not (control: one sample t test, $t_{(27)} = 3.15$, $p = 0.004$; $Sir2^-$: one sample t test, $t_{(26)} = 0.90$, difference was not

A

Ethanol Preference

B

Bitter Taste Aversion

C

Ethanol Reward
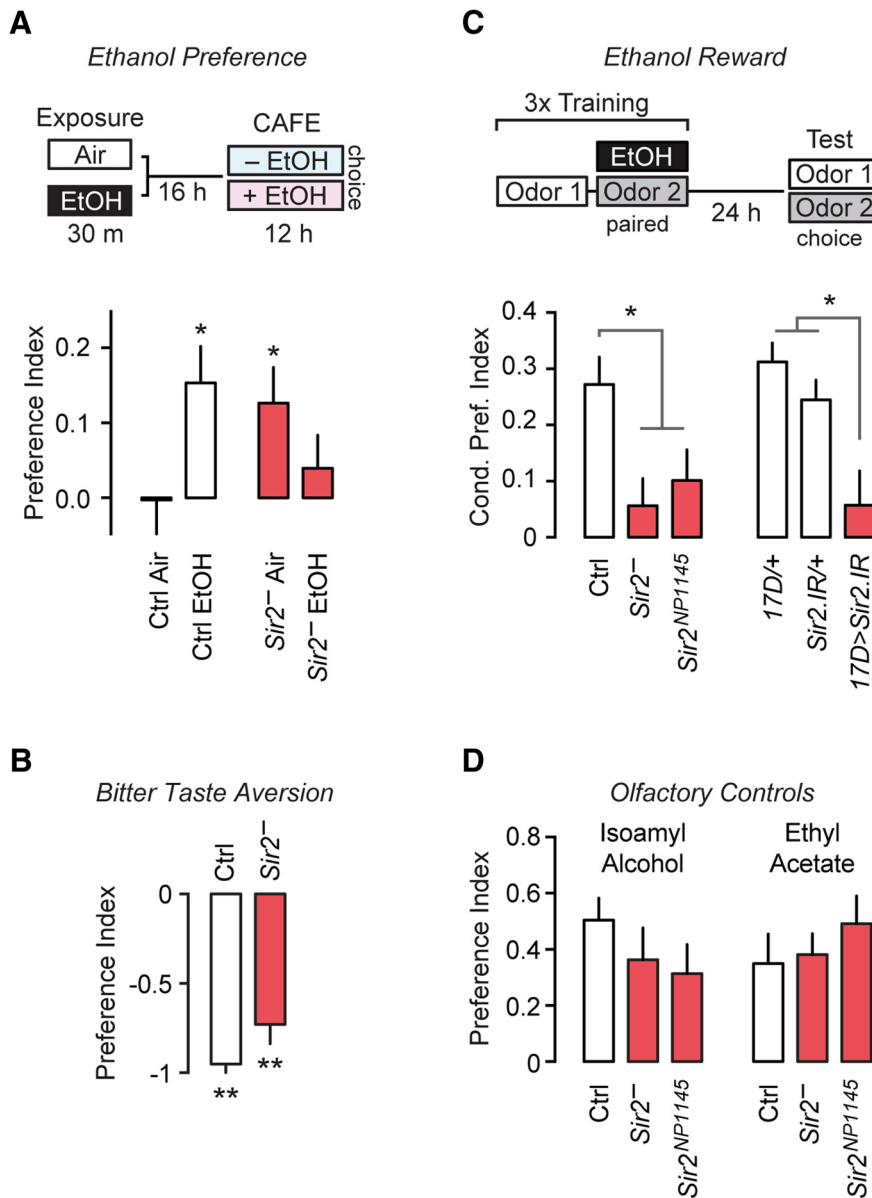

D

Olfactory Controls

Figure 5. Sir2 is required for ethanol preference and for reward in the mushroom bodies. ***A***, CAFE assay comparing the preference for liquid food with or without ethanol, with the schematic depicting the experimental design ($n = 27$–28). ***B***, Bitter taste aversion in flies given the choice of sucrose with and without quinine. A value of "−1" indicates complete aversion ($n = 7$). ***C***, Conditioned olfactory preference to measure ethanol reward, with a schematic diagram of the experimental design in *Sir2* mutants and flies expressing *Sir2* RNAi under the control of the mushroom body driver *17D-GAL4* ($n = 14$–16). ***D***, Preference for the odorants isoamyl alcohol and ethyl acetate in the absence of pairing with ethanol inebriation ($n = 8$). Error bars indicate the SE. *$p < 0.05$, **$p < 0.01$.

significant; Fig. 5*A*). In fact, *Sir2*⁻ flies preferred the ethanol-containing food without an ethanol pre-exposure, suggesting that animals lacking *Sir2* may be predisposed to drink ethanol (one-sample *t* test, $t_{(27)} = 2.65$, $p = 0.0135$; Fig. 5*A*). This decreased preference in ethanol pre-exposed *Sir2*⁻ flies may be a loss of preference, or a developing aversion. Similarly, *Sir2*⁻ unconditioned ethanol preference could be due to a loss of avoidance of aversive stimuli. To test this, we gave flies a choice between 100 mM sucrose with bitter quinine and 50 mM sucrose. *Sir2* mutants, like controls, showed a strong avoidance of the sweeter yet bitter quinine choice (control: one-sample *t* test, $t_{(6)} = 20$, $p < 0.001$; *Sir2*⁻: one-sample *t* test, $t_{(6)} = 6.708$, $p < 0.001$; Fig. 5*B*). These findings imply that Sir2 is more specifically involved in interpreting the value of ethanol intake.

Ethanol reward in *Drosophila* is measured using a conditioned olfactory preference assay that is conceptually analogous to conditioned place preference in mammals (Kaun et al., 2011). The assay measures reward by pairing an odor with ethanol intoxication, and assessing paired versus unpaired odor attraction 24 h later. *Sir2* mutant flies showed strongly reduced conditioned odor preference, similar to their loss of ethanol-primed preference (one-way ANOVA, $F_{(2,43)} = 5.67$, $p = 0.0065$; Fig. 5*C*). This manifested as a loss of the rewarding effect of ethanol. Alternatively, a second aversive process may develop to mask reward in *Sir2*⁻ flies. Importantly, mushroom body-specific *Sir2.IR* also decreased conditioned odor preference (one-way ANOVA, $F_{(2,29)} = 9.19$, $p = 0.0005$; Fig. 5*C*). Olfactory acuity for the pairing odors was unaffected in *Sir2* mutants (one-way ANOVA; isoamyl alcohol: $F_{(2,21)} = 0.99$, $p = 0.3875$; ethyl acetate: $F_{(2,21)} = 0.64$, $p = 0.5387$; Fig. 5*D*).

Together, these experiments demonstrate that Sir2 plays a more general role in mediating ethanol behavioral plasticity. However, whether this occurs through a common molecular target, shared behavior circuits, or a combination thereof is unknown. We hypothesize that the broad effects of Sir2 are suggestive of a shared molecular target known to affect behavioral plasticity in many different cell types.

Sir2-dependent ethanol regulation of presynaptic molecules: synapsin is required for tolerance
All known substrates for Sir2 deacetylase activity regulate gene transcription, including histone H3 acetylation. Ethanol-decreased expression of Sir2 may thus set in motion a transcriptional program that alters neuronal properties to change behavioral responses, such as those measured by assays for ethanol tolerance, preference, and reward. To identify genes that might contribute to Sir2-dependent ethanol behaviors, we performed an RNAseq analysis of RNA isolated from the heads of *Sir2*⁻ and wild-type control flies that were treated with ethanol vapor or humidified air alone. We curated a list of 749 genes that showed large (twofold or higher) changes in expression with ethanol treatment in either genotype and that were expressed at detectable levels. Interestingly, there was remarkably little overlap in the genes that respond to ethanol in control and *Sir2*⁻ flies; of the 492 genes upregulated in wild-type flies in response to ethanol, only 52 were also upregulated in *Sir2*⁻ flies treated with ethanol (Fig. 6*A*).

A marked change in *Synapsin* (*Syn*) RNA levels in *Sir2*⁻ suggested that Sir2 may regulate presynaptic function. Of genes encoding proteins predominantly localized to synapses, only *Syn* expression was affected (decreased) in the heads of *Sir2* mutants
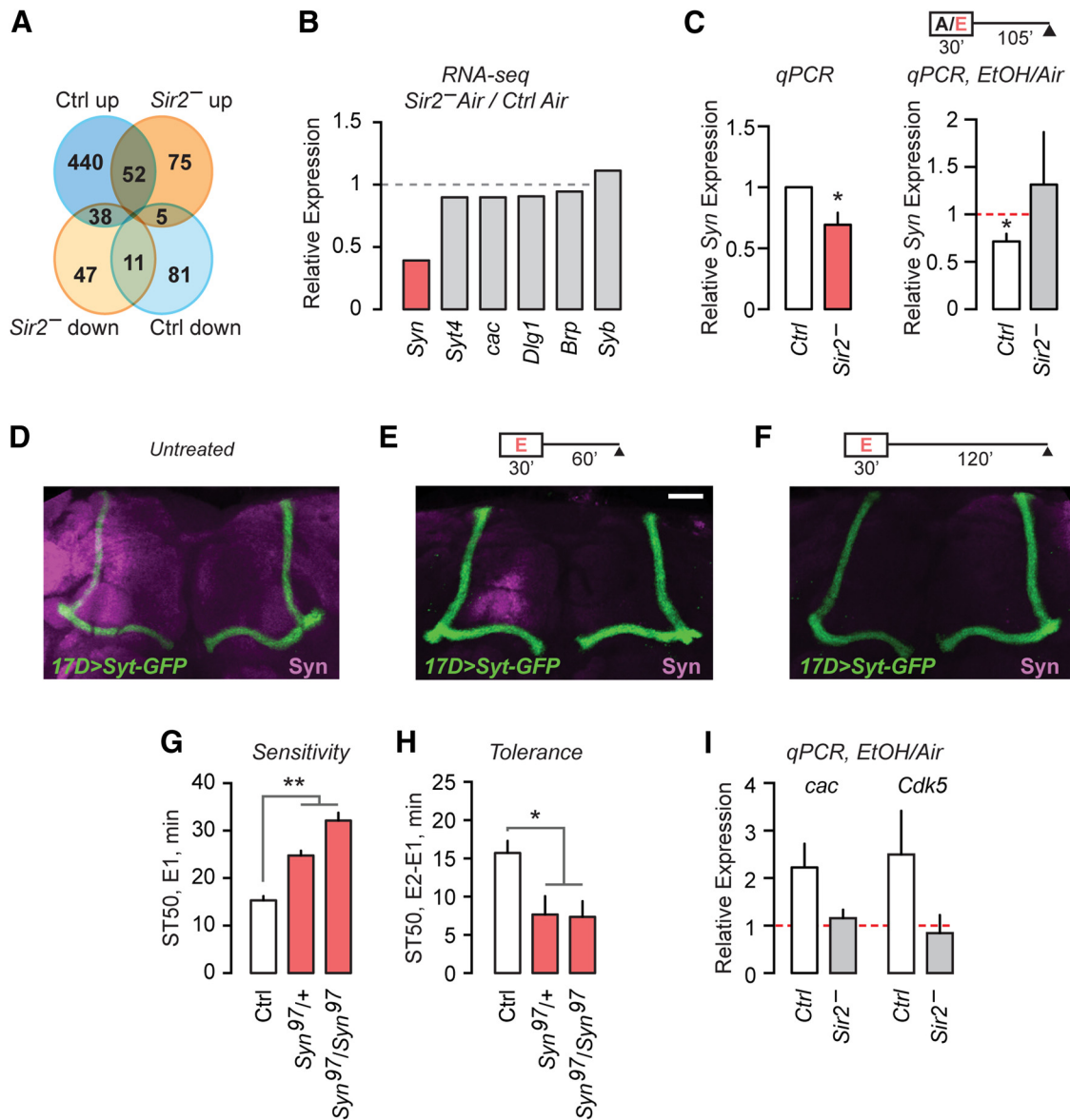
Figure 6. Presynaptic proteins are regulated by Sir2, and decreased Synapsin hinders ethanol tolerance. *A*, Diagram depicting genes upregulated and downregulated by ethanol exposure in control (blue) and *Sir2⁻* (orange) flies, as assayed by RNAseq. *B*, Relative expression of genes coding for synaptic proteins between control and *Sir2⁻* flies, assayed by RNA-seq. *C*, qPCR showing *Syn* expression changes in control and *Sir2⁻* flies, with and without ethanol exposure (left, $n = 9$; right, $n = 5$–6). *D–F*, Brains (25 μm projections) expressing synaptotagmin-GFP in 17d pattern (green) from untreated (*D*), 30 min ethanol plus 60 min recovery (*E*), and 30 min ethanol plus 120 min recovery (*F*) immunostained with Syn (magenta). Scale bar, 20 μm. *G*, *H*, Sedation sensitivity (*G*) and sedation tolerance (*H*) in *Syn⁹⁷* (*Syn-null*) flies vs controls ($n = 8$). *I*, Relative mRNA expression of the presynaptic proteins Cac and Cdk5 in control and *Sir2⁻* flies when assayed via qPCR in ethanol-treated vs untreated flies ($n = 3$). Error bars indicate the SE. *$p < 0.05$, **$p < 0.01$.

(Fig. 6*B*). *Syn* encodes abundant presynaptic phosphoproteins that bind synaptic vesicles and regulate vesicle dynamics (Südhof, 2012). *Syn* is important in flies for learning and memory, use-dependent synaptic growth, and the response to ethanol (Goden-schwege et al., 2004; Vasin et al., 2014). Additionally, the chromatin state of the *Syn* promotor changes in response to se-dation (Ghezzi et al., 2013). We verified *Syn* downregulation in *Sir2⁻* flies by qPCR (one-sample t test, $t_{(8)} = 3.12$, $p = 0.0142$; Fig. 6*C*, left). We then determined the effect of ethanol treatment on *Syn* RNA expression. In wild-type flies after recovery from ethanol exposure, *Syn* was significantly decreased compared with untreated controls (one-sample t test, $t_{(4)} = 3.49$, $p = 0.0251$; Fig. 6*C*, right). Importantly, there was no such decrease in ethanol-exposed *Sir2⁻* flies compared with untreated *Sir2⁻* (one-sample t test, $t_{(5)} = 0.57$, $p = 0.5946$; Fig. 6*C*, right). To determine whether

ethanol also decreases Syn protein expression, we immuno-stained control and ethanol-treated brains with a Syn antibody. Syn expression was strongly decreased throughout the brain by ethanol treatment (Fig. 6*D–F*). These data indicate that ethanol regulates *Syn* expression levels in a Sir2-dependent fashion.

We next investigated the role of *Syn* in behavior using a pre-viously characterized null mutant of *Syn* in the ethanol sedation tolerance assay. *Syn*-null mutant flies (*Syn⁹⁷*) showed strongly decreased sedation sensitivity (one-way ANOVA, $F_{(2,21)} = 46.47$, $p < 0.0001$; Fig. 6*G*) and tolerance (one-way ANOVA, $F_{(2,21)} = 5.41$, $p = 0.013$; Fig. 6*H*), mimicking the phenotype of *Sir2⁻* flies. Concordantly, a 50% reduction of Syn expression in *Syn*-null heterozygotes was sufficient to decrease ethanol sensitivity and tolerance, suggesting that changes in presynaptic function may explain the role of Sir2 in ethanol behavioral responses.

Expression of other presynaptic molecules may be regulated by ethanol in a Sir2-dependent manner. In wild-type and *Sir2⁻* fly heads treated with ethanol and allowed to recover, we assessed RNA transcript levels of the prominent presynaptic proteins cyclin-dependent kinase 5 (Cdk5), a cyclin-dependent kinase that influences presynaptic function, and Cacophony (Cac), the major presynaptic calcium channel. Like *Syn*, the *cac* genomic region shows changes in histone acetylation following sedation (Ghezzi et al., 2013). Both *cac* and *Cdk5* RNA transcript levels increased, albeit not to statistical significance, in ethanol-treated wild-type flies compared with air-treated controls, but no increase was apparent in *Sir2⁻* flies (Fig. 6*I*).

Discussion

Drugs of abuse engage epigenetic transcriptional programs that contribute to addiction-like behaviors (Wang et al., 2007; Renthal et al., 2009; Botia et al., 2012; Feng et al., 2014; Heller et al., 2014; Ferguson et al., 2015; Koo et al., 2015). We show that acute ethanol exposure, via the downregulation of Sir2, changes the expression of key presynaptic molecules and initiates behavioral adaptations in flies. Studies in mice demonstrate that SIRT1 is upregulated by long-term drug treatment. Further, experimentally increasing SIRT1 in the mouse nucleus accumbens enhances the rewarding effects of long-term cocaine and morphine administration, whereas reducing SIRT1 decreases it (Ferguson et al., 2013). The molecular mechanisms by which SIRT1 drives the reward for long-term drug exposure are partly understood. Our findings demonstrate that Sir2/SIRT1 functions across species for addictive drug action. More importantly, we tie the acute actions of ethanol to a transcriptional mechanism for effecting cellular and behavioral change. This pathway for early functional adaptations to ethanol may lay the molecular and circuit groundwork for the addictive liability of repeated drug intake. Further, it provides a molecular bridge between behavioral responses to ethanol, including sensitivity, tolerance, preference, and reward. A genetic relationship exists between ethanol tolerance and preference, but not sensitivity (Devineni et al., 2011). This is consistent with our findings that indicate separable roles for Sir2 in sensitivity and tolerance. However, Sir2 promotes the expression of Synapsin in ethanol-naive flies and permits its regulation in ethanol-treated flies. Therefore, Sir2 regulation of synaptic properties is likely a mechanism for the expression of both ethanol sensitivity and ethanol-induced behavioral plasticity. Sir2 works for sensitivity, tolerance, and reward in the *Drosophila* mushroom bodies, a central part of a circuit that appears to bear some functional equivalence to the vertebrate reward circuitry, tying these behaviors together anatomically, as well as molecularly.

How does Sir2 regulate behavioral plasticity? Ethanol, cocaine, opiates, and benzyl alcohol all lead to changes in covalent modifications in histones that likely affect gene transcription (Wang et al., 2007; Pandey et al., 2008; Renthal et al., 2009; Botia et al., 2012; Feng et al., 2014; Heller et al., 2014; Koo et al., 2015). However, only in rare cases are the enzymes that modify the histones or the functionally relevant target genes known for addictive drugs. Additionally, Sir2/SIRT1 deacetylates not only histones but also transcription factors that may impact ethanol-induced behavioral plasticity through changes in gene expression. For example, SIRT1 deacetylates the transcription factor FOXO3a, which is important for the development of conditioned place preference to chronic cocaine administration in mice (Ferguson et al., 2015). In flies, short-term exposure to ethanol and benzyl alcohol leads to changes in histone acetylation at many chromatin sites, including the region encoding the slowpoke BK-

type Ca^{2+}-activated K^+ channel that is involved in presynaptic function (Wang et al., 2007; Ghezzi et al., 2013). Both alcohols increase BK gene expression, and mutations in the channel decrease tolerance to the sedative effects of the drugs. The factors that regulate BK expression by ethanol and the causal link between BK gene regulation and the development of ethanol tolerance need further investigation. Of note, we were unable to detect ethanol- or Sir2-dependent changes in BK gene expression. However, our study used milder inebriation conditions and sampled gene expression earlier, so we cannot rule out a possible upstream role for Sir2 in BK channel regulation.

Our findings support a model whereby ethanol regulates the expression of presynaptic molecules through Sir2 activity. Whether ethanol inhibition of Sir2 increases H3K9 and possibly other histone residue acetylation to change chromatin accessibility, increases the acetylation of a transcription factor, or works through a more indirect mechanism will need to be determined. The unexpected promotion of H3K9 acetylation by Sir2 in ethanol-naive animals may also influence the pattern and extent of gene regulation by ethanol. However, our transcriptomic analysis detected remarkably little overlap of ethanol-regulated genes between wild-type control and *Sir2⁻* heads, suggesting that Sir2 is broadly responsible for ethanol-dependent gene regulation.

Ethanol-induced changes in presynaptic properties may be a key way in which the drug alters the brain to change behavior (Lovinger and Roberto, 2013). Acute ethanol exposure increases presynaptic GABAergic release probability in the ventral tegmental area and the central amygdala of mice (Melis et al., 2002; Roberto et al., 2003). Acute ethanol exposure also affects the activity of presynaptic BK and calcium channels, generally decreasing their activity, and attenuating ethanol inebriation in mice (Newton et al., 2008; Li et al., 2014). Furthermore, the presynaptic vesicle-associated protein Munc13–1 binds ethanol, and flies lacking the gene show increased ethanol preference (Das et al., 2013). Finally, polymorphisms of syntaxin-interacting Munc18–1 correlate with mouse strains preferring ethanol, and mutation of the *Caenorhabditis elegans* ortholog reduces ethanol sensitivity (Graham et al., 2009). Chronic ethanol exposure also alters presynaptic function in mice (Lovinger and Roberto, 2013). Interestingly, chronic ethanol use increases presynaptic calcium channel function, suggesting molecular adaptation in the presynaptic compartment in the transition from acute to chronic exposure (Newton et al., 2005). Together, these findings suggest that ethanol impacts presynaptic function and causes presynaptic adaptation.

We show that *Syn* is regulated by ethanol in a Sir2-dependent manner, and suggest that *cac* and *Cdk5* may also be regulated in the same manner. The direction of regulation—decreased *Syn* and increased *cac* and *Cdk5*—suggests that acute ethanol exposure may lead to presynaptic adaptations that increase neurotransmitter release. Syn binds to synaptic vesicles and to actin, and is proposed to maintain the reserve pool of synaptic vesicles (Bykhovskaia, 2011). Mutational disruption and ethanol-induced decreased levels of Syn may lead to greater dynamics of the reserve pool that manifest phenotypically with strong stimulation or in behavioral measurements of plasticity (Sun et al., 2006; Niewalda et al., 2015). Studies at the fly neuromuscular junction suggest that normal physiological synaptic homeostasis is essentially intact in Syn mutants, whereas the addition of new synaptic boutons due to strong stimulation is lost (Vasin et al., 2014). Further, Syn mutant flies are remarkably normal for spontaneous behavior, but show moderate to strong defects in various forms of learning and memory (Godenschwege et al., 2004; Mi-

chels et al., 2005; Knapek et al., 2010; Sadanandappa et al., 2013). Both Syn and Cac are phosphorylated by Cdk5 to regulate aspects of presynaptic function (Su et al., 2012; Verstegen et al., 2014). Acute ethanol exposure may increase Cdk5 to promote phosphorylation of a smaller pool of Syn, leading to greater vesicle dynamics in response to synaptic stimulation, and also may facilitate neurotransmitter release by phosphorylation of Cac. Behavioral plasticity initiated by acute ethanol exposure may be molecularly tied to changes in synaptic function or connectivity evoked by strong stimuli. Alternatively, Sir2 may allow a compensatory return of presynaptic molecules to prestimulus levels, a process required for the expression of behavioral plasticity.

The mushroom body intrinsic Kenyon cells of the α/β-lobe, where Sir2 is required for ethanol tolerance and reward, are important for both appetitive and aversive memory, and also for alcohol reward (Kaun et al., 2011; Aso et al., 2014). These Kenyon cells are connected synaptically to diverse input and output neurons, and they integrate sensory and internal-state inputs to drive innate and learned responses. Our findings imply that Sir2-dependent transcriptional regulation in the mushroom bodies is important for aspects of information processing that lead to the fly altering its behavioral responses to ethanol.

Our findings tie the gene regulatory effects of acute ethanol use to ethanol-induced behavioral plasticity in a brain region that associates context with innate approach-and-avoidance responses. How these ethanol-induced molecular changes impact neuronal function, and also interface with other molecular and cellular effects of acute ethanol exposure will help to define how circuits change to reinforce drug intake (Beckley et al., 2016). The mechanisms for ethanol-induced plasticity may be partly shared with those influencing natural and other pharmacologically induced neural plasticity, and it will be important to decipher how they integrate into the neural circuitry for addiction.

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