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Alcohol sensitivity and tolerance encoding in sleep regulatory circadian neurons in Drosophila

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Abstract

Alcohol tolerance is a simple form of behavioural and neural plasticity that occurs with the first drink. Neural plasticity in tolerance is likely a substrate for longer term adaptations that can lead to alcohol use disorder. Drosophila develop tolerance with characteristics similar to vertebrates, and it is a useful model for determining the molecular and circuit encoding mechanisms in detail. Rapid tolerance, measured after the first alcohol exposure is completely metabolized, is localized to specific brain regions that are not interconnected in an obvious way. We used a forward neuroanatomical screen to identify three new neural sites for rapid tolerance encoding. One of these was composed of two groups of neurons, the DN1a and DN1p glutamatergic neurons, that are part of the Drosophila circadian clock. We localized rapid tolerance to the two DN1a neurons that regulate arousal by light at night, temperature-dependent sleep timing, and night-time sleep. Two clock neurons that regulate evening activity, LNd6 and the 5th LNv, are postsynaptic to the DN1as, and they promote rapid tolerance via the metabotropic glutamate receptor. Thus, rapid tolerance to alcohol overlaps with sleep regulatory neural circuitry, suggesting a mechanistic link.

Keywords

alcohol tolerance; circadian rhythms; circuitry; Drosophila; sleep

1 | INTRODUCTION

Alcohol use disorder (AUD) is a progressive, chronic, and recurring brain disease that causes extraordinarily long-term changes to brain function. Multiple forms of behavioural

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AUTHOR CONTRIBUTIONS

Anthony P. Lange and Fred W. Wolf were responsible for the study concept and design. Anthony P. Lange performed all experiments. Anthony P. Lange and Fred W. Wolf performed data analysis. Anthony P. Lange and Fred W. Wolf drafted the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

adaptations to ethanol, the active ingredient in alcohol, occur that are mostly defined operationally. Determining their relative importance for AUDs and their interconnectedness will help determine the longitudinal and spatial pathways in addiction. Longer term forms of adaption to ethanol likely build on simpler early forms. Early adaptations are amenable to complete molecular and neural circuit definition.

Ethanol tolerance is a simple and early adaptation that is defined as the acquired resistance to the pharmacological effects of the drug. Tolerance can facilitate increased alcohol intake in an attempt to achieve the same pharmacological effect, a key factor in developing AUD.[1] Moreover, ethanol resistance is strongly correlated with the development of AUD.[2] Tolerance is classically divided into three forms: acute (acquired within a drinking session), rapid (expressed after the first drink is completely metabolized), and chronic.[3] The fly Drosophila exhibits all three forms of tolerance.[4,5] Rapid tolerance is currently the best characterized form in Drosophila (Figure 1A). An acute and moderately sedating dose of ethanol results in tolerance to its inebriating and sedating properties, sensitization to its locomotor activating properties, and it primes flies for developing ethanol preference (Figure 1B).[4,6,7] Molecular parallels exist to early forms of ethanol-induced neural plasticity in mammals.[6,8–14]

Defining the neural circuit that encodes rapid ethanol tolerance is essential for understanding the mechanisms and contexts of ethanol action. Current anatomical localization of rapid tolerance includes the α/β lobes of the mushroom bodies, the ellipsoid body, the perineurial glia, and some other less well-defined sites[12,15–19] (Figure 1C). These anatomical sites provide some insight into the nature of ethanol tolerance, based on their characterization in other behaviours. For example, the mushroom bodies are a major site of learning and memory in Drosophila.[20] The mushroom bodies play multiple roles in ethanol behaviours including the coding of preference and reward learning, consolidation, and retrieval.[21,22] The ellipsoid body is a compass for flight and locomotor navigation, and it also functions in sleep and arousal state.[23,24] The perineurial glia form the interface between the brain and the circulatory system; their role in brain physiology is less well understood. These early attempts at building a tolerance circuit were largely limited to well-studied neuropils in the fly brain, whereas 80%–90% of neurons in the fly brain are not in these neuropils.

Advances in genetic targeting of individual neurons in the fly brain, coupled with the advent of complete brain connectomics, promises to make the whole tolerance circuit available for characterization.[25,26] However, there exists no short path connectivity between the brain regions known to function in rapid tolerance, and it is not known how tolerance information flows in and out of the defined neurons. For example, output from the mushroom body intrinsic neurons is via the mushroom body output neurons (MBONs).[27] A subset of MBONs synapse onto fan-shaped body neurons, which are in turn synaptically connected to ellipsoid body neurons.[28] There are other paths that tolerance information could take between these brain structures, and the direction of information flow is not known. Alternatively, the currently known tolerance brain regions could parallel process separable aspects of tolerance. Finding additional tolerance circuitry can help us build better models of tolerance encoding.

We performed a functional anatomical screen for new rapid tolerance neurons in the Drosophila brain. We characterized one of three new sites, uncovering a role for the glutamatergic DN1a circadian clock neurons. The DN1a neurons regulate the timing and quality of evening sleep in relation to environmental inputs, and they promote rapid ethanol tolerance development. Additional clock neurons that control evening behaviour are postsynaptic to the DN1as and are required for rapid ethanol tolerance development. Both the mushroom bodies and the ellipsoid body function in sleep, suggesting a relationship between sleep and rapid tolerance.

2 | RESULTS

Rapid ethanol tolerance (hereafter referred to as tolerance) is thought to involve changes in presynaptic function, based in part on changes in expression of genes encoding the $Ca_V2.1$ channel Cacophony (*cac*), the presynaptic kinase Cdk5, and the synaptic vesicle regulator Synapsin.[12,15,29] The mushroom body α/β lobes require *Sirt1* for rapid tolerance, and acute ethanol regulation of presynaptic gene expression is lost in *Sirt1* null mutant flies. Hence, we used the mushroom body α/β lobes to test if decreasing expression of *cac* and *Cdk5* affects tolerance development. We first verified that silencing synaptic output from the α/β lobe neurons decreased tolerance, by expressing the tetanus toxin short chain (*UAS-TeTx*) specifically in the α/β lobes using the *17d-Gal4* transgene driver (Figure 1D). Silencing synaptic output decreased tolerance and did not affect naive ethanol sensitivity (Figure 1E,E′), as previously reported.[12] Decreasing expression of either *cac* or *Cdk5* in the α/β lobes also decreased tolerance (Figure 1F,F′,G,G′). Thus, presynaptic release from the α/β lobe neurons is important for tolerance development. Unlike tolerance, ethanol sensitivity was only affected with RNAi against Cdk5. Cdk5 plays multiple roles in neuronal maturation and function in addition to regulation of presynaptic release, suggesting distinct roles for the α/β lobe neurons in sensitivity and tolerance.[30] Finally, note that ethanol sensitivity and tolerance varies over days and weeks, due to unknown extrinsic factors (e.g., compare Figure 1E′–F′).

To identify additional components of the neural circuitry for tolerance, we reasoned that decreasing presynaptic release in sparse patterns of neurons that may contain tolerance neurons would impact tolerance development. We manually selected *enhancer-Gal4* strains from the FlyLight collection that appeared to express in sparse and distinct patterns in the Drosophila brain.[31] To improve our chances of discovering new tolerance circuitry, we selected against patterns with *Gal4* expression in either the mushroom bodies or the ellipsoid body. Decreasing *cac* expression in neurons in 112 different *enhancer-Gal4* patterns resulted in either decreased or increased tolerance (Figure 2A). We retested 24 of the top hits that were greater than one standard deviation from the mean tolerance difference score. The retests included the full suite of genetic controls and were tested over eight to 10 trials. Three *enhancer-Gal4* strains in retest resulted in robust reduction in tolerance when driving *cac* RNAi: *R82F12, R18H11*, and *R79H04* (Figure 2B,B′). Blockade of synaptic release with TeTx also reduced tolerance in all three *enhancer-Gal4* patterns, indicating that neurotransmission is required for rapid tolerance in multiple different neurons in the Drosophila brain (Figure 2C,C′). Ethanol sensitivity was decreased by *cac* RNAi in *R82F12* and TeTx expression in *R79H04*, whereas sensitivity was increased by TeTx expression

in *R18H11*. Thus, ethanol sensitivity regulatory neurons exist in all three *enhancer-Gal4* expression patterns, but the pattern of effect was distinct from tolerance, suggesting sensitivity and tolerance are differently encoded in these patterns.

We visualized the neurons in the three *enhancer-Gal4* patterns by expressing plasma membrane-bound GFP (*UAS-myr-GFP*) in each pattern and performing immunohistochemistry and confocal microscopy (Figure 2D–F). All three *enhancer-Gal4* patterns expressed in sparse patterns of neurons in the brain, with *R18H11* appearing the sparsest. None expressed in the mushroom bodies, the ellipsoid bodies, or the perineurial glia. *R18H11* contains a subset of the neurons that comprise the circadian circuitry, the DN1 neurons that have cell bodies in the dorsal region of the brain, and this *Gal4* transgene was used previously to characterize the function of these clock neurons (Figure 2E).[32] The DN1 neurons form two clusters consisting of two DN1a neurons and seven DN1p neurons.[33,34]

Neurons in both the DN1a and DN1p clusters are glutamatergic. To ask if glutamatergic neurons are responsible for promoting tolerance, we expressed an RNAi for the vesicular glutamate transporter vGlut in each of the three *enhancer-Gal4* patterns. *vGlut* RNAi in *R82F12* and *R18H11* reduced tolerance, whereas it did not in *R79H04* (Figure 3A,A′). A second RNAi directed against *vGlut* also reduced tolerance when expressed in *R82F12* neurons, indicating that the effect on tolerance is due to the reduction in vGlut expression (Figure 3B, B′). Hence, *R79H04* contains nonglutamatergic tolerance neurons that are distinct from those found in other known tolerance *enhancer-Gal4* patterns. Additionally, it is likely that the glutamatergic DN1 neurons are responsible for promoting ethanol tolerance. Ethanol sensitivity was reduced by *vGlut* RNAi in all three *enhancer-Gal4* patterns. Taken together with the effects on sensitivity of reducing *cac* expression and blockade of synaptic release with TeTx (Figure 2B′,C′), there may exist distinct glutamatergic ethanol sensitivity neurons in each *enhancer-Gal4* pattern. To ask if the role of glutamatergic transmission in tolerance is an adult role, we conditionally expressed *vGlut* RNAi in all adult neurons. *Gal80ts* encodes a temperature-sensitive repressor of GAL4, such that it represses GAL4 activity at 18°C and is inactive and allows GAL4 activity at 29°C.[35] Tolerance was reduced when GAL4 was allowed to be active and drive *vGlut* RNAi only in adult neurons (Figure 3C,C′).

Our findings indicated that glutamatergic tolerance neurons are present in both the *R82F12* and the *R18H11 enhancer-Gal4* patterns, and that it is possible that the glutamatergic DN1 circadian neurons are tolerance neurons. Thus, we asked if the *R82F12 enhancer-Gal4* expression pattern includes the DN1 neurons. The DN1p neurons co-express the neuropeptide DH31 and glutamate, and the DN1a neurons co-express the neuropeptide CCHa1 and glutamate.[32,36] *R82F12* did not express in either the DN1p or DN1a clusters (Figure 3D,D′,E, E′). To further test for possible overlap in *R82F12* and *R18H11* glutamatergic tolerance neurons, we created a *split-Gal4*, with *R82F12* driving expression of the GAL4 activation domain (AD) and *R18H11* driving expression of the GAL4 DNA-binding domain (DBD). If glutamatergic tolerance neurons are shared between *R82F12* and *R18H11*, then reconstituted functional GAL4 in the *R82F12∩R18H11 split-Gal4* overlap expressing *vGlut* RNAi should result in decreased tolerance. No decrease in tolerance was observed (Figure 3F). Ethanol sensitivity was reduced, suggesting that *R82F12* and

R18H11 share glutamatergic ethanol sensitivity neurons (Figure 3F′). Imaging GFP in the *split-Gal4* pattern revealed selective expression in a cluster of dorsolateral neurons that may be the site of ethanol sensitivity encoding (Figure 3G). Finally, we found that decreased glutamatergic signalling in the DN1 neurons did not affect the absorption or metabolism of ethanol, indicating that changes in ethanol pharmacokinetics cannot explain the ethanol behavioural phenotypes (Figure 3H). Thus, *R82F12* and *R18H11* contain different glutamatergic tolerance neurons. We chose to focus on the DN1 neurons, because tools exist to precisely separate the DN1 neuron groups, and because prior research has assigned functions to them.

A panel of three additional *enhancer-Gal4* transgenes was used to determine which, if any, of the DN1 neuron groups promote ethanol tolerance (Figure 4A). *tim-Gal4* expresses in all clock neurons, including the DN1a and DN1p neurons, whereas *R16C05-Gal4* expresses specifically in the DN1a neurons and *R51H05-Gal4* express specifically in the DN1p neurons.[32,37] RNAi against *vGlut* in *tim-Gal4* and *R16C05-Gal4* reduced tolerance, whereas expression in *R51H05-Gal4* did not (Figure 4B). Thus, the pair of DN1a circadian clock neurons promote rapid tolerance development through glutamatergic signalling. Ethanol sensitivity was only affected in *R16C05* and *R51H05*, suggesting that ethanol sensitivity may map to non-clock neurons in these *enhancer-Gal4* patterns (Figure 4B′). Alternatively, there exist competing ethanol sensitivity and resistance neurons in the clock, resulting in an apparent lack of phenotype with *tim-Gal4*.

DN1a neurons set arousal thresholds to brief pulses of light during evening sleep, transmit temperature information into the circadian circuitry, and may promote photoentrainment of the clock by the visual system.[38–40] The synaptic connectivity of the DN1a neurons is well characterized from electron micrograph reconstructions and computational detection of synapses in the adult Drosophila brain.[34,41] Fifty neurons make five or more input synapses with each of the two DN1a neurons per brain hemisphere, and 58–63 neurons are postsynaptic to the DN1a neurons by the same criteria. The top 10 presynaptic and postsynaptic neurons are listed in Figure 4C. In particular, two circadian neurons that control evening activity, the LNd6 and 5th LNv (previously named the 5th s-LNv) are postsynaptic to the DN1a neurons, providing a connection to circadian pacemakers. The DN1a neurons make synaptic connections to these E2 evening neurons in two regions of the brain, the accessory medulla (aMe) and the Lateral Horn Anterior/Posterior Ventral (LHPV/LHAV) region (Figure 4C, lower panel). The LNd6 and 5th LNv are similar anatomically and by synaptic connectivity patterns, and they both extend branches into the Superior Medial Protocerebrum (SMP), a higher order processing region of the brain.[34]

We performed a test to ask if glutamatergic transmission from the DN1a neurons to the E2 evening neurons might be critical for rapid ethanol tolerance. The single metabotropic glutamate receptor in Drosophila, mGluR, functions in the LNd neurons that includes LNd6.[42] We reduced *mGluR* expression in the LNd6 and 5th LNv neurons (as well as other lateral clock neurons) using the *DvPdf-Gal4* driver, and found that tolerance was reduced and ethanol sensitivity was moderately reduced (Figure 4D,D′). The DN1a neurons are reported to be postsynaptic to PDF peptidergic morning clock neurons through the PDF receptor PDFR.[40] Reduction of *Pdfr* expression in the DN1a neurons, however, had no

effect on tolerance (Figure 4E,E′). Thus, E2 evening circadian neurons are important for the promotion of tolerance development; tolerance information may be transmitted from the glutamatergic DN1a sleep regulatory neurons via the metabotropic glutamate receptor to the E2 neurons.

3 | DISCUSSION

Our main conclusion is that rapid ethanol tolerance is encoded in part in the two glutamatergic DN1a dorsal clock neurons and the postsynaptic E2 clock neurons LNd6 and 5th LNv. These four clock neurons organize locomotor activity states and aspects of sleep. There exist intriguing relationships between the effects of ethanol, the circadian clock, and sleep in Drosophila and mammals.[43–46] Our findings identify specific glutamatergic neurons that likely encode aspects of these relationships.

What is the role of sleep regulating central clock neurons in ethanol tolerance? The DN1a dorsal clock neurons regulate aspects of sleep, with their known sleep roles regulated by environmental input. First, cold temperature suppresses DN1a neural activity through activation of cold-temperature-encoding TPN-II second-order projection neurons, resulting in a shift to earlier daytime sleep.[39,47,48] This role requires a functioning clock in the DN1a neurons. Second, DN1a neuronal activity specifically promotes nighttime sensitivity to light-induced locomotor startle responses.[40] At night the DN1a neurons increase their arborization and synaptic number in the accessory medulla region, and this remodelling is critical for the circadian time that light can alter startle sensitivity. Acute ethanol exposure alters the expression level of genes encoding presynaptic proteins, and we showed that these genes are critical for rapid tolerance development.[12] Thus, a potential mechanism for tolerance encoding directly in the DN1a neurons is an impact on synaptic plasticity. Based on current knowledge, we suspect that ethanol, by co-opting circadian changes in neuronal morphology in the DN1a circuit or by altering pre-existing synapses, may regulate aspects of wakefulness or arousal state to increase resistance to ethanol sedation. Similarly, ethanol-induced changes in the DN1a circuit might lead to changes in sleep. In humans, ethanol primarily affects nighttime sleep, potentially paralleling the nighttime specificity of the arousal-like function of the DN1a neurons in Drosophila.[49]

The DN1p dorsal clock neurons also regulate sleep, however glutamatergic signalling from this group of neurons is not required for rapid tolerance. Distinct DN1p neurons are sleep promoting and sleep suppressing, and there may exist a parallel segregation for tolerance.[50,51] Moreover, despite being glutamatergic, DN1p wake promotion is driven by the neuropeptide CNMa and sleep promotion is driven by another neuropeptide, AstC.[51,52] Thus, while our current data does not support a potential connection between ethanol tolerance and sleep regulation in the DN1p neurons, more targeted experiments are needed to make a formal conclusion.

The E2 neurons were recently well-segregated as an anatomically, and thus likely functionally, separate group of clock neurons.[34,37] Combined with prior findings, it is now clear that the E2 neurons share very similar gene expression profiles. Ion transport peptide (ITP) is expressed in both E2 neurons—the only two clock neurons to express the peptide.[53]

ITP in the clock neurons promotes daytime siestas and nighttime sleep redundantly with the pigment dispersing factor (PDF) neuropeptide, and it separately suppresses nighttime locomotor activity. Thus, both the DN1a neurons and the E2 neurons control aspects of sleep and circadian regulation of locomotor activity levels, strengthening the likelihood that rapid tolerance is tied to one or both behaviours. The E2 neuron outputs include other clock neurons, including the E1 evening LNds and the DN1ps, but not the DN1as, as well as many nonclock neurons.[34] Interestingly, the E2 neurons are not tightly coupled to the M group circadian pacemaker cells that set morning behaviours, supporting the notion that the E2 neurons shape aspects of circadian behaviour other than the daily cycle itself.[54] Hence rapid tolerance encoding may involve a complex clock circuit, or it may exit the circadian clock network via the E2 LNd6 or 5th LNv. The mushroom bodies and the ellipsoid bodies, other sites of rapid ethanol tolerance encoding, also have specific roles in sleep, suggesting that rapid tolerance and sleep are strongly interrelated.[55–57]

Sleep and rapid tolerance are also genetically and behaviourally connected in flies. Mutation of the key pacemaker genes *per, tim*, and *cyc* completely blocks rapid tolerance, whereas mutations in the *Clk* pacemaker gene did not.[58] It was concluded that disruption to central pacemaker genes but not the pacemaker itself blocks rapid tolerance. Sleep and sleep rebound persist in *per* and *tim* mutants, but sleep is distributed differently over 24 h.[59] Mutation of the learning and memory gene *dunce* results in sleep deficits and a failure to develop rapid tolerance.[17] The anatomical site of action for *dunce* in tolerance is defined by the *NP6510-Gal4* transgene that is not yet characterized for its expression in the clock neurons. Additionally, a subset of histone demethylases of the JmjC class regulate both sleep and rapid tolerance.[60,61] Sleep deprivation for 1 day prior to tolerance induction appears to increase rapid tolerance measured at 4 h, but it reduces tolerance at 24 h.[62] However, rapid tolerance does not appear to be regulated by circadian time.[63,64] Thus, current evidence supports a role for ethanol in regulating sleep that is causally connected to rapid tolerance development, however the clock does not appear to reciprocally regulate rapid tolerance development.

Glutamatergic transmission in Drosophila was indirectly implicated in tolerance in prior findings. The fly HOMER1/HOMER2 ortholog Homer is reduced in expression by ethanol exposure, and Homer promotes rapid tolerance development.[65] Homer proteins function as scaffolding proteins at glutamate receptor postsynaptic densities.[66] In mammals, alterations to glutamatergic neurotransmission are critical for ethanol sensitivity and ethanol adaptations, including rapid tolerance.[67,68] Moreover, glutamatergic signalling in mammals is important for aspects of central circadian pacemaker function that is altered by ethanol.[69]

Finally, ethanol sensitivity appears to map broadly to anatomical sites in the Drosophila brain, but in a different manner as compared to rapid tolerance. For example, glutamatergic promotion of ethanol sensitivity appears to map to neurons that are shared between *R18H11-Gal4* and *R82F12-Gal4*, whereas the rapid tolerance neurons in these two *enhancer-Gal4* patterns mapped to distinct groups of neurons. Additionally, glutamatergic ethanol sensitivity appeared to map to non-clock neurons, since *tim-Gal4* reduction in *vGlut* expression did not affect ethanol sensitivity. These findings are in accordance with prior

studies indicating functional separation of the ethanol sensitivity and rapid tolerance neural circuitry.[70]

4 | METHODS

4.1 | Drosophila culturing and strains

Drosophila melanogaster strains were reared on food composed of molasses (9%), cornmeal (6.75%), yeast (1.7%), and agar (1.2%) food at 25°C and 60% humidity on a 16:8 hour light/dark cycle (Darwin Chambers, MO). All strains were outcrossed for at least five generations to the Berlin genetic background strain carrying the w^{1118} marker mutation. The RNAi transgenes used in this study were validated previously.[42,71–73] Strains are listed in Table S1.

4.2 | Behavioural studies

Parental crosses were set up in Drosophila culturing bottles containing 50 mL of food. After 2 days, the parents were removed. Fourteen days later, 0- to 3-day-old genetically identical adult male progeny were collected in groups of 20 ($n = 1$) and allowed to recover from CO_2 anaesthesia for 2 days. Ethanol sensitivity and rapid tolerance were measured as previously described.[12] Briefly, groups were exposed to 55% ethanol vapour or 100% humidified air for 30–40 min; 55% ethanol is an intermediate ethanol dose that results in submaximal rapid tolerance and in 50% sedation in 12–20 min.[6] The number of flies that lost the righting reflex was counted at 10-min intervals. The time to 50% sedation (ST50) was calculated for each group (E1). Flies were allowed to rest for 3.5 h and re-exposed to an identical concentration of ethanol vapour (E2). Rapid tolerance was calculated as the difference in ST50, E2-E1.

4.3 | Ethanol absorption and metabolism

Groups of 20 flies were exposed to 30 min of 20% ethanol to avoid sedation and then frozen in liquid nitrogen either immediately for “absorption” samples, or 30 min later for “metabolism” samples. After homogenization in 50-mM Tris-HCl, pH 7.5, ethanol concentrations were measured using the NAD-ADH Reagent kit following the manufacturer’s protocol (Sigma-Aldrich, N7160). The 340-nm spectrophotometric absorbance values were converted to mM and adjusted by the estimated 1 μL volume of an average fly to calculate ethanol concentrations.

4.4 | Immunohistochemistry

Adult fly brains were dissected in PBS with 0.05% Triton X-100 (PBT), fixed overnight at 4°C in PBT with 2% paraformaldehyde, blocked in PBS with 0.5% Triton X-100 with 5% normal goat serum and 0.5% bovine serum albumin (HDB), and immunostained as described previously.[74] Antibodies and their concentrations are listed in Table S1. The brains were mounted in Vectashield (Vector Laboratories) and imaged on a Zeiss LSM-880 confocal microscope. Image stacks were processed in Fiji, and brightness and contrast were adjusted in Photoshop CC 2022 (Adobe).

4.5 | Statistical analysis

Experimental and genetically-matched or treatment-matched controls were tested in the same session in a balanced experimental design. Experiments were repeated across days with progeny from repeat parental crosses, and data from all days and crosses were collated together without between-day adjustments. Untransformed (raw) data were used for statistical analysis. Where the experimental group was compared to two or more control groups, significance was only interpreted when all controls were different from the experimental. Graph-Pad Prism 9.5.0 was used for one-way analysis of variance (ANOVA) with Tukey's post hoc test for normally distributed data, Kruskal–Wallis test with Dunn's post hoc test for nonparametric data, and Brown–Forsythe test with Dunnett's post hoc for data that fails the Shapiro–Wilk normality test. Significance indicators on the figures indicate the results of post hoc tests for significant effects by ANOVA (**** for $p \leq 0.0001$; *** for $p \leq 0.001$; ** for $p \leq 0.01$; * for $p \leq 0.05$; and ns for $p > 0.05$). Error bars represent the standard error of the mean (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study will be made available in the supporting information of this article.

REFERENCES

1. Elvig SK, McGinn MA, Smith C, Arends MA, Koob GF, Vendruscolo LF. Tolerance to alcohol: a critical yet understudied factor in alcohol addiction. Pharmacol Biochem Behav. 2021;204:173155. doi:10.1016/j.pbb.2021.173155 [PubMed: 33631255]
2. Schuckit MA. Low level of response to alcohol as a predictor of future alcoholism. Am J Psychiatry. 1994;151(2):184–189. doi:10.1176/ajp.151.2.184 [PubMed: 8296886]
3. Fadda F, Rossetti ZL. Chronic ethanol consumption: from neuroadaptation to neurodegeneration. Prog Neurobiol. 1998;56(4):385–431. doi:10.1016/S0301-0082(98)00032-X [PubMed: 9775400]
4. Scholz H, Ramond J, Singh CM, Heberlein U. Functional ethanol tolerance in Drosophila. Neuron. 2000;28(1):261–271. doi:10.1016/S0896-6273(00)00101-X [PubMed: 11086999]
5. Berger KH, Heberlein U, Moore MS. Rapid and chronic: two distinct forms of ethanol tolerance in Drosophila. Alcohol Clin Exp Res. 2004;28(10):1469–1480. doi:10.1097/01.ALC.0000141817.15993.98 [PubMed: 15597078]
6. Kong EC, Allouche L, Chapot PA, et al. Ethanol-regulated genes that contribute to ethanol sensitivity and rapid tolerance in Drosophila. Alcohol Clin Exp Res. 2010;34(2):302–316. doi:10.1111/j.1530-0277.2009.01093.x [PubMed: 19951294]

7. Peru Y, Colón de Portugal RL, Ojelade SA, Penninti PS, et al. Longlasting, experience-dependent alcohol preference Drosophila. in Addict Biol. 2014;19(3):392–401. doi:10.1111/adb.12105 [PubMed: 24164972]
8. Cowmeadow RB, Krishnan HR, Atkinson NS. The slowpoke gene is necessary for rapid ethanol tolerance in Drosophila. Alcohol Clin Exp Res. 2005;29(10):1777–1786. doi:10.1097/01.alc.0000183232.56788.62 [PubMed: 16269907]
9. Morozova TV, Anholt RR, Mackay TF. Transcriptional response to alcohol exposure in Drosophila melanogaster. Genome Biol. 2006;7(10):R95. doi:10.1186/gb-2006-7-10-r95 [PubMed: 17054780]
10. Sakharkar AJ, Zhang H, Tang L, Shi G, Pandey SC. Histone deacetylases (HDAC)-induced histone modifications in the amygdala: a role in rapid tolerance to the anxiolytic effects of ethanol. Alcohol Clin Exp Res. 2012;36(1):61–71. doi:10.1111/j.1530-0277.2011.01581.x [PubMed: 21790673]
11. Ghezzi A, Krishnan HR, Lew L, Prado FJ, Ong DS, Atkinson NS. Alcohol-induced histone acetylation reveals a gene network involved in alcohol tolerance. PLoS Genet. 2013;9(12):e1003986. doi:10.1371/journal.pgen.1003986 [PubMed: 24348266]
12. Engel GL, Marella S, Kaun KR, et al. Sir2/Sirt1 links acute inebriation to presynaptic changes and the development of alcohol tolerance, preference, and reward. J Neurosci. 2016;36(19):5241–5251. doi:10.1523/JNEUROSCI.0499-16.2016 [PubMed: 27170122]
13. Berkel TDM, Pandey SC. Emerging role of epigenetic mechanisms in alcohol addiction. Alcohol Clin Exp Res. 2017;41(4):666–680. doi:10.1111/acer.13338 [PubMed: 28111764]
14. Ranson DC, Ayoub SS, Corcoran O, Casalotti SO. Pharmacological targeting of the GABAB receptor alters Drosophila's behavioural responses to alcohol. Addict Biol. 2020;25(2):e12725. doi:10.1111/adb.12725 [PubMed: 30761704]
15. Ghezzi A, Al-Hasan YM, Krishnan HR, Wang Y, Atkinson NS. Functional mapping of the neuronal substrates for drug tolerance in Drosophila. Behav Genet. 2013;43(3):227–240. doi:10.1007/s10519-013-9583-0 [PubMed: 23371357]
16. Park A, Ghezzi A, Wijesekera TP, Atkinson NS. Genetics and genomics of alcohol responses in Drosophila. Neuropharmacology. 2017;122:22–35. doi:10.1016/j.neuropharm.2017.01.032 [PubMed: 28161376]
17. Ruppert M, Franz M, Saratsis A, et al. Hangover links nuclear RNA signaling to cAMP regulation via the phosphodiesterase 4d ortholog dunce. Cell Rep. 2017;18(2):533–544. doi:10.1016/j.celrep.2016.12.048 [PubMed: 28076795]
18. Parkhurst SJ, Adhikari P, Navarrete JS, Legendre A, Manansala M, Wolf FW. Perineurial barrier glia physically respond to alcohol in an Akap200-dependent manner to promote tolerance. Cell Rep. 2018;22(7):1647–1656. doi:10.1016/j.celrep.2018.01.049 [PubMed: 29444420]
19. Kang YY, Wachi Y, Engdorf E, et al. Normal ethanol sensitivity and rapid tolerance require the G protein receptor kinase 2 in ellipsoid body neurons in Drosophila. Alcohol Clin Exp Res. 2020;44(8):1686–1699. doi:10.1111/acer.14396 [PubMed: 32573992]
20. Modi MN, Shuai Y, Turner GC. The Drosophila mushroom body: from architecture to algorithm in a learning circuit. Annu Rev Neurosci. 2020;43(1):465–484. doi:10.1146/annurev-neuro-080317-0621333 [PubMed: 32283995]
21. Kaun KR, Azanchi R, Maung Z, Hirsh J, Heberlein U. A Drosophila model for alcohol reward. Nat Neurosci. 2011;14(5):612–619. doi:10.1038/nn.2805 [PubMed: 21499254]
22. Xu S, Chan T, Shah V, Zhang S, Pletcher SD, Roman G. The propensity for consuming ethanol in Drosophila requires rutabaga adenylyl cyclase expression within mushroom body neurons. Genes Brain Behav. 2012;11(6):727–739. doi:10.1111/j.1601-183X.2012.00810.x [PubMed: 22624869]
23. Seelig JD, Jayaraman V. Neural dynamics for landmark orientation and angular path integration. Nature. 2015;521(7551):186–191. doi:10.1038/nature14446 [PubMed: 25971509]
24. Andreani T, Rosensweig C, Sisobhan S, Ogunlana E, Kath W, Allada R. Circadian programming of the ellipsoid body sleep homeostat in Drosophila. Elife. 2022;11:e74327. doi:10.7554/eLife.74327 [PubMed: 35735904]
25. Luan H, Diao F, Scott RL, White BH. The Drosophila Split Gal4 system for neural circuit mapping. Front Neural Circuits. 2020;14:603397. doi:10.3389/fncir.2020.603397 [PubMed: 33240047]

26. Galili DS, Jefferis GS, Costa M. Connectomics and the neural basis of behaviour. Curr Opin Insect Sci. 2022;54:100968. doi:10.1016/j.cois.2022.100968 [PubMed: 36113710]
27. Aso Y, Sitaraman D, Ichinose T, et al. Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. Elife. 2014;3:e04580. doi:10.7554/eLife.04580 [PubMed: 25535794]
28. Hulse BK, Haberkern H, Franconville R, et al. A connectome of the Drosophila central complex reveals network motifs suitable for flexible navigation and context-dependent action selection. Elife. 2021;10:e66039. doi:10.7554/eLife.66039 [PubMed: 34696823]
29. Godenschwege TA, Reisch D, Diegelmann S, et al. Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. Eur J Neurosci. 2004;20(3):611–622. doi:10.1111/j.1460-9568.2004.03527.x [PubMed: 15255973]
30. Shah K, Rossie S. Tale of the good and the bad Cdk5: remodeling of the actin cytoskeleton in the brain. Mol Neurobiol. 2018;55(4):3426–3438. doi:10.1007/s12035-017-0525-3 [PubMed: 28502042]
31. Pfeiffer BD, Jenett A, Hammonds AS, et al. Tools for neuroanatomy and neurogenetics in Drosophila. Proc Natl Acad Sci U S A. 2008;105(28):9715–9720. doi:10.1073/pnas.0803697105 [PubMed: 18621688]
32. Kunst M, Hughes ME, Raccuglia D, et al. Calcitonin gene-related peptide neurons mediate sleep-specific circadian output in Drosophila. Curr Biol. 2014;24(22):2652–2664. doi:10.1016/j.cub.2014.09.077 [PubMed: 25455031]
33. Shafer OT, Helfrich-Förster C, Renn SCP, Taghert PH. Reevaluation of Drosophila melanogaster's neuronal circadian pacemakers reveals new neuronal classes. J Comp Neurol. 2006;498(2):180–193. doi:10.1002/cne.21021 [PubMed: 16856134]
34. Shafer OT, Gutierrez GJ, Li K, et al. Connectomic analysis of the Drosophila lateral neuron clock cells reveals the synaptic basis of functional pacemaker classes. Elife. 2022;11:e79139. doi:10.7554/eLife.79139 [PubMed: 35766361]
35. McGuire SE, le PT, Osborn AJ, Matsumoto K, Davis RL. Spatiotemporal rescue of memory dysfunction in Drosophila. Science. 2003;302(5651):1765–1768. doi:10.1126/science.1089035 [PubMed: 14657498]
36. Fujiwara Y, Hermann-Luibl C, Katsura M, et al. The CCHamide1 neuropeptide expressed in the anterior dorsal neuron 1 conveys a circadian signal to the ventral lateral neurons in Drosophila melanogaster. Front Physiol. 2018;9:1276. doi:10.3389/fphys.2018.01276 [PubMed: 30246807]
37. Schubert FK, Hagedorn N, Yoshii T, Helfrich-Förster C, Rieger D. Neuroanatomical details of the lateral neurons of Drosophila melanogaster support their functional role in the circadian system. J Comp Neurol. 2018;526(7):1209–1231. doi:10.1002/cne.24406 [PubMed: 29424420]
38. Li MT, Cao LH, Xiao N, et al. Hub-organized parallel circuits of central circadian pacemaker neurons for visual photoentrainment in Drosophila. Nat Commun. 2018;9(1):4247. doi:10.1038/s41467-018-06506-5 [PubMed: 30315165]
39. Alpert MH, Frank DD, Kaspi E, et al. A circuit encoding absolute cold temperature in Drosophila. Curr Biol. 2020;30(12):2275–2288. doi:10.1016/j.cub.2020.04.038 [PubMed: 32442464]
40. Song BJ, Sharp SJ, Rogulja D. Daily rewiring of a neural circuit generates a predictive model of environmental light. Sci Adv. 2021;7(13):eabe4284. doi:10.1126/sciadv.abe4284 [PubMed: 33762336]
41. Scheffer LK, Xu CS, Januszewski M, et al. A connectome and analysis of the adult Drosophila central brain. Elife. 2020;9:e57443. doi:10.7554/eLife.57443 [PubMed: 32880371]
42. Guo F, Yu J, Jung HJ, et al. Circadian neuron feedback controls the Drosophila sleep-activity profile. Nature. 2016;536(7616):292–297. doi:10.1038/nature19097 [PubMed: 27479324]
43. Spanagel R, Pendyala G, Abarca C, et al. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. Nat Med. 2005;11(1):35–42. doi:10.1038/nm1163 [PubMed: 15608650]
44. Perreau-Lenz S, Zghoul T, de Fonseca FR, Spanagel R, Bilbao A. Circadian regulation of central ethanol sensitivity by the mPer2 gene. Addict Biol. 2009;14(3):253–259. doi:10.1111/j.1369-1600.2009.00165.x [PubMed: 19523042]

45. Parekh PK, Ozburn AR, McClung CA. Circadian clock genes: effects on dopamine, reward and addiction. Alcohol. 2015;49(4):341–349. doi:10.1016/j.alcohol.2014.09.034 [PubMed: 25641765]
46. Lóopez-Muciño LA, García-García F, Cueto-Escobedo J, Acosta-Hernández M, Venebra-Muñoz A, Rodríguez-Alba JC. Sleep loss and addiction. Neurosci Biobehav Rev. 2022;141:104832. doi:10.1016/j.neubiorev.2022.104832 [PubMed: 35988803]
47. Alpert MH, Gil H, Para A, Gallio M. A thermometer circuit for hot temperature adjusts Drosophila behavior to persistent heat. Curr Biol. 2022;32(18):4079–4087. doi:10.1016/j.cub.2022.07.060 [PubMed: 35981537]
48. Chen SC, Tang X, Goda T, et al. Dorsal clock networks drive temperature preference rhythms in Drosophila. Cell Rep. 2022;39(2):110668. doi:10.1016/j.celrep.2022.110668 [PubMed: 35417715]
49. Pabon E, Greenlund IM, Carter JR, de Wit H. Effects of alcohol on sleep and nocturnal heart rate: relationships to intoxication and morning-after effects. Alcohol Clin Exp Res. 2022;46(10):1875–1887. doi:10.1111/acer.14921 [PubMed: 35953878]
50. Lamaze A, Krätschmer P, Chen KF, Lowe S, Jepson JEC. A wake-promoting circadian output circuit in Drosophila. Curr Biol. 2018;28(19):3098–3105. doi:10.1016/j.cub.2018.07.024 [PubMed: 30270186]
51. Jin X, Tian Y, Zhang ZC, Gu P, Liu C, Han J. A subset of DN1p neurons integrates thermosensory inputs to promote wakefulness via CNMa signaling. Curr Biol. 2021;31(10):2075–2087.e6. doi:10.1016/j.cub.2021.02.048 [PubMed: 33740429]
52. Díaz MM, Schlichting M, Abruzzi KC, Long X, Rosbash M. Allatostatin-C/AstC-R2 is a novel pathway to modulate the circadian activity pattern in Drosophila. Curr Biol. 2019;29(1):13–22.e3. doi:10.1016/j.cub.2018.11.005 [PubMed: 30554904]
53. Hermann-Luibl C, Yoshii T, Senthilan PR, Dircksen H, Helfrich-Förster C. The ion transport peptide is a new functional clock neuropeptide in the fruit fly Drosophila melanogaster. J Neurosci. 2014;34(29):9522–9536. doi:10.1523/JNEUROSCI.0111-14.2014 [PubMed: 25031396]
54. Yao Z, Shafer OT. The Drosophila circadian clock is a variably coupled network of multiple peptidergic units. Science. 2014;343(6178):1516–1520. doi:10.1126/science.1251285 [PubMed: 24675961]
55. Sitaraman D, Aso Y, Rubin GM, Nitabach MN. Control of sleep by dopaminergic inputs to the Drosophila mushroom body. Front Neural Circuits. 2015;9:73. doi:10.3389/fncir.2015.00073 [PubMed: 26617493]
56. Sitaraman D, Aso Y, Jin X, et al. Propagation of homeostatic sleep signals by segregated synaptic microcircuits of the Drosophila mushroom body. Curr Biol. 2015;25(22):2915–2927. doi:10.1016/j.cub.2015.09.017 [PubMed: 26455303]
57. Aleman A, Omoto JJ, Singh P, et al. 2021. Opposing subclasses of Drosophila ellipsoid body neurons promote and suppress sleep. Published online October 19, 2021:2021.10.19.464469. 10.1101/2021.10.19.464469
58. Pohl JB, Ghezzi A, Lew LK, Robles RB, Cormack L, Atkinson NS. Circadian genes differentially affect tolerance to ethanol in Drosophila. Alcohol Clin Exp Res. 2013;37(11):1862–1871. doi:10.1111/acer.12173 [PubMed: 23808628]
59. Hendricks JC, Lu S, Kume K, Yin JCP, Yang Z, Sehgal A. Gender dimorphism in the role of cycle (BMAL1) in rest, rest regulation, and longevity in Drosophila melanogaster. J Biol Rhythms. 2003;18(1):12–25. doi:10.1177/0748730402239673 [PubMed: 12568241]
60. Pinzón JH, Reed AR, Shalaby NA, Buszczak M, Rodan AR, Rothenfluh A. Alcohol-induced behaviors require a subset of Drosophila JmjC-domain histone demethylases in the nervous system. Alcohol Clin Exp Res. 2017;41(12):2015–2024. doi:10.1111/acer.13508 [PubMed: 28940624]
61. Shalaby NA, Pinzon JH, Narayanan AS, et al. JmjC domain proteins modulate circadian behaviors and sleep in Drosophila. Sci Rep. 2018;8(1):815. doi:10.1038/s41598-017-18989-1 [PubMed: 29339751]
62. De Nobrega AK, Noakes EJ, Storch NA, Mellers AP, Lyons LC. Sleep modulates alcohol toxicity in Drosophila. Int J Mol Sci. 2022;23(20):12091. doi:10.3390/ijms232012091 [PubMed: 36292943]

63. van der Linde K, Lyons LC. Circadian modulation of acute alcohol sensitivity but not acute tolerance in Drosophila. Chronobiol Int. 2011;28(5):397–406. doi:10.3109/07420528.2011.577921 [PubMed: 21721855]

64. De Nobrega AK, Lyons LC. Circadian modulation of alcohol-induced sedation and recovery in male and female Drosophila. J Biol Rhythms. 2016;31(2):142–160. doi:10.1177/0748730415627067 [PubMed: 26833081]

65. Urizar NL, Yang Z, Edenberg HJ, Davis RL. Drosophila homer is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance. J Neurosci. 2007;27(17):4541–4551. doi:10.1523/JNEUROSCI.0305-07.2007 [PubMed: 17460067]

66. Castelli V, Brancato A, Cavallaro A, Lavanco G, Cannizzaro C. Homer2 and alcohol: a mutual interaction. Front Psych. 2017;8:268. doi:10.3389/fpsyt.2017.00268

67. Lindsay JH, Glass JD, Amicarelli M, Prosser RA. The mammalian circadian clock in the suprachiasmatic nucleus exhibits rapid tolerance to ethanol in vivo and in vitro. Alcohol Clin Exp Res. 2014;38(3):760–769. doi:10.1111/acer.12303 [PubMed: 24512529]

68. Burnett EJ, Chandler LJ, Trantham-Davidson H. Glutamatergic plasticity and alcohol dependence-induced alterations in reward, affect and cognition. Prog Neuropsychopharmacol Biol Psychiatry. 2016;65:309–320. doi:10.1016/j.pnpbp.2015.08.012 [PubMed: 26341050]

69. Lindsay JH, Prosser RA. The mammalian circadian clock exhibits chronic ethanol tolerance and withdrawal-induced glutamate hypersensitivity, accompanied by changes in glutamate and TrkB receptor proteins. Alcohol Clin Exp Res. 2018;42(2):315–328. doi:10.1111/acer.13554 [PubMed: 29139560]

70. Chvilicek MM, Titos I, Rothenfluh A. The neurotransmitters involved in Drosophila alcohol-induced behaviors. Front Behav Neurosci. 2020;14:607700. doi:10.3389/fnbeh.2020.607700 [PubMed: 33384590]

71. Aguilar JI, Dunn M, Mingote S, et al. Neuronal depolarization drives increased dopamine synaptic vesicle loading via VGLUT. Neuron. 2017;95(5):1074–1088.e7. doi:10.1016/j.neuron.2017.07.038 [PubMed: 28823729]

72. Nandi N, Tyra LK, Stenesen D, Krämer H. Stress-induced Cdk5 activity enhances cytoprotective basal autophagy in Drosophila melanogaster by phosphorylating acinus at serine437. Elife. 2017;6:e30760. doi:10.7554/eLife.30760 [PubMed: 29227247]

73. Newman ZL, Bakshinskaya D, Schultz R, et al. Determinants of synapse diversity revealed by super-resolution quantal transmission and active zone imaging. Nat Commun. 2022;13(1):229. doi:10.1038/s41467-021-27815-2 [PubMed: 35017509]

74. Kong EC, Woo K, Li H, et al. A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in Drosophila. PLoS ONE. 2010;5(4):e9954. doi:10.1371/journal.pone.0009954 [PubMed: 20376353]

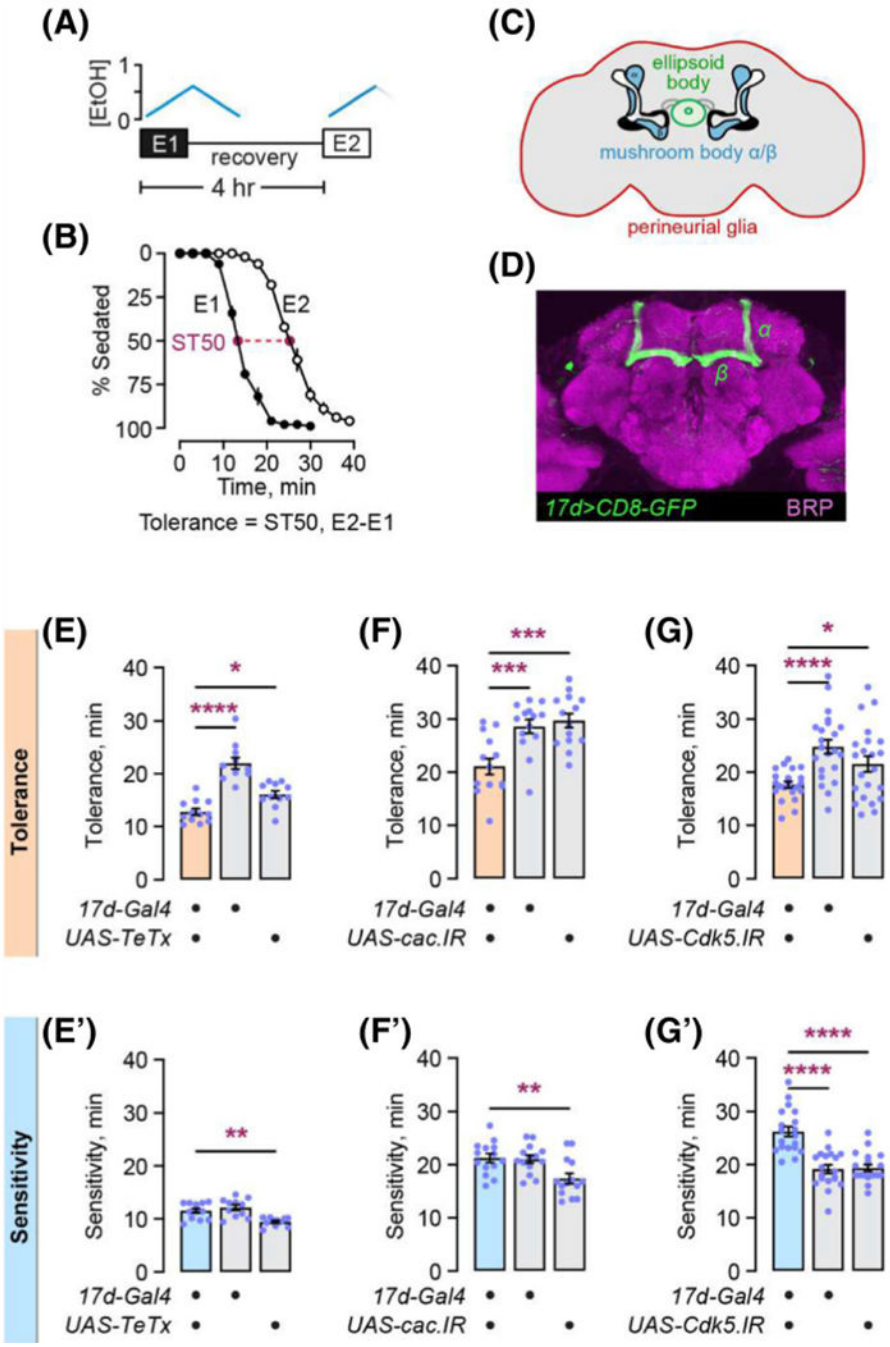


FIGURE 1.

Presynaptic genes promote rapid ethanol tolerance in the mushroom body α/β lobe intrinsic neurons. (A) Scheme for induction and detection of rapid tolerance. Identical ethanol vapour exposures, E1 and E2, are separated by 4 h and result in identical accumulation and dissipation kinetics for internal ethanol concentrations. (B) Time course for ethanol sedation during continuous ethanol vapour exposure, for E1 and E2, and the calculation of ethanol tolerance. (C) Diagram of the Drosophila brain, depicting the known sites for rapid tolerance encoding. (D) Expression pattern in the mushroom body α/β neurons of the

17d-Gal4 transgene, detected by the *UAS-CD8-GFP* reporter transgene, and counterstained for the ELKS/CAST ortholog BRP to reveal the synaptic neuropil. (E,E′) Tolerance (E) and sensitivity (E′) when presynaptic release is blocked by expression of the tetanus toxin light chain (*UAS-TeTx*) in *17d-Gal4* neurons. (F,F′) Effect of decreasing expression of the $Ca_V2.1$ Ca^{2+} channel *cac* in the *17d-Gal4* pattern. (G,G′) Effect of decreasing expression of the kinase *Cdk5* in the *17d-Gal4* pattern. *Statistics*: (E–G′) Quantitative data are mean ± SEM. (E) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: *$p = 0.0129$. (E′) One-way ANOVA ($p = 0.0002$) with Dunnett′s multiple comparisons, *Gal4/UAS* versus *Gal4*: $p = 0.4929$, *Gal4/UAS* versus *UAS*: **$p = 0.0025$. (F) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ***$p = 0.0007$, *Gal4/UAS* versus *UAS*: ***$p = 0.0001$. (F′) One-way ANOVA ($p = 0.0034$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: $p = 0.9781$, *Gal4/UAS* versus *UAS*: **$p = 0.0049$. (G) Brown–Forsythe ANOVA ($p = 0.0005$) with Dunnett's T3 multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: *$p = 0.0394$. (G′) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Here, and in subsequent figures, a dot represents an $n = 1$ of approximately 20 male flies.

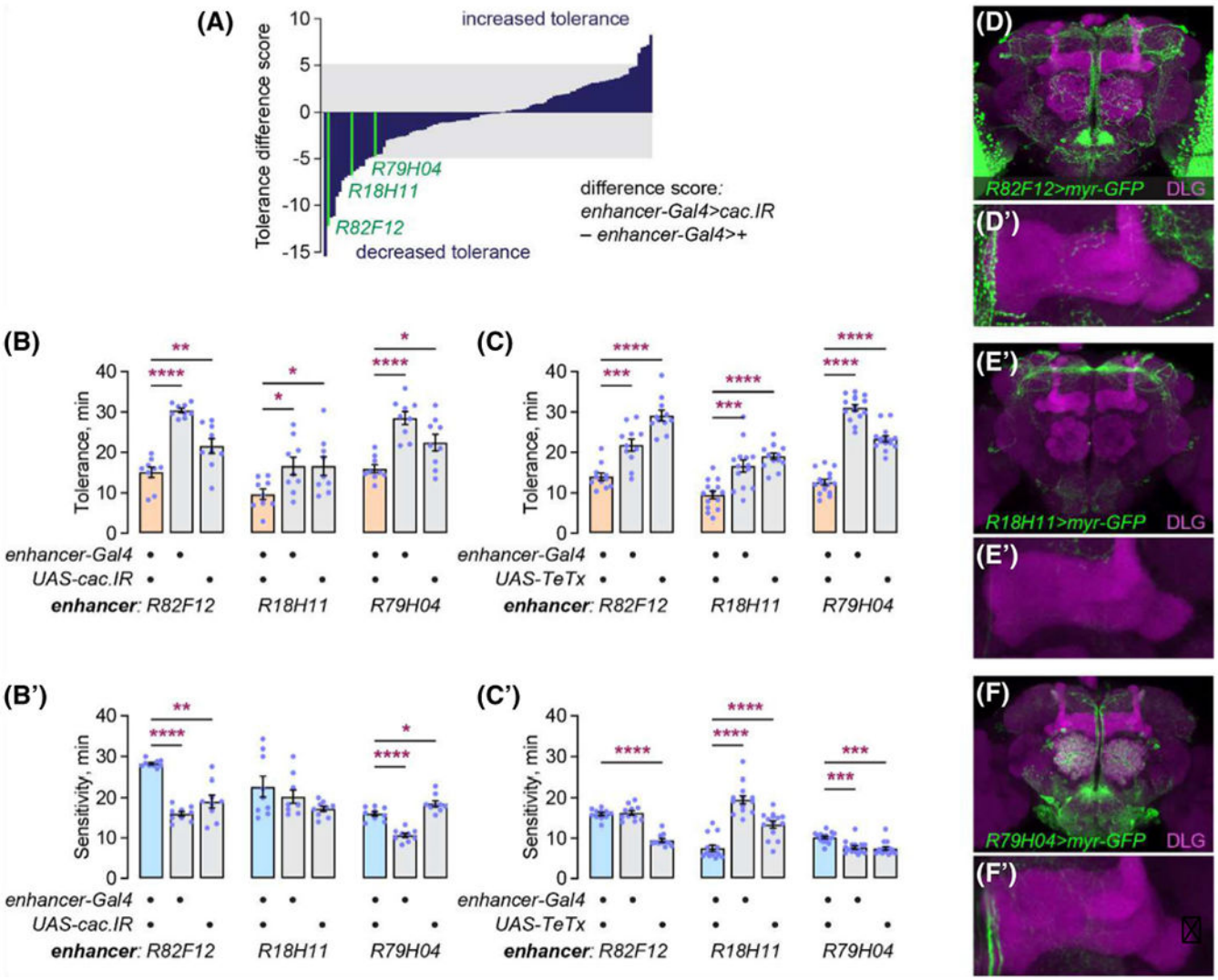


FIGURE 2.

A functional neuroanatomical screen identifies three patterns of neurons that promote rapid tolerance development through presynaptic release. (A) Tolerance difference score for 112 *enhancer-Gal4* strains expressing RNAi for *cac*. Grey region represents 1 standard deviation from the mean of the difference scores across all tested *enhancer-Gal4s*. Highlighted in green are three strains that passed secondary screens and that were further characterized. (B,B′) Reduction of *cac* expression in *R82F12-Gal4* (left), *R18H11-Gal4* (middle), and *R79H04-Gal4* (right) effects on rapid tolerance (B) and sensitivity (B′). (C,C′) Effect of tetanus toxin blockade of presynaptic release in the same three *enhancer-Gal4* strains for rapid tolerance (C) and sensitivity (C′). (D–F) Expression pattern of the *enhancer-Gal4* strains, revealed with the *UAS-myr-GFP* plasma membrane-tethered GFP, and counterstained with an antibody to the discs large (DLG) synaptic protein. (D′–F′) Enlargement of a substack from D–F encompassing the horizontal lobes of the mushroom bodies. *Statistics*: (B,C) Quantitative data are mean ± SEM. (B) Left panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus

Gal4: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: **$p = 0.0040$. Middle panel: One-way ANOVA ($p = 0.0293$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: *$p = 0.0376$, *Gal4/UAS* versus *UAS*: *$p = 0.0379$. Right panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: *$p = 0.0162$. (B′) Left panel: Kruskal–Wallis test ($p = 0.0001$) with Dunn's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: **$p = 0.0059$. Middle panel: One-way ANOVA ($p = 0.1120$). Right panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: *$p = 0.0126$. (C) Left panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ***$p = 0.0003$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Middle panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ***$p = 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Right panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. (C′) Left panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: $p = 0.8707$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Middle panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Right panel: Kruskal–Wallis test ($p < 0.0001$) with Dunn's multiple comparisons, *Gal4/UAS* versus *Gal4*: ***$p = 0.0007$, *Gal4/UAS* versus *UAS*: ***$p = 0.0001$.

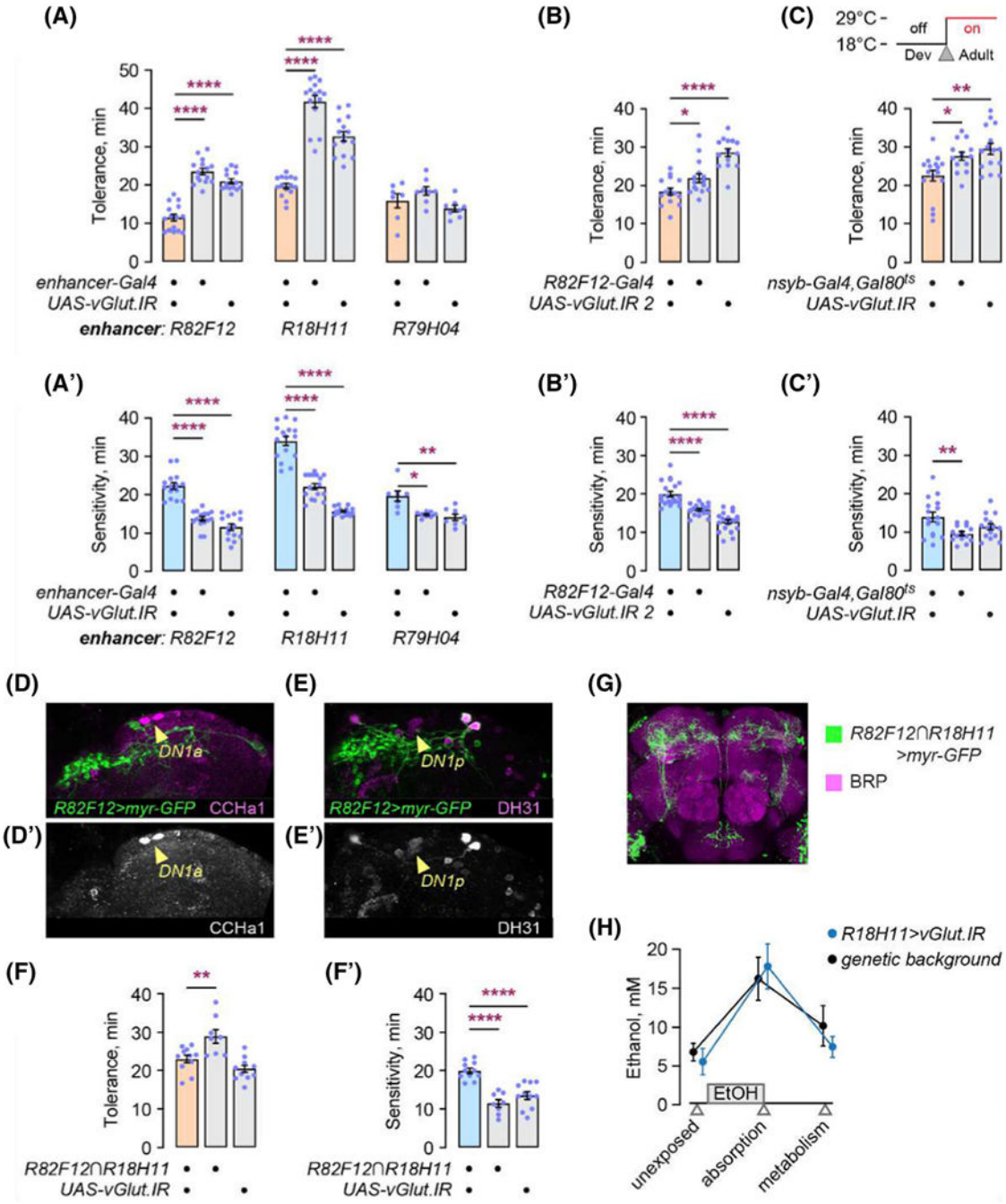
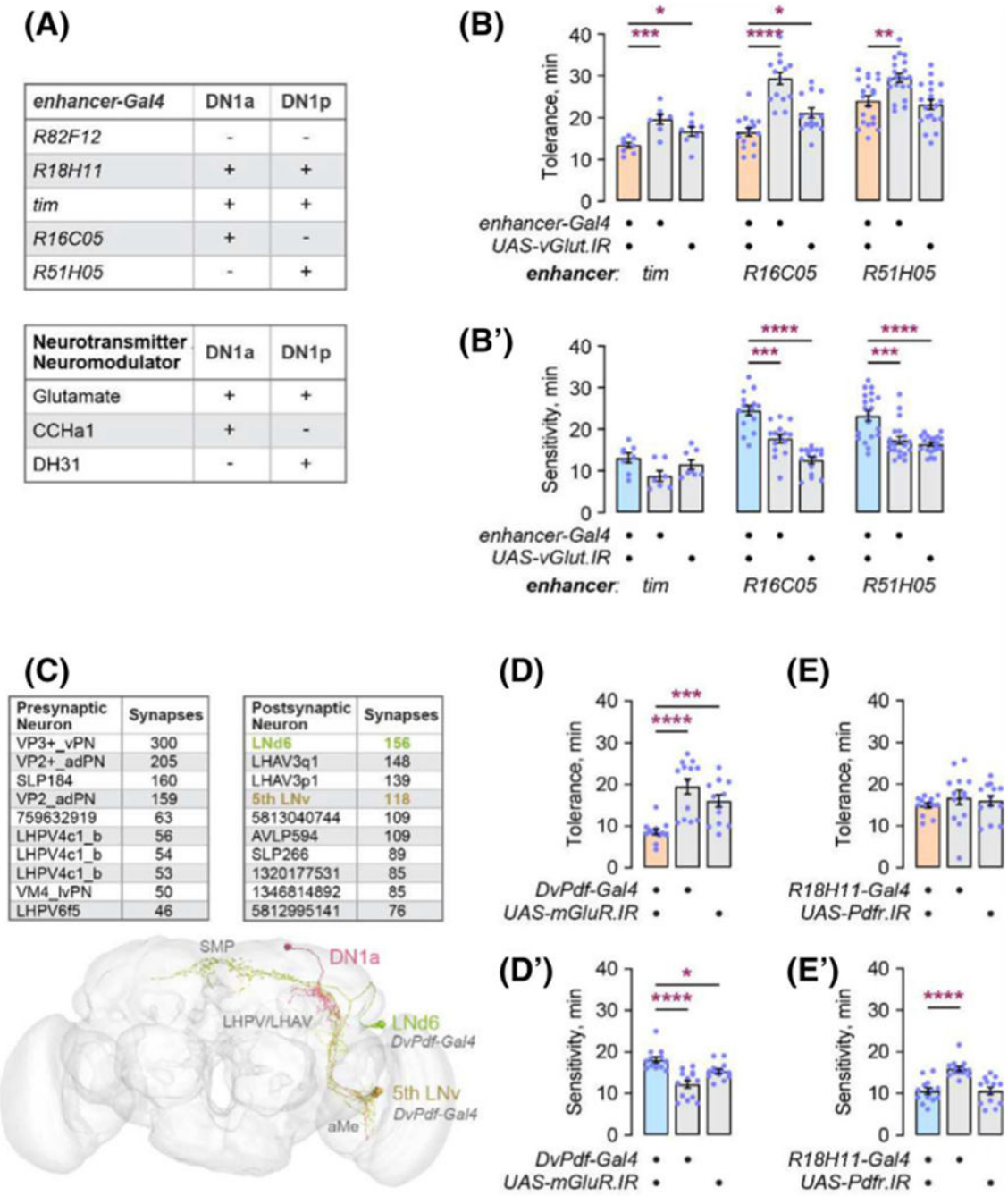


FIGURE 3.

Distinct rapid tolerance neurons exist in each *enhancer-Gal4*, including glutamatergic dorsal clock neurons in *R18H11-Gal4*. (A,A′) Vesicular glutamate transporter *vGlut* RNAi effects on rapid tolerance (A) and sensitivity (A′). (B,B′) Reduction of *vGlut* expression using a second independent RNAi transgene, effects on tolerance (B) and sensitivity (B′) in *R82F12-Gal4* expressing neurons. (C,C′) Adult specific *vGlut* RNAi in *R82F12-Gal4* expressing neurons, effect on rapid tolerance (C) and sensitivity (C′). (D,D′) Counterstaining of *R82F12>myr-GFP* brains with anti-CCHa1 to detect DN1a

neurons. (E,E′) Counterstaining of *R82F12>myr-GFP* brains with anti-DH31 to detect DN1p neurons. Micrographs in D and E depict the dorsal hemisphere of an adult brain. (F,F′) *Split-Gal4* driving expression of *vGlut* RNAi in neurons that are common between *R82F12* and *R18H11*, effect on rapid tolerance (F) and sensitivity (F′). (G) *Split-Gal4* expression pattern revealed with *UAS-myr-GFP*. (H) Effect of *vGlut* RNAi in *R18H11-Gal4* neurons on ethanol absorption and metabolism. Genetic background is the *w-* Berlin strain used to outcross all genetic reagents. *Statistics*: (A–C,F) Quantitative data are mean ± SEM. (A) Left panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Middle panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Right panel: One-way ANOVA ($p = 0.0588$). (A′) Left panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Middle panel: Brown–Forsythe ANOVA ($p < 0.0001$) with Dunnett's T3 multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Right panel: Kruskal–Wallis test (p = 0.0038) with Dunn's multiple comparisons, *Gal4/UAS* versus *Gal4*: *$p = 0.0141$, *Gal4/UAS* versus *UAS*: **$p = 0.0038$. (B) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: *$p = 0.0398$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. (B′) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. (C) One-way ANOVA ($p = 0.0018$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: *$p = 0.0209$, *Gal4/UAS* versus *UAS*: **$p = 0.0012$. (C′) Brown–Forsythe ANOVA ($p = 0.0078$) with Dunnett's T3 multiple comparisons, *Gal4/UAS* versus *Gal4*: **$p = 0.0090$, *Gal4/UAS* versus *UAS*: $p = 0.1720$. (F) One-way ANOVA ($p = 0.0002$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: **p = 0.0038, *Gal4/UAS* versus *UAS*: $p = 0.2004$. (F′) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. (H) One-way ANOVA ($p = 0.0067$) with šídák's multiple comparisons, no treatment: $p = 0.9716$, absorption: $p = 0.9423$, metabolism: $p = 0.7767$.



enhancer-Gal4	DN1a	DN1p
R82F12	-	-
R18H11	+	+
tim	+	+
R16C05	+	-
R51H05	-	+

Neurotransmitter Neuromodulator	DN1a	DN1p
Glutamate	+	+
CCHa1	+	-
DH31	-	+

Presynaptic Neuron	Synapses
VP3+_vPN	300
VP2+_adPN	205
SLP184	160
VP2_adPN	159
759632919	63
LHPV4c1_b	56
LHPV4c1_b	54
LHPV4c1_b	53
VM4_lvPN	50
LHPV6f5	46

Postsynaptic Neuron	Synapses
LNd6	156
LHAV3q1	148
LHAV3p1	139
5th LNv	118
5813040744	109
AVLP594	109
SLP266	89
1320177531	85
1346814892	85
5812995141	76

FIGURE 4.

DN1a and downstream evening circadian neurons promote rapid tolerance. (A) Presence (+) and absence (−) of expression of *enhancer-Gal4s* and neurotransmitter/neuromodulators in the DN1a and DN1p dorsal clock neurons. (B,B′) Reduction of glutamate release with vesicular glutamate transporter *vGlut* RNAi in *enhancer-Gal4* patterns that include one or both of the dorsal group clock neurons, effects on rapid tolerance (B) and sensitivity (B′). (C) Ten presynaptic (left) and postsynaptic (right) neurons that make the greatest number of synapses with the DN1a neurons, expressed as the sum total for the right

pair of DN1a neurons in the hemibrain electron microscopy reconstruction. The diagram below depicts the morphology of one of the pair of DN1a neurons and the LNd6 and 5th LNv postsynaptic neurons in the full adult female brain (FAFB) electron microscopy reconstruction. SMP: superior medial protocerebrum; LHPV/LHAV: lateral horn posterior/anterior ventral; aMe: accessory medulla. *DvPdf-Gal4* is expressed in the LNd6 and 5th LNv neurons. (D,D′) Reduction of metabotropic glutamate receptor *mGluR* expression in *DvPdf-Gal4* expressing neurons, effect on rapid tolerance (D) and sensitivity (D′). (E,E′) Reduction of PDF receptor Pdfr expression in *R18H11-Gal4* expressing neurons, effect on rapid tolerance (E) and sensitivity (E′). *Statistics*: (B,B′,D–E′) Quantitative data are mean ± SEM. (B) Left panel: One-way ANOVA ($p = 0.0013$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ***$p = 0.0007$, *Gal4/UAS* versus *UAS*: *$p = 0.0493$. Middle panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: *$p = 0.0195$. Right panel: One-way ANOVA ($p = 0.0002$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: **$p = 0.0018$, *Gal4/UAS* versus *UAS*: $p = 0.8003$. (B′) Left panel: One-way ANOVA ($p = 0.0561$). Middle panel: One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. Right panel: Brown–Forsythe ANOVA ($p < 0.0001$) with Dunnett's T3 multiple comparisons, *Gal4/UAS* versus *Gal4*: ***$p = 0.0009$, *Gal4/UAS* versus *UAS*: ****$p < 0.0001$. (D) Brown–Forsythe ANOVA ($p < 0.0001$) with Dunnett's T3 multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: ***$p = 0.0005$. (D′) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: *$p = 0.0221$. (E) Brown–Forsythe ANOVA ($p = 0.5724$). (E′) One-way ANOVA ($p < 0.0001$) with Dunnett's multiple comparisons, *Gal4/UAS* versus *Gal4*: ****$p < 0.0001$, *Gal4/UAS* versus *UAS*: $p = 0.9985$.