UNIVERSITY OF CALIFORNIA, MERCED

Memory-Like States of Rapid and Chronic Ethanol Tolerance

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

in

Quantitative and Systems Biology

by

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2024

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Professor Fred Wolf

University of California, Merced 2024

Dedication

I dedicate this to my partner Brielle, my parents Karen and Jory, my supportive family and friends, and my cat Matcha. Lastly, to my role model Dr. Mar Peter-Raoul.

Table of Contents

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I would like to acknowledge Dr. Fred Wolf and fellow lab members for their guidance, insight, and help with this body of work. I also acknowledge Dr. Galit Shohat-Ophir and their lab members for technical and conceptual advice. Lastly, I acknowledge co-authors Pratik Adhikari, Alex Del Toro, Ashley Valdez, Maria Nolazco, and Vanessa Sanchez for any data generation and/or assistance with experiments.

Agencies:

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List of Figures

Chapter 1 Figures

Chapter 2 Figures

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Publications

Larnerd C, Kachewar N, Wolf FW. *Drosophila* learning and memory centers and the actions of drugs of abuse. Learn Mem. **2024** Jun 11;31(5):a053815. DOI: 10.1101/lm.053815.123.

Larnerd C, Adhikari P, Valdez A, Del Toro A, Wolf FW. Rapid and Chronic Ethanol Tolerance Are Composed of Distinct Memory-Like States in *Drosophila*. J Neurosci. **2023** Mar 22;43(12):2210-2220. DOI: 10.1523/JNEUROSCI.1348-22.2023.

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Larnerd C, et al. Characterization of [Drug Product X] by LC-MS/MS Peptide Mapping. Bristol-Myers Squibb Technical Document. Internally published November **2018**.

Presentations

Encoding of ethanol tolerance in *Drosophila*, UC Merced Neuro/Developmental Joint Meeting, October 2023, **Talk**.

An alcohol-specific mushroom body circuit for chronic tolerance, Neurobiology of Drosophila Conference, Cold Spring Harbor Laboratory, October 2023, **Poster**.

Memory-like states induced by ethanol in *Drosophila*, Neuroscience Conference, Society for Neuroscience, November 2022, **Poster**.

Ethanol-specific memories in *Drosophila*, Alcohol and the Nervous System Conference, Gordon Research Conference, October 2022, **Poster**.

Ethanol-specific memories in *Drosophila*, Bar Ilan University Israel, July 2022, **Talk**.

Different forms of ethanol tolerance are encoded by distinct transcriptional programs and brain regions, UC Merced QSB Retreat, May 2022, **Talk**.

Different forms of ethanol tolerance are encoded by distinct transcriptional programs and brain regions, Neurobiology of Drosophila Conference, Cold Spring Harbor Laboratory, October 2021, **Poster**.

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Sequence Duplication as a Mechanism of RNA Evolution, NASA Ames Research Center, November 2017, **Poster**.

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Fundraising Coordinator, RadioBio	2021-2022
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Abstract

Memory-Like States of Rapid and Chronic Ethanol Tolerance

By

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Doctor of Philosophy in Quantitative and Systems Biology

University of California, Merced

Professor Fred Wolf, Dissertation Advisor

Ethanol tolerance is the first type of behavioral plasticity and neural plasticity that is induced by ethanol intake, and yet its molecular and circuit bases remain largely unexplored. Here, we characterize distinct forms of ethanol tolerance in *Drosophila*: rapid and chronic tolerance. Chronic tolerance, induced by continuous exposure, lasts for two days and depends on new protein synthesis and CREB. Unlike rapid, chronic tolerance is independent of the immediate early gene *Hr38/Nr4a*. Chronic tolerance is suppressed by the Sirtuin HDAC *Sirt1*, whereas rapid tolerance is enhanced by *Sirt1*. Moreover, rapid tolerance is composed of both labile and consolidated traces. Repeated ethanol exposures induce another type of chronic tolerance that is separately represented in the brain. Interestingly, rapid and chronic tolerance map to anatomically distinct regions of the *Drosophila* mushroom body learning and memory center, where they rely on mutually exclusive inhibitory circuits with large interneurons. Thus, depending on the initial dosage and pattern of intake, ethanol-induced neural plasticity underlies the longer-term brain changes associated with alcohol-use disorder.

Introduction

Alcohol Use Disorder (AUD) is a chronic, recurring medical condition that causes extraordinarily long-term changes to brain function. Understanding how ethanol affects the brain is complicated by the relative non-specificity of ethanol's molecular targets, the long time course from the first experience to developing AUD, the variation in definitions of AUD, and the myriad ways that genetics and experience interact with ethanol intake patterns. Multiple forms of behavioral adaptations to ethanol are defined operationally, and neither their relative importance for AUD nor their interconnectedness is clear. A useful approach to sort this complexity is to start from simpler, early forms of adaptation, where effects are more reproducible, a stimulus-response relationship is clearer, and where a complete description is more feasible. Some early forms of adaptation to ethanol are likely a basis upon which longer term forms build.

Tolerance is an early form of behavioral plasticity induced by ethanol intake. Tolerance is defined as the acquired resistance to ethanol's negative effects and sensitization to its positive or rewarding effects, facilitating increased intake (Fadda & Rossetti, 1998). Ethanol tolerance is classically divided into three forms, acute (acquired within a drinking session), rapid (expressed after the first drink is metabolized), and chronic. Some molecular mechanisms are known and are distinct for each form of tolerance, suggesting that the dose, time, and pattern of ethanol exposure can engage different plasticity mechanisms (Atkinson, 2009; Berger et al., 2008; Moore et al., 1998). It is not known if different forms of ethanol tolerance colocalize in brain circuits, if they share some common plasticity mechanisms, or their relative contribution to the progression towards AUDs.

One long-held view of substance use disorder is that maladapted behaviors towards drugs of abuse arise from maladapted learning and memory circuits (Carmack et al., 2017; Fadda & Rossetti, 1998; Hyman et al., 2006; Koob & Volkow, 2010; Robinson & Atkinson, 2013; Ryvkin et al., 2018). Indeed, even drugs that have separate mechanisms of action share common targets in memory circuitry. Behaviors associated with abusive drugs exacerbate the potential of further usage and relapse after breaks in usage. With ethanol as an example, tolerance contributes to an organism's need to consume more alcohol to achieve a target internal state. Thus, usage-dependent plasticity underlies drug representations in the brain, similar to how experience-dependent plasticity regulates learned events.

Drosophila is useful for defining the mechanisms of ethanol tolerance and of memory. Acute, rapid, and chronic tolerance all exist in flies, and they are separable genetically (Berger et al., 2004). Molecular parallels to early forms of ethanol plasticity in mammals indicate potential deep conservation of the basic mechanisms (Cowmeadow et al., 2005; Engel et al., 2016; Ghezzi et al., 2013; Kong et al., 2010; Morozova et al., 2006; Park et al., 2017; Ranson et al., 2019). Tolerance in *Drosophila* is functional, due to adaptive changes in behavior, and is not due to changes in ethanol metabolism (Berger et al., 2004; Scholz et al., 2000). How *Drosophila* learn, remember, and forget classically-conditioned experiences, plus where these relevant molecular and cellular mechanisms exist, is well understood (Aso et al., 2014; Davis, 2023). The mushroom bodies (MBs) function as the primary learning and memory structure for the fly, plus ethanol-related behaviors have been mapped there, hence these neurons may share tolerance-encoding and memory-encoding functions.

The MBs are composed of thousands of intrinsic Kenyon cell neurons (KCs) whose axonal tracts project anteriorly and bifurcate into vertical and horizontal lobes: α/β, α'/β', and γ. Sensory information, such as olfaction, is relayed to Kenyon cells via sparse representation, possibly integrated with other sensory cues or motivational states, then transmitted to a small number of MB output neurons (MBONs) that regulate approach and avoidance behaviors. Further, each MB lobe has multiple zones with distinct MBON tiling and innervation by dopamine neurons (DANs). DANs relay unconditioned stimuli and information about internal state so that sensory information is assigned value and the appropriate downstream MBONs are selected. Last, two large interneurons, the anterior paired lateral (APL) and the dorsal paired medial (DPM), synapse broadly on the entire KC population and can innervate these neurons at multiple locations. The feedback loops governed by the APLs and DPMs serve additional roles in learning and memory.

Drosophila exhibit multiple distinguishable forms of memory through the course of time after a classically-conditioned event, such as short-term memory (STM), intermediate-term memory (ITM) that is composed of anesthesia-sensitive memory (ASM) and anesthesia-resistant memory (ARM), and protein-synthesis dependent long-term memory (PSD-LTM) (Margulies et al., 2005). Decades of studies have determined that these memory encoding mechanisms are separable in terms of both genes and circuits, however memory pathways can also interact with each other. For example, ASM and ARM pathways converge on the Rgk1 small GTPase in the MBs (Murakami et al., 2017), and the Ras/Raf/ROCK pathway suppresses ARM to permit PSD-LTM in the MBs (Noyes et al., 2020a). There are multiple memory encoding mechanisms that contribute to all memory phases, and early memory mechanisms are required for later phases of memory (Davis, 2011; Tully et al., 1994), however initial processes can also be dispensable for later memory phases. For example, mutations in Rugose, an A-kinase anchor protein (AKAP), cause an STM deficit but not ARM or LTM deficits (J. Zhao et al., 2013). Therefore, *Drosophila* memory mechanisms are mostly independent, but can interact via shared genes and circuits. Here, we present additional overlaps, distinctions, and interactions between forms of ethanol tolerance and ethanol memory.

Chapter 1: Rapid and Chronic Ethanol Tolerance are Composed of Distinct Memory-Like Traces

Summary

The pattern of initial ethanol intake engages distinct molecular pathways for plasticity that are encoded into different neural circuits. Rapid tolerance requires the induction of the immediate early gene (IEG) *Hr38*, ortholog of mammalian *Nr4a1-3*, and the HDAC *Sirt1* in the α/β lobes of the adult mushroom bodies, the major learning and memory center of the *Drosophila* brain (Adhikari et al., 2019; Engel et al., 2016). Chronic tolerance, induced by a prolonged exposure to a low concentration of ethanol, is *Hr38* independent and is inhibited by *Sirt1* in the γ lobes of the adult mushroom bodies. Chronic tolerance enables the induction of the *c-fos*-like IEG *kayak* by subsequent ethanol exposures, whereas rapid tolerance does not. Chronic exposure specifically forms a long-term memory (LTM) that is distinct from both appetitive and aversive forms of LTM, whereas rapid tolerance is an intermediate term memory (ITM) composed of labile and consolidated components. There is a second form of chronic tolerance, induced by repeated exposure to moderately inebriating doses of ethanol that results in a third pattern of gene expression and lasting behavioral outcomes.

Results

Different ethanol exposure paradigms induce different forms of behavioral plasticity

Flies were given ethanol exposures of different length, concentration, or pattern (or matched humidified air controls) and they were then tested for ethanol behavioral responses with a uniform challenge exposure (**Fig. 1A**). The uniform challenge exposure was 55% ethanol vapor, to determine the time to 50% sedation (ST50) for a population of about 20 flies; ST50 typically occurs at 15-20 min for ethanol-naive flies. Rapid tolerance is induced by a just sedating ethanol exposure; its expression is measured 4hrs later, after the initial dose is fully metabolized, with an identical exposure (Kong et al., 2010; Scholz et al., 2000). This level of ethanol exposure is operationally similar to the tolerance-inducing effects of binge drinking in humans (Schuckit, 1994). Chronic tolerance is induced by prolonged exposure to a low dose of ethanol; it is intended to mimic aspects of maintenance drinking by individuals with alcohol use disorder (Berger et al., 2004). Finally, we also gave flies repeated exposures to an inebriating but not sedating ethanol dose (42% ethanol vapor for 20 min), once per day for four consecutive days, similar to limited access paradigms with mice (Rhodes et al., 2005).

Chronic ethanol exposure induced resistance to ethanol sedation (chronic tolerance). Increasing concentrations of ethanol during the chronic exposure increased the extent of tolerance (**Fig. 1B**). Pretreatment with chronic ethanol did not alter ethanol absorption or metabolism, measured with a challenge dose (**Fig. 1C**). Chronic ethanol also did not cause tissue damage. Specifically, repeated brief exposures to high concentration ethanol causes necrosis of the third antennal segment olfactory organ (French & Heberlein, 2009). Chronic exposure to 21% ethanol vapor, the highest concentration we tested, resulted in necrosis of 0 of 56 antennae. Chronic tolerance dissipated over time and persisted for at least 48hrs (**Fig. 1D**). To determine if rapid and chronic tolerance could co-occur in flies, we subjected flies serially to a chronic exposure and then 24hrs later to a rapid tolerance induction paradigm (**Fig. 1E**). Rapid tolerance was decreased in flies previously given a chronic ethanol exposure, suggesting a mechanistic link between rapid and chronic tolerance. Ethanol preference, measured in the CAFÉ two choice assay, is a learned behavior that can be induced by an acute inebriating ethanol dose (Ja et al., 2007; Peru Y Colón de Portugal et al., 2014). Binge-like acute pre-exposure and chronic pre-exposure to ethanol both potentiated ethanol preference (**Fig. 1F**). These results indicate that there exists behavioral and functional links between rapid and chronic ethanol tolerance, and that chronic exposure can induce a positive valence towards ethanol.

Repeated ethanol exposure also reduced sensitivity to ethanol sedation (repeated tolerance), measured after a 24hr recovery (**Fig. 1G**). Like chronic exposure, repeated exposure decreased subsequent rapid tolerance development (**Fig. 1H**), suggesting a link between rapid and repeated tolerance mechanisms. However, repeated exposure induced a mild ethanol aversion instead of ethanol preference (**Fig. 1I**). Thus, repeated ethanol exposure may create a distinct form of neuronal plasticity.

Rapid and chronic tolerance are transcriptionally distinct

We asked if tolerance-inducing ethanol exposure paradigms cause distinct transcriptional responses by measuring the expression levels of several IEG transcriptional regulators implicated in neural plasticity. We found that there are very

different IEG response profiles for rapid and chronic tolerance (**Fig. 2A**). The *Nr4a1-3* ortholog *Hr38* and the *Egr1-4* ortholog Stripe (*Sr*) were selectively induced by the rapid tolerance-inducing acute exposure, whereas the *c-Fos* ortholog *kayak* (*kay*) was selectively induced by chronic tolerance. By contrast, *Hr38* remained inducible by ethanol following repeated inebriating ethanol exposures. Interestingly, two sedating ethanol doses blocked *Hr38* inducibility, possibly indicating molecular distinctions between inebriation and sedation, or a ceiling to rapid tolerance.

Hr38 induction by acute ethanol exposure is necessary and sufficient for rapid tolerance development (Adhikari et al., 2019). Because *Hr38* is no longer inducible by ethanol following chronic exposure, we asked if it was required for chronic tolerance. Chronic tolerance was normal in *Hr38* loss-of-function mutants, indicating that *Hr38* functions in rapid but not in chronic tolerance (**Fig. 2B**). Thus, rapid, chronic, and repeated tolerance are molecularly distinct forms of alcohol memory.

Histone deacetylation maintains chronic tolerance and sets IEG inducibility

The distinct patterns of IEG induction in rapid and chronic tolerance paradigms suggests that chronic exposure creates a specific chromatin state to set a transcriptional response pattern, that is likely a plasticity encoding mechanism for ethanol and addiction (Walker et al., 2015). Rapid tolerance in flies is encoded into chromatin: histone acetylation is quickly increased by a sedating ethanol exposure, and rapid tolerance is completely blocked by chemical inhibition of the NAD-dependent Sirtuin class of HDACs, and by genetic deletion of the HDAC *Sirt1* (Engel et al., 2016). To test if there is a chromatin state component to chronic tolerance, we fed flies two broadly acting HDAC inhibitors that act similarly in flies and mammals (Bitterman et al., 2002; Foglietti et al., 2006). Trichostatin A (TSA) inhibits Class I/II HDACs, and nicotinamide inhibits the NAD-dependent Sirtuins, including *Sirt1* that promotes rapid tolerance (Engel et al., 2016). Flies were given a chronic ethanol exposure, transferred to HDAC inhibitor-containing food during the withdrawal period, and then given an ethanol challenge dose. TSA erased and nicotinamide reduced chronic tolerance without affecting ethanol sensitivity, indicating that multiple HDACs encode the experience of chronic ethanol exposure (**Fig. 3A, 3B**). Interestingly, TSA treatment after chronic ethanol exposure restored *Hr38* inducibility by ethanol, whereas nicotinamide did not (**Fig. 3C**). Thus, Class I/II HDACs are responsible for blocking *Hr38* induction by ethanol following chronic exposure. Because chronic ethanol pre-exposure decreases the expression of rapid tolerance (**Fig. 1E**), we asked if TSA erased this interaction between forms of tolerance. TSA treatment during chronic ethanol exposure withdrawal restored the expression of normal rapid tolerance (**Fig. 3D**). We conclude that chronic ethanol exposure alters chromatin to impede rapid tolerance development. Chronic ethanol exposure likely promotes chromatin compaction through histone deacetylation to encode the state of chronic ethanol tolerance.

Sirt1 limits chronic tolerance development in the mushroom bodies

The NAD-dependent Sirtuin encoding of chronic tolerance prompted us to ask if a Sirtuin may regulate chronic tolerance development. We chose *Sirt1* because it is strongly regulated by a rapid tolerance-inducing acute ethanol exposure and it promotes rapid tolerance development (Engel et al., 2016; Kong et al., 2010). Surprisingly, *Sirt1* null mutants exhibited increased chronic tolerance (**Fig. 4A**). Thus, *Sirt1* inhibits chronic

tolerance, whereas it promotes rapid tolerance, providing further evidence that chronic and rapid tolerance are molecularly distinct.

We used RNAi to decrease *Sirt1* in specific tissues and cell types to map its site of action. *Sirt1* RNAi in all neurons but not in all glia resulted in increased chronic tolerance, the same behavioral phenotype as in the *Sirt1* null mutant (**Fig. 4B, 4C**). The mushroom bodies are the major learning and memory center in the fly brain, and they are critical for multiple forms of ethanol-induced behavioral plasticity including rapid tolerance, ethanol preference, and ethanol reward. We used a panel of GAL4 drivers that express in the entire mushroom bodies or specifically in one of the three mushroom body lobes (Noyes et al., 2020b). *Sirt1* RNAi in all mushroom body lobes or specifically in the mushroom body γ lobes increased chronic tolerance (**Fig. 4D**). *Sirt1*-dependent sensitivity to acute ethanol inebriation mapped to neurons in all three mushroom body lobes, suggesting that sensitivity is encoded through mechanisms that are fundamentally distinct from either form of tolerance (**Fig. 4D'**). In rapid tolerance, *Sirt1* functions in the mushroom body α/β lobes (Engel et al., 2016). To verify that rapid and chronic tolerance map to distinct neurons of the mushroom bodies, we also decreased *Sirt1* in the α/β lobes with the *17d-Gal4* driver that was used previously to localize rapid tolerance (Engel et al., 2016). *Sirt1* RNAi in the *17d-Gal4* α/β neurons did not affect chronic tolerance development (**Fig. 4E**). Thus, both rapid and chronic ethanol tolerance require *Sirt1* in the mushroom bodies, with *Sirt1* limiting chronic tolerance in the γ lobes, and promoting rapid tolerance in the α/β lobes. This indicates that there exist distinct circuits for different forms of ethanol tolerance.

The adult mushroom bodies require Sirt1 and neuronal activity for chronic tolerance

To determine when *Sirt1* acts to limit chronic tolerance, we used a temperature-sensitive form of the GAL4 inhibitor GAL80 to limit expression of *Sirt1* RNAi to adults. Adult-specific *Sirt1* RNAi in all mushroom body neurons increased chronic tolerance (**Fig. 5A**), indicating a regulatory role of *Sirt1* on ethanol plasticity in adult flies. There was no effect of adult-specific *Sirt1* RNAi on sensitivity to an acute inebriating dose of ethanol (**Fig. 5A'**). Thus, *Sirt1*-dependent inhibition of chronic tolerance is an adult function of the HDAC, whereas *Sirt1*-dependent promotion of ethanol sensitivity is likely to arise during development.

We next asked if neuronal activity of the mushroom bodies is necessary to produce chronic tolerance in adults. Flies expressing the inward rectifying potassium channel Kir2.1 to hyperpolarize the adult mushroom bodies showed decreased chronic tolerance development (**Fig. 5B**). Thus, mushroom body neurons promote chronic tolerance via Kenyon cell activity. Considering that chronic tolerance is negatively regulated by *Sirt1* levels and positively regulated by mushroom body activity, we asked if simultaneously manipulating these two factors interact. Silencing mushroom body neurons in *Sirt1* null mutant flies decreased chronic tolerance (**Fig. 5C**). Thus, neuronal activity is critical for *Sirt1*-dependent inhibition of chronic tolerance development.

Rapid tolerance is a multi-trace memory of ethanol

We asked if chronic tolerance shares molecular features with forms of *Drosophila* memory. It is possible to distinguish different types of labile and consolidated memories using specific mutants and tests for anesthesia sensitivity. *Drosophila* intermediate term

memories are composed of anesthesia sensitive memory (ASM) and anesthesia resistant memory (ARM) traces. *Drosophila* also form longer-lasting forms of LTM. The gene *radish* encodes a GTPase that sustains ARM, a quickly consolidated form of memory, as well as appetitive but not aversive LTM. Mutant flies expressing a truncated Radish protein displayed decreased ethanol sensitivity, yet chronic tolerance was unaffected (**Fig. 6A, 6A'**). The *amnesiac* (*amn*) gene encodes a PACAP-like neuropeptide required to form labile ASM. Flies mutant for *amn* showed decreased sensitivity to ethanol sedation and unhindered chronic tolerance (**Fig. 6A, 6A'**). An orthogonal test of ASM performs memory disruption via cold-induced hypothermia. Control flies cold-shocked after chronic ethanol showed a mild increase in sedation sensitivity, indicating sufficient cold-shock conditions, but chronic tolerance measured at 24hrs was unaffected (**Fig. 6B, 6B'**). Thus, the genetic pathways for intermediate term memory, ASM and ARM, are dispensable for lasting chronic tolerance. We also directly tested for labile and consolidated intermediate term memories, ASM and ARM, by measuring tolerance after a 3hr recovery, when ASM and ARM are typically tested. Again, there was no effect of either cold shock anesthesia or mutation of *radish*, indicating that chronic exposure does not result in the formation of either ASM or ARM (**Fig. 6C**). In contrast, rapid tolerance was partially affected by both manipulations, and was nearly abolished with the combination (**Fig. 6D**). Thus, a binge-like ethanol exposure forms rapid tolerance that consists of labile ASM and consolidated ARM.

Chronic tolerance is a persistent, non-canonical memory of ethanol

Finally, we tested if chronic tolerance depended on CREBB, a transcriptional regulator of LTM that is required in the mushroom bodies for LTM (Yin et al., 1994). We expressed an inhibitor form, CREB2b, in all adult neurons, or specifically in all mushroom body neurons. Brain-wide but not mushroom body specific suppression of CREBB interfered with chronic tolerance (**Fig. 7A**). Thus, chronic ethanol exposure engages CREBB-dependent transcription outside the mushroom bodies, potentially creating an interplay of mushroom body epigenetic and other brain circuit memory traces. A model for how rapid and chronic tolerance are separably encoded into the *Drosophila* brain is shown (**Fig. 7B**).

Figure 1

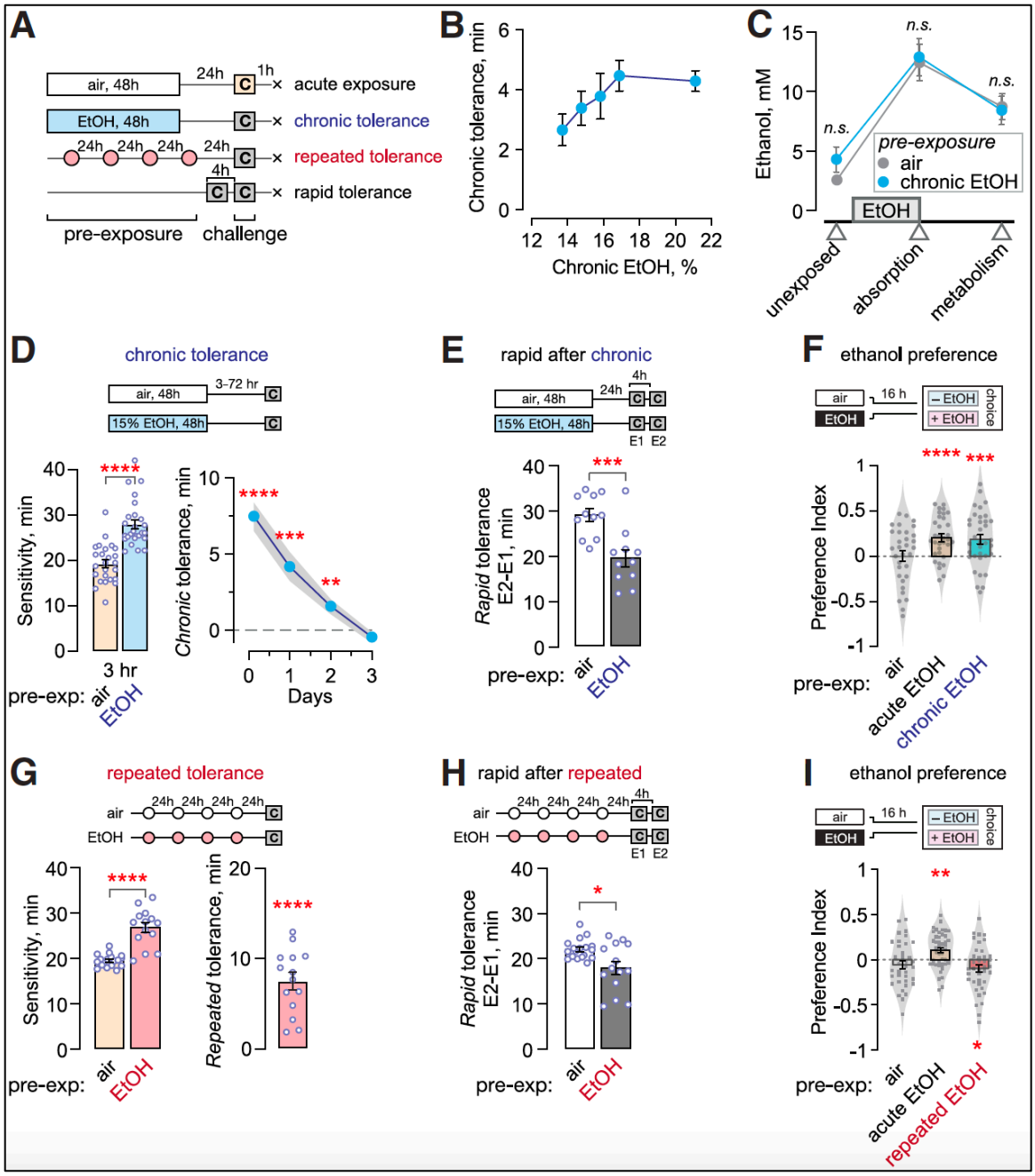

Figure 1. Different ethanol exposure paradigms induce different forms of behavioral plasticity.

A) Ethanol exposure schemes to induce and measure ethanol sensitivity and tolerance. Challenge doses (C) are 55% ethanol unless noted otherwise. Gene expression measurements were sampled 1 h after the ethanol challenge dose.

B) Dose response for chronic ethanol pre-exposure. Chronic tolerance is ST50, chronic minus acute.

C) Ethanol absorption and metabolism with air or 16% chronic ethanol pre-exposure. Flies were exposed to 30 min of 20% ethanol to avoid sedation. Absorption was measured immediately afterward, and metabolism was measured 30 min later. Pharmacokinetics: Brown–Forsythe ANOVA.

D) Left, Chronic ethanol pre-exposure caused resistance to sedation. Right, Chronic ethanol exposure induces tolerance that lasts for 2 d. Sensitivity: Mann–Whitney test. Tolerance: One-sample t test (theoretical mean= 0).

E) Chronic ethanol exposure interfered with the subsequent development of rapid tolerance. Flies were either given 48 h of chronic ethanol exposure or humidified air, and 24 h later were subjected to a rapid tolerance test, two 30min 60% EtOH exposures, E1 and E2, 4 h apart. Rapid tolerance is ST50, E2–E1. Tolerance: Unpaired t test.

F) Chronic ethanol exposure induced ethanol preference, measured in the CAFÉ two-choice assay. Preference: Wilcoxon signed-rank test (theoretical mean = 0).

G) Left, Repeated inebriating doses of ethanol caused resistance to ethanol sedation. Right, Repeated ethanol exposure induced tolerance that lasts for at least 1 d. Tolerance is from data shown in the left panel, repeated minus acute. Sensitivity: Welch's t test. Tolerance: One-sample t test (theoretical mean= 0).

H) Repeated ethanol exposure inhibited the subsequent development of rapid tolerance. Tolerance: Welch's t test.

I) Repeated ethanol exposure induced ethanol aversion in the CAFÉ two-choice assay. Preference: Wilcoxon signed-rank test (theoretical mean = 0).

Figure 2

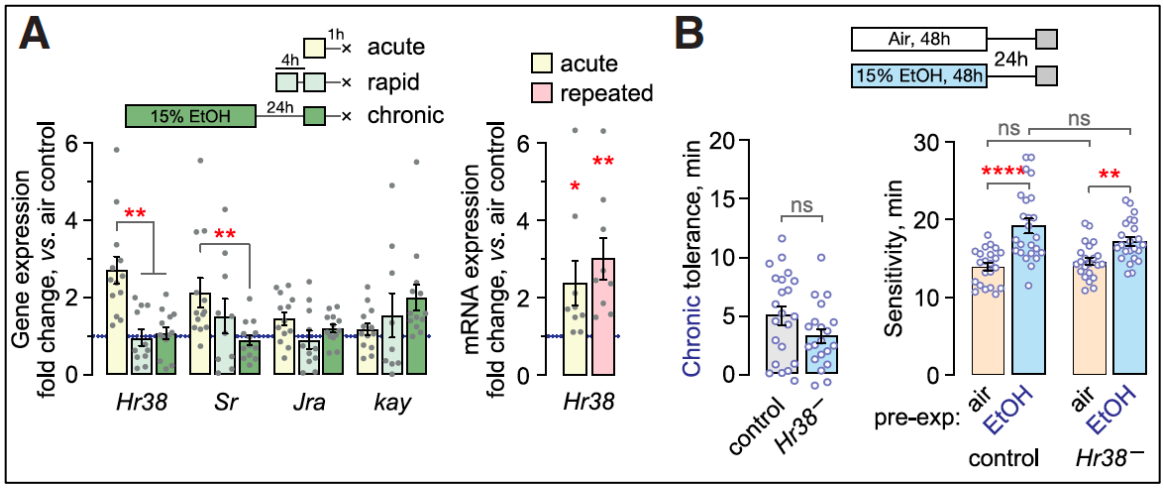

Figure 2. Rapid and chronic tolerance are transcriptionally distinct.

A) Quantitative PCR of transcription factor IEGs induced by acute dose, or a challenge dose following acute (rapid), chronic, or repeated exposure, expressed as the fold change versus humidified air control exposure. Left, Different ethanol pre-exposures induce different IEG response profiles. Note: *kay* is significantly induced when acute and chronic exposures are directly compared ($p = 0.0221$, Mann–Whitney test). Right, *Hr38* is inducible following acute and repeated ethanol exposure conditions. *Hr38*: Kruskal–Wallis ANOVA. *Sr*: Kruskal–Wallis ANOVA. *Jra*: One-way ANOVA. *kay*: Kruskal–Wallis ANOVA. *Hr38* Repeated: Wilcoxon signed-rank test (theoretical mean = 1).

B) Left, Chronic tolerance is unaffected in *Hr38* mutants. Right, Chronic ethanol pre-exposure causes sedation resistance in control and *Hr38* mutants. Ethanol sensitivity is unaffected in *Hr38* mutants. Tolerance: Unpaired t test. Sensitivity: Brown–Forsythe ANOVA.

Figure 3

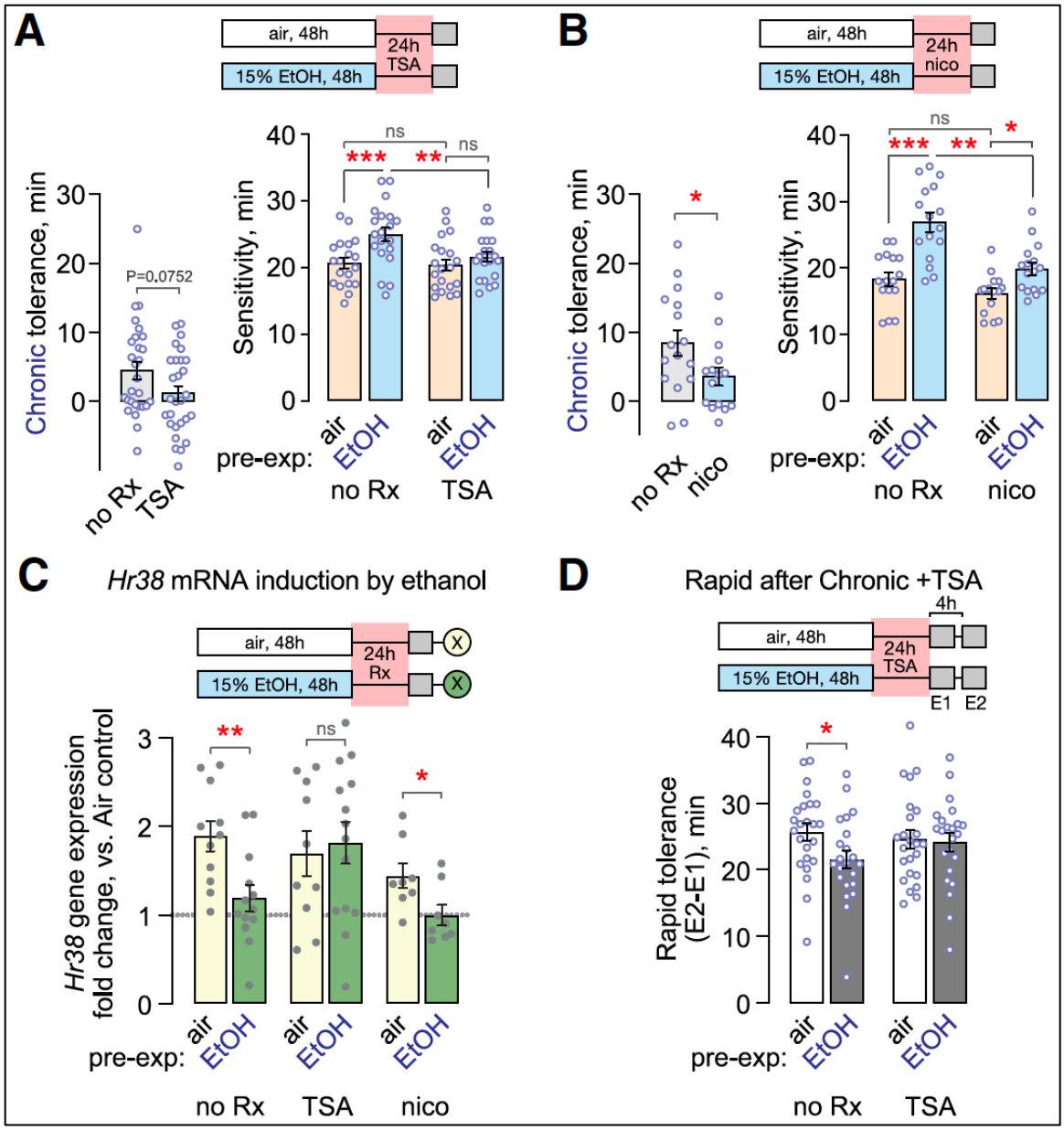

Figure 3. Histone deacetylation maintains chronic tolerance and sets IEG inducibility.

A) Left, TSA, which inhibits Class I/II histone deacetylases, trends toward decreased chronic tolerance. Right, TSA does not affect ethanol sensitivity. Tolerance: Mann–Whitney test. Sensitivity: One-way ANOVA.

B) Left, Nicotinamide, which inhibits NAD-dependent sirtuin class histone deacetylases, decreases chronic tolerance. Right, Nicotinamide does not affect ethanol sensitivity. Tolerance: Unpaired t test. Sensitivity: Brown–Forsythe ANOVA.

C) TSA but not nicotinamide restores *Hr38* ethanol inducibility by an ethanol challenge following chronic ethanol exposure, measured by quantitative PCR. No Rx: Unpaired t test. TSA: Unpaired t test. Nico: Mann–Whitney test.

D) TSA restores rapid tolerance following a chronic exposure. No Rx: Unpaired t test. TSA: Unpaired t test.

Figure 4

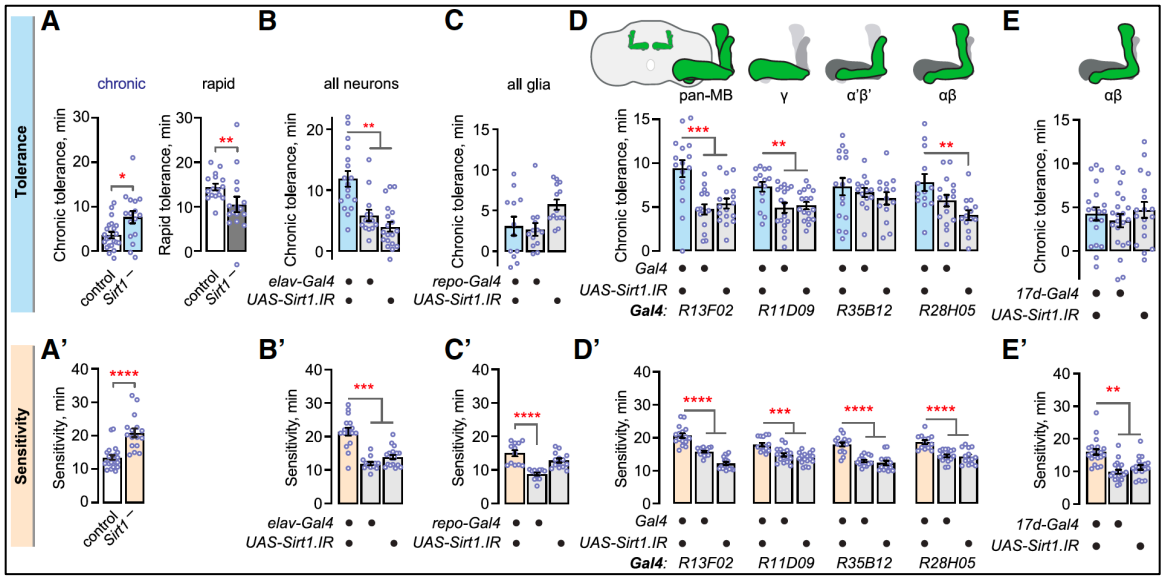

Figure 4. *Sirt1* limits chronic tolerance development in the mushroom bodies.

A) Left, *Sirt1*-null mutant flies develop more chronic tolerance. Right, *Sirt1*-null mutant flies develop less rapid tolerance. **A')** *Sirt1*-null mutants have decreased sensitivity to ethanol sedation. Tolerance: both Welch's t tests. Sensitivity: Mann-Whitney test.

B) Reduction of *Sirt1* by RNAi specifically in all postmitotic neurons increases chronic tolerance. **B')** Reduction of Sirt1 expression in all neurons causes decreased ethanol sensitivity. Tolerance: Kruskal–Wallis ANOVA. Sensitivity: Kruskal–Wallis ANOVA.

C) Reduced *Sirt1* expression in all glia does not affect chronic tolerance. **C')** Reduced Sirt1 expression in all glia does not affect ethanol sensitivity. Tolerance: One-way ANOVA. Sensitivity: Brown–Forsythe ANOVA.

D) *Sirt1* RNAi in all neurons of the mushroom bodies (*R13F02*), or specifically in the mushroom body γ lobes (*R11D09*), increases chronic tolerance. Reduction of *Sirt1* in either the mushroom body α'/β' lobes (*R35B12*) or the α/β lobes (*R28H05*) does not affect chronic tolerance. **D')** Reduced Sirt1 in the mushroom bodies or in each lobe causes decreased sensitivity. Tolerance: One-way ANOVA, Kruskal–Wallis ANOVA, Brown–Forsythe ANOVA, One-way ANOVA. Sensitivity: all One-way ANOVAs.

E) Chronic tolerance was unaffected when *Sirt1* was reduced in *17d-Gal4* neurons, the site of action for *Sirt1* in rapid tolerance. **E')** Reduced *Sirt1* in *17d-Gal4* neurons causes decreased sensitivity. Tolerance: One-way ANOVA. Sensitivity: Kruskal–Wallis ANOVA.

Figure 5

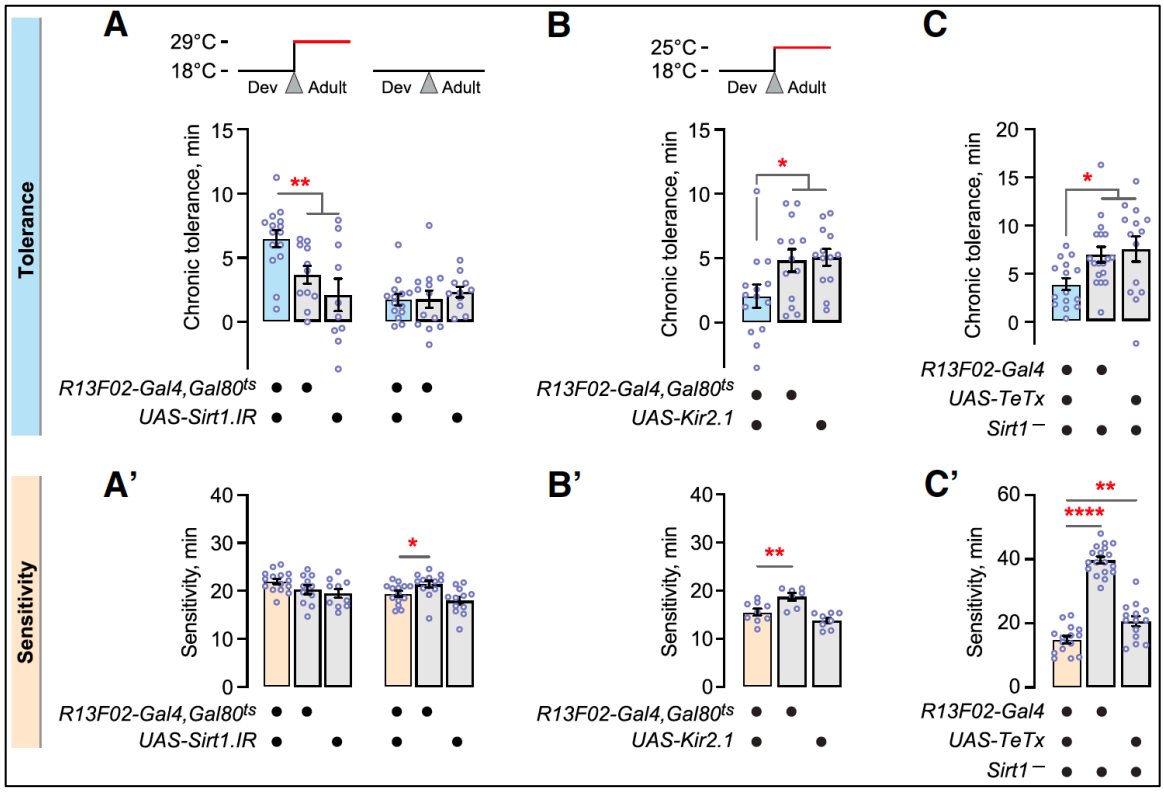

Figure 5. The adult mushroom bodies require *Sirt1* and neuronal activity for chronic tolerance.

A) *Sirt1* is required in the adult mushroom bodies for chronic tolerance development. GAL4 was suppressed by temperature-sensitive GAL80 throughout development (Dev) by rearing the flies at 18°C (GAL80 on, GAL4 blocked), and shifting them to 29°C (GAL80 off, GAL4 active) after eclosion (Adult). A 50% ethanol challenge dose was used to account for increased ethanol sedation at 29°C. **A')** Adult-specific decrease of *Sirt1* in the mushroom bodies did not affect sensitivity to a 50% ethanol challenge, independent of temperature during adulthood. Tolerance: both One-way ANOVAs. Sensitivity: One-way ANOVA, Kruskal–Wallis ANOVA.

B) Hyperpolarization of the mushroom bodies in adults with the inwardly rectifying potassium channel Kir2.1 blocks chronic tolerance development. A 50% ethanol challenge dose was used. **B')** Adult-specific hyperpolarization of the mushroom bodies had no effect on sensitivity to a 50% ethanol challenge. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

C) Blocking synaptic vesicle release [tetanus toxin light chain (TeTx)] in the mushroom bodies in *Sirt1*-null mutants results in decreased chronic tolerance. **C')** Blocking synaptic vesicle release in the mushroom bodies in *Sirt1*-null mutant flies increased ethanol sensitivity. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

Figure 6

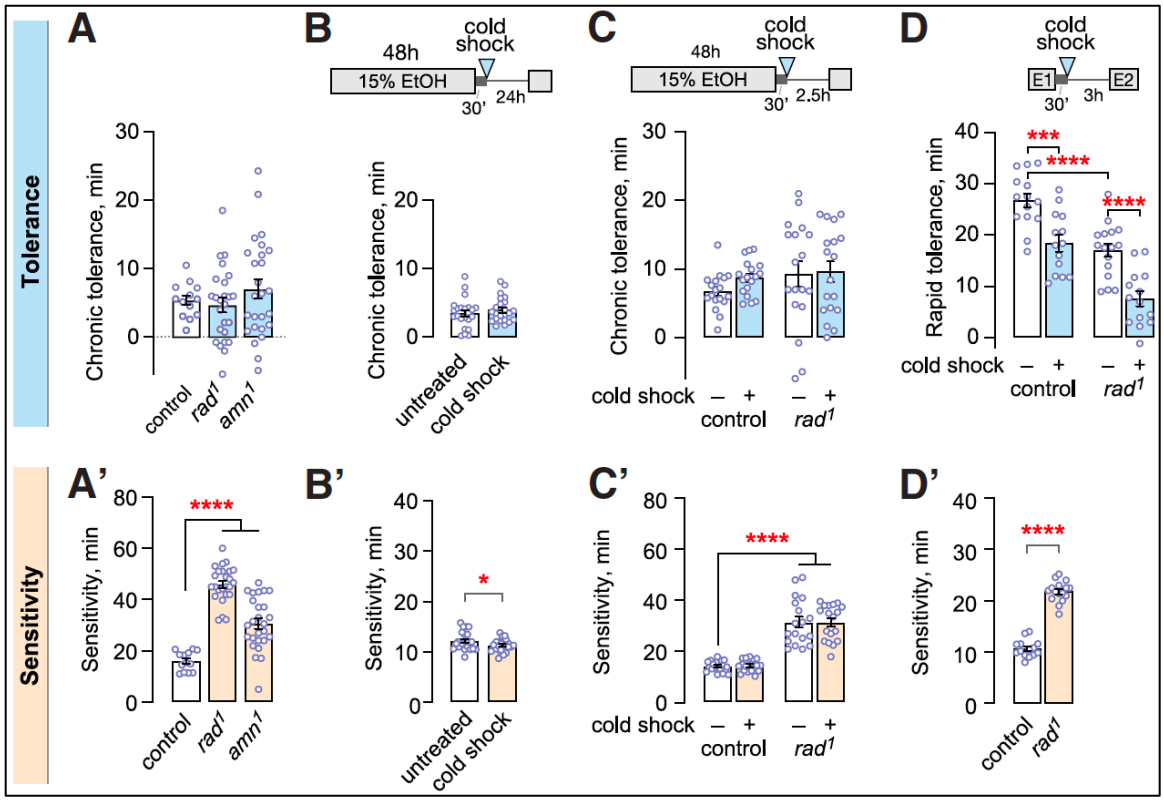

Figure 6. Rapid tolerance is a multi-trace memory of ethanol.

A) Mutants that abolish anesthesia-resistant memory (*rad*[1]) and anesthesia-sensitive memory (*amn*[1]) develop normal chronic tolerance at 24 h. **A')** Decreased ethanol sensitivity in mutants for early forms of memory. Tolerance: Brown–Forsythe ANOVA. Sensitivity: Brown–Forsythe ANOVA.

B) Cold-shock anesthesia, blocking anesthesia-sensitive memories, does not affect chronic tolerance. **B')** Increased ethanol sensitivity following cold-shock anesthesia. Tolerance: Unpaired t test. Sensitivity: Unpaired t test.

C) Chronic tolerance does not include 3 h anesthesia-sensitive memory-like or 3 h anesthesia-resistant memory-like states. **C')** Decreased ethanol sensitivity in *radish* mutants, no effect of cold shock. Tolerance: Brown–Forsythe ANOVA. Sensitivity: Brown–Forsythe ANOVA.

D) Rapid tolerance is composed of 3 h anesthesia-sensitive memory-like and 3 h anesthesia-resistant memory-like states. **D')** *radish* mutants have decreased ethanol sensitivity, as measured from the rapid tolerance inducing exposure. Tolerance: One-way ANOVA. Sensitivity: Unpaired t test.

Figure 7

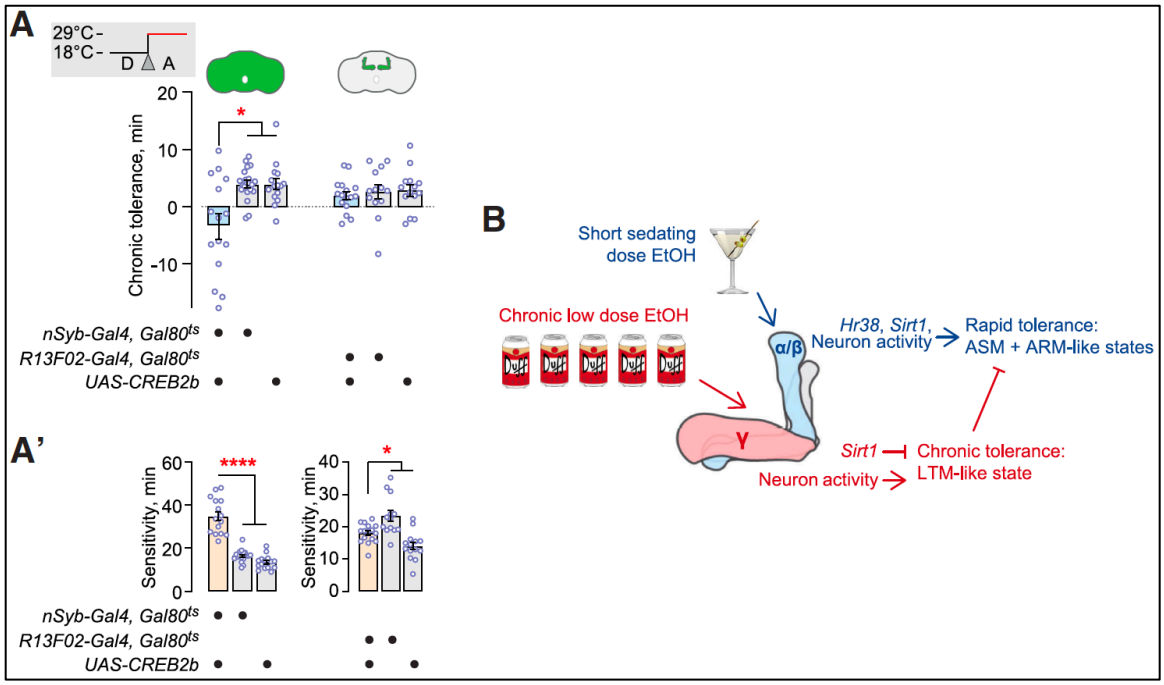

Figure 7. Chronic tolerance is a persistent, non-canonical memory of ethanol.

A) Inhibition of CREBB blocks chronic tolerance when expressed in all adult neurons (left), but not when expressed in all adult mushroom body neurons (right). **A')** Adult-specific inhibition of CREBB signaling in all neurons (left), but not in mushroom body neurons (right), decreased sensitivity to a 50% ethanol challenge. Tolerance: Brown–Forsythe ANOVA, One-way ANOVA. Sensitivity: Brown–Forsythe ANOVA, One-way ANOVA.

B) Summary diagram of the encoding of chronic and rapid tolerance in the adult *Drosophila* mushroom bodies.

Discussion

Chronic and rapid ethanol tolerance are encoded by distinct molecular programs in distinct neural circuits. Moreover, repeated tolerance is a second, independent form of chronic tolerance in *Drosophila*. Thus, the pattern of initial ethanol intake selects different molecular encoding mechanisms of neural plasticity with different durations and circuitry. Here we discuss the properties of chronic tolerance, how chronic and rapid tolerance may contribute to the progression towards AUD, and evidence that chronic tolerance is an ethanol-specific form of long-term memory.

Chronic ethanol exposure creates a unique, ethanol-specific memory, differentiating ethanol experience from other experience-dependent memories. Here we compare chronic tolerance to experience dependent memories in flies. We note that chronic ethanol experience is not explicitly paired with a neutral cue: the flies may form a lasting non-associative memory, or they may use environmental cues, including ethanol olfactory cues, to form a lasting associative memory. The perdurance of chronic tolerance, two to three days, overlaps with two types of memories created by associative conditioning: the shorter ARM, and the longer LTM (Margulies et al., 2005). Chronic tolerance shares some features of ARM and LTM, but differs from both in critical ways. First, our chronic exposure more closely resembles massed training, where animals are given multiple training trials without rest and that results in ARM. However, both chronic ethanol tolerance and spaced training LTM depend on de novo protein synthesis, whereas ARM does not (Berger et al., 2004). Like LTM, chronic ethanol tolerance is dependent on CREB signaling in the brain. However, unlike in LTM CREB functions outside the mushroom bodies for chronic tolerance, indicating that additional as yet undiscovered neural circuitry harbors the site of learning for chronic ethanol (Miyashita et al., 2018). Moreover *kayak* (*c-fos*) is selectively induced following the chronic challenge dose. In classical aversive conditioning *kayak* is induced by spaced training but not massed training, and *kayak* is required for LTM expression (Miyashita et al., 2018). We do not yet know if *kay* is required for the expression of chronic tolerance. We suggest that chronic ethanol in flies primes neural circuits for new learning via licensing of *kayak* inducibility, and supports the progress of ethanol experience towards the longer term debilitating effects of ethanol. In support of this, chronic intermittent ethanol exposure in rats causes changes in subsequent non-ethanol learning and memory (Shields & Gremel, 2021).

Ethanol likely creates complex memories. For example, ethanol in flies and mammals is both appetitive and aversive, relative to the time, pattern, and dose of the ethanol experience (Kaun et al., 2011; Koob & Le Moal, 2001; Lynch & Carroll, 2001; Nunez et al., 2018). Moreover, both appetitive and aversive traces can be created by the same ethanol experience. Unlike most stimuli that elicit learning and memory, ethanol has direct access to the entire nervous system, where it can exert its pharmacological and metabolic effects. Chronic ethanol exposure does create appetitive memories, since it induces ethanol preference. Appetitive conditioning in flies, with food when hungry or water when thirsty, forms protein synthesis dependent LTM after a single associative training (Krashes & Waddell, 2008; Shyu et al., 2017). However appetitive LTM is distinct from chronic ethanol tolerance as well as aversive LTM in that it requires *radish*. Thus, the appetitive memories created by ethanol and natural rewards are distinct. Taken together, chronic ethanol encoding shares features with but is distinct from appetitive and aversive LTM.

Additional complexity of ethanol memories is hinted at by the distinct forms of memory in rapid tolerance as compared to chronic tolerance. Drinking patterns in humans can be complex, mixing binge drinking and chronic intake over relatively short time periods (Kaprio et al., 1987; Sudhinaraset et al., 2016). Thus, ethanol may create multiple interacting memory traces that can shape future intake.

Epigenetic mechanisms, important for both rapid and chronic ethanol tolerance expression, likely ensure that distinct patterns of ethanol intake are separably encoded and expressed as tolerance. Histone acetylation state and thus chromatin structure maintains the experience of chronic ethanol exposure, with differential involvement of Class I/II HDACs and the Sirtuins. Chromatin-encoded chronic tolerance is manifest in the altered inducibility of IEGs, the selective suppression of *Hr38* inducibility by Class I/II HDACs, and by the active occlusion of rapid tolerance development. Epigenetic suppression of *Hr38* inducibility may be a molecular mechanism by which chronic ethanol exposure suppresses rapid tolerance development, since rapid tolerance development requires *Hr38* induction and chronic tolerance development does not (Adhikari et al., 2019). An as yet unidentified Class I/II HDAC, and not *Sirt1*, is likely the effector of *Hr38* suppression by chronic ethanol exposure. Adult *Sirt1* promotion of rapid tolerance and inhibition of chronic tolerance underscores the fundamentally different states created by different patterns of initial ethanol exposure. Moreover, our findings with *Sirt1* broadly fit with prevailing mammalian models of increased chromatin accessibility with initial ethanol inebriation, and chromatin compaction with prolonged ethanol use and with ethanol withdrawal (Berkel & Pandey, 2017b).

Chronic ethanol suppression of rapid tolerance is likely due to circuit-level communication from the γ lobes to the α/β lobes of the mushroom body learning and memory centers. Chronic tolerance is limited by *Sirt1* specifically in the mushroom body γ lobes, and neural activity in the mushroom bodies is required for chronic tolerance development. Thus, we predict that *Sirt1* dampens mushroom body γ lobe neural activity in response to chronic low dose ethanol. Although the mechanism is currently not understood, it is possible that *Sirt1* regulates GABA receptors in the γ lobe in response to chronic ethanol exposure, to effect a homeostatic maintenance of mushroom body activity through the broadly mushroom body-innervating APL GABAergic neurons; chronic ethanol in rodents causes marked changes in GABAergic neurotransmission (Liu & Davis, 2009; Petrie et al., 2001; Roberto et al., 2003). By contrast, rapid tolerance is promoted by *Sirt1* in the mushroom body α/β lobes, and neural activity in the mushroom bodies is required for rapid tolerance development. Thus, we predict that *Sirt1* promotes mushroom body α/β lobe neural activity in response to acute inebriating ethanol. Moreover, the mushroom bodies are the site for ethanol reward memories produced from a spaced associative training protocol (Kaun et al., 2011). The association is established in the γ lobes, consolidation occurs in the α'/β' lobes, and expression requires the α/β lobes. Thus, circuit-level communication between mushroom body lobes is also a feature of ethanol reward learning and memory. Interestingly, short-term rapid tolerance and ethanol reward memory expression colocalize in the α/β lobes, and long-term chronic tolerance and ethanol reward learning colocalize in the γ lobes. Furthermore, the initiating ethanol exposures for rapid and chronic tolerance both potentiate ethanol preference, indicating they include appetitive memory traces that may indicate reward-like actions. Thus, ethanol tolerance uses some of the same brain

circuitry as ethanol reward, but tolerance uses the circuitry differently. Recent advances in memory mapping indicates that there exists some functional specialization of the mushroom body intrinsic neurons; distinct, as yet uncharacterized lobe subregions may encode tolerance vs. other forms of memory (W.-P. Lee et al., 2020a).

Repeated tolerance in *Drosophila* is induced by a paradigm that is similar to the regular intermittent drinking in humans that the National Institute on Alcohol Abuse and Alcoholism defines as heavy alcohol use. Repeated or intermittent ethanol exposure is widely used in vertebrate models of alcohol use disorders to study ethanol plasticity and model features of alcohol use disorders (Carnicella et al., 2014; Morales et al., 2018; Nimitvilai et al., 2016). Repeated exposure to inebriating but not sedating ethanol doses in *Drosophila* resulted in molecular and behavioral responses that are distinct from both rapid and chronic tolerance. For example, rapid and chronic pre-exposures both prime flies for ethanol preference, whereas repeated pre-exposure primes flies for mild ethanol aversion. Thus, repeated exposure appears to create a third form of ethanol plasticity in flies. In rodents the length of the interstimulus interval determines whether ethanol experience is appetitive or aversive (Cunningham et al., 1997). Optimization of *Drosophila* repeated exposures may reveal conditions that favor ethanol preference.

Chapter 2: Mushroom Body Circuits for Rapid and Chronic Ethanol Tolerance

Summary

Multiple independent memory traces underscore the tolerance that forms from an acute sedating dose of ethanol. Specifically, rapid tolerance is composed of both labile anesthesia-sensitive tolerance (AST) and consolidated anesthesia-resistant tolerance (ART) at similar scales and genetic components as classically-conditioned ASM and ARM. However, rapid tolerance requires the GABAergic actions of APL neurons to limit mushroom body function. Conversely, a persistent and protein-synthesis dependent tolerance trace is induced by chronic ethanol. Chronic tolerance requires a GABAergic DPM circuit with KCs to limit mushroom body function, and thus LTM-like encoding in typical LTM neurons.

Results

An acute, sedating dose of ethanol forms AST and ART

After an acute sedating dose of ethanol, Anesthesia-Sensitive rapid Tolerance (AST) persists at least 3hrs but dissipates by 24hrs (**Fig. 1A**). Since AST does not contribute to the 24hr rapid tolerance score, it is an independent phase of tolerance, and the 24hr rapid tolerance score is primarily composed of Anesthesia-Resistant rapid Tolerance (ART). Moreover, a cold shock timed to block AST does not impact ART (**Fig. 1A**). Thus, the temporal aspects of AST are consistent with those of an aversively-conditioned ASM.

Cold shock-induced deficits in rapid tolerance may arise in part from non-specific disruptions of neural activity. Thus, as an orthogonal test for AST, we investigated the role of the neuropeptide *amnesiac* (*amn*), that is necessary and sufficient for aversively-conditioned ASM (DeZazzo et al., 1999; Keene et al., 2006; Tamura et al., 2003; Waddell et al., 2000). *Amn*[1] mutants display reduced rapid tolerance at 4hrs after the initiating dose, but not at 24hrs (**Fig. 1B**), suggesting that *amn*-dependent pathways support AST. *Amn*[1] mutants show resistance to ethanol sedation, as quantified in our loss of the righting reflex assay (**Fig. 1B**). This sedation response is opposite from historical findings on *amn* alleles using inebriometers that measure postural control, for example the *amn* allele *cheapdate* that shows high ethanol sensitivity (Moore et al., 1998). *Amnesiac* is a predicted activator of adenylyl cyclase (AC), that generates cAMP and is encoded by the *rutabaga* (*rut*) gene in *Drosophila*. *Rut* supports coincidence detection for associative learning paradigms that pair conditioned and unconditioned stimuli (Davis, 2005; Thum et al., 2007). In our assay, *Rut*[1] mutants display a rapid tolerance deficit at the 4hr timepoint, not the 24hr timepoint, and they show reduced sedation sensitivity (**Fig. 1C**). Thus, the *rut* AC specifically supports the earlier labile form of rapid tolerance, similar to its role in olfactory conditioning.

Pan-neuronal expression of an RNAi against *amn* reduced rapid tolerance and sensitivity (**Fig. 1D**). Adding a cold shock after the inducing exposure significantly reduced tolerance in controls, but not the experimental group, suggesting that neuronal *amn* acts in the same pathway as cold shock anesthesia. Cold shock does not erase aversively-conditioned ARM that is supported by the GTPase *radish* (*rad* or *rsh*) (Bourouliti & Skoulakis, 2022a; P.-T. Lee et al., 2011; Wu et al., 2013; Yang et al., 2016). *Rad*[1] mutants show reduced rapid tolerance, with or without amnestic conditions that erase AST, suggesting that *rsh*-dependent rapid tolerance is ART (**Chap. 1 Fig. 6D**) (Larnerd et al., 2023). Consistent with this, pan-neuronal expression of an RNAi against *rsh* decreased rapid tolerance and sedation sensitivity, even under cold shock conditions (**Fig. 1E**). Thus, *rsh*-dependent ART and cold shock-dependent AST additively form rapid tolerance (**Fig. 1F**).

In conclusion, ethanol-induced AST and ART are operationally and temporally distinct. AST resembles ASM through its temporal features and usage of *amnesiac* and *rutabaga*. Likewise, ethanol-induced ART resembles ARM through its usage of *radish*.

AST resides in Kenyon cells

The KCs and DPMs are positive for *amn* expression (Shih et al., 2019; Turrel et al., 2018, 2020; Waddell et al., 2000). *Amn* knockdown in all KCs reduced rapid tolerance but did not affect sensitivity (**Fig. 2A**). Moreover, a cold shock did not further decrease

rapid tolerance in flies lacking *amn* in the KCs, suggesting that these manipulations redundantly decrease AST (**Fig. 2A**). Conversely, *amn* knockdown in the DPMs was dispensable for rapid tolerance and sensitivity (**Fig. 2B**). Thus, rapid tolerance requires *amn* in KCs, not DPMs, similar to aversively-conditioned ASM (Turrel et al., 2018). Cell-type specific sequencing of the *Drosophila* MBs has revealed that all seven KC subtypes are positive for *amn* (Shih et al., 2019). To localize its role in rapid tolerance, we knocked down *amn* in each of the three KC subtypes that form the three anatomical lobes of the MBs. *Amn* acts in specifically the γ lobe to support rapid tolerance (**Fig. 2C**). Meanwhile, *amn* functions in the γ and α/β KCs to support sedation sensitivity (**Fig. 2C**). Taken together, the *amn*-dependent aspect of rapid tolerance is similar but distinct from aversively-conditioned ASM. Both AST and ASM form independently of DPM *amn*, but they require *amn* in separate KC populations.

Aversive ASM, but not ARM, requires NMDA receptors in the MBs, consistent with the role of Ca^{2+}-dependent synaptic plasticity in olfactory conditioning (Wu et al., 2007). Moreover, mutants for *Nmdar* or *Dlg1*, one of its scaffolding proteins, show decreased rapid tolerance phenotypes (Maiya et al., 2012). Thus, we used an RNAi against the *NR1* subunit of all NMDARs to test their function in rapid tolerance. *Nmdar* knockdown in all KCs caused a decrease in the 4hr timepoint, but not the 24hr timepoint, indicating that NMDARs specifically support AST (**Fig. 2D**). Pan-KC NMDARs also support sedation sensitivity (**Fig. 2D**).

To conclude, AST utilizes KC-specific *amn* and NMDAR signaling mechanisms that are common to ASM. However, *amn* separately acts in γ or α/β neurons to support rapid ethanol tolerance or aversive olfactory conditioning, respectively.

Rsh-dependent ART resides outside the mushroom bodies

The genes and circuits of the MBs have a demonstrated role in supporting ARM (Bourouliti & Skoulakis, 2022a; Davis, 2023). Considering that neuronal *rsh* supports rapid tolerance (**Fig. 1E**), we hypothesized that MB neurons hold *rsh*-dependent ART. However, targeting KCs with multiple *rsh* RNAis did not affect rapid tolerance, but did reduce sedation sensitivity (**Fig. 3A, 3B**). To rule out the possibility that a *rsh* knockdown causes a gain in AST and thus masks a deficit in ART, we also tested the manipulation under conditions that lack AST. Even when AST was blocked by cold shock or dissipated with time, ART remained unaffected by *rsh* knockdown in all KCs (**Fig. 3A, 3B**). To search for a site of action for *rsh*-dependent rapid tolerance, we next investigated extrinsic MB neurons. *Rsh* is dispensable in the APLs for rapid tolerance, but may support sensitivity there (**Fig. 3C**). Similarly, *rsh* is dispensable in the DPMs for rapid tolerance and sensitivity (**Fig. 3D**). Thus, the bilateral pairs of DPM and APL neurons, that each broadly innervate the mushroom bodies (**Fig. 3E**), lack coding capacity for *rsh*-dependent ART.

To conclude, *rsh* supports rapid tolerance in neurons outside the MBs. Despite that functional localization of *rsh* has not been well characterized for ARM, a known site of action is in α/β neurons (Yang et al., 2016). Thus, not only does *rsh*-dependent ART differ from ARM, *rsh*-dependent ART and *amn*-dependent AST arise from separate circuits in the *Drosophila* brain.

Temporally precise APL activity induces rapid tolerance

The APLs support learning and memory via broad innervation of KCs, providing GABAergic feedback inhibition to support sparse encoding of olfactory stimuli (Amin et al., 2020; Liu & Davis, 2009; Pitman et al., 2011). We tested the role of these large neurons in controlling ethanol behaviors by inactivating them via overexpression of temperature-sensitive *Shibire* (*Shi*) – a dynamin that regulates vesicular endocytosis and thus presynaptic release in *Drosophila*. Inactivating adult neurons marked by the *GH146-Gal4* driver decreases rapid tolerance, except under *Gad1-Gal80* conditions that permit normal function of GABAergic cells in the pattern (**Fig. 4A**). Moreover, inactivating adult neurons marked by the *VT43924-Gal4* driver, that does not express in olfactory projection neurons (**Fig. 3E**), also decreases rapid tolerance, but does not affect sensitivity (**Fig. 4A**). Taken together, APL neurons support rapid tolerance.

We temporally dissected our rapid tolerance paradigm into separately testable phases, namely acquisition, consolidation, and expression, based on the paradigmatic distinctions established in the associative learning and memory literature for *Drosophila*. These descriptors have been used to temporally parse the stages of ethanol associative preference and aversion (Kaun et al., 2011). For a rapid ethanol tolerance paradigm, we reason that Acquisition represents the first ethanol exposure “E1” as the acute, sedating dose that induces the observed tolerance; Consolidation represents the period of ethanol deprivation and recovery from sedation; and Expression represents the second ethanol exposure “E2” that reveals the magnitude of tolerance development.

Shi-mediated APL activity is required only during the acquisition phase (**Fig. 4B**), hence the APLs regulate the initial development of tolerance, but not the consolidation or expression of tolerance behaviors. Even tolerance measured at the 24hr timepoint is supported by APL activity during the acquisition phase (**Fig. 4C**), suggesting that APL neurons either specifically induce ART or they generally induce tolerance. However, APL-specific knockdown of either *Gad1*-mediated GABA synthesis or *Tbh*-mediated octopamine synthesis had no effect on ART (data not shown). Thus, we favor the interpretation that APL activity during acquisition induces any trace of rapid tolerance.

When *Shi*-mediated inactivation of either APL driver occurred during the “E1” sedation measurement, sensitivity was by and large unaffected (**Fig. 4A, 4B, 4C**). However, inactivation occasionally caused sensitivity to mildly decrease (*VT43924*; **Fig. 4C**) or increase (*GH146*; **Fig. 4A**), possibly suggesting that APL neurons marginally support sensitivity while other neurons in the *GH146* pattern weakly support resistance. More likely, the observed variable effects on sedation sensitivity resulted from the ectopic nature, leaky expression, and/or short activation window of the *Shi* transgene.

Considering a role for the APLs in tolerance induction, we next looked to DPM neurons, that aid in consolidating associative learning of aversive stimuli (Keene et al., 2004, 2006; P.-T. Lee et al., 2011; Yu et al., 2005). Surprisingly, *Shi*-mediated activity of the adult DPMs was dispensable for rapid tolerance and sensitivity when tested during the entire paradigm and specifically consolidation (**Fig. 4D**). Consistent with this, GABA synthesis in the DPMs was dispensable for rapid, but did support sedation sensitivity (**Fig. 4D**). Thus, unlike ITM, rapid tolerance does not require activity of the DPM extrinsic neurons.

APL and DPM neurons are adjoined by a gap junction that is required for aversively-conditioned ASM, specifically via the heterotypic pairing of Innexin 7 in the APL and

Innexin 6 in the DPM (Wu et al., 2011). We tested this gap junction for ethanol behaviors. Knockdown of either *inx7* in the APL or *inx6* in the DPM was dispensable for rapid tolerance, but required for sedation sensitivity (**Fig 4E, 4F**). Thus, the Innexin-dependent gap junction that supports ASM is dispensable for rapid tolerance.

In conclusion, APL activity during an acute ethanol dose induces tolerance to that dose. Since DPM activity and APL-DPM gap junctions are dispensable, rapid tolerance has distinct circuit features from ITM.

AST requires GABAergic repression of Kenyon cells

Considering that adult-specific APL vesicle release supports rapid tolerance, we searched for GABA receptivity in the MBs. *Drosophila* have both ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors, both of which have been implicated in tolerance (Dzitoyeva et al., 2003). And, the *Resistance to dieldrin* (*Rdl*) subunit of $GABA_A$ has been found to regulate memory (Liu et al., 2007, 2009). Thus, we knocked down *Rdl* in the adult KCs to test for GABA reception. The *Rdl* subunit supports rapid tolerance measured at 4hrs, but not at 24hrs (**Fig. 5A**), suggesting that signaling through $GABA_A$ is critical to express the earlier labile phase of rapid tolerance, AST. *Rdl* knockdown did not affect sedation sensitivity (**Fig. 5A**). Taken together, KCs show temporal responsiveness to GABA release at the MBs.

Next, using the *Shi* transgene that blocks presynaptic release, we investigated the consequences of lobe-specific KC blockade on sensitivity and tolerance to acute ethanol. Inactivation of all KCs did not affect rapid tolerance or sensitivity (**Fig. 5B**). Consistently, inactivating any of the three KC subtypes that project to the three anatomical lobes did not affect rapid tolerance or sensitivity (**Fig. 5B**). This was surprising because the activity of α/β lobes was previously found to support rapid tolerance, using constitutive inactivation via tetanus toxin and other vesicular release mechanisms (Engel et al., 2016; Lange & Wolf, 2023). We repeated our *Shi* test in the α/β lobes under conditions that only inactivate them during the expression phase, under the hypothesis that the final step of expressing tolerance would critically depend on neuronal activity. However, tolerance and sensitivity remained normal under these specific conditions (**Fig. 5C**). Thus, *Shi*-mediated activity of the KCs is dispensable for rapid tolerance.

To conclude, adult KCs require $GABA_A$ receptors to support AST and do not require *Shi*-mediated activity, suggesting that rapid tolerance occurs via GABAergic inhibition of KCs. Despite that ITM has been well-mapped to KC subtypes, for example *Shi*-mediated activity of γ KCs supports ASM and not ARM (Yang et al., 2016), rapid tolerance is wholly independent of *Shi*-mediated KC function.

KCs are dispensable for protein synthesis-dependent chronic tolerance

LTM of an associatively-trained event requires new protein synthesis. Cycloheximide pharmacologically inhibits translation and has been used to inhibit aversive olfactory memory (Davis, 2011; Tully et al., 1994; Wu et al., 2007; Yin et al., 1994), as well as chronic ethanol tolerance (Berger et al., 2004). We followed up on this translation-dependent tolerance using a genetically encoded eukaryotic ribosome inhibitor, the Ricin toxin, that was developed into a cold-sensitive version and used for spatiotemporal inducibility (Allen et al., 2002; Chen et al., 2012; W.-P. Lee et al., 2020b; B. Zhao et al., 2019). Neuronal Ricin activation is functionally equivalent to cycloheximide in blocking

LTM (Chen et al., 2012). We first expressed Ricin in all adult neurons and only yielded testable flies when combined with *Gal80^{ts}*, another thermogenetic GAL4/UAS repressive system. Adult-specific, pan-neuronal inhibition of translation caused decreased chronic tolerance, but no change in sedation sensitivity (**Fig. 6A**), thus redemonstrating that chronic tolerance is protein synthesis-dependent (PSD). Expressing Ricin specifically in all adult KCs marked by the *R13F02* driver yielded testable flies without the additional *Gal80^{ts}* system. Blocking new protein synthesis during the entire chronic tolerance paradigm in all adult KCs did not affect chronic tolerance or sensitivity (**Fig. 6B**). Similarly, blocking adult KC translation specifically during the tolerance-inducing dose of chronic ethanol, or during the 24hrs after it, caused no changes in chronic tolerance or sensitivity (**Fig. 6B**). Thus, a PSD trace for chronic tolerance exists in a non-KC site in the *Drosophila* nervous system.

CREB functions outside the mushroom bodies to support chronic tolerance (**Chap. 1 Fig. 7A**) (Larnerd et al., 2023). To briefly verify that the transcriptional and post-translational pathways participating in CREB encoding are also dispensable in KCs, we tested the role of Mef2, a transcription factor that induces CREB expression, and Ca^{2+}-associated genes in our chronic tolerance assay. Mef2 signaling has a demonstrated role in supporting rapid tolerance via induction of the IEG *Hr38* (Adhikari et al., 2019). Expressing dominant negative Mef2 in all KCs did not affect chronic tolerance or sensitivity (**Fig. 6C**), thus ruling out Mef2-mediated signaling, and further distinguishing rapid and chronic ethanol tolerance. The phosphorylation of CREB protein is a consequence of Ca^{2+} influx, cAMP generation, and activation of the MAPK signaling cascade. Intracellular calcium levels are regulated in part by entrance through NMDA receptors, thus knockdown of the constituent subunit *NR1* abolishes NMDA-mediated synaptic plasticity. KC-specific knockdown of *NR1* was dispensable for chronic tolerance and sensitivity (**Fig. 6D**), ruling out a possible CREB activation route. Interestingly, aversive LTM also does not require NMDARs in the MBs, instead they act in ellipsoid body neurons (Wu et al., 2007). Next, to test Ca^{2+}-sensitive kinase activation in KCs, we expressed a constitutively active version of CaMKII, *CaMKII.T287* (Thornquist et al., 2020). However, no change in chronic tolerance or sensitivity was observed (**Fig. 6E**), indicating that a canonical post-calcium kinase response is dispensable in all KCs. Last, we asked if any cAMP generation is important for chronic tolerance using a mutant for the *Drosophila* adenylyl cyclase *rut* gene. *Rut^{1}* mutants exhibit strongly reduced chronic tolerance and sensitivity (**Fig. 6F**), consistent with its role in supporting associative learning.

Taken together, the CREB and PSD mechanisms that encode classically conditioned LTM do not occur in KCs to create tolerance to chronic ethanol. However, Ca^{2+}-dependent events, such as activation of adenylyl cyclase and non-KC NMDARs, are common to both of these long-term responses.

Temporally precise DPM activity induces chronic tolerance

Because genetic and circuit features of chronic ethanol tolerance have been implicated in and mapped to KCs (Berger et al., 2004; Larnerd et al., 2023), we searched for possible roles of other neuronal constituents of the MBs. Expression of temperature-sensitive *Shi* in the DPMs using the *2721-Gal4* driver to block dynamin-mediated activity caused a reduction in chronic tolerance, but not sedation sensitivity (**Fig. 7A**). Moreover, DPM blockade timed to occur during the consolidation phase did not affect either ethanol

behavior (**Fig. 7A**). To confirm this, and to ask if the DPMs temporally regulate chronic tolerance at all, we used the highly specific *VT64246-Gal4* driver (**Fig. 3E**). *Shi*-mediated inactivation of the DPMs during acquisition, not consolidation or expression, caused decreased chronic tolerance and sedation sensitivity (**Fig. 7B**). Thus, vesicle release from the DPMs only during the initiating chronic ethanol dose helps create tolerance to that dose.

Next, blocking *Shi*-mediated activity in the APLs (**Fig. 7C**) or all KCs (**Fig. 7D**) during the entire paradigm did not affect chronic tolerance or sensitivity. Moreover, individually blocking each KC lobe with *Shi* revealed no change in chronic tolerance (**Fig. 7E**). Sedation sensitivity increased upon long-term inactivation of α/β neurons, but not other lobes (**Fig. 7E**). To conclude, the DPMs use *Shi*-mediated activity to induce chronic tolerance.

The DPM GABAergic repression of KCs is compartmentalized to support chronic tolerance

DPM neurons are positive for GABA, serotonin, and the neuropeptide *amnesiac* (Haynes et al., 2015; P.-T. Lee et al., 2011; Waddell et al., 2000). The knockdown of the GABA synthesis gene *Gad1* within multiple drivers that mark DPM neurons, *VT64246-Gal4* and *2721-Gal4*, caused decreases in chronic tolerance and sedation sensitivity (**Fig. 8A**). Thus, the DPMs synthesize GABA in support of ethanol behaviors. Next, adult-specific knockdown of the serotonin synthesis gene *Ddc* in the DPMs was dispensable for both ethanol behaviors (data not shown), ruling out serotonergic signaling. Last, flies harboring the amn^{1} mutant allele display normal chronic tolerance (**Chap. 1 Fig. 6A**) (Larnerd et al., 2023). Thus, the DPMs specifically regulate chronic tolerance through GABA, not through other known signaling mechanisms.

To map possible targets of this GABA signaling, we tested the role of the *Rdl* subunit that constitutes ionotropic $GABA_A$ receptors. In theory, the loss of GABA reception would recapitulate the loss of GABA synthesis and signaling. Indeed, adult-specific knockdown of *Rdl* in all KCs under the *R13F02-Gal4* driver reduced chronic tolerance, without affecting sedation sensitivity (**Fig. 8B**). Furthermore, expressing *Rdl* RNAi in α/β KCs, represented by *R28H05-Gal4* and *17d-Gal4*, each caused a decrease in chronic tolerance and in sensitivity, but no difference was observed when *Rdl* RNAi was expressed in the remaining KC lobes (**Fig. 8C**). Therefore, the GABAergic actions of the DPMs may compartmentalize to α/β KCs and be received there by $GABA_A$ *Rdl*.

DPMs require protein synthesis, not CREB, to support chronic tolerance

Because CREB functions in a non-KC site for chronic tolerance (**Chap. 1 Fig. 7A**) (Larnerd et al., 2023), we hypothesized that it acts in the DPMs to aid in generating chronic tolerance. However, overexpressing a dominant negative form of CREB in the DPMs caused no change in chronic tolerance or sedation sensitivity (**Fig. 9A**). Interestingly, blocking protein synthesis in the DPMs during the acquisition stage, but not consolidation, caused reduced chronic tolerance without affecting sensitivity (**Fig. 9B**). Thus, a PSD but not CREB-dependent chronic tolerance trace exists in the DPMs. Our data suggest that α/β KCs are a likely target of DPM regulation, therefore we specifically tested the PSD encoding capacity of these cells. Ricin-mediated inhibition of translation in α/β neurons was dispensable for chronic tolerance and sensitivity when activated during acquisition or consolidation (**Fig. 9C**), consistent with our pan-KC findings (**Fig 6B**). To test another LTM encoding mechanism, we investigated the IEG *kay* – homolog

of mammalian *c-fos* – that marks engram neurons and is responsible for the persistent CREB expression that sustains LTM (Miyashita et al., 2018). *Kay* is inducible after chronic ethanol exposure (**Chap. 1 Fig. 2A**). Expressing a dominant negative *kay* transgene in multiple drivers that target α/β KCs to block *kay* signaling did not affect chronic tolerance or sensitivity (**Fig. 9D**). Thus, separate from traditional LTM, chronic tolerance does not utilize α/β signaling mechanisms.

Figure 1

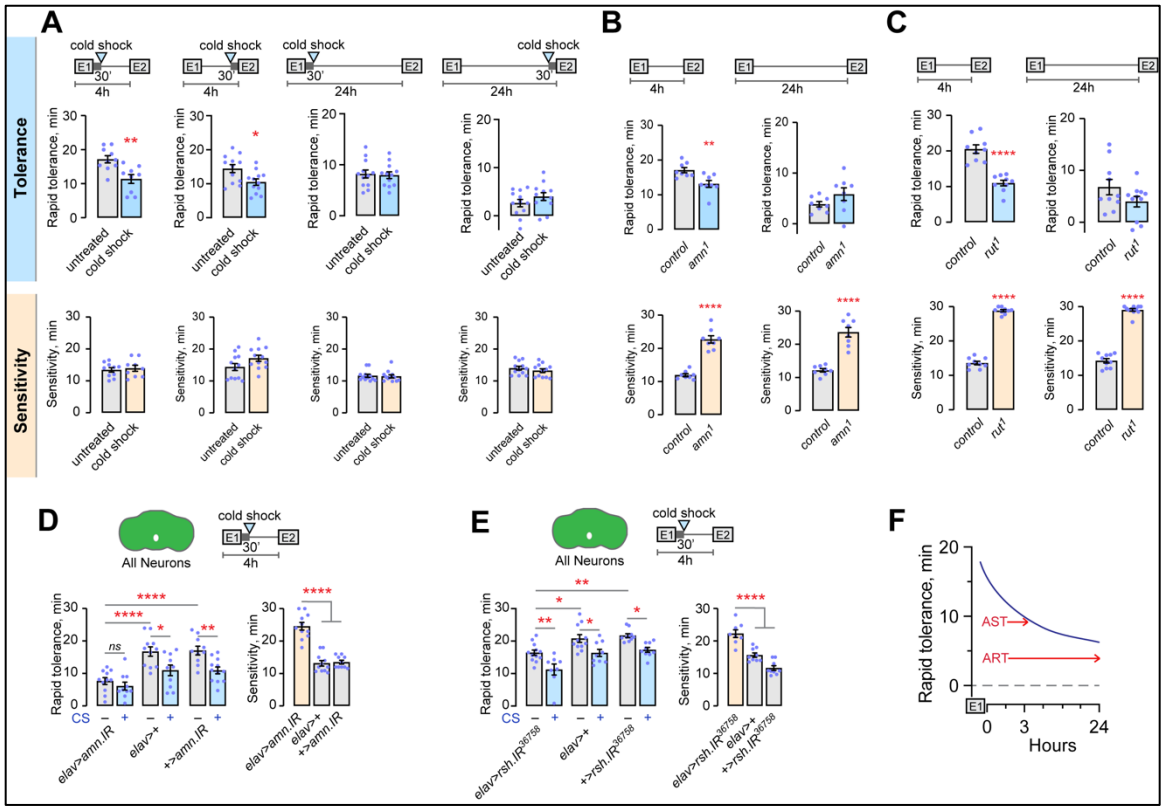

Figure 1. An acute, sedating dose of ethanol forms AST and ART.

A) Anesthetic cold shock erases labile, not consolidated, rapid tolerance. Left and Center-left: Labile AST persists at least 3 h after acute ethanol. Right and Center-right: Consolidated ART persists at least 24 h after acute ethanol. Sedation sensitivity is unaffected as the cold shock occurs post-measurement. Tolerance: all Unpaired t tests. Sensitivity: Unpaired t test, Unpaired t test, Mann-Whitney test, Mann-Whitney test.

B) *Amn* supports AST and sedation sensitivity. Tolerance: Unpaired t test, Welch's t test. Sensitivity: both Welch's t tests.

C) *Rut* supports AST and sedation sensitivity. Tolerance: all Unpaired t tests. Sensitivity: all Unpaired t tests.

D) Neuronal *amn* supports AST and sedation sensitivity. Tolerance: all One-way ANOVAs. Sensitivity: all One-way ANOVAs.

E) Neuronal *rsh* supports ART and sedation sensitivity. Tolerance: all One-way ANOVAs. Sensitivity: all One-way ANOVAs.

F) Representative graph of the time-dependent properties of AST and ART after acute ethanol.

Figure 2

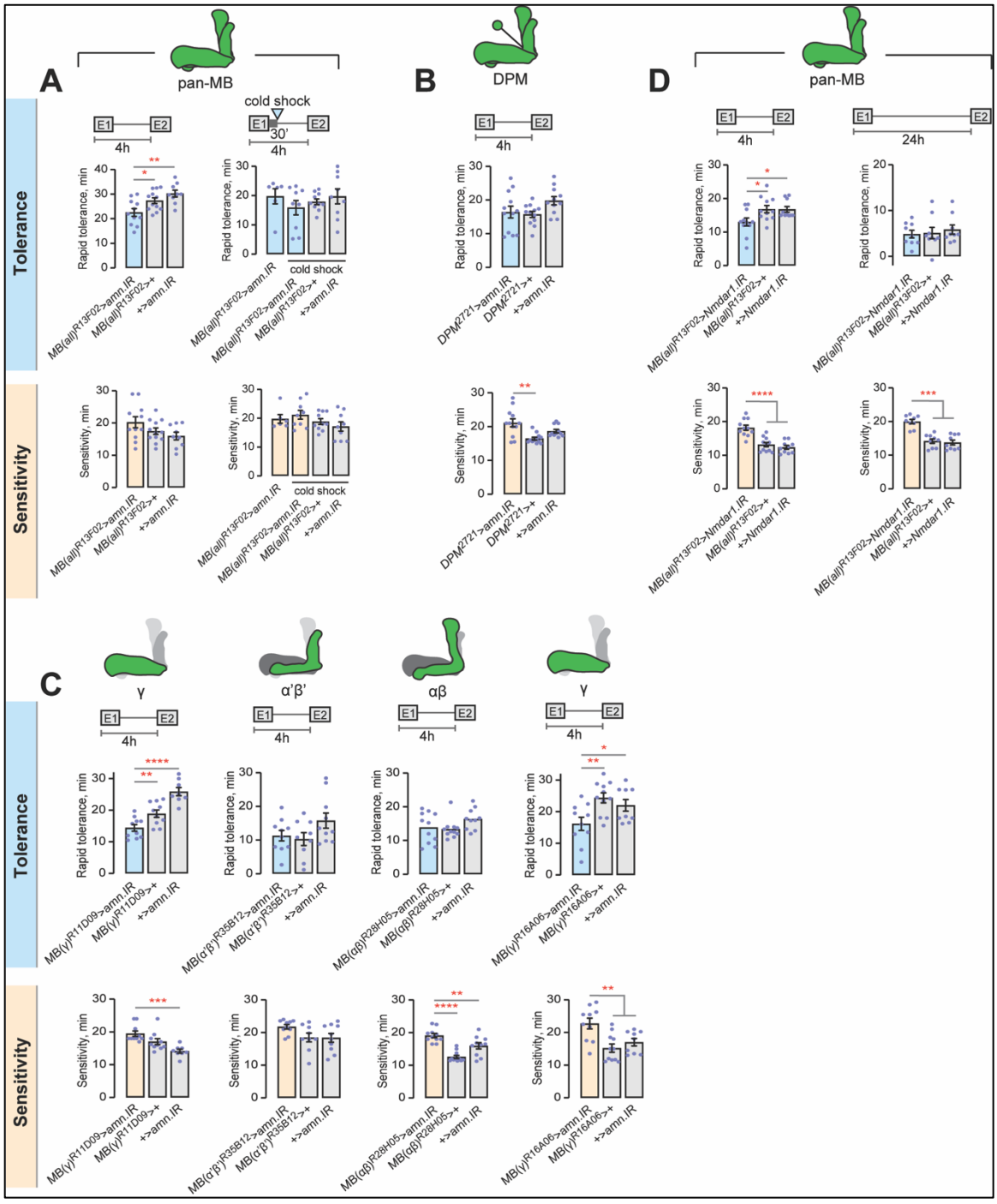

Figure 2. AST resides in Kenyon cells.

A) KC *amn* supports AST, not sedation sensitivity. Tolerance: both One-way ANOVAs. Sensitivity: both One-way ANOVAs.

B) DPM *amn* is dispensable for rapid tolerance and sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: Kruskal-Wallis ANOVA.

C) KC γ *amn* supports rapid tolerance. KC γ or α/β *amn* supports sedation sensitivity. Tolerance: all One-way ANOVAs. Sensitivity: Kruskal-Wallis ANOVA, One-way ANOVA, One-way ANOVA, One-way ANOVA.

D) KC *Nmdar1* supports AST and sedation sensitivity. Tolerance: both One-way ANOVAs. Sensitivity: One-way ANOVA, Kruskal-Wallis ANOVA.

Figure 3

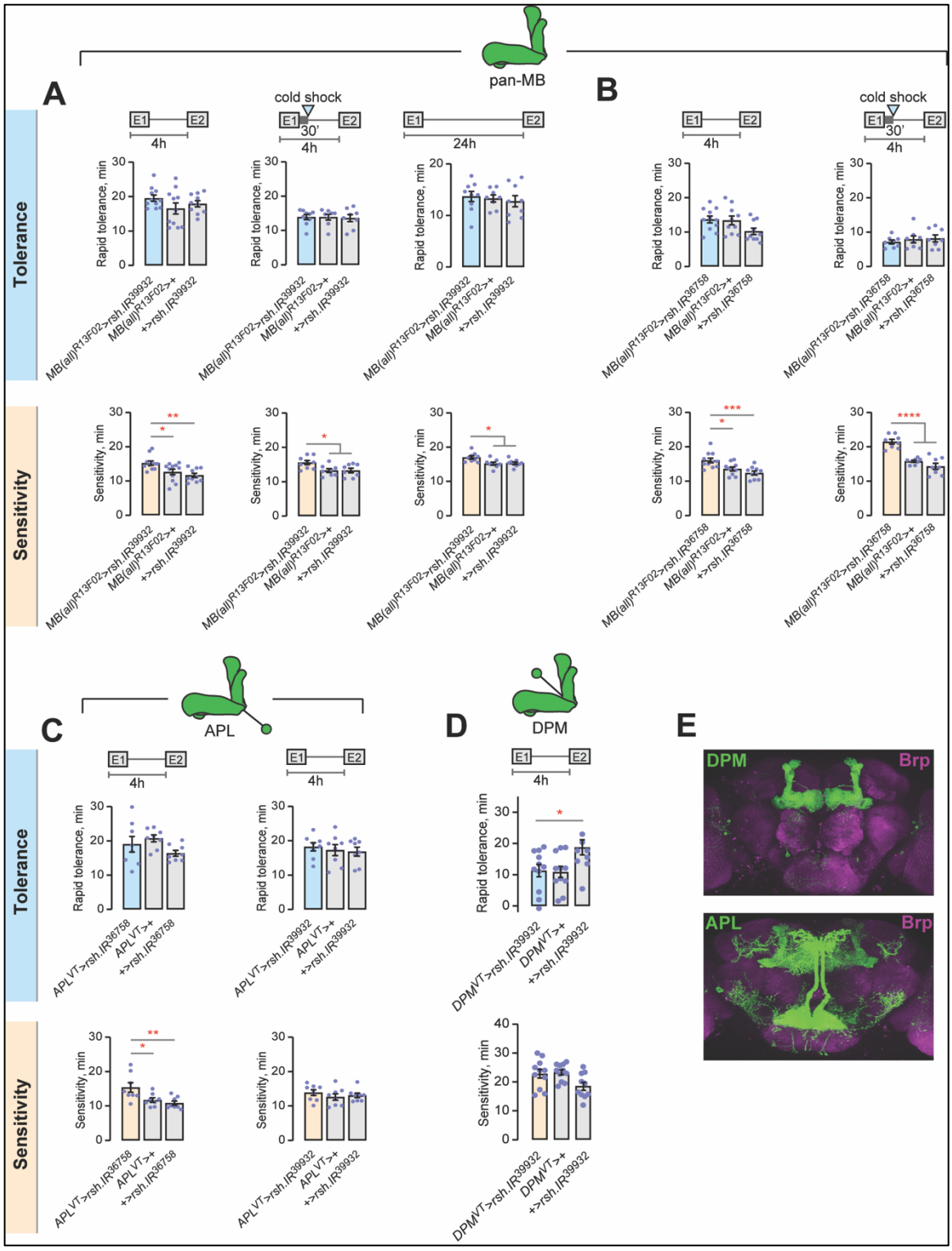

Figure 3. *Rsh*-dependent ART resides outside the mushroom bodies.

A) KC *rsh*, as targeted by RNAi^{39932}, is dispensable for ART, but supports sedation sensitivity. Tolerance: Brown-Forsythe ANOVA, Kruskal-Wallis ANOVA, One-way ANOVA. Sensitivity: all One-way ANOVAs.

B) KC *rsh*, as targeted by RNAi^{36758}, is dispensable for ART, but supports sedation sensitivity. Tolerance: both One-way ANOVAs. Sensitivity: One-way ANOVA, Brown-Forsythe ANOVA.

C) Left: APL *rsh*, as targeted by RNAi^{36758}, is dispensable for rapid tolerance, but supports sedation sensitivity. Right: APL *rsh*, as targeted by RNAi^{39932}, is dispensable for rapid tolerance and sedation sensitivity. Tolerance: Brown-Forsythe ANOVA, One-way ANOVA. Sensitivity: both One-way ANOVAs**.**

D) DPM *rsh*, as targeted by RNAi^{39932}, is dispensable for rapid tolerance and sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

E) The DPMs are composed of a bilateral pair of neurons that each broadly arborize the ipsilateral mushroom body. Top: Expression pattern of *DPM^{VT64246}-Gal4* via immunostaining GFP (green) and bruchpilot (magenta). Bottom: Expression pattern of *APL^{VT43924}-Gal4* via immunostaining GFP (green) and bruchpilot (magenta).

Figure 4

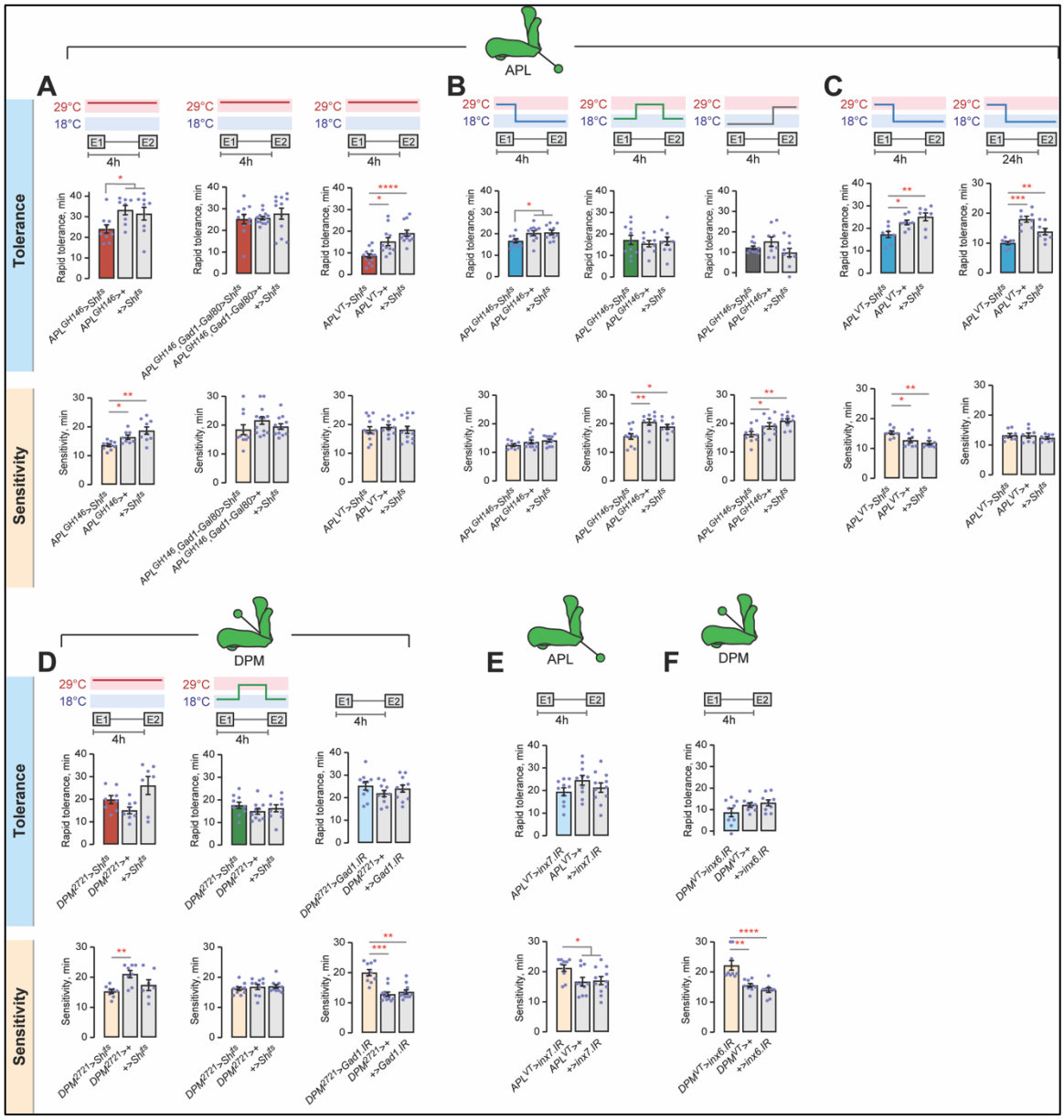

Figure 4. Temporally precise APL activity induces rapid tolerance.

A) Left: APL *Shi*-mediated activity, as targeted by *GH146-Gal4*, supports rapid tolerance at any phase and limits sedation sensitivity. Center: APL *Shi*-mediated activity, as targeted by *GH146-Gal4* but excluded in *Gad1*-positive cells, is dispensable for rapid tolerance at any phase and sedation sensitivity. Right: APL *Shi*-mediated activity, as targeted by *VT43924-Gal4*, supports rapid tolerance at any phase, not sedation sensitivity. Tolerance: One-way ANOVA, Kruskal-Wallis ANOVA, Kruskal-Wallis ANOVA. Sensitivity: Brown-Forsythe ANOVA, Kruskal-Wallis ANOVA, One-way ANOVA.

B) Left: APL *Shi*-mediated activity, as targeted by *GH146-Gal4*, supports rapid tolerance acquisition, not sedation sensitivity. Center: APL *Shi*-mediated activity, as targeted by

GH146-Gal4, is dispensable for rapid tolerance consolidation, but limits sedation sensitivity. Right: APL *Shi*-mediated activity, as targeted by *GH146-Gal4*, is dispensable for rapid tolerance expression, but limits sedation sensitivity. Tolerance: One-way ANOVA, One-way ANOVA, Brown-Forsythe ANOVA. Sensitivity: all One-way ANOVAs.

C) Left: APL *Shi*-mediated activity, as targeted by *VT43924-Gal4*, supports 4hr rapid tolerance acquisition and sedation sensitivity. Right: APL *Shi*-mediated activity, as targeted by *VT43924-Gal4*, supports 24hr rapid tolerance acquisition, not sedation sensitivity. Tolerance: both One-way ANOVAs. Sensitivity: both One-way ANOVAs.

D) Left: DPM *Shi*-mediated activity, as targeted by *2721-Gal4*, is dispensable for rapid tolerance at any phase and sedation sensitivity. Center: DPM *Shi*-mediated activity, as targeted by *2721-Gal4*, is dispensable for rapid tolerance consolidation and sedation sensitivity. Right: DPM *Gad1*-mediated activity, as targeted by *2721-Gal4*, is dispensable for rapid tolerance, but supports sedation sensitivity. Tolerance: Brown-Forsythe ANOVA, One-way ANOVA, One-way ANOVA. Sensitivity: One-way ANOVA, One-way ANOVA, Kruskal-Wallis ANOVA.

E) APL *inx7*, as targeted by *VT43924-Gal4*, is dispensable for rapid tolerance, but supports sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

F) DPM *inx6*, as targeted by *VT64246-Gal4*, is dispensable for rapid tolerance, but supports sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: Kruskal-Wallis ANOVA.

Figure 5

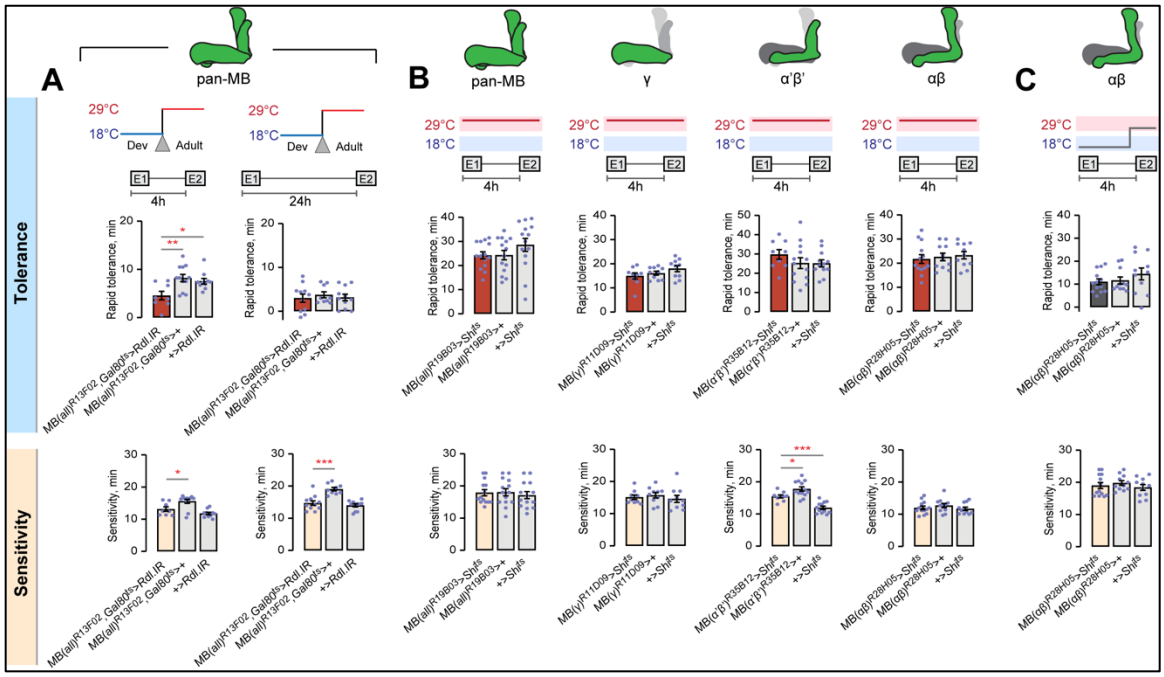

Figure 5. AST requires GABAergic repression of Kenyon cells.

A) KC *Rdl* supports AST, not sedation sensitivity. Tolerance: both One-way ANOVAs. Sensitivity: both One-way ANOVAs.

B) KC *Shi*-mediated activity, as targeted by multiple KC lobe drivers, is dispensable for rapid tolerance at any phase and sedation sensitivity. Tolerance: Kruskal-Wallis ANOVA, Brown-Forsythe ANOVA, One-way ANOVA, One-way ANOVA. Sensitivity: Kruskal-Wallis ANOVA, One-way ANOVA, One-way ANOVA, One-way ANOVA.

C) KC *Shi*-mediated activity is dispensable for rapid tolerance at expression and sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: Kruskal-Wallis ANOVA.

Figure 6

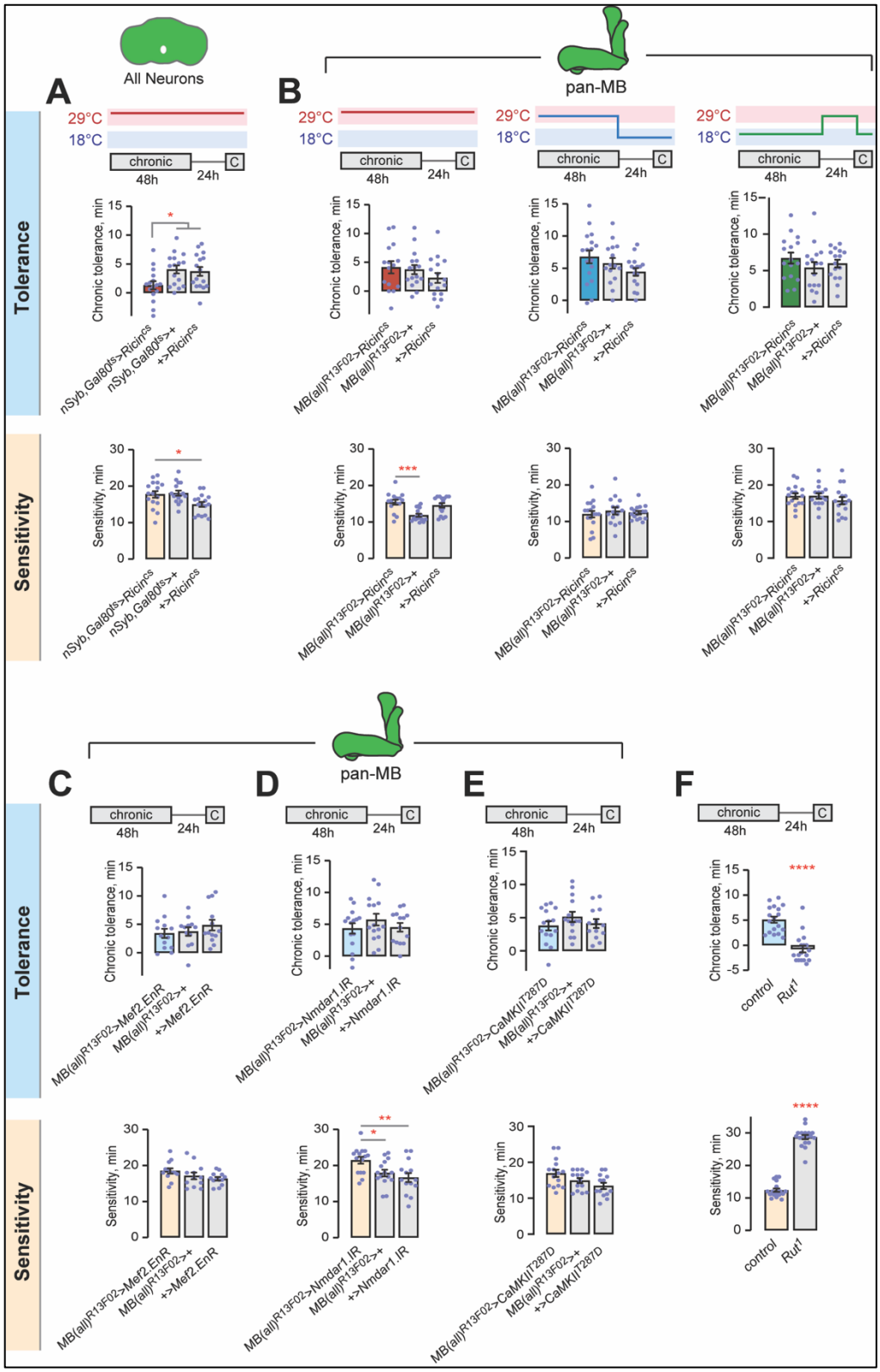
All Neurons
pan-MB
A
B
C
D
E
F
Tolerance
Sensitivity
29°C
18°C
chronic
48h
24h
C
Chronic tolerance, min
Sensitivity, min
nSyb,Gal80ts>Ricincs
nSyb,Gal80ts>+
+>Ricincs
MB(all)R13F02>Ricincs
MB(all)R13F02>+
MB(all)R13F02>Mef2.EnR
+>Mef2.EnR
MB(all)R13F02>Nmdar1.IR
+>Nmdar1.IR
MB(all)R13F02>CaMKIIT287D
+>CaMKIIT287D
control
Rut1

Figure 6. KCs are dispensable for protein synthesis-dependent chronic tolerance.

A) Neuronal protein synthesis is required for chronic tolerance at any phase, not sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

B) KC protein synthesis, as targeted by *R13F02-Gal4*, is dispensable for chronic tolerance at multiple phases and sedation sensitivity. Tolerance: all One-way ANOVAs. Sensitivity: One-way ANOVA, Brown-Forsythe ANOVA, One-way ANOVA.

C) KC Mef2 signaling is dispensable for chronic tolerance and sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

D) KC *Nmdar1* is dispensable for chronic tolerance, but required for sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

E) KC *CaMKII* is dispensable for chronic tolerance and sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: One-way ANOVA.

F) *Rut* supports chronic tolerance and sedation sensitivity. Tolerance: Mann-Whitney test. Sensitivity: Unpaired t test.

Figure 7

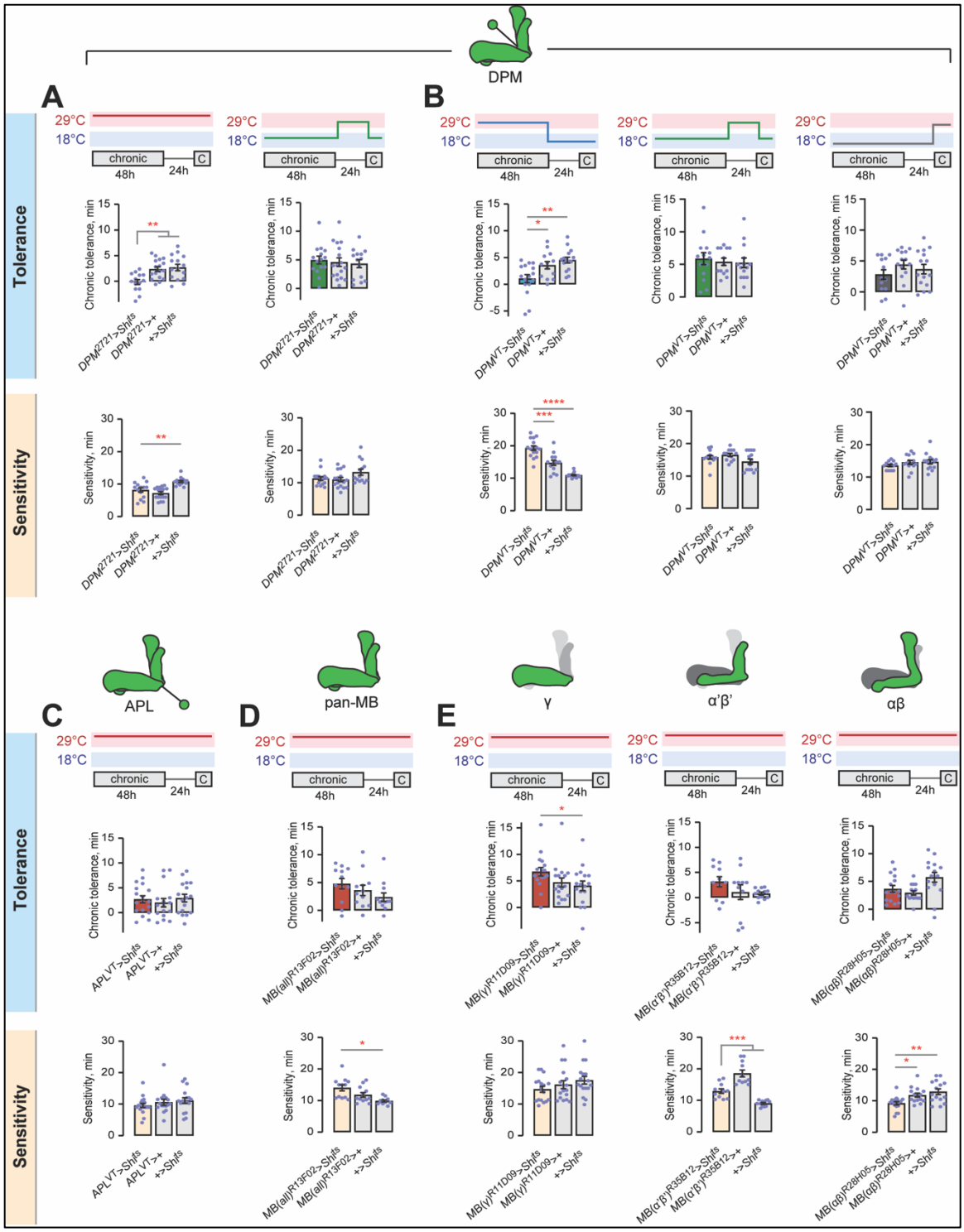

Figure 7. Temporally precise DPM activity induces chronic tolerance.

A) Left: DPM *Shi*-mediated activity, as targeted by *2721-Gal4*, supports chronic tolerance at any phase, not sedation sensitivity. Right: DPM *Shi*-mediated activity, as targeted by

2721-Gal4, is dispensable for chronic tolerance at consolidation and sedation sensitivity Tolerance: both One-way ANOVAs. Sensitivity: Brown-Forsythe ANOVA, Kruskal-Wallis ANOVA.

B) Left: DPM *Shi*-mediated activity, as targeted by *VT64246-Gal4*, supports chronic tolerance acquisition and sedation sensitivity. Center: DPM *Shi*-mediated activity, as targeted by *VT64246-Gal4*, is dispensable for chronic tolerance consolidation and sedation sensitivity. Right: DPM *Shi*-mediated activity, as targeted by *VT64246-Gal4*, is dispensable for chronic tolerance expression and sedation sensitivity. Tolerance: all One-way ANOVAs. Sensitivity: Brown-Forsythe ANOVA, Brown-Forsythe ANOVA, One-way ANOVA.

C) APL *Shi*-mediated activity is dispensable for chronic tolerance at any phase and sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: Kruskal-Wallis ANOVA.

D) KC *Shi*-mediated activity, as targeted by *R13F02-Gal4*, is dispensable for chronic tolerance at any phase and sedation sensitivity. Tolerance: One-way ANOVA. Sensitivity: Brown-Forsythe ANOVA.

E) KC *Shi*-mediated activity, as targeted by multiple KC lobe drivers, is dispensable for chronic tolerance at any phase and sedation sensitivity. Tolerance: Kruskal-Wallis ANOVA, Brown-Forsythe ANOVA, Brown-Forsythe ANOVA. Sensitivity: Kruskal-Wallis ANOVA, Brown-Forsythe ANOVA, One-way ANOVA.

Figure 8

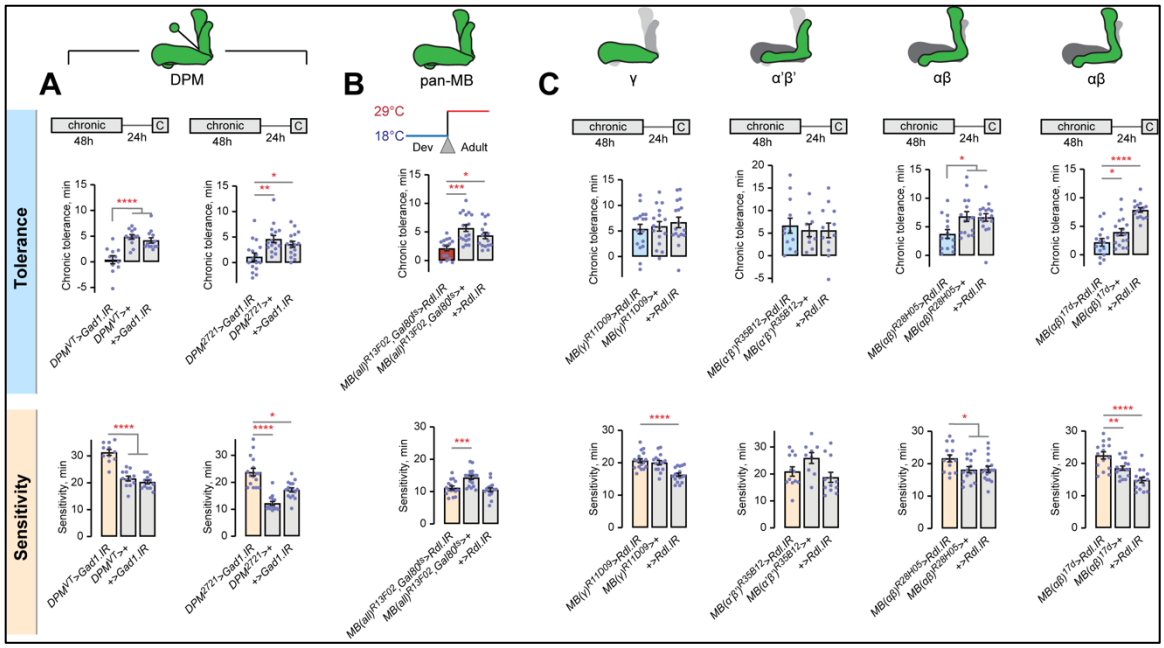

Figure 8. The DPM GABAergic repression of KCs is compartmentalized to support chronic tolerance.

A) Left: DPM GABA synthesis, as targeted by *VT64246-Gal4*, supports chronic tolerance and sedation sensitivity. Right: DPM GABA synthesis, as targeted by *2721-Gal4*, supports chronic tolerance and sedation sensitivity. Tolerance: One-way ANOVA, Kruskal-Wallis ANOVA. Sensitivity: One-way ANOVA, Kruskal-Wallis ANOVA.

B) KC *Rdl*, as targeted by *R13F02-Gal4*, supports chronic tolerance, not sedation sensitivity. Tolerance: Kruskal-Wallis ANOVA. Sensitivity: One-way ANOVA.

C) KC α/β *Rdl* supports chronic tolerance and sedation sensitivity. Tolerance: all One-way ANOVAs. Sensitivity: all One-way ANOVAs.

Figure 9

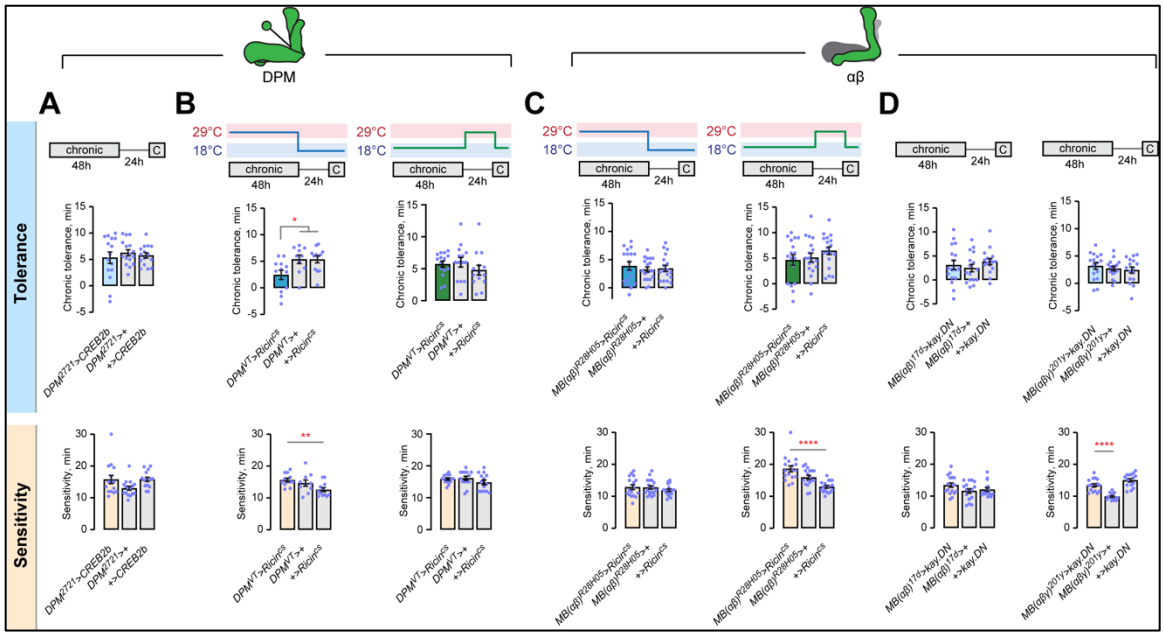

Figure 9. DPMs require protein synthesis, not CREB, to support chronic tolerance.

A) DPM CREB is dispensable for chronic tolerance and sedation sensitivity. Tolerance: Brown-Forsythe ANOVA. Sensitivity: Kruskal-Wallis ANOVA.

B) Left: DPM protein synthesis, as targeted by *VT64246-Gal4*, supports chronic tolerance acquisition, not sedation sensitivity. Right: DPM protein synthesis, as targeted by *VT64246-Gal4*, is dispensable for chronic tolerance consolidation and sedation sensitivity. Tolerance: Kruskal-Wallis ANOVA, One-way ANOVA. Sensitivity: One-way ANOVA, Brown-Forsythe ANOVA.

C) Left: KC α/β protein synthesis is dispensable for chronic tolerance acquisition and sedation sensitivity. Right: KC α/β protein synthesis is dispensable for chronic tolerance consolidation and sedation sensitivity. Tolerance: Brown-Forsythe ANOVA, One-way ANOVA. Sensitivity: Brown-Forsythe ANOVA, Kruskal-Wallis ANOVA.

D) KC α/β *kay* signaling, as targeted by multiple drivers, is dispensable for chronic tolerance and sedation sensitivity. Tolerance: both One-way ANOVAs. Sensitivity: One-way ANOVA, Brown-Forsythe ANOVA.

Figure 10

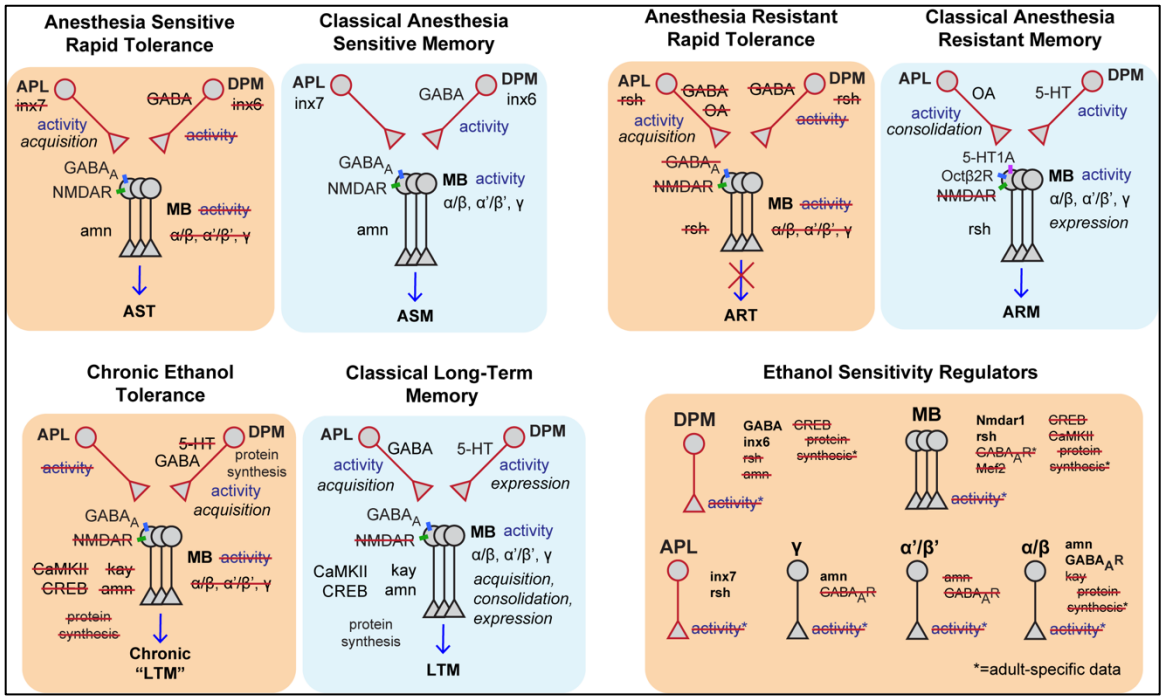

Figure 10. Summary diagrams of mushroom body genes and circuits for *Drosophila* memory and ethanol behaviors.

Orange boxes contain known pathways for ethanol tolerance and sensitivity. Blue boxes contain known pathways for associative memory.

Discussion

Here, we present a model describing how the type of initial ethanol experience not only induces separate forms of tolerance, but also engages unique memory encoding pathways (**Fig. 10**). For rapid tolerance, an acute, sedating dose of ethanol induces multiple time-dependent components of tolerance that are distributed across the *Drosophila* brain. First, an AST trace occurs in KCs, as it requires *Rdl* and *Nmdar* in all lobes, and *amn* in just the γ lobe. One way that AST differs from ASM is the latter requires *amn* in α/β KCs (Turrel et al., 2018). Second, an ART trace occurs via both APL induction and *rsh* in a non-KC site. ART is induced by the APLs separately from aversively-conditioned ARM; *Tbh*-mediated octopamine release from the APLs is dispensable for ART, yet required for ARM and targets α'/β' *OctB2* receptors (Wu et al., 2013). Moreover, *rsh* acts in KCs for ARM, not ART. For chronic tolerance, a continuous low dose of ethanol engages a separate inhibitory circuit in the *Drosophila* mushroom bodies. The DPMs release GABA to inhibit α/β KCs during the induction of chronic tolerance, and this silencing likely prevents their activity, PSD encoding, and *kay*-encoding capacity. This is one major difference between chronic tolerance and traditional learning and memory considering that α/β KCs classically hold LTM of associative stimuli (Miyashita et al., 2018; Yu et al., 2006).

We recognize that other extrinsic MB neurons, for example DANs and MBONs, remain untested in this body of work. The actions of these neurons may directly regulate tolerance, or do so indirectly via modification of APL and DPM signaling or alteration of the value of their transmitted information. Compared with previous studies, here the usage of spatiotemporally precise tools to dissect ethanol behaviors have clarified that Kenyon cells play a less central role than previously thought on tolerance formation and storage. For example, original evidence that hyperpolarization of all KCs with Kir2 abolishes chronic tolerance (**Chap. 1 Fig. 5B**) (Larnerd et al., 2023) is met with evidence that *Shi*-mediated inactivation of the same population is dispensable (**Chap. 2 Fig. 7D**). These results can coexist, considering the separate methods of neuronal inactivation, and that hyperpolarization may affect the voltage-sensitive $GABA_A$ receptors that are required for chronic tolerance (**Chap. 2 Fig. 8B**). Similarly for rapid tolerance, there is a neural synaptobrevin-dependent role (Engel et al., 2016; Lange & Wolf, 2023), *amnesiac* neuropeptidergic role (**Chap. 2 Fig. 2A, 2C**), but *Shi*-independent role (**Chap. 2 Fig. 5B**) for Kenyon cells. Further research, development of the effector tools that probe function, and refinement of the neuronal drivers that target key brain regions, will make clearer the genetic and circuit functions of intrinsic and extrinsic MB neurons on ethanol behaviors.

Of particular interest for future mechanistic studies on MB tolerance encoding pathways is the AST-specific role of *amn*, *rut*, and *Nmdar1*. Indeed, many cAMP signaling components display both deficits in learning and memory and in ethanol behaviors when mutated, such as *rut*, *PKA*, and *elm* (Berger et al., 2004; LaFerriere et al., 2008; Moore et al., 1998). Dysregulated cAMP signaling alters both consolidated and labile forms of ITM. Protein Kinase A in the MBs specifically inhibits ARM, and the *dunce* phosphodiesterase, that degrades cAMP, acts at multiple nodes in the olfactory pathway to support ARM, not ASM (Horiuchi et al., 2008; Scheunemann et al., 2012). Conversely, AKAPs and the *rut* AC function in the MBs to support ASM, not ARM (Scheunemann et al., 2012; Schwaerzel et al., 2007). Uncovering how these cAMP pathway components are activated or inhibited by the pattern of ethanol intake will inform and help predict what specific downstream changes are important for tolerance.

Both ART and chronic tolerance exemplify consolidated tolerance because they are unaffected by amnestic cold shock. So, could chronic tolerance have any ARM components to it? Our chronic ethanol paradigm mirrors a massed training protocol that creates ARM, however current evidence suggests that chronic tolerance is distinct from ARM in key ways. First, there are two types of ARM: ARM #1 is produced by single cycle training and is not sensitive to cold shock, while ARM #2 is produced by massed training and is sensitive to cold shock (Bourouliti & Skoulakis, 2022b). Both ARM types do not require protein synthesis. Both ARM types do require the *radish* GTPase (Bourouliti & Skoulakis, 2022a). In contrast, chronic ethanol creates tolerance that is resistant to amnestic cold shock at 3hr and 24hr timepoints (**Chap. 1 Fig. 6A, 6B**) (Larnerd et al., 2023). Also, chronic tolerance does require protein synthesis (**Chap. 2 Fig. 6A**) (Berger et al., 2004), and it does not require *radish* (**Chap. 1 Fig. 6A, 6C**) (Larnerd et al., 2023). Therefore, chronic tolerance shares the operational definitions, but lacks the mechanistic components, of ARM. Interestingly, transferring the amnestic operations that erase labile memories into the alcohol behavior field has revealed a possible mode of intervention for AUD too. For example, psychiatric procedures that reduce the intensity of persistent fear memories employ reconsolidation-mediated lability on these inappropriately learned events. Forcing tolerance states to become labile to intentionally erase them may rescue ethanol sensitivity and ultimately reduce further drinking.

Inhibitory circuits in the mushroom bodies are a candidate site for ethanol's pharmacological actions and they are critical for olfactory learning, partly because APL inhibition contributes to odor discrimination (Amin et al., 2020; Liu & Davis, 2009; Zhang & Roman, 2013). Even if the APLs do sparsely encode ethanol as an olfactory stimulus, it is unlikely that this olfactory discrimination solely induces tolerance. First, the APLs are dispensable for chronic ethanol tolerance (**Chap. 2 Fig. 7C**). Second, cross tolerance to the sedating effects of alcohol exists between ethanol and other odorants with sedative properties, such as benzyl alcohol (Cowmeadow et al., 2006), suggesting pharmacological action. The ability of ethanol to diffuse through membranes and indiscriminately affect neuronal processes likely presents a unique challenge for the brain to encode the drug experience. For chronic tolerance, the distinct encoding into the DPMs perhaps occurs due to both ethanol's volatility and the paradigm's long duration. Because, compared to stimuli like OCT that use canonical olfactory pathways, volatile benzaldehyde has multiple sensory routes and requires DPM activity during acquisition, not other stages of ITM (Keene et al., 2004). Also, the longer form of training that creates LTM, spaced conditioning, causes increased odor responses in the DPMs (Yu et al., 2005). These criteria may hold true for chronic ethanol tolerance.

Regardless of why rapid and chronic tolerance utilize mutually exclusive encoding by APL and DPM neurons, clearly these large interneurons permit normal tolerance through inhibition. Silenced KCs may prevent a coincidence detection mechanism from occurring, that would otherwise drive associative learning of unimportant internal and/or environmental cues associated with the inebriated state. Indeed, locating a calcium response (TRIC signal) in the MBs from acute ethanol has proven challenging (Merriman & Petruccelli, 2021). Regardless, ethanol does cause behavioral plasticity in the MBs, considering that associative preference or aversion for alcohol requires multiple, even sequential, circuits there (Kaun et al., 2011). Moreover, shutdown of the MBs may be important for tolerance encoding by favoring a separate circuit in the *Drosophila* brain.

For example, the DPMs promote sleep by inhibiting wake-promoting α'/β' neurons with GABA (Haynes et al., 2015). Thus, it may be that DPM-driven PSD chronic tolerance interacts with circadian circuitry, as does rapid tolerance (Lange & Wolf, 2023). Testing these relationships and cataloguing the roles of other neuroanatomical and transmitter systems on tolerance will inform how ethanol instigates maladapted behavior.

Chapter 3: Conclusions

Conclusions

The pattern and concentration of initial ethanol exposure causes operationally distinct types of ethanol tolerance to form. We identified and characterized separate molecular or neural circuit mechanisms for four types of ethanol tolerance: 1) anesthesia-sensitive rapid tolerance, 2) anesthesia-resistant rapid tolerance, 3) chronic tolerance, and 4) repeated tolerance. The multiple forms of ethanol memory traces described here are genetically tractable for understanding how initial forms of ethanol-induced neural plasticity form a substrate for the longer-term brain changes associated with AUD.

Quantitatively, the rules for ethanol tolerance follow the logic of a mathematical piecewise function. This is because the extent and site of action of tolerance (the y function) differs based on the initial dosage and timing of ethanol (the x input). For example, when x is an acute high dose, a multi-trace type of *Sirt1*-mediated rapid tolerance occurs at distinct sites of the mushroom bodies. When x is a chronic low dose, a consolidated and long-lasting type of *Sirt1*-suppressed chronic tolerance occurs in sites other than the mushroom bodies. This equational description of tolerance is likely incomplete, but demonstrates the point that tolerance is complex.

The APL- and DPM-mediated inhibitory pathways for mushroom body shutdown may be an underlying contributor for state-dependent learning, the observation that ethanol-adapted organisms learn best in either the naïve or adapted state only (Jung et al., 2023; Robinson & Atkinson, 2013). *Shi*-independent KC function may be a feature, not a bug, of the broad neural circuits required to produce normal tolerance and learning mechanisms. It is unknown whether ethanol itself, or its associated cues, definitively provide sufficient context for associative pairing. It is clear however that the reactivation of maladapted circuits drives drug seeking and relapse, thus externally trained factors play a role in triggering ethanol behaviors even after long durations. Unlike traditional LTM, context-dependent LTM in flies occurs immediately without the need for protein synthesis or retrieval from the mushroom bodies. Instead, context-dependent LTM requires the original training context and recall from the lateral horn that regulates innate behaviors (B. Zhao et al., 2019). So, alternate memory sites are being characterized that may harbor the distinct features needed for ethanol associations.

Unbiased approaches will be key to identify other brain regions critical for tolerance, whether associative or not. Using RNA-seq to understand the transcriptional landscape and Mass Spectrometry to understand the translational landscape of different ethanol experiences will significantly advance the field. Also, employing unbiased approaches to probe the chromatin state, like 4-C, will provide complementary resolution of the genetic programs recruited for ethanol behaviors. Interestingly, chromatin regulators and transcriptional machinery shifts over the course of initial LTM storage and long-term maintenance (Hirano et al., 2016; Xu et al., 2014), and this same premise may hold true for tolerance. In particular, probing *Sirt1* will be important, since we speculate that this HDAC can be altered by experience and thus epigenetically set the ability for ethanol memories to be encoded.

The appetitive aspects of tolerance described here likely contribute to the increased setpoint of drinking that is required to achieve target internal states. Repeated tolerance showed aversive properties, but likely has key neural features beyond what this study explored. Repeated tolerance likely remains a risk factor for AUD considering that chronic

intermittent exposures have successfully modelled sustained drinking in mammalian models. Thus, there are likely multiple routes toward AUD, even if operationally distinct patterns of ethanol use induce unique genes and circuits to create the appropriate tolerance response. The future is bright for the value of these models.

Materials and Methods

Drosophila Culturing and Strains

Culturing: Strains used in this study were outcrossed for five generations to the Berlin genetic background carrying either the vermillion or w^{1118} marker mutation. The w^{1118} Berlin strain served as a control for loss-of-function mutants. The outcrossing of *CaMKII.T287D* occurred thrice and that of ITM mutants included *FM7* balancers. Flies were cultured on standard cornmeal/molasses/yeast medium at 25°C and 60% relative humidity under a 12 hr light/dark schedule. Flies for thermogenetic experiments were cultured the same, except at 18°C. All experiments used adult male flies that recovered from CO_2 collection at least 1d before any behavioral paradigms.

Strains: The following fly strains were used:

Strain Name	Source	Stock Number
UAS-Gad1.IR	BDSC	28079
UAS-amn.IR	VDRC	5606
UAS-rsh.IR	VDRC	39932
UAS-rsh.IR	BDSC	36758
repo-Gal4	BDSC	7415
UAS-TeTx	Sean Sweeney	
UAS-Kir2::eGFP	BDSC	6595
Rad^{1}	BDSC	79209
Amn^{1}	BDSC	5954
Rut^{1}	BDSC	9404
$Hr38^{y214}$	Carl Thummel	
elav(c155)-Gal4	BDSC	458
R13F02-Gal4	BDSC	48571
R11D09-Gal4	BDSC	48456
R35B12-Gal4	BDSC	49822
R28H05-Gal4	BDSC	49472
2721-Gal4	BDSC	2721
GH146-Gal4	BDSC	30026
VT43924-Gal4	VDRC	201194
UAS-Tbh.IR	BDSC	27667
VT64246-Gal4	VDRC	204311
UAS-inx6.IR	VDRC	8638
UAS-inx7.IR	VDRC	22948
UAS-Rdl.IR	Ron Davis	
$UAS\text{-}Shibire^{ts}$	Janelia	1117405
GAD1-Gal80	Tim Lebestky	
$tub\text{-}Gal80^{ts}$	BDSC	7019
R19B03-Gal4	BDSC	49830

Sirt1$^{2A-7-11}$	BDSC	8838
UAS-Sirt1.IR 32481	BDSC	32481
R16A06-Gal4	BDSC	48709
17d-Gal4	BDSC	51631
201y-Gal4	BDSC	4440
UAS-Nmdar1.IR	Chia-Lin Wu	
UAS-Mef2.EnR	Justin Blau	
UAS-Creb2b	BDSC	7219
UAS-Ricin^{cs}	BDSC	38624
UAS-kay.DN	Minoru Saitoe	
UAS-myr::GFP	Janelia	1116842
UAS-Ddc.IR	BDSC	27030
nSyb-Gal4	Julie Simpson	
UAS-CaMKII^{T287D}	Michael Crickmore	

Ethanol Behaviors

Rapid Tolerance: Ethanol sensitivity and rapid tolerance were measured as previously described (Engel et al., 2016). Briefly, groups of 20 genetically identical flies (n=1) were exposed to 55% ethanol vapor or 100% humidified air, and the number of flies that lost the righting reflex were counted at 6min intervals. The time to 50% sedation (ST50) was calculated for each group, and the experiment was repeated across different days and from different parental crosses. Flies were allowed to rest for 3.5hrs and re-exposed to an identical concentration of ethanol vapor. Rapid tolerance was calculated as the difference in ST50 between the two exposures.

Chronic tolerance: Flies in perforated 50 mL conical tubes with 5 mL of food were placed in a temperature-controlled chamber that was perfused with 15% ethanol vapor or humidified air for 48hrs. Chronic tolerance was measured between groups pre-exposed to ethanol minus air, with random assignment within day.

Repeated tolerance: Flies were exposed to 42% ethanol vapor or 100% humidified air for 20min every 24hr for 4d. Repeated tolerance was measured between groups pre-exposed to ethanol minus air, with random assignment within day.

Ethanol preference: The capillary feeding assay (CAFE) was used as previously described (Devineni & Heberlein, 2009; Ja et al., 2007). Groups of eight flies were exposed to either 55% ethanol or 100% humidified air alone for 20min. After 16hr 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.

Cold Shock Anesthesia

A brief anesthetic cold shock was administered to flies either 30min after a tolerance-inducing dose of ethanol (Larnerd et al., 2023), or 30min before E2. Standard fly vials housing ~20 flies were placed in a 4°C ice bath for 3min to achieve anesthetic cold shock. Observationally, flies quickly lose locomotion on ice then regain it upon return to 25°C.

Drug Treatments

Nicotinamide (70 mM, Sigma-Aldrich, St Louis, MO USA) or Trichostatin A (8 μM, Sigma-Aldrich) was fed to flies dissolved in 5% sucrose/2% yeast extract on Whatman filter paper for 24hrs.

RNA Measurement

RNA was extracted from heads using Trizol (Thermo Fisher Scientific), treated with DNase (Promega), and reverse-transcribed using MultiScribe (Applied Biosystems). Quantitative PCR reactions were performed on the qTOWER^{3}84 machine (Genesee Scientific) using the SYBR Green method (Bio-Rad) and custom designed primers (Integrated DNA Technologies). RpL32 was used to normalize Ct values, expression of genes of interest was calculated using the ΔΔCt method, and the mean expression was calculated from multiple independent biological replicates.

Oligonucleotide primer sequences:
Hr38: GAGTGGCTCAACGACATCAT (F), CGTTCTGTGATCAGGGTTAGG (R);
Sr: CCGAGTATGCCGCTCAATTA (F), GGCGTATGGTGGTGATAAGG (R);
Jra: GTTCCCACCCACTGATTGA (F), GCTTGTTCTTGGCACTCTTG (R);
kay: CCGATACTTCAAGTGCCCATAC (F), CCAGGACATTGGAGAAGTTGTT (R);
Sirt1: CGGTGGCCGTTACTGAGGAGGA (F), TACTCATCCGGCAGTCCCTCGC (R);
RpL32: GTTCGATCCGTAACCGATGT (F), CCAGTCGGATCGATATGCTAA (R).

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 NAD-ADH Reagent kit following the manufacturer's protocol (Sigma-Aldrich, N7160). To calculate the ethanol concentration in flies, the volume of one fly was estimated to be 1μL.

Statistics

For all experiments, the experimental manipulation was tested in the same session as the genetically matched or treatment-matched controls. Data was collected across multiple days with progeny from repeat parental crosses, then collated together without between-day adjustments. Untransformed (raw) data were inputted into GraphPad Prism 10 and used for the following statistical analyses: One-sample t test; Wilcoxon signed-rank test; Unpaired t test for normally distributed data; Welch's t test for data that fails the F test for variance; Mann-Whitney test for nonparametric data that fails the Shapiro–Wilk normality test; One-way ANOVA with Tukey's post hoc test for normally distributed data; Brown–Forsythe test with Dunnett's post hoc test for data with unequal standard deviations; and Kruskal–Wallis test with Dunn's post hoc test for nonparametric data that fails the Shapiro–Wilk normality test. Each figure legend lists the type of statistical analysis performed on graphed data from left to right. For experiments designed with more than one control, statistical significance is only interpreted when each control is different from the experimental. These interpretations are shown as significance

indicators on the figures based on the results of t tests or ANOVA post hoc tests (****$p \leq 0.0001$; ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$; and ns, $p > 0.05$). Quantitative data are the mean and error bars represent the SEM.

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