ARTICLE

Interneuronal δ-GABA$_A$ receptors regulate binge drinking and are necessary for the behavioral effects of early withdrawal

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Extensive evidence points to a role for GABAergic signaling in the amygdala in mediating the effects of alcohol, including presynaptic changes in GABA release, suggesting effects on GABAergic neurons. However, the majority of studies focus solely on the effects of alcohol on principal neurons. Here we demonstrate that δ-GABA$_A$Rs, which have been suggested to confer ethanol sensitivity, are expressed at a high density on parvalbumin (PV) interneurons in the basolateral amygdala (BLA). Thus, we hypothesized that δ-GABA$_A$Rs on PV interneurons may represent both an initial pharmacological target for alcohol and a site for plasticity associated with the expression of various behavioral maladaptations during withdrawal from binge drinking. To investigate this, we used a mouse model of voluntary alcohol intake (Drinking-in-the-Dark-Multiple Scheduled Access) to induce escalating heavy binge drinking and anxiety-like behavior in mice. This pattern of intake was associated with increased δ protein expression on parvalbumin positive interneurons in both the BLA and hippocampus. Loss of δ-GABA$_A$Rs specifically in PV interneurons (PV:δ$^{-/-}$) increased binge drinking behavior, reduced sensitivity to alcohol-induced motor incoordination, enhanced sensitivity to alcohol-induced hyperlocomotion and blocked the expression of withdrawal from binge drinking. This study is the first to demonstrate a role for δGABA$_A$Rs specifically in PV-expressing interneurons in modulating binge alcohol intake and withdrawal-induced anxiety.

INTRODUCTION

Binge drinking is a dangerous pattern of alcohol consumption that is increasing in prevalence across the world [1]. This is of concern, given the strong association between heavy episodic drinking and the development of alcohol use disorders. Though only 1 in 9 of these binge drinkers will be diagnosed with an alcohol use disorder [2], this pattern of heavy episodic use is also implicated in the development of negative affect and anxiety [3, 4]. Preclinical findings also support the development of anxiety-like behavior during withdrawal from this pattern of alcohol use [5–7]. Given the increasing prevalence of binge drinking, there is a critical need to clarify the biological underpinnings of this type of alcohol intake.

The GABAergic system has long been considered a relevant molecular target for alcohol, given the GABAmimmetic characteristic of many of alcohol's effects, including changes in motor coordination, locomotion and anxiety. Indeed, concentrations of ethanol relevant to an active heavy binge drinking session (i.e., 17–30 mM) have been shown to augment tonic inhibition mediated by extrasynaptic GABA$_A$ receptors [8–16]. Consistent with a role for δ-GABA$_A$Rs in mediating the effects of alcohol, both genetic [17] and pharmacological [18] manipulation of these receptors has been shown to alter alcohol drinking behaviors. Specific to binge drinking, activation of δ-GABA$_A$Rs using THIP has been shown to reduce this pattern of alcohol intake in mice [19–21]. The well-established relationship between neurosteroids that modulate δ-GABA$_A$ receptor activity and alcohol [22–24], also

supports a role for δ-GABA$_A$Rs as a neural substrate for the behavioral effects associated with this pattern of alcohol use.

A number of independent labs have found long-lasting, functionally relevant δ-GABA$_A$R adaptations associated with alcohol exposure. Together, their efforts suggest that persistent δ-GABA$_A$R adaptations may be seen following exposure to alcohol during adolescence [14, 25–27], or alcohol exposure that leads to significant as seen in chronic intermittent ethanol (CIE) paradigms [12, 13, 28]. This persistent downregulation of δ mRNA and protein is even found in the postmortem brains of alcohol-dependent patients [29]. Importantly, though this downregulation in δ-GABA$_A$ is seen following just a single exposure to ethanol, this plasticity is short-lived [30]. This transience raises a question about the role adaptations in this system may play in the relationship between binge drinking and the development of problems with alcohol use and whether δ-GABA$_A$R plasticity is most relevant after multiple cycles of exposure and withdrawal has transitioned the adult binger from restrained and irregular use to an alcohol use disorder.

Interestingly, much of the preclinical work assessing the role for δ-GABA$_A$Rs in mediating the effects of low to moderate alcohol doses have focused on δ-GABA$_A$Rs expressed by principal cells. Though excitatory cells represent the major population of neurons in many circuits relevant to reward and intoxication, inhibitory interneurons play a critical role in regulating the activity of these principal cells and, therefore, may also play a role in mediating the effects of alcohol.

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In support of this hypothesis, the GABA receptor composition in interneurons, which incorporate the δ subunit, has been demonstrated to confer unique sensitivity to low dose ethanol in the hippocampal dentate gyrus [10]. In the hippocampal dentate gyrus, all parvalbumin positive interneurons express δ containing GABA$_A$ receptors [31], while less than 10% of somatostatin interneurons and no calbindin or calretinin positive interneurons expresses this GABA$_A$ receptor subtype. The increased sensitivity to ethanol's effects on tonic inhibition in the molecular layer of the hippocampal dentate may involve interneurons expressing unique δ-GABA$_A$ receptors [10]. Interneurons in the basolateral amygdala (BLA), a region known to express interneurons that play a role in GABAergic regulation of anxiety-like behavior [32], may also express these δ-GABA$_A$ receptors[31]. Indeed, elegant work in the central amygdala(CeA), an amygdalar sub-nucleus with a significant population of interneurons, demonstrates that subpopulations of neurons in the CeA expressing δ- GABA$_A$ receptors have an enhanced sensitivity to ethanol effects on tonic current [33] that may be altered by chronic alcohol exposure [34]. Though present evidence supports changes in the BLA and anxiety associated with alcohol dependence [35], it is unclear whether regulation of δ-GABA$_A$ receptors in this region is involved in the development of anxiety-like behavior following binge.

Based on these findings, we hypothesized that δ-GABA$_A$Rs on interneurons play a role in mediating the effects of ethanol exposure seen following voluntary consumption. To test this hypothesis, we investigated changes in the expression of δ-GABA$_A$Rs on parvalbumin positive interneurons (PV + INs) following binge drinking. To further support a role for δ-GABA$_A$Rs on PV + INs in binge drinking and its behavioral consequences, we utilized mice with selective deletion of δ on PV interneurons (PV:δ$^{-/-}$). Here we demonstrate that these mice consume more alcohol in a limited-access paradigm that models binge-like consumption, display less intoxication to alcohol, and display reduced anxiety-like behaviors during withdrawal from alcohol. These data highlight δ-GABA$_A$Rs on PV + INs as an important target for alcohol and support a role in both the maintenance and consequences of binge alcohol consumption.

METHODS

Animals
Adult (>P60) male and female mice were housed at the Tufts University School of Medicine in a temperature and humidity controlled environment on a reversed 12 h light/dark cycle. Food was available ad libitum in the home cage. Water was available ad libitum in the home cage except when ethanol was made available (see Drinking-in-the-Dark-Multiple Scheduled Access protocol, below). Animals were handled according to protocols approved by the Tufts University Institutional Animal Care and Use Committee.

Animals with parvalbumin-specific deletion of the GABA$_A$-δ subunit (PV:δ$^{-/-}$) were generated by crossing floxed Gabrd mice [36] with PV-Cre mice obtained from Jackson's Lab (Stock #012358). These PV:δ$^{-/-}$ mice have recently been characterized [37, 38]. Genotyping for PV-Cre was performed in-house using the primers listed below. Genotyping for the floxed Gabrd was performed in-house as previously described [36] with the primers listed below to distinguish the wild-type Gabrd (449 bp) or floxed-Gabrd (543 bp) product.
PV-Cre:
5′: CATGAGGAGTGGCATACACG
3′: TTCGCGATTTGAGGTCTTCT
Gabrd:
5′: GACTCCAGTTGCCAAGCCTTTAATTCC
3′: CATCTGCCTGTACCTCCAATGCCTG

DID-MSA
To induce a pattern of escalating binge drinking, the Drinking-in-the-Dark-Multiple Scheduled Access (DID-MSA) procedure was adapted from refs. [39, 40]. Briefly, beginning at lights out, mice received access to water or a 20% unsweetened alcohol solution (95% ethanol Pharmco Products Inc., Brook- field, CT) during three, 1-h drinking sessions. Each drinking session was separated by 2-h breaks where regular water bottles were returned. Volumes consumed were recorded for each 1 h binge-drinking session and summed for the day.

Sucrose preference test
A separate cohort of PV:δ$^{-/-}$ and wild-type mice received 24 h access to sucrose (2% w/v) and water in a two-bottle choice paradigm for 4 days. Fluids were delivered in modified 50 mL conical tubes fitted with balled bearing sippers and were weighed at the same time (ZT 15–16) daily. Tube positions were rotated after 2 days to account for potential side preferences. Sucrose preference was calculated as change in sucrose bottle weight over sum of sucrose and water bottle weight changes ($g_{sucrose}$/$g_{sucrose} + g_{water\ volume\ (mL)}$) of water consumed was also calculated for each individual mouse (1 g_{H2O} = 1 mL$_{H2O}$).

Balance beam
To measure intoxication, mice were trained to traverse a balance beam (122 cm long × 2 cm wide × 4 cm tall). Following the final drinking session on the final day of alcohol access (experiment 2) or 10 min following an intraperitoneal (i.p.) injection of 1.75 g/kg ethanol (experiment 3), each mouse traversed the same beam and the number of hind footslips were recorded.

Open field activity
To measure the locomotor response to ethanol, mice were administered an i.p. injection of saline or ethanol (20% v/v) and tested for 10 min in an open field assay as previously described [41]. Briefly, mice were placed in the center of a 40.64 × 40.64 cm open field photobeam frame lined with 16 photocells spaced 2.54 cm apart (Hamilton-Kinder). Photocell beam-breaks were measured using MotorMonitor software (Hamilton-Kinder) to determine entries into the center zone (20.32 × 20.32 cm square in center of maze) and distance traveled (cm).

Light/dark box
This light/dark box test was performed as previously described [41]. Mice were placed in the dark compartment of a two chambered box enclosed by a 22 × 43 cm photobeam frame with 8 equally spaced photocells (Hamilton-Kinder interfacing with MotorMonitor software) with the open compartment illuminated by a red bulb lamp. The distance traveled in the light compartment and emergence delay was measured using Motor-Monitor software.

Blood ethanol concentration
For mice in experiments 3, submandibular blood samples were collected at the end of the balance beam test (within 12 min of 1.75 g/kg ethanol (20%v/v; i.p). Blood samples were collected via submandibular bleeds into heparinized tubes and centrifuged before storing plasma supernatant at −20 °C. Samples were later analyzed using an Analox Alcohol Analyzer (AM1, Analox Instruments, Lunenburg, MA) and accompanying reagents as described by manufacturer.

Immunohistochemistry
Immunohistochemistry was performed as previously described [42]. Twenty-four hours following behavioral testing, mice were anesthetized with isoflurane and euthanized by rapid decapitation. Brains were removed and immersion fixed by overnight incubation (at 4 °C) in 4% paraformaldehyde. Brains were
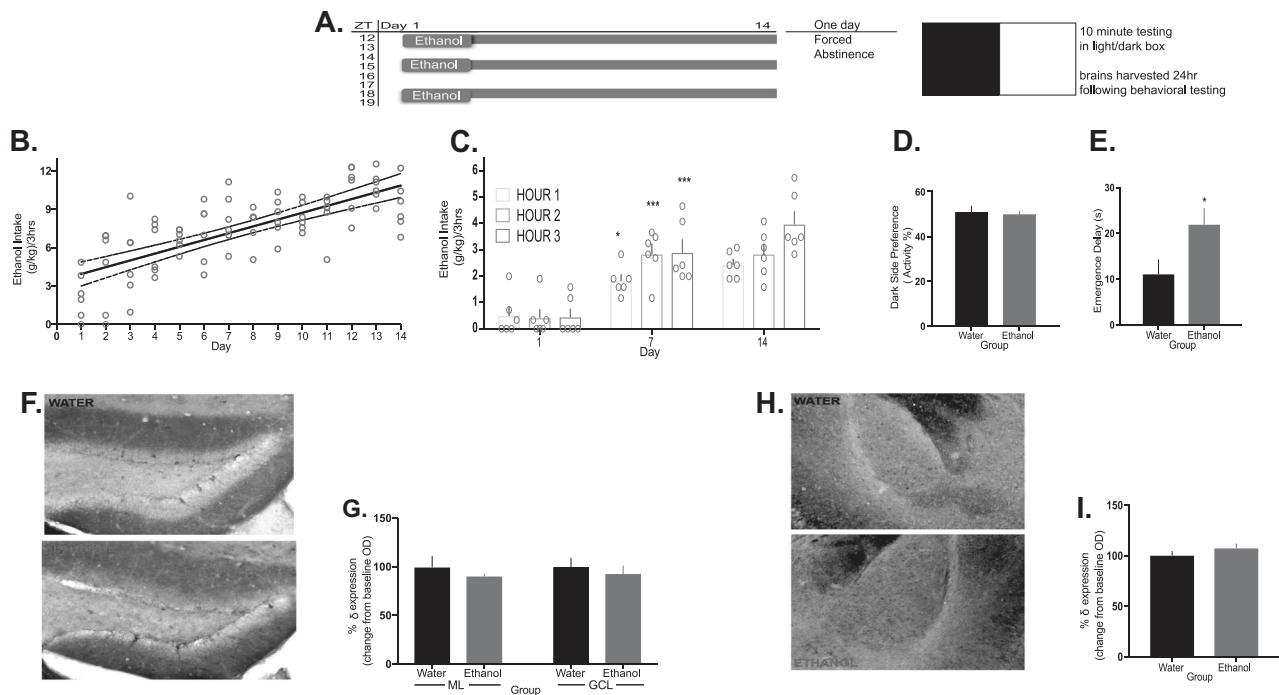
Fig. 1 Escalated heavy binge drinking is associated with anxiety-like behavior during early withdrawal and enhanced δ protein expression in the basolateral amygdala. **a** Female C57BL/6J mice had 2 weeks of access to alcohol in three separate hourly binge sessions (DID-MSA) and 1 day of forced abstinence before testing for anxiety-like behavior using the light/dark box. Brains were collected the day following behavioral testing. **b** Mice steadily increased the total ethanol consumed during the DID-MSA protocol across two weeks ($r^2 = 0.88$, $p < 0.0001$, $n = 6$ mice). **c** This enhanced intake involved increases in the hourly consumption of alcohol by day 7 (*$p < 0.05$, **$p = <0.001$ as compared to hourly intake from day 1) and modest enhancement in the final binge session by day 14. **d** There was no change in exploration of the dark side following binge drinking. **e** There was a significant increase in the time to emerge from the dark side of the box following binge drinking ($t(9) = 2.37$, *$p < 0.05$, $n = 6$ mice per treatment group). Photomicrographs of δ protein immunoreactivity in the hippocampal dentate gyrus (**f**) and BLA (**i**). Overall optical density of δ subunit expression remains unchanged in the molecular or granule cell layer (**g**), and the BLA (**h**) in mice with a 2 week binge drinking history

cryoprotected using overnight incubations in 10 and 30% sucrose, snap frozen in dry-ice chilled isopentane, and maintained at −80 °C until cryosectioning.

For δ immunolabeling with DAB, endogenous peroxidase was quenched in free-floating 40 μm coronal sections with 3%H_2O_2 in methanol for 30 min then incubated in 0.05 M citrate buffer at 90 °C for 45 min to improve antigen retrieval. Slices were blocked in 10% normal goat serum in 1X PBS with 0.3% Triton X-100 for 1 h then incubated with a 1° antibody against δ-GABA_AR (1:250, Millipore AB9752) overnight at 4 °C followed by a 2-h incubation with a biotinylated anti-rabbit antibody and a HRP-conjugated streptavidin (ABC Elite, Vector Laboratories). Immunolabeling was visualized with diaminobenzidine (DAB) and quantified by bright-field microscopy by measuring optical density in the region of interest using Image J-based FIJI software [43]. The tracer tool was used to outline a given region (Basolateral amygdala or hippocampal dentate gyrus) and total optical density was recorded using the software's results manager and saved to Excel for later analysis.

For parvalbumin and δ co-immunofluorescence, free floating 40 μm coronal sections were incubated in 0.5M citrate buffer for 45 min at 90 °C. Slices were blocked in 10% normal goat serum in 1X PBS with 0.3% Triton X-100 for 1 h, then incubated with 1° antibodies against δ-GABA_AR (1:250, Millipore AB9752) and parvalbumin (1:1000, Sigma P3088) for 4 days at 4 °C. Slices were then incubated with a biotinylated goat anti-rabbit (1:1000, Vector Laboratories BA1000) and Alexa-Fluor 647 conjugated goat anti mouse (1:200,ThermoFisher Scientific A28181) and Alexa-Fluor 488-streptavidin (1:200, ThermoFisher Scientific S32354).

Immunolabeling was visualized on a Nikon a1r confocal and quantified using Image J software. A series of 1 μm step images were acquired using a ×40 oil-immersion objective and the same laser power and camera settings for samples across each treatment group. The 1 μm step containing the highest immunoreactivity in the channel corresponding to PV was used to identify PV positive cells and to verify that the δ co-immunofluorescence measured in the respective channel corresponds to that same cell. Specifically, cell bodies within a stack series were traced and saved to the ROI manager and the multi-measure tool was used to measure immunoreactivity in all steps in the stack for later assessment. For analysis, the cell/stack with the highest PV immunoreactivity was used to determine the co-expression of δ, reported as integrated optical density to account for any changes in cell size across cells selected.

Corticosterone ELISA

For mice in experiment 2, corticosterone (CORT) was quantified as previously described [41]. Briefly, following behavioral testing, trunk bloods were collected and centrifuged to isolate plasma. Duplicate 5 μL samples were assayed using an enzyme immunoassay according to manufacturer's specifications (Enzo Life Sciences, Farmingdale, New York) and compared to a standard curve of known CORT concentrations.

Statistical measures

Data were analyzed using Graphpad Prism 7. A linear regression was used to assess escalation in binge drinking across days of access. Student's t-test was used to compare the effects of binge
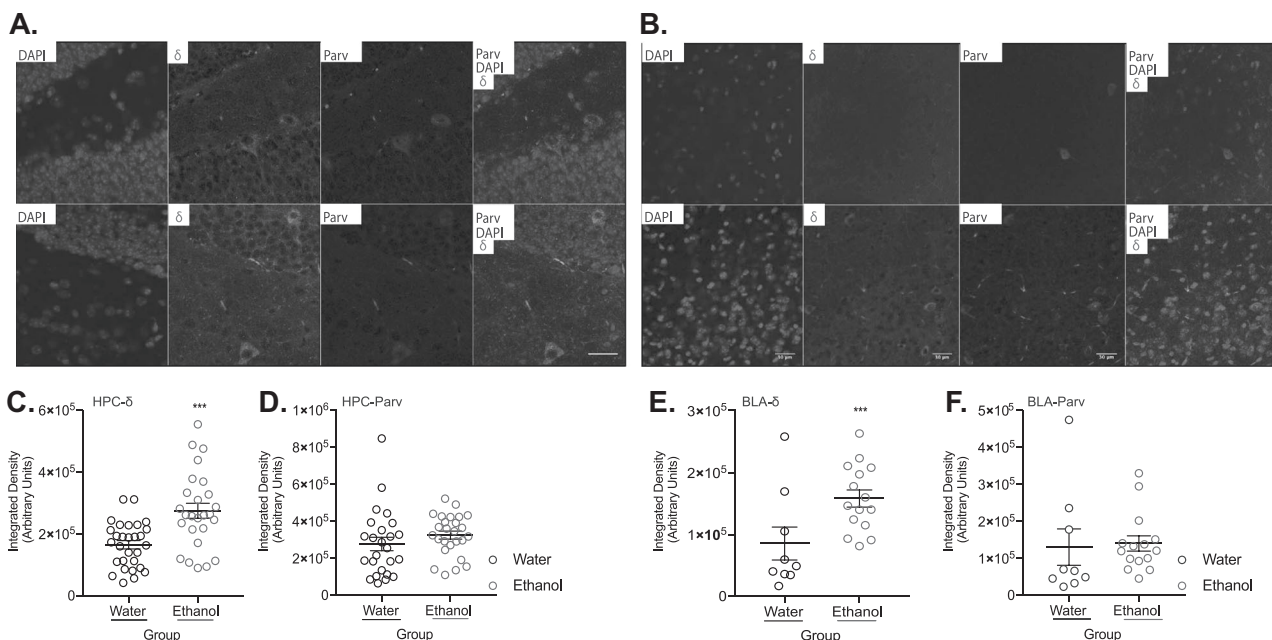
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Fig. 2 Binge drinking increases δ expression on parvalbumin positive interneurons in the hippocampus and basolateral amygdala. **a** Photomicrographs of DAPI (blue), δ (green) and parvalbumin (red) immunoreactivity in the hippocampal dentate gyrus of water (top) or ethanol (bottom) drinkers. **b** There was an increase in δ immunoreactivity in parvalbumin positive interneurons in the hippocampal dentate gyrus in ethanol drinkers ($t(53) = 4.1$, $p < 0.001$; $n = 26$–29 cells per treatment group). **c** There was no significant change in parvalbumin expression levels in the same cells **d**. Photomicrographs of DAPI (blue), δ (green), and parvalbumin (red) immunoreactivity in the basolateral amygdala of water (top) or ethanol (bottom) drinkers. **e** There was an increase in δ immunoreactivity in parvalbumin positive cells in the basolateral amygdala in ethanol drinkers (*$p < 0.05$, 9–15 cells per treatment group). **f** There was no significant change in parvalbumin in the same cells

drinking history (on anxiety-like behavior, δ expression) across groups (ethanol vs. water). For locomotor activity, area under the curve was generated to reflect changes in the distance traveled in each 1 min bout across the 10-min test. To assess the effects of genotype (PV:δ$^{-/-}$ vs. wild type) on binge drinking behavior, anxiety-like behavior, intoxication or locomotor activity, a one-way ANOVA was used within each sex. Bonferroni adjusted post hoc analyses or Dunnett's post hoc tests were used to compare across all groups or to compare groups to an ultimate control (saline treatment, for example) as indicated. For all tests, $p < 0.05$ was used for significance.

RESULTS

Experiment 1: Are changes in δ expression associated with escalated binge drinking and anxiety-like behavior during early withdrawal?

Binge drinking escalates using DID-MSA. Mice received 14 days of limited access to alcohol in a manner shown to produce escalated intake. Over the 14 days of access, females escalated their binge drinking, with a significant linear relationship between day and amount consumed [$r^2 = 0.88$, $p < 0.0001$; Fig. 1b]. Bonferroni adjusted planned comparisons were used to assess degree of change in drinking behavior over the 2 weeks of access (Fig. 1c). By the end of the first week of drinking (day 7) mice increased the amount of alcohol they consumed in hourly binge 1 by 260% [t (20) = 2.96; $p < 0.05$], hourly binge 2 by 575% [$t(20) = 5.42$; $p < 0.0001$] and hourly binge 3 by 532% [$t(20) = 5.5$; $p < 0.0001$].

Early withdrawal from escalated binge drinking associated with anxiety-like behavior. At the end of the 14-day binge drinking period, mice were given 1 day of forced abstinence before testing for anxiety-like behavior during early withdrawal using the light

dark box. There was no significant difference in activity in the dark side of the box (ratio of "dark side distance traveled" to "total distance traveled in the apparatus"; Fig. 1d) between ethanol [$M = 50.27\% \pm 1.17$] or water drinkers [$M = 51.3 \pm 2.45$]. Analysis of the secondary index of anxiety, emergence delay (Fig. 1e), showed that binge ethanol drinkers [$M = 22 \pm 3.44$] took significantly longer to emerge from the dark side of the box at the start of the test, as compared to water drinking controls [$M = 11.17 \pm 3.05$; $t(9) = 2.37$, $p < 0.05$].

Escalated binge drinking does not change overall δ expression in the hippocampus or basolateral amygdala. Following anxiety testing, mice were returned to their home-cages and brains harvested the following day (72 h following final binge) for immunohistochemical analysis as described. In the hippocampus, overall δ expression was not significantly changed by binge drinking history in either the molecular layer [water $M = 99.81 \pm 10.91$., ethanol $M = 90.51 \pm 1.52$, $p = 0.51$; n.s.], or granule cell layer [water $M = 100.06 \pm 8.75$, ethanol $M = 92.90 \pm 7.79$; $p = 0.58$; Fig. 1g] of the dentate gyrus. Similarly, there was no significant change in the overall expression of δ protein in the BLA of ethanol drinkers [$M = 107.88 \pm 3.88$] when compared to controls [$M = 100.7 \pm 3.66$].

Escalated binge drinking associated with specific increase of δ protein on parvalbumin interneurons (PV + INs) in hippocampus and basolateral amygdala. We did a cell body restricted assessment of δ protein expression co-localized on parvalbumin positive interneurons in confocal images of the hippocampal dentate gyrus and BLA. In the hippocampus, we found a significant increase in δ expressed on PV + INs in the hippocampus [$t(53) = 4.1$, $p < 0.001$; Fig. 2c] of ethanol drinkers [$M = 274,459 \pm 24,290$ IOD] when compared to water drinking controls [$M = 164,675 \pm 13523$ IOD]. Similarly, in the BLA we
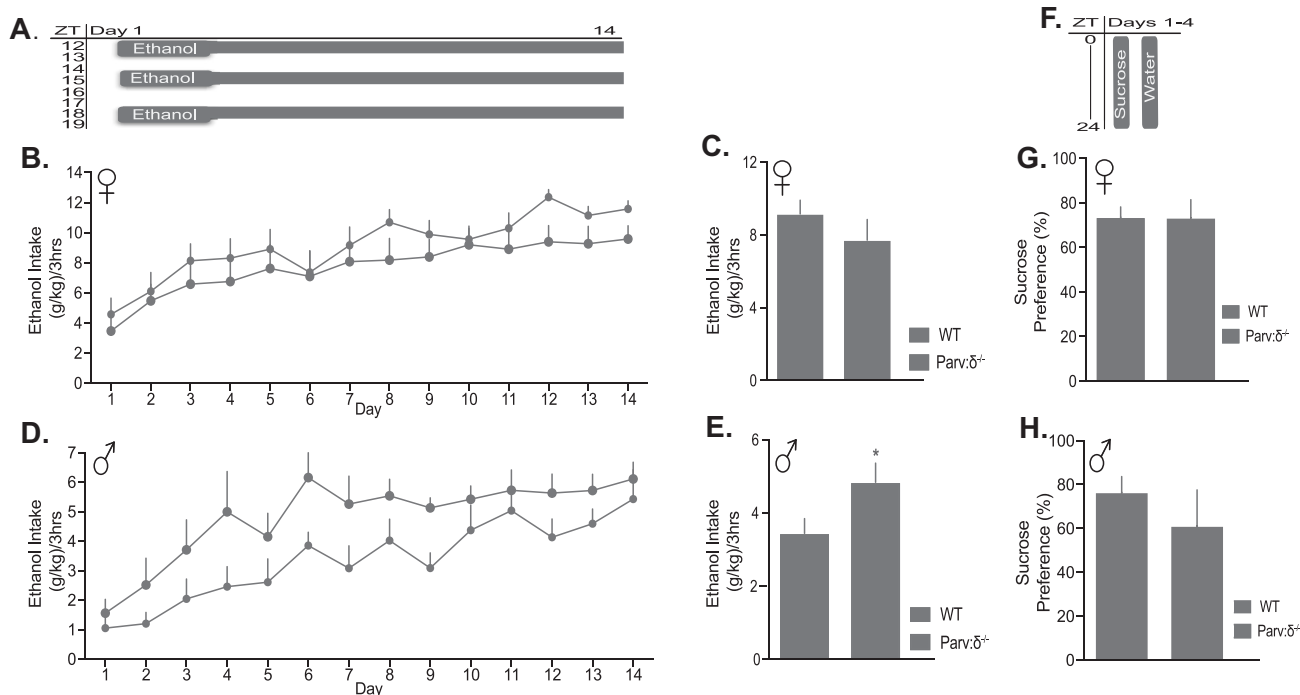
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Fig. 3 Parvalbumin selective deletion of δ GABA$_A$ receptor subunit associated with enhanced drinking in males. **a** Mice had access to ethanol or water using the DID-MSA protocol. **b** Females enhanced intake over days similarly across genotypes **c**. Females consumed similar amounts of alcohol across the access period($n = 8$–9 per genotype). **d** Males enhanced drinking over the 2 weeks ($F(1,13) = 12.57$, $p < 0.0001$) but PV: $\delta^{-/-}$ males showed greater daily intake ($F(1,13) = 5.03$, $p < 0.05$). **e** Average intake across the 2 weeks of access was significantly greater for PV: $\delta^{-/-}$ males ($t(14) = 2.16$, *$p < 0.05$, $n = 7$–8 per genotype). **f** A separate cohort of mice has 24 h access to two bottles, one containing sucrose (2% w/v) or water for 4 days. **g** Females did not show any effect of genotype on the average sucrose preference over these 4 days. **h** Males did not show any significant effect of genotype on preference for sucrose the average sucrose preference over these 4 days

found a significant increase in δ expressed by PV + INs [$t[22] = 2.67$, $p < 0.05$; Fig. 2f] of ethanol drinkers [ethanol $M = 158{,}275 \pm 13{,}995$ IOD] when compared to water controls [$M = 85{,}524 \pm 26{,}643$ IOD]. Parvalbumin expression did not change in the BLA (Fig. 2f; $t[4] = 0.46$; $p = 0.66$) or hippocampus (Fig. 2f) as a function of ethanol exposure.

Experiment 2: Are δ-GABA$_A$Rs on PV-positive interneurons a necessary target for binge drinking?
Males with PV-specific knockout of GABA$_A$-δ binge drink more ethanol. Mice were generated with PV + IN-specific deletion of the GABA$_A$R δ subunit. These PV:$\delta^{-/-}$ mice and their wild-type PV: $\delta^{+/+}$(WT) littermates were singly housed as adults and received limited-access to alcohol or water using DID-MSA protocol for 2 weeks. The total daily alcohol consumed across the 14 days of access is shown in Fig. 3. Females increased drinking over time [$F(13,286) = 18.86$, $p < 0.0001$; Fig. 3b] but showed no effect of genotype. In contrast, males also increased their drinking over time [$F(1,13) = 12.57$, $p < 0.0001$; Fig. 3d], but did show a significant effect of genotype on intake [$F(1,13) = 5.03$, $p < 0.05$] across these days. An assessment of the average ethanol consumed over this two week period showed that PV:$\delta^{-/-}$ males ($M = 4.84 \pm 0.52$, $n = 7$) consumed over 40% more alcohol than their wild-type counterparts ($M = 3.44 \pm 0.40$, $n = 9$) during these 2 weeks of access [$t[14] = 2.16$, $p < 0.05$; Fig. 3e]. A separate cohort of mice given 24 h access to sucrose (2% w/v) and water for 4 days showed no significant effect of genotype on sucrose preference (Fig. 3f–h). Female PV:$\delta^{-/-}$ had a similar preference for sucrose ($M = 73.33 \pm 8.06$, $n = 6$) as wild-type females (73.55 ± 4.57, $n = 6$). Male PV:$\delta^{-/-}$ mice also had similar preference for sucrose ($M = 61.09 \pm 16.43$, $n = 6$) than wild-type males ($M = 76.5 \pm 7.13$, $n = 6$; $p = 0.41$).

Males with parvalbumin-specific knockout of GABA$_A$-δ are less sensitive to motor incoordination associated with binge drinking. Footslips on the balance beam following the final binge session were used as an index of motor incoordination. As our findings confirmed no difference in footslips between the genotypes at baseline, these data were consolidated for water drinkers and a one-way analysis was used to assess data. Females showed no effect of group on the number of footslips measured on the balance beam on this day (water $M = 1.78 \pm 0.26$, $n = 23$; wild-type bingers $M = 2.6 \pm 0.72$, $n = 10$; PV:$\delta^{-/-}$ $M = 3 \pm 0.67$, $n = 9$; Fig. 4b). Males showed a significant effect of group on footslips following the final access of alcohol or water [$F(2,27) = 16.15$, $p < 0.0001$; Fig. 4e]. Wild-type males ($M = 3.67 \pm 0.62$, $n = 9$) were significantly intoxicated on this day of access, demonstrating an increase in the number of footslips; whereas, PV:$\delta^{-/-}$ males that drank ethanol ($M = 1.29 \pm 0.29$, $n = 7$) did not exhibit a difference in footslips compared to water drinking controls ($M = 0.79 \pm 0.21$, $n = 14$).

Mice with parvalbumin-specific knockout of GABA$_A$-δ are less sensitive to ethanol-induced motor incoordination and sedation and more sensitive to ethanol-associated hyperlocomotion. An alcohol naive cohort of PV:$\delta^{-/-}$ mice and their WT counterparts were administered 1.75 g/kg ethanol (20%v/v; i.p.) and tested 10 min later on the balance beam. There was no effect of genotype on blood ethanol levels and mice displayed an ethanol content of 208.21 ± 32.37 mg/dL 10 min following drug administration. During the 10-min wait for balance beam testing, mice were placed in the open field and locomotor activity was recorded. Females showed a significant interaction of group and time on locomotor activity across the 10 min test [$F(18,243) = 4.58$, $p < 0.0001$]. Dunnett post hoc test confirmed that PV:$\delta^{-/-}$ females maintained a more robust hyperlocomotor response to ethanol than wild-type mice, showing
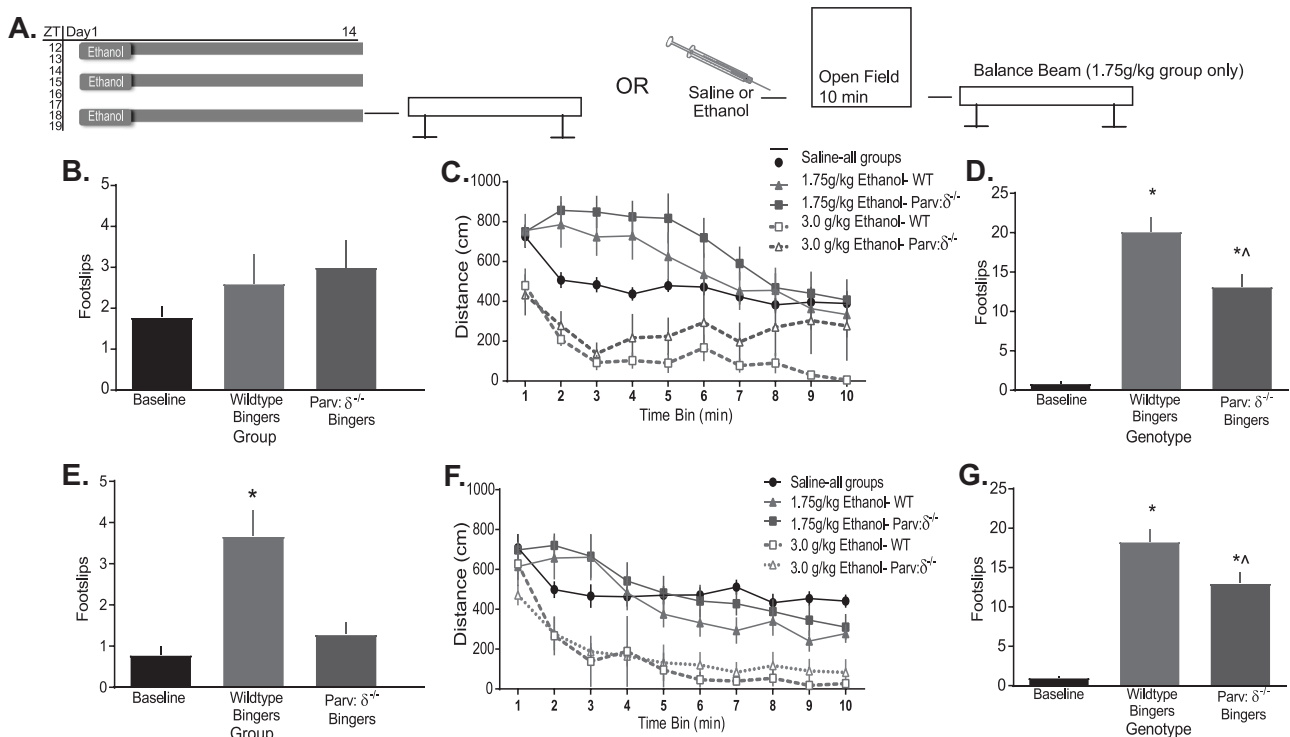
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Fig. 4 PV:δ$^{-/-}$ mice are less sensitive to the sedative effects but more sensitive to the stimulant effects of alcohol. **a** Mice either had access to ethanol (or water) using DID-MSA for 14 days and were tested on the balance beam immediately following the final binge session or naive mice were administered ethanol placed in an open field for 10 min to assess locomotor activity and tested on the balance beam to assess motor incoordination. **b** Females did not show significant increases in footslips following binge drinking ($p = 0.15$; n.s.). **c** Following acute administration of 1.75 g/kg ethanol, wild-type females show increased activity compared to baseline for the first 3 min ($p < 0.05$ when compared to saline) following drug infusion, while PV:δ$^{-/-}$ females maintain a stimulant response for twice as long ($p < 0.05$ when compared to saline) and similarly, wild-type females administered 3.0 g/kg ethanol show significant hypolocomotion for the entire duration of the task, while PV:δ$^{-/-}$ begin to return to baseline activity sooner. **d** PV:δ$^{-/-}$ females also display dampened footslips following exposure to ethanol when compared to ethanol-treated wild-type females (*$p < 0.05$ compared to baseline; ^$p < 0.05$ compared to similarly treated wildtypes). **e** Wild-type males are significantly impaired following binge drinking (*$p < 0.05$ as compared to wildtypes) but PV:δ$^{-/-}$ males do not demonstrate similar motor incoordination **f** Though wild-type males display discrete minutes of hypolocomotion (*$p < 0.05$ as compared to saline), Area Under Curve Analysis (inset) show males do not display significant change in locomotor activity following this dose of ethanol **g**. PV:δ$^{-/-}$ males display dampened motor incoordination when compared to ethanol-treated wild-type males. (*$p < 0.05$, compared to baseline, ^$p < 0.05$, compared to similarly treated wildtypes)

increased locomotion through the first 6 min of testing ($p < 0.05$) compared to the baseline activity of saline treated mice. Males also showed a significant interaction of group and time [$F(18,252) = 2.997$, $p < 0.0001$]. Post hoc test showed that PV:δ$^{-/-}$ males had a unique but transient hyperlocomotor response to ethanol ($p < 0.05$ for second minute) not seen for wild-type mice administered alcohol. Similarly, during the final minutes of testing, when activity for wild-type mice administered alcohol was significantly lower than baseline activity of saline treated mice ($p < 0.05$), PV:δ$^{-/-}$ males did not show this hypolocomotor response. To further evaluate this genotype-specific hypolocomotor response, a separate cohort of mice were included that received 0.5 g/kg and 3.0 g/kg in a random order design. There was an effect of genotype on hypolocomotion following 3.0 g/kg ethanol for females, though not for males. Specifically, PV:δ$^{-/-}$ females show a reduction in activity following just the first minute following ethanol at 3.0 g/kg ($p < 0.05$) while wild-type females demonstrate significant hypolocomotion throughout the 10 min test ($p < 0.05$). Both PV:δ$^{-/-}$ and wild-type males show significant hypolocomotion at 3.0 g/kg ($p < 0.05$). Neither males nor females of either genotype showed a treatment effect of 0.5 g/kg ethanol on activity in the open field (data not shown).

Following testing in the open field apparatus, mice were placed on the balance beam and footslips recorded. Females showed a significant effect of group [$F(2,26) = 81.4$, $p < 0.0001$; Fig. 4d]. Post hoc tests revealed that although this 1.75 g/kg administration of

ethanol significantly increased footslips for both wildtype ($M = 20.13 \pm 1.90$, $n = 8$) and PV:δ$^{-/-}$ females ($M = 13.14 \pm 1.61$, $n = 7$) as compared to saline controls ($M = 0.86 \pm 0.27$, $n = 14$; $p < 0.0001$), the level of intoxication for PV:δ$^{-/-}$ females was significantly lower than that of similarly treated wild-type mice ($p < 0.01$). Males also showed a significant effect of group [$F(2,24) = 68.4$, $p < 0.0001$; Fig. 4g], with both groups of mice administered ethanol showing significant intoxication when compared to saline treated controls ($M = 1 \pm 0.27$, $n = 11$; $p < 0.0001$). However, this dose of ethanol resulted in significantly fewer footslips in PV:δ$^{-/-}$ males ($M = 13 \pm 1.4$, $n = 9$) as compared to the wild-type males administered the same dose of ethanol ($M = 18.29 \pm 1.57$, $n = 7$; $p < 0.05$).

Experiment 3: Do δ-GABA$_A$Rs on PV-positive interneurons play a role in the behavioral consequences of binge drinking?
Mice with parvalbumin-specific knockout of GABA$_A$-δ do not display anxiety-like behaviors after a history of heavy binge drinking. Following a 2-week history of binge drinking, mice were given 1 day of forced abstinence before assessing anxiety-like behaviors using the light dark emergence task. Time in the dark, distance traveled in the dark, percent of activity in the dark (distance traveled in the dark/total distance) and the time (s) to first emerge from the dark side of the light/dark box were used as indices anxiety-like behavior.
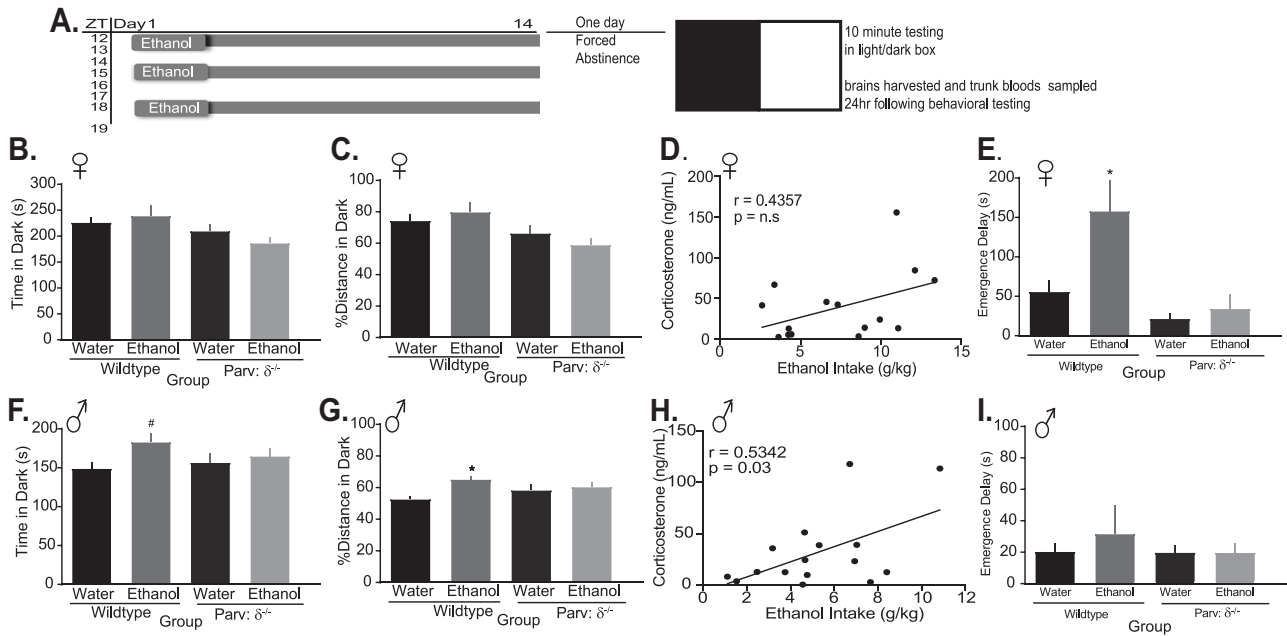
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Fig. 5 PV:δ$^{-/-}$ mice do not display anxiety-like behavior during withdrawal. **a** Mice had access to ethanol (or water) using DID-MSA for 14 days and were tested following 1 day of forced abstinence in the light dark box for 10 min. Brains were collected and trunk bloods sampled 24 h after behavioral testing. **b** There was no effect on time in the dark side of the chamber in any group for female mice. **c** Female wild-type mice do not display change in exploration of the dark box during early withdrawal, though PV:δ$^{-/-}$ females have reduced dark box exploration (reduced anxiety) when compared to similarly treated wild-type females. **d** Females did not demonstrate a relationship between corticosterone and average ethanol consumed during the 2 week access period. **e** Wild-type females show significant increase in time it takes to emerge into the light side of the box (more anxiety-like behavior) after 2 weeks of binge drinking whereas PV:δ$^{-/-}$ females do not. **f** Though there was no group effect on time in the dark, wild-type males do show a marginal increase in time spent in the dark side of the box following binge drinking ($p = 0.06$). **g** Only wild-type males show increased exploration of the dark box (increased anxiety-like behavior) following 2 weeks of binge drinking. **h** Males show significant relationship between circulating corticosterone during early withdrawal and the average amount of daily alcohol consumed during DID-MSA. **i** Emergence delay was not altered by alcohol exposure. (*$p < 0.05$, compared to baseline, ^$p < 0.05$, compared to similarly treated wildtypes)

There was a significant effect of group on preference for the dark [$F(3,40) = 3.44$, $p < 0.05$; Fig. 5]. However, Bonferroni adjusted multiple comparisons showed that wild-type females ($M = 79.84\% \pm 6.33$) did not display increased preference for the dark when compared to their water drinking controls ($M = 74.34\% \pm 3.83$) during this early withdrawal period. Instead, group differences were driven by PV:δ$^{-/-}$ females with a history of binge drinking ($M = 59\% \pm 4.08$) who showed significantly lower preference for the dark side than wild-type mice with a similar history ($p < 0.05$). There was a marginal effect of group on the time spent in the dark during the task [$F(3,40) = 2.54$, $p = 0.06$; Fig. 5b], with wild-type binge drinkers ($M = 239.2 \pm 20.38$) spending similar time in dark as wild-type water drinkers ($M = 226.3 \pm 11.22$) and PV:δ$^{-/-}$ water drinkers but 20% more time in the dark than similarly treated PV:δ$^{-/-}$ females ($M = 186.6 \pm 11.33$). There was a significant effect of group on emergence delay [$F(3,42) = 7.14$, $p < 0.0001$; Fig. 5e]. In particular, wild-type females with a history of binge drinking ($M = 158 \pm 39.65$) took almost three times as long as their water drinking controls ($M = 55.92 \pm 14.59$) to emerge from the dark side of the box ($p < 0.01$), supporting anxiety-like behavior during withdrawal. PV:δ$^{-/-}$ females who consumed alcohol ($M = 34.36 \pm 18.04$) took a similar amount of time to emerge from the dark and explore the anxiogenic side of the box as their water drinking counterparts ($M = 21.8 \pm 7.37$). As with dark side activity, wild-type females with a history of binge drinking took significantly longer than similarly treated PV:δ$^{-/-}$ females to emerge from the dark side of the box than wild-type females ($p < 0.001$).

There was a significant effect of group on preference for the dark side of the box for males [$F(3,26) = 3.9$, $p < 0.02$; Fig. 5].

Bonferroni adjusted comparisons confirmed that wild-type males with a history of binge drinking displayed significantly more preference for the dark side of the box ($p < 0.05$; $M = 65.19\% \pm 2.34$; $n = 6$) than their water drinking controls ($M = 52.68\% \pm 1.86$; $n = 9$), while PV:δ$^{-/-}$ males who consumed ethanol displayed similar dark side activity preference ($M = 60.47\% \pm 3$; $n = 6$) as PV:δ$^{-/-}$ males who consumed water ($M = 58.41\% \pm 3.63$; $n = 7$). Unlike females, males did not show a significant group effect on time to emerge from the dark side of the box ($p = 0.16$; n.s.) nor an effect of group on time in the dark ($p = 0.81$; n.s.).

DISCUSSION
The data presented herein support a significant role for δ containing GABA$_A$ receptors (δ-GABA$_A$Rs) in interneurons, specifically PV-positive interneurons, in both the maintenance of binge alcohol drinking and in the maladaptive development of anxiety that follows this pattern of alcohol use. We demonstrate for the first time that voluntary binge drinking associated with the development of anxiety-like behavior during early withdrawal causes an interneuron restricted increase in the expression of the GABA$_A$R δ subunit in female mice. Our findings also show that the restricted deletion of δ-GABA$_A$Rs from PV-positive interneurons results in an enhanced heavy binge drinking phenotype in males, alters response to the effects of ethanol on locomotor behavior and motor coordination in both males and females. Deletion of the GABA$_A$R δ subunit from PV-positive interneurons also protects mice from the development of anxiety-like behavior during withdrawal from heavy drinking in both sexes. Together, these data strongly support δ-GABA$_A$Rs in PV interneurons as an

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integral target for binge alcohol drinking and its behavioral consequences.

Evidence supporting δ-GABA$_A$Rs as a target for alcohol

Although alcohol has not been shown to directly interact with δ-GABA$_A$Rs, a growing body of evidence shows that the drug can augment tonic inhibition in a number of systems. Experiments using hippocampal slice preparations confirmed that this increase in tonic inhibition is mediated by extrasynaptic δ containing GABA$_A$ receptors (δ-GABA$_A$R), as it may be seen in the dentate gyrus granule cells, but not in CA1 pyramidal cells, where tonic inhibition is mediated by α5 containing extrasynaptic GABA$_A$ receptors that do not express the δ subunit [16]. Relatedly, acute alcohol may increase synthesis and translocation of steroidogenic acute regulatory protein [44–46], an integral protein in the rate-limiting step in the steroid synthesis pathway. This may underlie the increase in synthesis and availability of neurosteroids following acute ethanol ([47]; [22, 23, 48–50]). With this, acute ethanol may augment δ-mediated tonic inhibition and that chronic ethanol exposure may lead to long-term adaptations in this system. Indeed, adult rats chronically exposed to ethanol show reduced expression of δ protein in the hippocampus [12, 51] and BLA [13]. Whether these latter changes are relevant to the effects of ethanol seen in non-dependent binge drinkers is unclear, as these studies used significant exposure paradigms. On the other hand, studies modeling binge drinking have all failed to support changes in this GABA$_A$ receptor protein in adult exposed rats. In these studies, only adolescents exposed to ethanol show long-lasting downregulation in δ protein in the hippocampus and correspondingly, downregulation of δ-mediated tonic inhibition in this region [25, 26]. In this latter case, shorter term ethanol exposure also increases the ethanol-induced enhancement of δ-mediated tonic conductance in dentate gyrus granule cells. The focus solely on principal neurons may overlook the potential role that interneurons may play in the effects of alcohol. In support of this, we find that voluntary binge drinking in mice leads to significant increases in δ protein expression specific to parvalbumin interneurons in the hippocampus and basolateral amygdala, where no changes were found using a broader region-based assessment. Thus, we demonstrate that short term but heavy binge drinking in adults leads to both anxiety-like behavior and changes in δ expression on interneurons.

The development of the global δ knockout mouse was instrumental in our understanding of the significant role that δ-GABA$_A$R mediated tonic inhibition may play in the effects of alcohol. These mice displayed lower sensitivity to the handling-induced convulsions associated with ethanol withdrawal and had lower sensitivity to ethanol-induced motor incoordination [52]. Though this reduced sensitivity would suggest a greater capacity for the consumption of ethanol, male δ$^{-/-}$ mice display lower intake of alcohol in a 24 h preference paradigm, while females show modest reductions in their ethanol preference behavior [52]. Since then, a number of pharmacological studies have confirmed that δ-GABA$_A$Rs are a relevant target for alcohol consumption, as activating this receptor population with THIP prior to drinking reduces ethanol intake behaviors in mice [18–21]. Interestingly, genetic ablation of this receptor system-either locally in regions like the nucleus accumbens [17] or globally as in the case of δ$^{-/-}$ knockout mice [52], also reduces alcohol drinking. This first appears to be a contradiction in the effects of pharmacological activation and genetic ablation of the same receptor subtype. However, when one considers that δ-GABA$_A$Rs may be expressed by both excitatory cells and by interneurons that regulate these excitatory cells, it is clear that the cellular subtype matters significantly to the overall effect that modulation of δ-GABA$_A$Rs may have on a given behavior. Indeed, our PV-specific deletion of δ, unlike global deletion of this GABA$_A$R subunit, is associated with

an increase in intake. Interestingly, similar to previous findings, our data suggests that the effects of δ manipulation on intake may be sex specific. Global deletion of δ was associated with a greater reduction in alcohol preference for males than females [52]. Our results here demonstrate that PV-specific deletion of δ produces a male-specific increase in binge drinking. Recent work supporting sex differences in the expression of δ mRNA and protein show lower δ mRNA in females [53]. Relatedly, studies on δ-GABA$_A$ regulation across the estrous cycle in females show that δ-mediated tonic current [54] and δ mRNA and protein [20, 53–56] are all lower in the estrus phase as compared to the diestrus phase. Interestingly δ-mediated tonic current during this diestrus phase-when δ expression is high- is more similar to that seen in males than the other phases [54]. Together, these findings suggest that the higher δ expression in males could play a role in noted sex differences in ethanol intake [57]. However, it is unclear whether the male-specific increase in intake following deletion of δ in PV interneurons noted here truly is related to biological sex, or a product of a ceiling effect; as females in the DID-MSA limited access paradigm consume significantly more ethanol than males, there may be little room to see greater intake using short periods of ethanol access. Future studies clarifying the role that δ-GABA$_A$ receptors may play in sex differences in ethanol preference and voluntary consumption are warranted.

Ethanol has a biphasic effect on locomotion, with lower blood ethanol concentrations causing an initial stimulant response and higher concentrations producing hypolocomotion or sedation. Our data suggest a role for δ-GABA$_A$Rs in sensitivity to the stimulant side of this biphasic response curve, given that the change in locomotor activity following ethanol is greater for female PV:δ$^{-/-}$. We do not see this for males, although their stimulant response appears to be reduced during this period than females, as others have found [58]. Evidence supports a positive relationship between the stimulant response to alcohol during the rising phase of the alcohol concentration curve and future binge drinking [59], progression of alcohol use disorder [60, 61] and current heavy drinking status [62]. Future work should clarify a potential role for interneuronal δ-GABA$_A$Rs in ethanol-induced motor incoordination and/or sedation. Whether changes in δ expression demonstrated at 48 h following cessation of binge drinking in wild-type mice are relevant to their behavioral dysfunction measured at 24–32 h following cessation of binge drinking and the role that these differences in the acute reactivity to the drug across these genotypes warrants further investigation.

Maladaptive behaviors following binge drinking

Much of the concern regarding the increasing prevalence of binge drinking comes from the relationship between this pattern of alcohol use and the development of an alcohol use disorder (AUD), with about 10% of binge drinkers having an AUD [2]. However, studies showing mood disturbances and anxiety in non-dependent binge drinkers suggest other psychiatric disorders may precede the development of an AUD in this population and proper treatment associated with these affective dysfunctions may offer an opportunity to block the development of further problem drinking.

Extended binge drinking may produce anxiety-like behavior [6]. Previous studies demonstrate that acute alcohol may reduce the time rodents take to emerge from the dark to the light [63]. Here we demonstrate anxiety-like behavior during early withdrawal from binge drinking and that this maladaptation was blocked in PV:δ$^{-/-}$ mice. Together, these data support δ on PV-interneurons as a significant mechanism driving anxiety-like behavior following binge drinking. Though this also suggests a potential role for δ-GABA$_A$Rs on parvalbumin interneurons in mediating the anxiolytic effects of acute alcohol, this remains to be explored.

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Together, the data presented herein demonstrate that δ-GABA$_A$Rs in PV-positive interneurons are not only a relevant target for the long-term effects of binge drinking, but may regulate the behavior itself, potentially via its role in mediating the sedative effects of alcohol or the rewarding/stimulating effects of the compound. Identifying molecular differences in this subtype of δ-GABA$_A$Rs vs δ-GABA$_A$Rs expressed by other cell types offers a potential therapeutic target for the reversal of anxiety associated with heavy drinking.

ADDITIONAL INFORMATION
Competing interests: LCM, JTN, ASJ, and KM report no competing financial interests or conflicts of interest. JLM is a member of the Scientific Advisory Board and consultant for SAGE Therapeutics, a relationship overseen by Tufts University and does not represent a conflict with the current study.

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