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High ethanol preference and dissociated memory are co-occurring phenotypes associated with hippocampal $GABA_AR$-δ receptor levels

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Abstract

Alcohol use disorder (AUD) frequently co-occurs with dissociative disorders and disorders with dissociative symptoms, suggesting a common neurobiological basis. It has been proposed that facilitated information processing under the influence of alcohol, resulting in the formation of dissociated memories, might be an important factor controlling alcohol use. Access to such memories is facilitated under the effect of alcohol, thus further reinforcing alcohol use. To interrogate possible mechanisms associated with these phenotypes, we used a mouse model of dissociative amnesia, combined with a high-alcohol preferring (HAP) model of AUD. Dissociated memory was induced by activation of hippocampal extrasynaptic GABA type A receptor delta subunits ($GABA_AR$-δ), which control tonic inhibition and to which ethanol binds with high affinity. Increased ethanol preference was associated with increased propensity to form dissociated memories dependent on $GABA_AR$-δ in the dorsal hippocampus (DH). Furthermore, the DH level of $GABA_AR$-δ protein, but not mRNA, was increased in HAP mice, and was inversely correlated to the level of miR-365–3p, suggesting an miRNA-mediated post-transcriptional mechanism contributing to elevated $GABA_AR$-δ. The observed changes of DH $GABA_AR$-δ were associated with a severe reduction of excitatory projections stemming from $GABA_AR$-δ-containing pyramidal neurons in the subiculum and terminating in the mammillary body. These results suggest that both molecular and circuit dysfunction involving hippocampal $GABA_AR$-δ receptors might contribute to the co-occurrence of ethanol preference and dissociated information processing.

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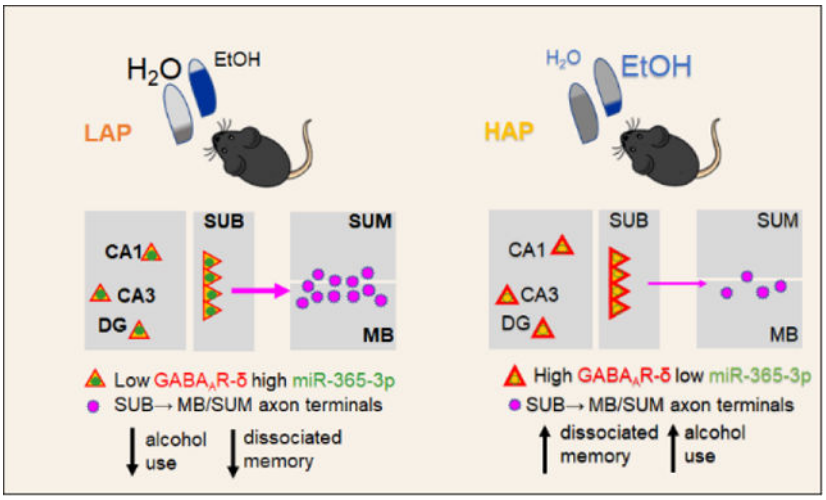
CRediT authorship contribution statement

Vladimir Jovasevic: Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Resources; Validation; Visualization; Writing - original draft; Writing - review & editing. **Jelena Radulovic:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Validation; Visualization; Writing - review & editing.

Declaration of Competing Interest

The authors declare no financial or non-financial conflicts of interest associated with this manuscript.

Graphical Abstract



Keywords

memory; alcohol use disorder; microRNA; GABA receptors; hippocampus; mammillary body

1. Introduction

Alcohol profoundly and adversely affects episodic memory, mainly through direct action on hippocampal extrasynaptic GABA type A receptors ($GABA_AR$), often resulting in the complete loss of recollection of events occurring during alcohol intoxication (White, 2003). In contrast, through its action on $GABA_AR$ in the mesolimbic system, alcohol mediates its rewarding properties (Koob, Rassnick, Heinrichs, & Weiss, 1994), and supports appetitive conditioning to alcohol-associated spatial contexts (Lisman & Grace, 2005), thereby leading to persistent alcohol seeking, use, and addiction. These two effects of alcohol are largely seen as independent. However, very high comorbidity between alcohol use disorder (AUD) and mental disorders stemming from maladaptive processing of traumatic memories (Gilpin & Weiner, 2017; Whitaker, Gilpin, & Edwards, 2014) suggests otherwise. Dissociative disorders and other psychiatric disorders with dissociative symptoms, including post-traumatic stress disorder (PTSD), anxiety disorders, major depressive disorder, and schizophrenia (Lanius et al., 2010; Molina-Serrano, Linotte, Amat, Souery, & Barreto, 2008; Renard, Pijnenborg, & Lysaker, 2012; Sar & Ross, 2006), have a particularly high prevalence among those suffering from AUD (Kessler et al., 1996; Regier et al., 1990). Alcohol use disorder is observed in 20–40% of individuals suffering from PTSD, making alcoholism and PTSD the most commonly co-occurring mental health disorders (Whitaker et al., 2014). While many dissociative symptoms are intrinsic to human PTSD sufferers, and therefore cannot be modeled in rodents, this is not the case with dissociative amnesia, which can be successfully modeled in rodents using dissociated memory (Girden & Culler, 1937), also known as state-dependent memory.

Extrasynaptic $GABA_A$Rs have a well-established contribution to dissociated memory. These receptors are key regulators of tonic inhibition mediated by ambient GABA levels but also by low doses of alcohol. Extrasynaptic $GABA_A$Rs containing δ, α1, and α4 subunits are particularly sensitive to alcohol, resulting in increased tonic inhibition at doses corresponding to blood alcohol levels during moderate consumption (Sundstrom-Poromaa et al., 2002; Wallner, Hanchar, & Olsen, 2003). Correspondingly, *Gabrd* knockout (Mihalek et al., 2001) or an shRNA-mediated decrease in the level of α4 or δ subunits (Nie, Rewal, Gill, Ron, & Janak, 2011; Rewal et al., 2009) result in decreased ethanol preference. Extrasynaptic $GABA_A$Rs containing δ or α4 subunit are enriched in the hippocampus, especially in neurons sending long-range projections to other cortical and subcortical areas (Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000).

Whereas the role of hippocampal extrasynaptic $GABA_A$R in dissociated memory has been established (Jovasevic et al., 2015; Meyer et al., 2017), it is not clear whether levels of these subunits in memory circuits are related to alcohol use, and what the mechanisms are by which memory circuits might influence alcohol-motivated behavior. It has been proposed (Donald A. Overton, 1972) that facilitated information processing under the influence of alcohol, resulting in dissociated memory, might reinforce alcohol use due to facilitated access to such memories under the effect of alcohol. To address the relationship between these phenotypes, we compared the regulation of dorsohippocampal $GABA_A$R-δ receptors and $GABA_A$R-δ receptor-regulated DH circuits in mice bred for high (HAP) and low (LAP) alcohol preference.

2. Material and Methods

2.1. Animals

9-week-old male C57BL/6N mice were obtained from a commercial supplier (Envigo). The vGlut1-Cre (Harris et al., 2014) (Slc17a7-IRES2-Cre, Vglut1-IRES2-Cre-D), vGlut2-Cre (Vong et al., 2011) (Slc17a6tm2-cre, VGlut2-ires-Cre), and GAD2-Cre (Taniguchi et al., 2011) ($Gad2^{tm2(cre)Zjh}$/J) mouse lines were obtained from the Jackson Laboratory (Bar Harbor, ME). Male HAP2 and LAP2 (replicate line 2) mice (Oberlin, Best, Matson, Henderson, & Grahame, 2011) were obtained from Dr. Nicholas Grahame (Indiana University–Purdue University Indianapolis). All animals were individually housed on a 12-h light/dark cycle (lights on at 7 AM) and allowed ad libitum access to food and water. All procedures were approved by Northwestern University's Animal Care and Use Committee in compliance with US National Institutes of Health standards.

2.2. Surgery and cannulation

Double guided cannulas (Plastic One) were implanted in the dorsal hippocampus (DH) as described previously (Radulovic, Ruhmann, Liepold, & Spiess, 1999). Mice were anesthetized with 1.2% tribromoethanol (vol/vol, Avertin) and implanted with bilateral 26-gauge cannulas using a stereotaxic apparatus. Stereotaxic coordinates for the dorsal hippocampus were 1.8 mm posterior, ±1.0 mm lateral and 2.0 mm ventral to bregma, according to the mouse brain atlas (Franklin & Paxinos, 2013). Cannula placements were

verified in coronal sections through DH at the end of the behavioral experiments, and only animals with the correct placement were included in the analysis.

2.3. Viral vectors and infusions

The viral vector AAV8.hSyn.hM4D(Gi)-mCherry (University of North Carolina Vector Core), used for anterograde tracing, was bilaterally infused into the dorsal hippocampus (1.8 mm posterior, ±1.0 mm lateral and 2.0 mm ventral to bregma). Cre-dependent retrograde AAV (AAVrgpAAV-hSyn-Cre-P2A-dTomato, Addgene, 28306), used for retrograde tracing, was unilaterally infused into SUM (2.8 mm posterior, ± 0.5 mm lateral and 4.85 mm ventral to bregma) or MB (2.8 mm posterior, ± 0.5 mm lateral and 5.3 mm ventral to bregma). The viral vector AAV-DJ-CAG-eGFP-2A-TENT (Stanford Medicine Vector Core) expressing tetanus toxin light chain, or serotype/promoter matched control AAV-DJ-CAG-eGFP vector expressing GFP (Vector Biolabs, 7078) were infused bilaterally into SUM. Infusions were performed using an automatic microsyringe pump controller (Micro4-WPI) connected to a Hamilton microsyringe. The viral vectors were infused in a volume of 0.5 μl (DH) or 0.2 μl (SUM, MB) per site, at titer > 8×10^{12} GC/ml, over 2 min, and syringes were left in place for 5 min prior to removal to allow for virus diffusion. Mice were allowed 6 weeks for virus expression prior to behavioral testing or histological analyses.

2.4. Pharmacological treatments

Gaboxadol (0.25 or 0.5 μg, dissolved in artificial cerebrospinal fluid (ACSF), Sigma-Aldrich) was injected intrahippocampally (i.h.), at a volume of 0.5 μl per side, at a rate of 0.15 μl/min.

2.5. Fear conditioning

Contextual fear conditioning was performed in an automated system (TSE Systems) as previously described (Radulovic et al., 1999). Briefly, mice were exposed for 3 min to a novel context, followed by a foot shock (2 s, 0.7 mA, constant current). 24 h later, mice were tested for memory retrieval. Testing consisted of 3 min in the conditioning context, during which freezing was measured every 10 s. Freezing was expressed as a percentage of the total number of observations during which the mice were motionless. Activity was recorded automatically by an infrared beam system and expressed as cm/s. The individual experiments were not performed on littermates, so we did not apply randomization procedures, but all behavioral tests were performed by experimentalists who were unaware of the treatments. During training, blinding was performed so that a laboratory member not involved in the experiments would prepare and color code the solution. In addition, the experimenter performing the tests was not aware of the numbering code.

2.6. Ethanol preference

Ethanol preference was determined using two bottle paradigm, as previously described (Oberlin et al., 2011). During preference testing, mice were housed individually in polycarbonate cages with microisolator tops. Mice were allowed to drink from two 50 ml graduated bottles. One bottle contained 10% ethanol (v/v) in distilled water, and the other bottle contained distilled water. Mass of liquid consumed was measured to a resolution of

±0.01 g every two days. Water and ethanol bottle locations were alternated after every measurement to eliminate position bias. Mice were weighed after each ethanol intake measurements. Ethanol preference over one week was calculated at the end of the procedure as average score in g/kg/day (Oberlin et al., 2011), or by dividing the volume of 10% ethanol consumed by the volume of total fluid (water + 10% ethanol) consumed, and is expressed as a percentage (Mill, Bito-Onon, Simms, Li, & Bartlett, 2013).

2.7. Tissue collection

For all analyses, mice were sacrificed by cervical dislocation, dorsal hippocampi immediately dissected and frozen in liquid nitrogen. Frozen tissue was stored at −80ºC until protein or RNA extractions were performed. Tissue from one side of the brain was used for RNA analyses, and from the other for protein analyses. The side used for each analysis was alternated to eliminate the side bias.

2.8. Quantitative PCR analysis

Dorsal hippocampi were collected around the tips of the hippocampal cannulas. Total RNA was extracted using miRCURY RNA Isolation Kit-Tissue (Qiagen). Reverse transcription was performed on 20 ng of total RNA using Universal cDNA Synthesis Kit (Exiqon-Qiagen) for the analysis of microRNAs; on 100 ng of total RNA using First Strand cDNA Synthesis Kit (Applied Biosystems) for the analysis of mRNAs. Real-time PCR analysis was performed on an Applied Biosystems 7600 instrument using SYBR green detection system (Applied Biosystems) and primers specific for $GABA_AR$-δ, miR-615–3p, miR-299a-3p, miR-365–3p (all from Qiagen).

2.9. Immunohistochemistry

Mice were anesthetized with an i.p. injection of 240 mg/kg Avertin and transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4, 150 ml per mouse). Brains were removed and post-fixed for 24 h in the same fixative and then immersed for 24 h each in 20 and 30% sucrose in phosphate buffer. Brains were frozen and 50 μm sections were cut for use in free-floating immunohistochemistry (Jovasevic et al., 2015) with primary antibodies against mCherry (1:1000, rabbit, Abcam, AB167453), Tyrosine hydroxylase (1:2000, Immunostar, 22941), Calretinin (1:4,000, Swant, CG1), $GABA_AR$-δ (1:2000, Alomone, AGA-014). Secondary antibodies were obtained from Jackson ImmunoResearch (1:200, Alexa Fluor® 594 AffiniPure Donkey Anti-Mouse IgG [H+L]). GFP was visualized by its intrinsic fluorescence. Nuclei were counterstained with Hoechst 33342 (ThermoFisher), except in double staining experiments, where blue color was used for one of the antibodies. Sections were mounted using FluorSave (Millipore-Sigma) and observed with Leica microscope equipped with a CCD (Olympus) camera, using 5 ×, 10 ×, or 20 × objectives. For light microscopy, signals were visualized with diaminobenzidine (Sigma).

2.10. Western blot

Dorsal hippocampi were collected around the tips of the hippocampal cannulas. Tissue was lysed in modified RIPA buffer, incubated 15 min on ice, and centrifuged for 15 min

(15,000g) at 4 °C. Samples were subjected to SDS-PAGE (10 μg per well) and transferred to PVDF membrane (Biorad). Membranes were blocked with I-block (Tropix), incubated with primary antibody overnight at 4 °C, and with secondary antibody for 1 h at room temperature. Primary antibodies used were against $GABA_AR$-α1 (1:500, Sigma-Aldrich, G4416), $GABA_AR$-α4 (1:500, sc-20917, Santa Cruz), and $GABA_AR$-δ (1:500, Alomone, AGA-014). Secondary antibodies were DyLight 800 (Bio Rad). Bands were visualized using ChemiDoc MP Imaging System (Bio-Rad). All antibodies gave bands at the predicted molecular sizes. Specific band intensities were normalized to the total protein bands intensity of each lane.

2.11. Statistics

Statistical analyses were performed using GraphPad Prism software. One-way ANOVA was followed by Tukey's test for post hoc comparisons of three or more experimental groups (only when ANOVA was significant) or Student's t test for comparison of two experimental groups. Homogeneity of variance was confirmed with Levene's test for equality of variances. All comparisons were conducted using two-tailed tests and the *P* value for all cases was set to <0.05 for significant differences. Group sizes were determined using power analysis assuming a moderate effect size of 0.5.

3. Results

3.1. Susceptibility to dissociated memory is associated with ethanol preference

We first tested whether the level of ethanol preference is associated with the susceptibility to form memories in a dissociated manner. For these experiments we used male high- and low-alcohol preferring (HAP and LAP, respectively) outbred mice (Oberlin et al., 2011) as well as male C57BL/6N (C57) mice, a commonly used inbred strain. HAP and LAP mice had consistently uniform high or low alcohol preference, respectively. In comparison to HAP and LAP mice, C57 mice showed a more moderate alcohol preference. All three strains of mice have comparable total fluid intake (Fig. 1A). To determine the susceptibility of these mice to dissociated memory, we evaluated the ability of different doses of gaboxadol, an agonist of extrasynaptic $GABA_A$ receptors (Jovasevic et al., 2015; Meyer et al., 2017), to produce dissociated memory effects. When memory is encoded as dissociated, it can only be retrieved under the influence of gaboxadol, but not when mice are infused with vehicle (Jovasevic et al., 2015). Therefore, the ability of a dose of gaboxadol to produce dissociated memory effect is determined by impaired memory recall when animals are infused with vehicle prior to memory test. We showed previously that 0.5 μg/side of gaboxadol efficiently produced dissociated memory effects in C57 mice, whereas 0.25 μg/side did not (Jovasevic et al., 2015). Here, we tested the ability of these two doses to produce dissociated memory effects in HAP and LAP mice. We trained C57, HAP, and LAP mice on vehicle (0 μg), 0.25 or 0.5 μg/side of gaboxadol, and tested them under vehicle 24 h later (Fig. 1B). As we demonstrated previously (Jovasevic et al., 2015), C57 mice were susceptible to dissociated memory effects of 0.5 μg, but not 0.25 μg dose (Fig. 1C). In contrast, HAP mice were affected by both higher and lower doses of gaboxadol, while LAP mice showed no dissociated memory effect under any of these conditions, indicating that animals with higher alcohol intake require a lower dose of gaboxadol to produce dissociated memory effects.

These results show that the predisposition for high alcohol preference is associated with a predisposition to state (tonic inhibition)-dependent encoding and recall of contextual information.

3.2. $GABA_AR$-δ are differentially expressed in the hippocampus of mice with high- or low-ethanol preference

Pharmacological approaches reveal that dissociated memory most commonly occurs during enhanced activity of extrasynaptic $GABA_AR$ (Jovasevic et al., 2015; D. A. Overton, 1964), and GABAergic activity shows marked differences between HAP and LAP mice (Saba et al., 2011; Tabakoff et al., 2009), suggesting that dysregulation of extrasynaptic $GABA_AR$ activity may be a shared genetic trait contributing to both susceptibility to dissociated memory processing and enhanced alcohol preference. Since extrasynaptic $GABA_ARs$ containing δ, α1, and α4 subunits are particularly sensitive to alcohol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003) and gaboxadol (Boehm, Homanics, Blednov, & Harris, 2006; Chandra et al., 2006; Meera, Wallner, & Otis, 2011), we compared the protein levels of δ, α1, and α4 subunits in DH of HAP, LAP, and C57 mice. We observed increased levels of $GABA_AR$-δ, but not α4, or α1 in HAP, relative to LAP mice (Fig. 2). C57 mice, whose alcohol preference falls between LAP and HAP mice (Fig. 1A), had $GABA_AR$-δ levels that were also between LAP and HAP mice. These results suggest that δ subunit-containing extrasynaptic $GABA_ARs$ might contribute to the co-occurrence between increased dissociated memory processing and ethanol preference.

We went on to determine the possible regulatory mechanisms responsible for these differences in $GABA_AR$-δ expression in DH. Changes in transcriptional regulation are frequently the cause of altered protein levels in individuals affected by conditions with a genetic component, including AUD. Studies in both humans and animal models of AUD have identified numerous mutations in promoter regions that are responsible for aberrant protein levels (Domart et al., 2012; Hoffman et al., 2014; Manzardo, Henkhaus, & Butler, 2012). However, we did not observe any difference in *Gabrd* mRNA levels between C57, HAP, and LAP mice (Fig. 3A), suggesting that the observed differences in protein levels are the result of altered post-transcriptional regulation. One very prominent mechanism of post-transcriptional regulation is microRNA (miRNA)-mediated silencing. We examined the 3'UTR of *Gabrd* mRNA and identified three miRNA binding sites (Fig. 3B, Supplementary Fig. S2). Of these three miRNAs, only miR-365–3p was significantly decreased in the DH of HAP mice, compared to LAP (Fig. 3C). The level of miR-365–3p in C57 mice was between that seen in LAP and HAP mice. The regression analysis showed significant correlation between miR-365–3p and $GABA_AR$-δ protein levels (Fig. 3D). These results suggest that *Gabrd* mRNA is a target of miR-365–3p, and that altered levels of $GABA_AR$-δ in the DH of HAP mice are the result of dysregulation of miR-365–3p expression.

3.3. HAP mice have severely reduced hippocampal projections to mammillary body

It is well-established that DH is involved in memory storage (Fanselow & Dong, 2010), and therefore very likely that $GABA_AR$-δ-mediated susceptibility to dissociated memory is established at the local, hippocampal level. However, we hypothesized that $GABA_AR$-δ-expressing neurons of DH regulate ethanol preference through projections to reward

processing regions. We mapped efferent projections from DH in C57, LAP, and HAP mice to identify DH circuits that may contribute to the difference in ethanol preference by infusing adeno-associated virus vector (AAV) expressing hM4D(Gi)-mCherry into DH. We observed a striking difference in the projection from DH to the region encompassing the border between MB and SUM. A projection stemming from neurons in the subiculum (SUB) was prominent in C57 and LAP mice but severely reduced in HAP mice (Fig. 4A). The main terminals of this projection were in the acellular region of the mammillary body (MB) bordering the supramammillary nucleus (SUM), with fine terminals also penetrating SUM and surrounding calretinin-positive and dopaminergic neurons (Fig. 4B). While both regions have a role in memory (S. D. Vann, 2010; Vertes, 2015), SUM has also been implicated in reward processing (Ikemoto, 2005; Ikemoto, Witkin, Zangen, & Wise, 2004; Shin & Ikemoto, 2010). Therefore, we determined whether SUM mediates ethanol preference using tetanus toxin-expressing AAV to inactivate SUM in HAP mice. This manipulation resulted in reduced ethanol preference (Fig. 4C), demonstrating that SUM activity promotes ethanol preference. Other DH projections, including those to the septal area and thalamic nuclei, were intact (Supplementary Fig. S3).

To further characterize the projections from DH to SUM/MB, we identified the type of DH neurons projecting to SUM/MB using Cre-dependent anterograde tracer AAV8-hSyn-DIO-mCherry in vGlut1-, vGlut2-, and GAD2-Cre mice. The mCherry staining was strong in vGlut1-, and vGlut2-Cre mice, and barely detectable in GAD2-Cre mice, indicating that these DH efferents are predominantly excitatory (vGlut1 and vGlut2), with minimal GABAergic inputs (Fig. 5A).

To identify hippocampal subfield and the cell population that sends projections to MB, we infused Cre-dependent retrograde AAV (rgAAV-hSyn-Cre-P2A-dTomato) into MB of vGlut1-Cre mice. We performed double immunostaining for dTomato, to identify MB-projecting cells, and for $GABA_AR$-δ, to determine whether these cells express this $GABA_AR$ subunit. Retrograde tracing from MB showed a large number of labeled cells in SUB, and none in the denate gyrus (DG) (Fig. 5B), consistent with earlier observations (Meibach & Siegel, 1977; Swanson & Cowan, 1977), indicating that the hippocampal projections to MB originate in SUB, and not in DG. Double immunofluorescence for the retrograde tracer dTomato and $GABA_AR$-δ showed that most of the SUB neurons projecting to MB express $GABA_AR$-δ (Fig. 5B). Retrograde tracing from SUM showed that there were fewer SUM- than MB-projecting cells in SUB (Supplementary Fig. S4), which is consistent with our observation of sparser SUB axon terminals in SUM (Fig. 4B). These results suggest that $GABA_AR$-δ expressed on MB/SUM projecting SUB excitatory neurons are well positioned to control the co-regulation of these regions.

4. Discussion

Here, we investigated molecular and circuit mechanisms that may contribute to high comorbidity between AUD and dissociative disorders. We used a mouse model of dissociative memory we developed (Jovasevic et al., 2015), along with HAP and LAP mouse lines. We chose these lines as they avoid several limitations of other animal models developed to study various aspects of alcohol addiction (Belknap, Richards, O'Toole,

Helms, & Phillips, 1997; Colombo, 1997; Eriksson, 1968; Li, Lumeng, & Doolittle, 1993; Mardones & Segovia-Riquelme, 1983; McBride & Li, 1998). Most other lines are derived from inbred strains, resulting in a unique genotype that cannot be extrapolated to the population as a whole, unless compared to multiple inbred lines (Crabbe, Phillips, Kosobud, & Belknap, 1990). We performed our experiments on male mice in order to define fundamental molecular and circuit principles that may be the underlying cause of comorbidity between AUD and dissociative disorders. Although dissociative amnesia occurs at similar rates in both sexes (Foote, Smolin, Kaplan, Legatt, & Lipschitz, 2006), it is well established that men and women cope with traumatic events differently (Schmied et al., 2015). Clear sex bias also exists with respect to AUD (Erol & Karpyak, 2015; Nelson, Heath, & Kessler, 1998). We do not know at present whether molecular and circuit differences between mouse strains we describe here also exist between sexes, and whether they contribute to sex differences in dissociative symptoms and AUD in humans.

We demonstrated that the level of $GABA_AR$-δ expression in DH is associated with ethanol preference and with susceptibility to dissociated memory, indicating that $GABA_AR$-δ-mediated tonic inhibition of DH neurons may be a basis of a neurobiological mechanism contributing to the co-regulation of ethanol preference and dissociated memory processing. Our results also show that the differences in $GABA_AR$-δ levels, observed in HAP and LAP mice, are not the result of changes in gene expression, but rather the result of dysregulation of miRNA-mediated post-transcriptional control. In addition to gene-coding regions, miRNAs are also implicated in regulating ethanol preference. Several human and animal studies have shown that alcohol-induced changes in miRNA levels are associated with regulation of alcohol consumption, episodes of binge drinking, dependence, and withdrawal (Lewohl et al., 2011; Manzardo, Gunewardena, & Butler, 2013; Mayfield, 2017; Osterndorff-Kahanek et al., 2018). All studies thus far examine the changes in miRNA levels with ethanol exposure; therefore, it is not known whether these miRNAs are dysregulated prior to the development of AUD and are predisposition factors for high ethanol preference, or if their expression changes as a compensatory mechanism to alcohol consumption. Here, we show that miR-365–3p expression is dysregulated in naive mice, prior to ethanol exposure, and likely contributes to predisposition to high ethanol preference. We do not know at present why HAP and LAP mice have different levels of miR-365–3p expression. Analyses of corresponding human miRNA, miR-365a-3p, show that the expression is regulated through its own promoter, and requires NF-κB, SP1, as well as MAPK signaling pathway (Xu et al., 2011). It is conceivable that genetic or epigenetic changes in the binding sites for the corresponding transcriptional factors result in altered expression of miR-365–3p in HAP mice, relative to LAP. It cannot be ruled out, however, that these changes can be consequent to the observed circuit deficits.

Our results suggest that miR-365–3p-mediated differences in expression of $GABA_AR$-δ in DH of HAP and LAP mice may be responsible for both enhanced susceptibility to dissociated memory and enhanced ethanol preference of HAP mice, a possibility that remains to be experimentally confirmed. DH has been implicated in memory storage (Fanselow & Dong, 2010); however, it is also thought to be involved in the processing of contextual information on reward expectation and availability, and its entry into long-term memory (Lisman & Grace, 2005). Thus far it has been shown that DH is connected to the

mesolimbic system through a circuit to the nucleus accumbens (Trouche et al., 2019) and to the ventral tegmental area, via the lateral septum (Luo, Tahsili-Fahadan, Wise, Lupica, & Aston-Jones, 2011). Here, we describe an additional long-range projection to a reward processing region, to SUM via SUB. As the major output of the hippocampal formation (O'Mara, Commins, Anderson, & Gigg, 2001) SUB projects directly to the diencephalon, with MB receiving especially dense inputs (Meibach & Siegel, 1977; Swanson & Cowan, 1977). We show that some of these efferents terminate in SUM (or branch from MB), albeit much more sparsely. It is likely that these fine terminals in SUM were missed in previous tracing studies, since the tracing methods used in these studies were not as sensitive as the ones used in our experiments. SUM has been extensively studied for its role in regulating hippocampal theta rhythm and memory processing (Vertes, 2015); however, it has also been implicated in reward processing (Ikemoto, 2005; Ikemoto et al., 2004; Shin & Ikemoto, 2010). This function of SUM is mediated by its $GABA_A$Rs (Ikemoto, 2005), and involves activation of the mesolimbic dopamine system, as well as other brain areas involved in motivational processes including the prefrontal cortex, septal area, preoptic area, lateral hypothalamic area, and dorsal raphe nucleus (Shin & Ikemoto, 2010). Our results suggest that SUM may be regulated by DH, through the SUB excitatory afferents, and this regulation is achieved through the two complementary mechanisms: (1) the level of $GABA_A$R-δ-mediated tonic inhibition, and (2) the density of excitatory terminals in SUM. DG and SUB are among the regions with the highest levels of $GABA_A$R-δ expression (Pirker et al., 2000; Wei, Zhang, Peng, Houser, & Mody, 2003), therefore the regulation of SUM through hippocampal tonic inhibition can be mediated by either of the regions, or both, simultaneously. We do not know whether increased level of $GABA_A$R-δ expression in HAP mice, and reduced SUB efferent terminals density in SUM, are causatively related. It is, however, important to note that GABAergic signaling promotes synapse elimination during development (Nakayama et al., 2012; Wu et al., 2012), and it is possible that persistent enhancement in tonic inhibition of HAP mice would also result in increased reduction of axon terminals in an innervating region.

The SUB efferents to SUM and MB may also contribute to dissociated memory, since the reciprocal connections, from SUM and MB back to DH, are known to regulate hippocampal functions important for memory processing (Seralynne D. Vann & Aggleton, 2004; Vertes, 2015). Of the two regions, MB has been shown to regulate dissociated memory by alternating δ-oscillations, as the silencing of MB neurons enhances cortical δ-oscillations and generates dissociated memory (Jiang, Wang, Luo, Xie, & Guan, 2018). Similarly, we found that gaboxadol infusion into DH also results in enhanced δ power (Meyer et al., 2017), suggesting a possible functional link. It is important to note that any memory role of the SUB efferents to SUM/MB would likely be specific for dissociated memory, since LAP and HAP mice do not show any differences in freezing to context under normal (vehicle) conditions (Fig. 1C), despite the differences in the density of SUB inputs. Therefore, SUB excitatory neurons may exert a dual role: in dissociated memory, through projections to MB, and in ethanol preference, through projections to SUM.

While our results demonstrate the role of SUM in the regulation of ethanol preference, the precise contribution of SUB afferents to this regulatory role of SUM remains to be determined. Furthermore, in order to demonstrate the specific contribution of the excitatory

vGlut1 and vGlut2 SUB-MB/SUM projections in dissociated memory and ethanol preference differences between HAP and LAP mice, it will be important to perform functional analyses in which these projections would be silenced or stimulated. However, these analyses would require cre-expressing mouse lines in order to perform cell type-specific manipulations. Since HAP and LAP mice are outbred strains these analyses are not possible at this time, but our data indicate the need for developing models that would allow for combined genetic and circuit analyses of complex behavioral phenotypes.

Despite the common view that the optimal therapeutic approach for the treatment of comorbid AUD and dissociative disorders requires the concurrent treatment of both, in order to prevent the untreated disorder aggravating the one being treated (Greenfield et al., 1998; Haver, 2003; Kushner et al., 2005), very little research is focused on the treatment of patients with dual diagnosis. Here, we investigated molecular and circuit traits that may contribute to both pathologies. Our findings that predisposition to dissociated memory and high ethanol preference is likely regulated by miRNAs have strong translational potential. Levels of $GABA_AR$-δ in the brain are difficult to measure; however, that may not be the case with miR-365–3p. MiRNAs are released into the circulation, where they can be found within extracellular vesicles (Valadi et al., 2007), or in complex with lipoproteins or RNA-binding proteins (Boon & Vickers, 2013). Many studies demonstrate the relevance of miRNAs as biomarkers for numerous diseases (Anfossi, Babayan, Pantel, & Calin, 2018), including PTSD (Martin et al., 2017; Zhou et al., 2014), with some data suggesting a correlation between the levels of some miRNAs in the brain and plasma (Balakathiresan et al., 2014). Therefore, blood levels of miR-365–3p may be used as a biomarker to predict the development of AUD and dissociative disorders. MiRNAs are also attractive for the development of therapeutic treatments as they are much easier to selectively target then proteins, and manipulation of a single miRNA can affect the function of numerous functionally related proteins, resulting is a strong physiological effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Ethanol preference is associated with susceptibility to form dissociated memories
- High-alcohol preferring mice have elevated $GABA_AR$-δ in dorsal hippocampus
- The level of $GABA_AR$-δ is regulated post-transcriptionally
- High-alcohol preferring mice have reduced subicular efferents to mammillary body

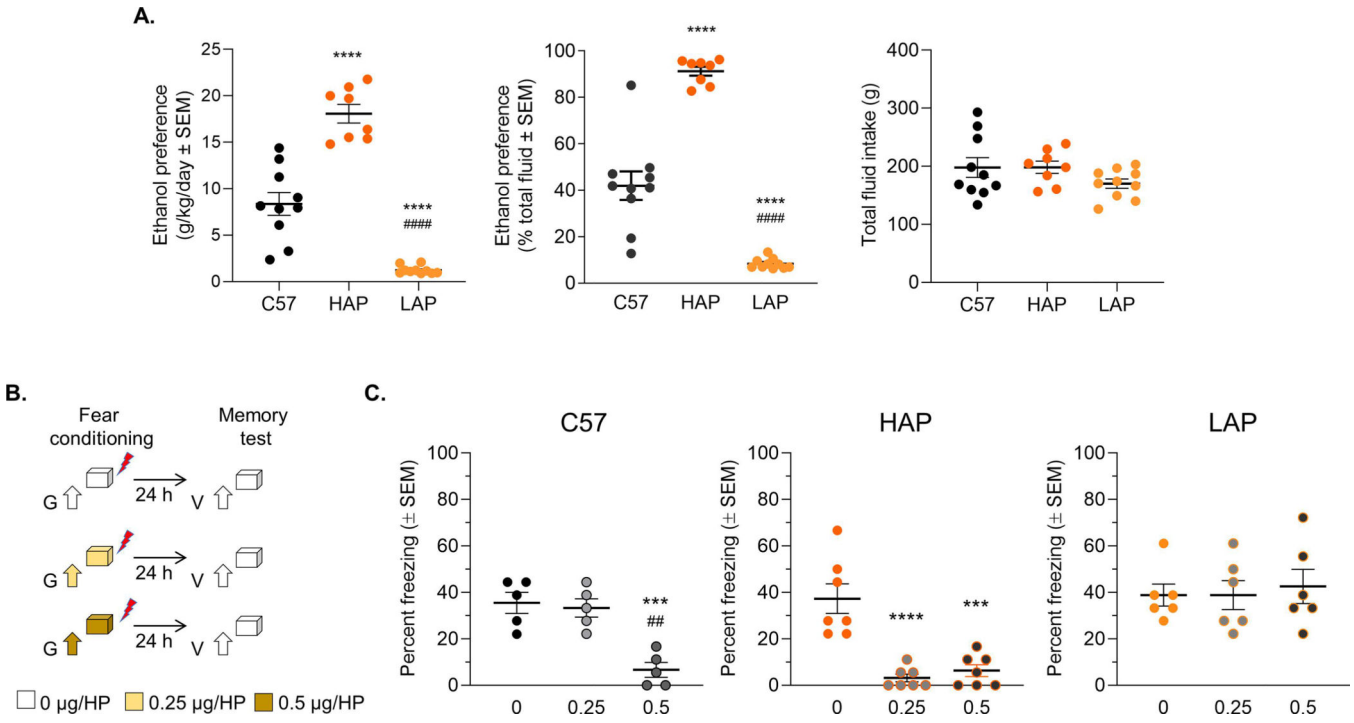

Figure 1:

Ethanol preference association with dissociated memory. A) Ethanol preference measured in two bottle choice procedure. ***P < 0.001 vs. C57, ####P < 0.0001 vs. HAP (one-way ANOVA, n = 10 C57, LAP; 8 HAP; g/kg/day: $F_{2, 25} = 81.28$, $P < 0.0001$; % total fluid: $F_{2, 25} = 105.8$, $P < 0.0001$; total fluid intake: $F_{2, 25} = 1.637$, $P = 0.2148$). B) Experimental outline: indicated amounts of gaboxadol injected i.h. 20 min prior to fear conditioning. Mice were injected i.h. with vehicle and tested 24 h later. Separate groups of mice were used for each condition. C) Effect of gaboxadol injected i.h. before fear conditioning on freezing of C57, HAP and LAP mice. ***$P < 0.001$, ****$P < 0.0001$ vs vehicle, ##$P < 0.01$ vs. 0.35 µg (one-way ANOVA, C57: n = 5/group, $F_{2, 12} = 16.61$, $P = 0.0003$; HAP: n = 7, $F_{2, 18} = 21.34$, $P < 0.0001$; LAP: n = 6/group, $F_{2, 15} = 0.1176$, $P = 0.8898$).

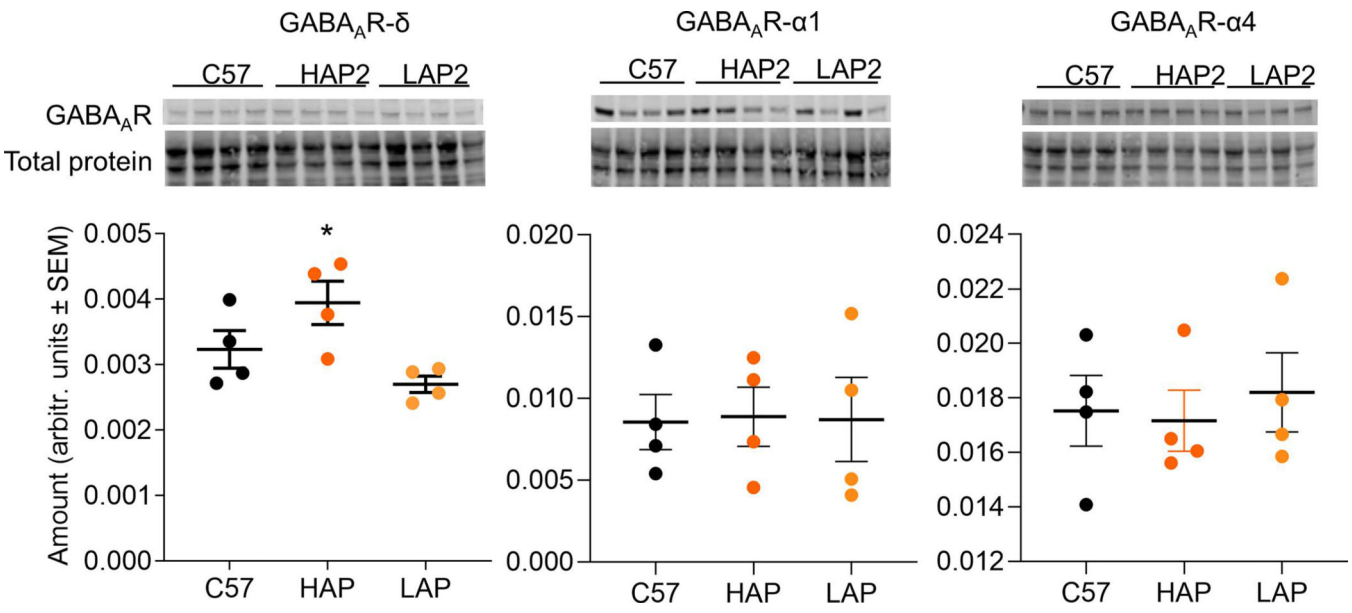

Figure 2:

Extrasynaptic GABA$_A$Rs in DH of naïve C57, HAP and LAP mice. Representative blots (upper panel) and quantified data (lower panel) of the amounts of GABA$_A$R-δ, GABA$_A$R-α1, and GABA$_A$R-α4, determined by western blot. The amounts of individual GABA$_A$Rs were normalized relative to total protein amount. *P < 0.05, vs LAP (one-way ANOVA, n = 4/group; GABAAR-δ: $F_{2, 9} = 5.626$, $P = 0.0260$; GABAAR-α1: $F_{2, 9} = 0.006456$, $P = 0.9936$; GABAAR-α4: $F_{2, 9} = 0.1663$, $P = 0.8493$).

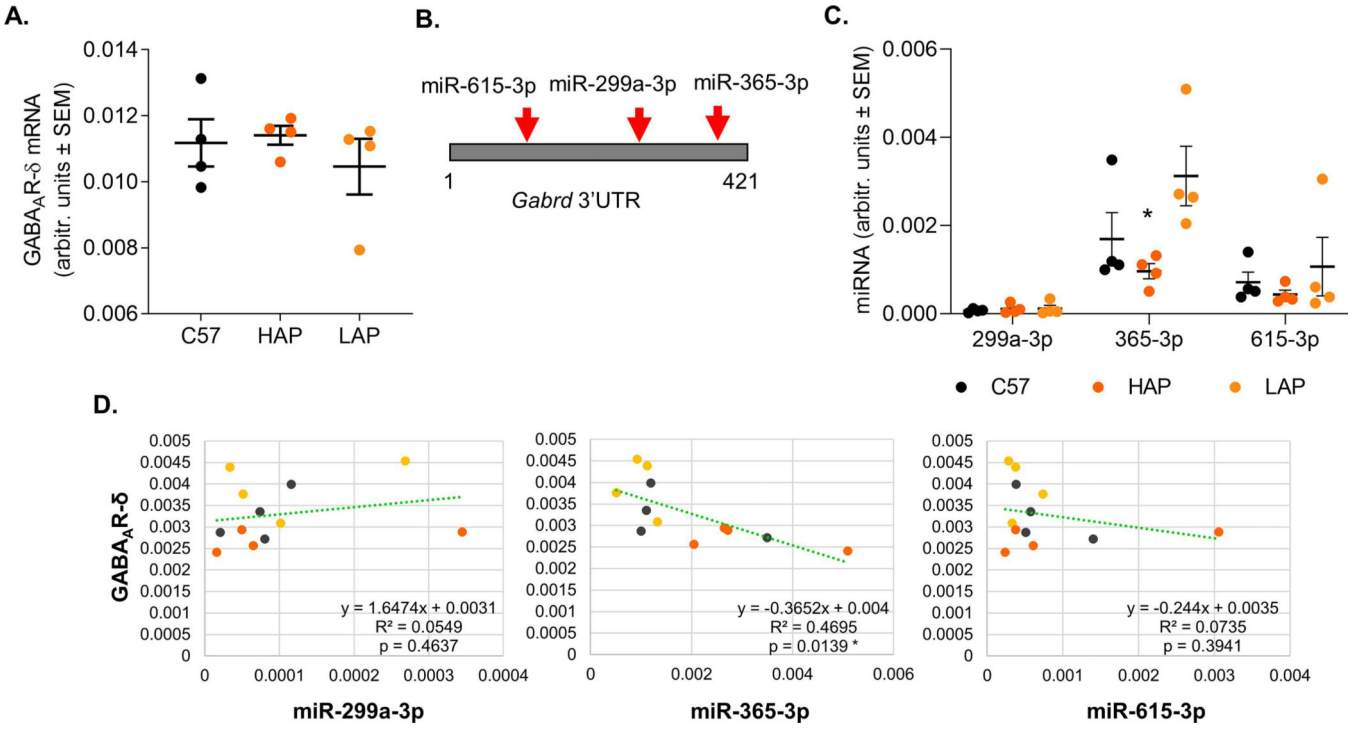

Figure 3:

GABA$_A$R-δ levels in DH are regulated by miR-365–3p. A) *Gabrd* mRNA levels in DH of C57, HAP and LAP mice. The amount was normalized relative to GAPDH. B) Map of *Gabrd* mRNA 3'UTR, with miRNA binding sites marked. Length expressed as number of nucleotides. C) Expression of miRNAs targeting *Gabrd* mRNA 3'UTR in DH of C57, HAP and LAP mice. **P* < 0.05 vs LAP (one-way ANOVA, n = 4/group; miR-299a-3p: $F_{2, 9}$ = 0.2035, *P* = 0.8195; miR-365–3p: $F_{2, 9}$ = 4.292, *P* = 0.0491; miR-615–3p: $F_{2, 9}$ = 0.6017, *P* = 0.5685). D) Linear regression analysis of miRNA and GABA$_A$R-δ protein levels in DH. **P* < 0.05 (miR-299a-3p: $F_{1, 10}$ = 0.5804, *P* = 0.4637; miR-365–3p: $F_{1, 10}$ = 8.852, *P* = 0.0139; miR-615–3p: $F_{1, 10}$ = 0.7931, *P* = 0.3941).

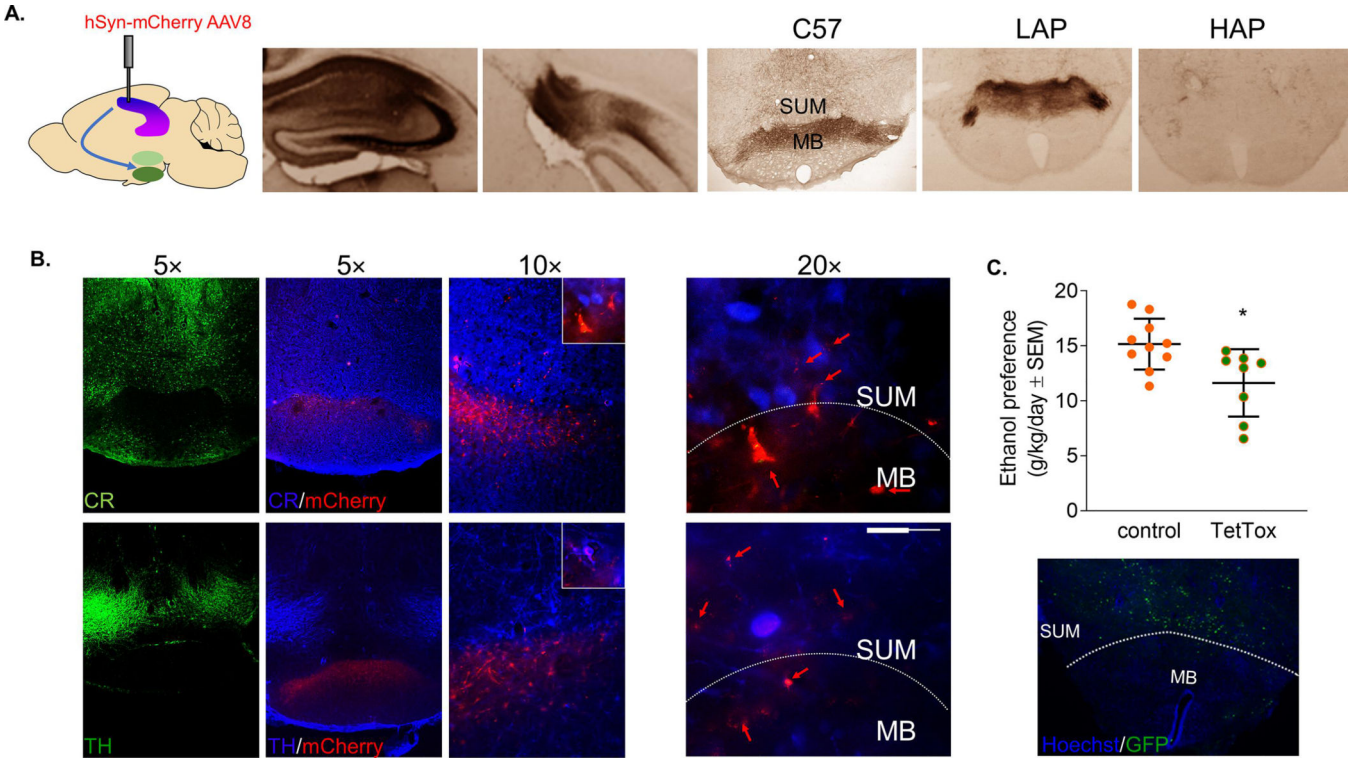

Figure 4:

DH projections to MB are severely reduced in HAP mice. A) hM4D(Gi) [hSyn.hM4D(Gi)-mCherry AAV8] injected into DH of C57, HAP or LAP mice. Six weeks later projections traced by IHC for mCherry. B) Double staining for calretinin (CR) or tyrosine hydroxylase (TH) and mCherry. hM4D(Gi) [hSyn.hM4D(Gi)-mCherry AAV8] injected into DH of C57 mice. Tissue was dissected 6 weeks later, and stained with indicated antibodies. Size bar: 12 μm. C) Ethanol preference of HAP mice injected with control, or tetanus toxin light chain-expressing AAV vector into SUM. Lower panel: representative image showing AAV vector spread. *$P < 0.05$ (unpaired t-test, two-tailed, n = 10 (control), 8 (TetTox)/group; $t_{16} = 2.787$, $P = 0.0132$). SUM, supramammillary nucleus; MB, mammillary body.

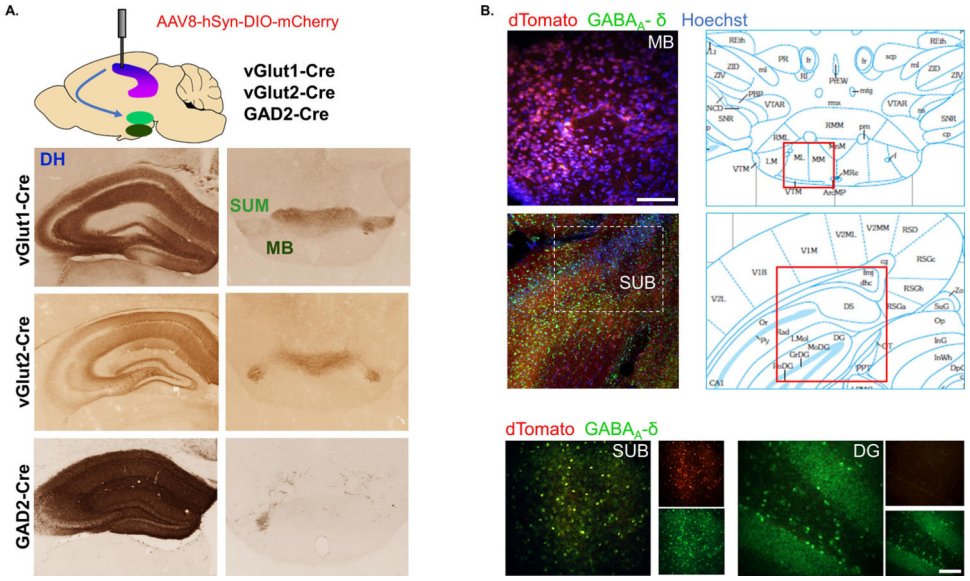

Figure 5:

DH projections to MB stem from GABA$_A$R-δ-containing excitatory neurons in SUB. A) hSyn.DIO.hM4D(Gi)-mCherry AAV8 injection bilaterally into DH of vGlut1-Cre, vGlut2-Cre, or GAD2-Cre transgenic mice. Projections traced by IHC for mCherry. B) Unilateral injection of AAVrgpAAV-hSyn-Cre-P2A-dTomato retrograde tracer into MB of vGlut1-Cre mice. Projecting cells visualized by dTomato intrinsic fluorescence. GABA$_A$R-δ-expressing cells identified by IHC. Upper panels: Expression of the AAV at the injections site (top), and in SUB (bottom). Regions shown in the images are marked in red in the diagrams from the mouse brain atlas (Paxinos). Lower panels: Overlap between dTomato and GABA$_A$R-δ in SUB and DG. Size bar: 25 μm.