Behavioral/Systems/Cognitive

α4-Containing $GABA_A$ Receptors in the Nucleus Accumbens Mediate Moderate Intake of Alcohol

Mridula Rewal,[1] Rachel Jurd,[1] T. Michael Gill,[1] Dao-Yao He,[1] Dorit Ron,[1,2,3,4] and Patricia H. Janak[1,2,3,4]

[1]Ernest Gallo Clinic and Research Center, University of California, San Francisco, Emeryville, California 94608, and [2]Program in Neuroscience, [3]Department of Neurology, and [4]Wheeler Center for the Neurobiology of Addiction, University of California, San Francisco, San Francisco, California 94143

Alcohol has subjective and behavioral effects at the pharmacological levels typically reached during the consumption of one or two alcoholic drinks. Here we provide evidence that an α4-subunit-containing $GABA_A$ receptor contributes to the consumption of low-to-moderate levels of alcohol. Using viral-mediated RNA interference (RNAi), we found that reduced expression of the α4 subunit in the nucleus accumbens (NAc) shell of rats decreased their free consumption of and preference for alcohol. The time course for the reduced alcohol intake paralleled the time course of *α4* mRNA reductions achieved after viral-mediated RNAi for *α4*. Furthermore, the reduction in drinking was region- and alcohol-specific: there was no effect of reductions in α4 expression in the NAc core on alcohol intake, and reductions in α4 expression in the NAc shell did not alter sucrose or water intake. These results indicate that the $GABA_A$ receptor α4 subunit in the NAc shell mediates alcohol intake.

Key words: **ethanol; addiction; extrasynaptic; self-administration; tonic current; RNA interference**

Introduction

The molecular site(s) of action that support the voluntary consumption of alcohol are not known, but a major contribution of GABAergic systems, especially the $GABA_A$ receptor ($GABA_AR$), has long been postulated (Grobin et al., 1998; Chester and Cunningham, 2002; Koob, 2004; Lobo and Harris, 2008). Recently, there has been great interest in whether $GABA_ARs$ might mediate the pharmacological effects of low-to-moderate (≤30 mM) alcohol brain concentrations attained following the consumption of one or a few alcoholic drinks (cf. Lovinger and Homanics, 2007). $GABA_ARs$ are pentamers assembled from a variety of subunits to form multiple isoforms that are likely to differ in their alcohol sensitivity. Notably, pharmacological actions of low-to-moderate concentrations of alcohol (1–30 mM) have been reported at the α4βδ $GABA_AR$ isoform (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004), and, hence, this receptor is a potential candidate for the mediation of the reinforcing effects of oral alcohol. These findings, obtained in heterologous expression systems or brain slices, are highly controversial (Borghese et al., 2006; Yamashita et al., 2006; Borghese and Harris, 2007; Lovinger and Homanics, 2007). Therefore, to assess the relevance of the potentially alcohol-sensitive α4βδ $GABA_AR$ to alcohol's actions, we used viral-mediated RNAi, which allows for region-specific reductions of target gene mRNA levels in adult animals (Hommel et al., 2003; Lasek et al., 2007), to determine the effect of reductions in α4 expression on oral alcohol intake *in vivo*. If the α4βδ $GABA_AR$ is required for the pharmacological actions of alcohol that support alcohol intake, then reduction of the expression of the α4 subunit should reduce alcohol intake.

The nucleus accumbens (NAc) contributes to the rewarding and reinforcing effects of drugs including alcohol (Koob et al., 1998; McBride et al., 1999; Weiss and Porrino, 2002; Everitt and Robbins, 2005). Studies suggest that the GABAergic system in the NAc is an important mediator of alcohol self-administration. For example the GABA antagonists, SR-95531 (Hyytiä and Koob, 1995) and bicuculline (Hodge et al., 1995), reduce operant alcohol self-administration following NAc microinfusion. Notably, both the mRNA and protein for the α4βδ $GABA_AR$ subunits are detected in the NAc (Pirker et al., 2000; Schwarzer et al., 2001). Therefore we determined the effects of α4 subunit reductions in the NAc on alcohol intake. Because the subregions of the NAc, the core and the shell, have somewhat distinct afferent and efferent projections and correspondingly distinct behavioral functions (Robbins and Everitt, 2002), their role in alcohol intake was examined separately.

Materials and Methods

Design and cloning of short hairpin RNA constructs. Two 21 nt small interfering RNA (siRNA) sequences of the rat α4 subunit (GenBank accession NM080587) were designed using the GenScript web-based program. Specificity of the siRNA sequences was verified by a BLAST search. The two siRNA sequence are as follows: siRNA1 (α4-1), 5′-AUAACAUGACAGCUCCAAAUA; siRNA2 (α4-2), 5′-UGAGUUUGCUGCUGUCAACUA. A nonrelated 19 nt sequence (5′-AUGAACGUGAAUUGCUCAA) was used as a negative siRNA control. To employ viral delivery of double-stranded siRNA, the adenoviral shuttle vector pRNAT-H1.1 (GenScript Corporation) and the adenoviral vector Adeno-X (Clontech) were used. pRNAT-H1.1 is a vector for vector-

Received July 9, 2008; revised Nov. 13, 2008; accepted Dec. 10, 2008.

This work was supported by National Institutes of Health Grant R01 AA016835 and by funds from the State of California for medical research on alcohol and substance abuse through the University of California, San Francisco (P.H.J., D.R.). We thank Dr. Victor Kharazia for expert help with histological assessments.

Correspondence should be addressed to Patricia H. Janak, Ernest Gallo Clinic and Research Center, University of California, San Francisco, 5858 Horton Street, Suite 200, Emeryville, CA 94608. E-mail: pjanak@gallo.ucsf.edu.

R. Jurd's present address: Department of Neuroscience, Tuft University, Boston, MA 02111.

DOI:10.1523/JNEUROSCI.3199-08.2009

 0270-6474/09/290543-07$15.00/0

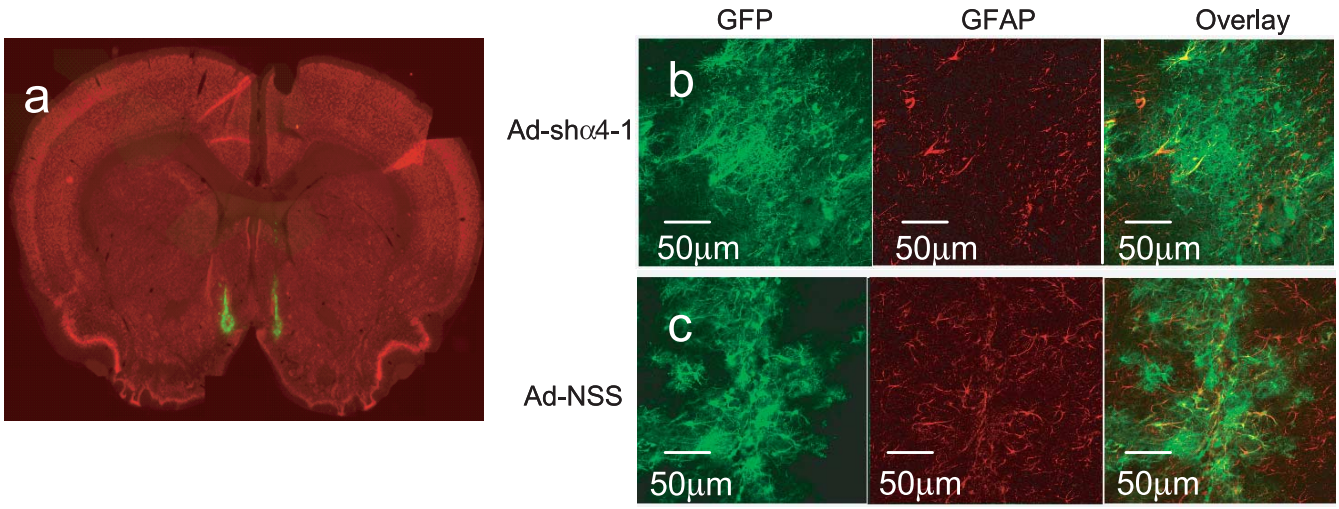

Figure 1. Infection of NAc shell with Ad-NSS and Ad-shα4-1. ***a***, Presence of GFP in NAc 10 d after microinjection of virus, as revealed by anti-GFP antibodies (green). Infection is limited to medial shell region targeted by our infusions. 60 μm slices, neutral red stain. ***b***, ***c***, Section of NAc shell taken 18 d after microinfusion of Ad-shα4-1 (***b***) or Ad-NSS (***c***) stained with anti-GFP (green) and anti-GFAP (red) antibodies. Note minimal overlap (yellow) of staining for GFP and the astrocytic marker, GFAP.

based siRNA cloning with an H1 promoter to express short hairpin RNA (shRNA), and contains GFP under control of the CMV promoter. For each siRNA sequence, two complementary DNA oligos, containing sense and antisense siRNA sequences with a stem-loop structure, were synthesized, annealed, and ligated into *Bam*HI and *Hin*dIII sites of pRNAT-H1.1 vector following the vector cloning protocol. The pRNAT-shRNA recombinants were confirmed by sequencing before subcloning into the cloning sites of I-*Ceu*I and PI-*Sce*I of Adeno-X vector.

Adenovirus production. Preparation of adenoviruses, Ad-shα4-1, Ad-shα4-2, and Ad-NSS, was initiated by transfection of recombinant adenoviral constructs into HEK293 cells using Lipofectamine 2000 (Invitrogen). Recombinant viruses were amplified in HEK293 cells, followed by purification using Adeno-X Virus Purification Kit (Clontech). Viruses with shRNA-recombinants were used to infect SHSY5Y neuroblastoma cells with 20 viruses per cell, which produced almost 100% infection (data not shown).

Subjects. Male Long–Evans rats (Harlan) weighing ~350 g were singly housed with *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee of the Ernest Gallo Clinic and Research Center.

Stereotaxic surgery. Rats anesthetized with isoflurane were stereotaxically infused with Ad-NSS or Ad-shα4 bilaterally in the NAc shell (AP: +1.6 mm, ML: ±0.78 mm, DV: −6.5 mm) or core (AP: +1.2 mm, ML: ±1.9 mm, DV: −6.8 mm; coordinates relative to bregma). A stainless-steel infuser (30 gauge) connected via polyvinyl chloride tubing to a 10 μl Hamilton Gastight syringe was used to infuse 1 μl of virus (1×10^{10} to 3×10^{10} TU/ml) at a rate of 0.1 μl/min for 10 min. After an additional 10 min, the infuser was removed and the scalp closed with sutures. Rats were allowed to recover for 5 d.

Two-bottle preference test (continuous access paradigm). Two bottles, one filled with tap water and the other 10% alcohol (ethanol; v/v), were placed on subjects' cages for 2 weeks before viral infusion. Intake of alcohol and water, as well as body weights, were measured daily, and the left–right position of the bottles was alternated daily to avoid side bias. Five days after viral infusion, rats were again allowed access to 10% alcohol and water for 30 d. To determine whether the alterations in drinking were alcohol specific, a two-bottle preference test comparing 2% sucrose (w/v) and water was conducted in separate rats, as described for alcohol.

Two-bottle preference test (limited access paradigm). To test the role of the $\alpha 4\beta\delta$ GABA$_A$R under conditions likely to induce high intakes, along with corresponding higher blood alcohol levels, a separate group of rats was given limited alchol access for 1 h/d for 21 d following 2 weeks of 24 h *ad libitum* access (as described above). Intake of alcohol and water, as well as body weights, were measured before and after 1 h access every day, and the left–right position of the bottles was alternated daily to avoid side bias. Five days after viral infusion, rats were again allowed access to 10% alcohol (1 h) and water for 30 d.

Immunohistochemistry. Vibratome cut sections (50 μm) of fixed tissue were subjected to immunohistochemistry as described (Kharazia et al., 2003). Primary antibodies used were rabbit anti-GFP polyclonal antibody (1:10,000, Abcam) and anti-GFAP (1:1000, Promega). Secondary antibodies for immunofluorescence were donkey anti-rabbit or anti-mouse antibodies (1:300, Jackson ImmunoResearch). Images were acquired using a Zeiss confocal microscope and visualized using LSM software.

Western blot analysis. Samples were separated by 4–12% SDS-PAGE (Bis-Tris Gel system; Invitrogen) and transferred onto a nitrocellulose membrane (Millipore). The membrane was blocked in milk solution (5% milk in PBS containing 0.05% Tween 20), and incubated with specific primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. The primary antibodies used were anti-α4 antibody (1:500, Novus), anti-α1 antibody (1:500, Phosphosolutions), anti-β2/3 antibody (1:500, Millipore), anti-γ2 antibody (1:500, Alpha Diagnostics), anti-δ antibody (Santa-Cruz Biotechnology), and anti-actin antibodies (1:1000, Santa Cruz Biotechnology). Actin immunoreactivity was used as an internal control. Immunoreactivity was detected by enhanced chemiluminescent reaction and processed using the Typhoon PhosphorImager (Amersham Biosciences). Results were quantified with ImageQuant 5.0 (Amersham Biosciences).

TaqMan quantitative reverse transcriptase PCR. Rats infused with Ad-NSS or Ad-shα4-1 were killed at 5, 10, 18, or 25 d after viral infusion (n = 2–3 per treatment, per time point). Tissue punches from the NAc were collected and processed for measuring GABA$_A$R α4, α1, β2, γ2, or δ mRNA levels by TaqMan quantitative PCR using primer/probe kits (Applied Biosystems) with *GAPDH* as an internal control.

Blood alcohol level measurement. Blood alcohol concentration was determined by gas chromatographic (GC) procedures as described previously (Bowers et al., 2008) with a 0.01 mM limit of detection. Briefly, rats were anesthetized with isoflurane and ~300–500 μl of blood was collected from the lateral, rostral tail vein by venous puncture. Blood was vigorously mixed with inversion and centrifuged at 5000 × g at 4°C for 20 min. Serum was decanted, vortexed, and 10 μl of serum was sealed in a GC autosampler vial (National Scientific) with 10 μl of 0.05% n-propanol as an external pipetting standard. Samples, in triplicate, were heated to 65°C for 20 min, agitated for 30 s, and allowed to settle for 1 min

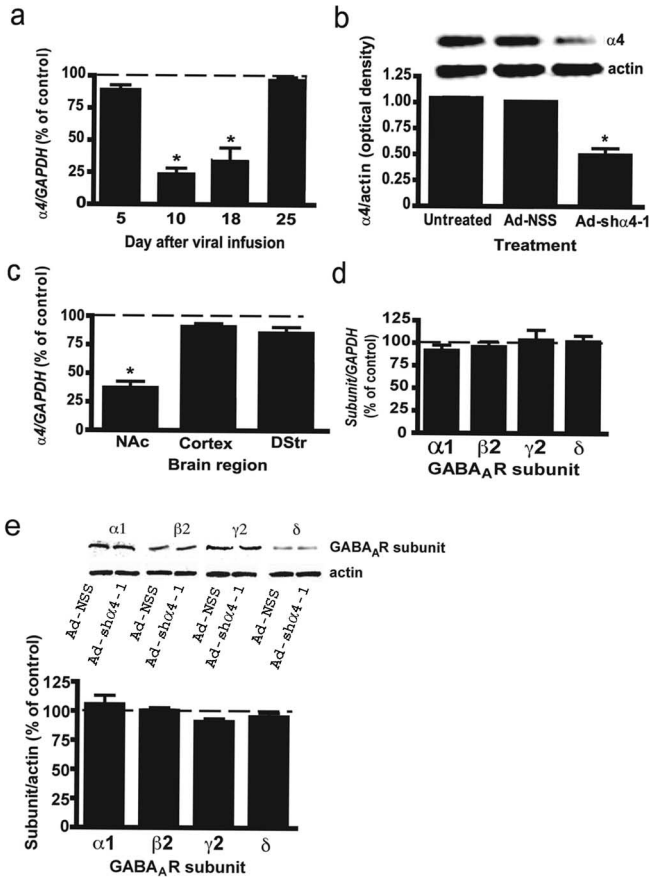

Figure 2. Ad-shα4-1 infection in the NAc shell decreases GABA$_A$R *α4* mRNA and protein levels *in vivo*. GABA$_A$R α4, α1, β2, γ2, and δ subunits' mRNA or protein levels were measured in tissue punches from rat brains after *in vivo* infusion of Ad-NSS or Ad-shα4-1. ***a***, Time course of *α4* mRNA levels in dissected NAc. GABA$_A$R *α4* subunit mRNA levels were decreased 10 and 18 d after infusion. Data presented as mean ratio values (±SEM) expressed as a percentage of the Ad-NSS-treated group (100%). *$p < 0.01$. $n = 3$/group. ***b***, Ad-shα4-1 decreased α4 protein levels in NAc 18 d after infusion *$p < 0.01$, compared with Ad-NSS. $n = 2$ for untreated and $n = 5$ for Ad-NSS and Ad-shα4-1 groups. ***c***, *α4* subunit mRNA levels are reduced in NAc ($n = 5$), but not cortex ($n = 2$) or dorsal striatum ($n = 2$), 18 d after Ad-shα4-1 NAc shell infusion. Data presented as mean ratio values (±SEM) expressed as a percentage of untreated animals (100%). *$p < 0.01$. ***d***, ***e***, No changes in *α1*, *β2*, *γ2*, and δ subunits' mRNA (***d***) or protein (***e***) levels are observed in NAc 18 d after infusion with Ad-NSS or Ad-shα4-1. $n =$ 3/group. Data presented as mean ratio values (±SEM) expressed as a percentage of the control ratio (100%).

before pressurizing for headspace extraction into a 2 ml, depolarized loop (Tekmar Control Systems). Samples were immediately passed through a 220° deactivated, glass-lined inlet (Hewlett-Packard) and subjected to gas chromatography (He, 5 kPa) on a megabore 30 m, 1 μm film Innowax column (Agilent Technologies) at a 45°C isotherm and quantified via flame ionization at 310°C (HP5890, Hewlett-Packard). The column was purged after each sample by holding at 210°C for 1.5 min before cooling to 45°C over 5 min. The alcohol area under the curve (AUC) was divided by the external *n*-propanol standard AUC and compared with known standards from 300 to 0.003 mM.

Data analysis. Gene expression data calculated as a percentage of control were analyzed by one-sample *t* test, which infers variation for a control population based on variability in the sample set. Protein expression data were tested using a two-sample *t* test. Behavioral data were analyzed using one- or two-way ANOVA, followed by Bonferroni's *post hoc* test when indicated by significant ($\alpha = 0.05$) main effects or interactions. Blood alcohol data were analyzed using linear regression.

Results

Reductions in GABA$_A$R *α4* subunit mRNA and protein in NAc by Ad-shα4-1 microinfusion

To test the role of the α4 subunit in alcohol intake, the technique of viral-mediated RNA interference was applied (Hommel et al., 2003; Lasek et al., 2007). We designed two 21 nt siRNA sequences of the rat α4 subunit. These siRNAs were effective in suppressing *α4* mRNA expression in HEK293 cells following transfection of the siRNA constructs recombined in a pRNAT-H1.1 vector, which express the shRNA (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), as well as in striatal neuronal cultures by infection of the siRNA-recombinant adenovirus (Ad-shα4-1) in comparison with the control siRNA-recombinant adenovirus (Ad-shNSS) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Next, we tested the *in vivo* efficacy of the Ad-shα4-1 virus, targeting the α4 subunit, and of the Ad-NSS control virus that expresses an RNA sequence that shares no known homology with any sequence in the rat genome. After infusion into the NAc shell, examination of GFP immunohistochemistry revealed that infection of cells within this region was obtained (Fig. 1). We determined the time course of *α4* mRNA alterations following dissection of the NAc at multiple time points after Ad-shα4-1 infusion. We found a transient decrease in GABA$_A$R α4 subunit mRNA levels at 10 and 18 d after infusion ($p < 0.01$) but not 5 or 25 d (Fig. 2*a*). Ad-shα4-1 also downregulated α4 protein levels in NAc at day 18 ($p < 0.01$) (Fig. 2*b*). To test whether the effect of Ad-shα4-1 was localized to the area of microinfusion within the NAc, we measured *α4* subunit mRNA levels in frontal cortex and dorsal striatum and found that *α4* subunit mRNA levels were reduced only within the NAc ($p < 0.01$) 18 d after NAc shell infusion of Ad-shα4-1 (Fig. 2*c*). To test for compensatory changes in other related GABA$_A$R subunits due to downregulation of the α4 subunit, we measured mRNA and protein levels of the α1, β2, γ2, and δ subunits in NAc and detected no change in the levels of these subunits' mRNA (Fig. 2*d*) or protein (Fig. 2*e*) in the NAc 18 d after viral infusion.

Viral-mediated GABA$_A$R *α4* subunit knockdown in NAc shell, but not core, reduces alcohol intake and preference in a continuous access paradigm

To test the effects of NAc knockdown of the α4 GABA$_A$R subunit on alcohol intake, Ad-shα4-1 or Ad-NSS was infused into the NAc shell of alcohol-experienced rats. Five days later, subjects were allowed continuous access to 10% alcohol and water. Infusion of the Ad-shα4-1 virus reduced alcohol intake (Fig. 3*a*) ($F_{(3,44)} = 17.98$, $p < 0.0001$) and preference (Fig. 3*b*) (main effect of treatment, $F_{(1,22)} = 3.12$, $p = 0.09$; main effect of day, $F_{(3,66)} = 5.24$, $p = 0.002$; treatment × day interaction, $F_{(3,66)} = 16.30$, $p < 0.001$) on the 18th day after infusion ($p < 0.001$, for both intake and preference), a time at which *α4* mRNA is significantly reduced (Fig. 2*a*). Examination of the time course (Fig. 3*c*) of Ad-shα4-1 effects on alcohol intake revealed a main effect of day ($F_{(26,572)} = 7.10$, $p < 0.0001$) and a significant treatment × day interaction ($F_{(26,572)} = 44.94$, $p < 0.0001$) with no main effect of treatment ($F_{(1,22)} = 2.60$, $p = 0.12$). The time course of the onset and offset of the reduction in alcohol intake after Ad-shα4-1 infusion (Fig. 3*c*) paralleled the change in *α4* mRNA expression (Fig. 2*a*). Water consumption was not significantly altered (Fig. 3*d*) ($F_{(3,44)} = 1.36$, $p = 0.54$).

Although we did not take blood alcohol measurements from these particular subjects, we took blood samples 3 h after the start of the dark cycle in a separate group of rats maintained on an

identical two-bottle 24 h access protocol and found measurable alcohol levels (range, 2.3–46.5 mg/dl, $n = 10$).

It is possible that observed effects of short interfering RNA sequences result from a disruption of cellular function, and subsequently, behavior, because the sequence interferes with one or more gene products instead of, or in addition to, the targeted gene. We therefore tested the effects of a second viral vector, Adshα4-2, that expresses a different shRNA sequence that is effective in downregulating the expression of *α4* mRNA and α4 protein levels (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). We again observed a decrease in alcohol intake and preference (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), supporting the conclusion that the decrease in alcohol self-administration is due to a decrease in the expression of the GABA$_A$R α4 subunit in the NAc shell and not to off-target effects.

The NAc shell is proposed to mediate the primary reinforcing effects of drugs of abuse, whereas the NAc core may contribute to conditioned stimulus-supported drug-seeking behavior (Everitt and Robbins, 2005). In line with this view, we found no effect of Ad-shα4-1 infusion into the NAc core on alcohol intake ($F_{(3,44)} = 2.27$, $p = 0.10$) (Fig. 4*a*).

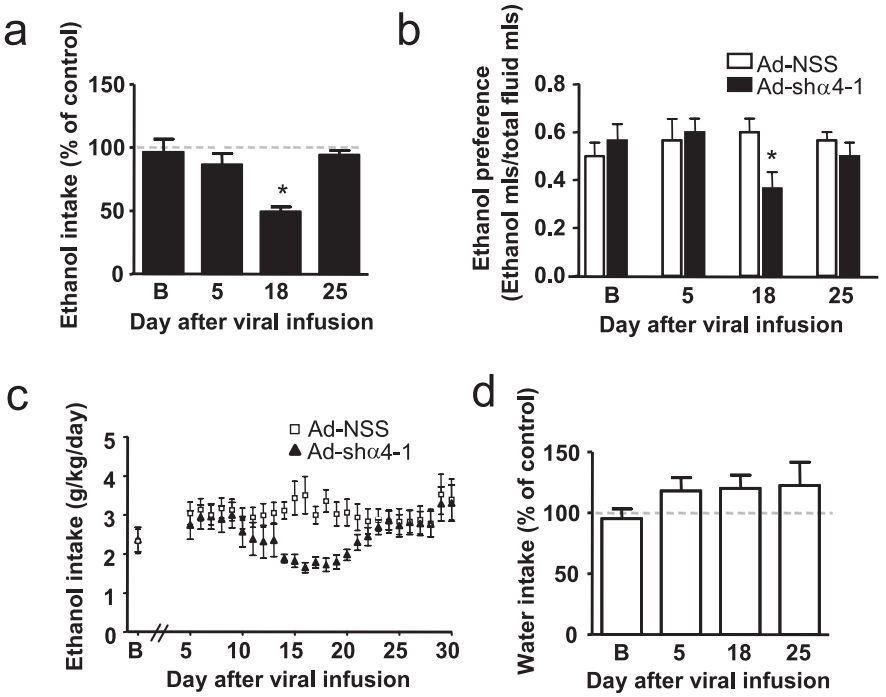

Figure 3. Viral-mediated GABA$_A$R α4 subunit knockdown in NAc shell in rats reduces alcohol intake and preference. Rats were infused in the NAc shell with Ad-shα4-1 ($n = 12$) or Ad-NSS ($n = 12$) bilaterally. ***a***, Estimated alcohol intake (g/kg) for Ad-shα4-1-treated rats expressed as a percentage of Ad-NSS-treated rats. *$p < 0.001$, compared with B (baseline). ***b***, Alcohol preference for Ad-shα4-1-treated and Ad-NSS-treated rats (*$p < 0.001$, compared with Ad-NSS). ***c***, Time course of alcohol intake after Ad-shα4-1 or Ad-NSS treatment. ***d***, Water intake for Ad-shα4-1-treated rats expressed as a percentage of Ad-NSS-treated rats. For all panels, "B" refers to baseline, the last day before virus infusion. Values depict mean ± SEM.

Viral-mediated GABA$_A$R α4 subunit knockdown in NAc shell does not alter sucrose intake

GABA$_A$R activation within the NAc shell is implicated in the control of food preference (Baldo and Kelley, 2007); therefore, we tested whether the α4 subunit contributes to intake of the preferred substance, sucrose. Ad-shα4-1 microinfusion into the NAc shell did not affect intake of a 2% sucrose solution ($F_{(3,44)} = 1.30$, $p = 0.28$) (Fig. 4*b*), suggesting that the effects of GABA$_A$R α4 subunit reduction are specific to alcohol.

Viral-mediated GABA$_A$R α4 subunit knockdown in NAc shell reduces alcohol intake in a limited access paradigm

The effects of NAc knockdown of the α4 GABA$_A$R subunit on limited access alcohol intake was tested following virus microinfusion into the NAc shell. Ad-shα4-1 infusion reduced alcohol intake (Fig. 5*a*) ($F_{(3,44)} = 5.34$, $p < 0.01$) on the 18th day after infusion. Examination of the time course of the effect of the Ad-shα4-1 virus (Fig. 5*b*) revealed a main effect of day ($F_{(26,546)} = 3.36$, $p < 0.0001$) and a significant treatment × day interaction ($F_{(26,546)} = 2.5$, $p < 0.0001$) with no main effect of treatment ($F_{(1,21)} = 4.03$, $p = 0.058$). There was no change in water intake ($p = 0.534$), although the amounts of water consumed in the 1 h measurement period are so low as to approach the limits of our measurement reliability.

The limited access paradigm results in higher blood alcohol levels than we observed within the continuous access paradigm. Blood alcohol levels were measured immediately after 1 h ethanol access from a subset of 10 rats from the above study 1 week before the viral infusions. The blood alcohol levels significantly correlated with oral alcohol intake (alcohol intake: 0.55–1.59 g/kg; blood alcohol levels: 25–83 mg/dl; *$p < 0.05$, $R^2 = 0.413$) (supplemental Fig. 4, available at www.jneurosci.org as supplemental material).

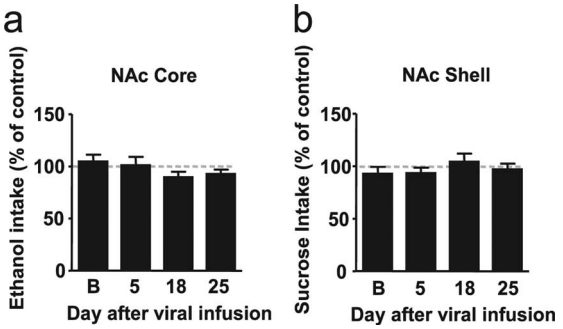

Figure 4. Effect of viral-mediated GABA$_A$R α4 subunit knockdown in NAc shell on alcohol intake is brain region-specific and alcohol-specific. ***a***, Alcohol intake after Ad-shα4-1 infusion into the NAc core. Ad-shα4-1 ($n = 12$); Ad-NSS ($n = 12$). ***b***, Two percent sucrose intake after Ad-shα4-1 infusion into the NAc shell. Ad-shα4-1, $n = 12$; Ad-NSS, $n = 12$. For both panels, "B" refers to baseline, the last day before virus infusion. Data are presented as mean ratio values (±SEM) expressed as a percentage of Ad-NSS-treated rats (100%).

Discussion

We found that alcohol intake and preference were decreased by reductions in GABA$_A$R α4 subunit expression in the NAc shell, but not NAc core. In addition, sucrose intake was not altered by decreases in GABA$_A$R α4 subunit expression in the shell. Together, these results support a role for α4-containing GABA$_A$Rs in voluntary intake of alcohol at the moderate levels experienced after one or a few drinks.

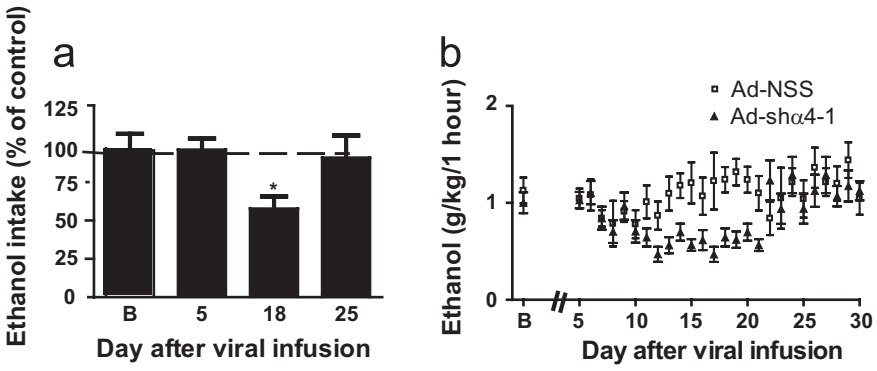

Figure 5. Viral-mediated GABA$_A$R α4 subunit knockdown in NAc shell in rats reduces alcohol intake in a limited access paradigm. Rats were infused in the NAc shell with Ad-shα4-1 ($n = 11$) or Ad-NSS ($n = 11$) bilaterally. ***a***, Estimated alcohol intake (g/kg) for Ad-shα4-1-treated rats expressed as a percentage of Ad-NSS-treated rats. *$p < 0.01$, compared with B (baseline). ***b***, Time course of alcohol intake after Ad-shα4-1 or Ad-NSS treatment. For all panels, "B" refers to baseline, the last day before virus infusion. Values depict mean ± SEM.

GABA$_A$R α4 subunits typically partner with β and δ subunits to form the $\alpha4\beta\delta$ GABA$_A$R isoform. Thus, our findings together with a previous study in which δ knock-out mice consumed less alcohol than wild-type mice (Mihalek et al., 2001) provide strong evidence that $\alpha4\beta\delta$ GABA$_A$Rs contribute to mechanisms mediating alcohol intake. GABA$_A$R α4 subunit knock-out mice have no alteration in acute behavioral effects of alcohol in tests of anxiety, motor behavior, analgesia, and sedation (Chandra et al., 2008). It is interesting to speculate that the α4 subunit may contribute to select behavioral effects of alcohol, including alcohol's reinforcing effects.

Because we altered α4 subunit expression in the NAc, it was possible that decreases in alcohol intake could reflect a role of the subunit in reward or reinforcement in general. Indeed, GABAergic mechanisms in the medial shell have been implicated in the intake of preferred substances; specifically, NAc medial shell microinjection of the direct GABA agonist, muscimol, increases intake of sucrose (Basso and Kelley, 1999). However, our finding of no change in sucrose consumption after knocking down the GABA$_A$R α4 subunit in the NAc shell suggests that this result is specific to pharmacological effects of alcohol and not related to general decreases in reward processing, or in motivation for or ingestion of preferred substances. Instead, this result in combination with the lack of effect of α4 knockdown in the NAc core, is congruent with the proposed role of the NAc shell in the direct reinforcing effect of drugs (Everitt and Robbins, 2005; Ikemoto, 2007). In support of this idea, rats will self-administer cocaine (Rodd-Henricks et al., 2002), amphetamine (Ikemoto et al., 2005), and dopamine agonists (McBride et al., 1999) directly into the medial shell/medial olfactory tubercle, the same subregion that we targeted for our infusions.

α4-containing GABA$_A$Rs in some brain regions have been found to be sensitive to low (1–30 mM) concentrations of alcohol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004). Alcohol concentrations in this range are attained after a few drinks in humans and impair cognition, judgment and motor abilities (Eckardt et al., 1998). We also obtained blood alcohol levels in this range in rats consuming alcohol in both the continuous and limited access paradigms (see Results).

The reduced alcohol intake we observed after knockdown of the α4 subunit in the NAc shell can be explained by a decrease in the expression of an α4-containing GABA$_A$R population at which alcohol exerts direct actions. However, our findings cannot exclude the possibility that α4-containing GABA$_A$Rs mediate indirect effects of alcohol through an intermediary, such as endogenous GABAergic neurosteroids, which act potently at α4-containing GABA$_A$Rs, and are proposed to mediate some GABAergic effects of alcohol (Morrow et al., 2001). In agreement with this possibility, alcohol intake increases the synthesis of the GABAergic neurosteroid, allopregnanolone, in the nervous system (Finn et al., 2004), and allopregnanolone administration alters alcohol self-administration in nondependent rodents (Janak et al., 1998; Sinnott et al., 2002; Janak and Gill, 2003; Ford et al., 2005). An additional possibility is that α4-containing GABA$_A$Rs contribute to drinking via alcohol-induced increases in presynaptic GABA release. Concentrations of alcohol as low as 20 mM may increase presynaptic GABA release in the cerebellum (Carta et al., 2004), as well as in the basolateral nucleus (Zhu and Lovinger, 2006) and, at 44 mM, in the central nucleus (Roberto et al., 2003), of the amygdala. The presynaptic effects of low concentrations of alcohol on GABA release in the NAc shell are not known.

Whether the actions of alcohol at α4-containing GABA$_A$Rs are direct or indirect, the primary effect of activation of these receptors is likely to be mediated by enhanced tonic inhibition. Studies of subunit expression in the thalamus and hippocampus indicate that α4-containing GABA$_A$Rs are primarily extrasynaptic or perisynaptic (Chandra et al., 2006; Liang et al., 2006). These receptors have high affinity for GABA, slowly desensitize, and mediate a tonic inhibitory current that regulates neuronal excitability (Farrant and Nusser, 2005; Chandra et al., 2006). The GABAergic tonic currents mediated by α4-containing GABA$_A$Rs are reported to be alcohol sensitive (Glykys et al., 2007; Liang et al., 2008), although those of the thalamus require higher alcohol concentrations (Jia et al., 2008). Together, these results suggest the compelling hypothesis that alcohol at moderate levels normally produces reinforcing/rewarding effects by enhancing GABAergic tonic current. Consequently, by this hypothesis, reductions in alcohol-induced GABAergic tonic currents via the knockdown of NAc shell α4 levels would reduce drinking. In the current study, it is possible that a decrease in tonic GABAergic inhibition changed ethanol drinking behavior by inducing compensatory changes in excitatory and/or inhibitory synaptic transmission or in voltage-gated ion channel function. However, given the demonstrated receptor specificity of alcohol's enhancement of tonic currents, we favor a direct modulation of α4-containing GABA$_A$Rs by alcohol, rather than a more general mechanism. Of note, we found no evidence for compensatory changes in the expression of the mRNA or protein for the δ, α1, β2, and γ2 subunits after *in vivo* injection of the Ad-shα4-1 virus into the NAc shell.

As the GABA$_A$R α4 subunit partners with the δ subunit at extrasynaptic locations, it is likely that decreasing the expression of δ subunit in NAc shell also would alter alcohol drinking behavior, in line with the findings from $\delta-/-$ knock-out mice (Mihalek et al., 2001). Other GABA$_A$R subunits have been implicated in alcohol intake: GABA$_A$R α1 and α5 subunit knock-out mice each show decreased alcohol preference (Blednov et al., 2003; Boehm et al., 2004), whereas GABA$_A$R β2 (Blednov et al.,

2003) and α2 (Boehm et al., 2004) subunit knock-out mice show no change in their alcohol drinking behavior. The $GABA_AR$ α1 results are particularly interesting in light of recent reports of an unexpected α1- and δ-containing $GABA_AR$ identified in hippocampal interneurons that mediates an extrasynaptic current sensitive to low alcohol concentrations (Glykys et al., 2007). Understanding the contributions of the $GABA_AR$ subunits in the NAc shell other than the α4 awaits future studies of shell-specific reductions in their expression.

In conclusion, we have provided empirical evidence that α4-containing $GABA_ARs$ in the NAc shell play an important role in alcohol drinking behavior, strengthening the hypothesis that the $\alpha4\beta\delta$ $GABA_AR$ in the NAc shell is a key brain substrate for the reinforcing properties of oral alcohol.

References

Baldo BA, Kelley AE (2007) Discrete neurochemical coding of distinguishable motivational processes: insights from nucleus accumbens control of feeding. Psychopharmacology 191:439–459.

Basso AM, Kelley AE (1999) Feeding induced by GABAA receptor stimulation within the nucleus accumbens shell: regional mapping and characterization of macronutrient and taste preference. Behav Neurosci 113:324–336.

Blednov YA, Walker D, Alva H, Creech K, Findlay G, Harris RA (2003) GABAA receptor alpha 1 and beta 2 subunit null mutant mice: behavioral responses to ethanol. J Pharmacol Exp Ther 305:854–863.

Boehm SL 2nd, Ponomarev I, Jennings AW, Whiting PJ, Rosahl TW, Garrett EM, Blednov YA, Harris RA (2004) gamma-Aminobutyric acid A receptor subunit mutant mice: new perspectives on alcohol actions. Biochem Pharmacol 68:1581–1602.

Borghese CM, Harris RA (2007) Studies of ethanol actions on recombinant delta-containing gamma-aminobutyric acid type A receptors yield contradictory results. Alcohol 41:155–162.

Borghese CM, Stórustovu S, Ebert B, Herd MB, Belelli D, Lambert JJ, Marshall G, Wafford KA, Harris RA (2006) The delta subunit of gamma-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. J Pharmacol Exp Ther 316:1360–1368.

Bowers MS, Hopf FW, Chou JK, Guillory AM, Chang SJ, Janak PH, Bonci A, Diamond I (2008) Nucleus accumbens AGS3 expression drives ethanol seeking through G betagamma. Proc Natl Acad Sci U S A 105:12533–12538.

Carta M, Mameli M, Valenzuela CF (2004) Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in Golgi cell excitability. J Neurosci 24:3746–3751.

Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, Harrison NL, Homanics GE (2006) GABAA receptor alpha 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. Proc Natl Acad Sci U S A 103:15230–15235.

Chandra D, Werner DF, Liang J, Suryanarayanan A, Harrison NL, Spigelman I, Olsen RW, Homanics GE (2008) Normal acute behavioral responses to moderate/high dose ethanol in GABAA receptor alpha 4 subunit knockout mice. Alcohol Clin Exp Res 32:10–18.

Chester JA, Cunningham CL (2002) GABA(A) receptor modulation of the rewarding and aversive effects of ethanol. Alcohol 26:131–143.

Eckardt MJ, File SE, Gessa GL, Grant KA, Guerri C, Hoffman PL, Kalant H, Koob GF, Li TK, Tabakoff B (1998) Effects of moderate alcohol consumption on the central nervous system. Alcohol Clin Exp Res 22:998–1040.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci 8:1481–1489.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6:215–229.

Finn DA, Sinnott RS, Ford MM, Long SL, Tanchuck MA, Phillips TJ (2004) Sex differences in the effect of ethanol injection and consumption on brain allopregnanolone levels in C57BL/6 mice. Neuroscience 123:813–819.

Ford MM, Nickel JD, Phillips TJ, Finn DA (2005) Neurosteroid modulators of GABA(A) receptors differentially modulate ethanol intake patterns in male C57BL/6J mice. Alcohol Clin Exp Res 29:1630–1640.

Glykys J, Peng Z, Chandra D, Homanics GE, Houser CR, Mody I (2007) A new naturally occurring GABA(A) receptor subunit partnership with high sensitivity to ethanol. Nat Neurosci 10:40–48.

Grobin AC, Matthews DB, Devaud LL, Morrow AL (1998) The role of GABA(A) receptors in the acute and chronic effects of ethanol. Psychopharmacology 139:2–19.

Hodge CW, Chappelle AM, Samson HH (1995) GABAergic transmission in the nucleus accumbens is involved in the termination of ethanol self-administration in rats. Alcohol Clin Exp Res 19:1486–1493.

Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ (2003) Local gene knockdown in the brain using viral-mediated RNA interference. Nat Med 9:1539–1544.

Hyytiä P, Koob GF (1995) GABAA receptor antagonism in the extended amygdala decreases ethanol self-administration in rats. Eur J Pharmacol 283:151–159.

Ikemoto S (2007) Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. Brain Res Rev 56:27–78.

Ikemoto S, Qin M, Liu ZH (2005) The functional divide for primary reinforcement of D-amphetamine lies between the medial and lateral ventral striatum: is the division of the accumbens core, shell, and olfactory tubercle valid? J Neurosci 25:5061–5065.

Janak PH, Gill TM (2003) Comparison of the effects of allopregnanolone with direct GABAergic agonists on ethanol self-administration with and without concurrently available sucrose. Alcohol 30:1–7.

Janak PH, Redfern JE, Samson HH (1998) The reinforcing effects of ethanol are altered by the endogenous neurosteroid, allopregnanolone. Alcohol Clin Exp Res 22:1106–1112.

Jia F, Chandra D, Homanics GE, Harrison NL (2008) Ethanol modulates synaptic and extrasynaptic GABAA receptors in the thalamus. J Pharmacol Exp Ther 326:475–482.

Kharazia VN, Jacobs KM, Prince DA (2003) Light microscopic study of GluR1 and calbindin expression in interneurons of neocortical microgyral malformations. Neuroscience 120:207–218.

Koob GF (2004) A role for GABA mechanisms in the motivational effects of alcohol. Biochem Pharmacol 68:1515–1525.

Koob GF, Roberts AJ, Schulteis G, Parsons LH, Heyser CJ, Hyytiä P, Merlo-Pich E, Weiss F (1998) Neurocircuitry targets in ethanol reward and dependence. Alcohol Clin Exp Res 22:3–9.

Lasek AW, Janak PH, He L, Whistler JL, Heberlein U (2007) Downregulation of mu opioid receptor by RNAi in the VTA reduces ethanol consumption in mice. Genes Brain Behav 6:728–735.

Liang J, Zhang N, Cagetti E, Houser CR, Olsen RW, Spigelman I (2006) Chronic intermittent ethanol-induced switch of ethanol actions from extrasynaptic to synaptic hippocampal $GABA_A$ receptors. J Neurosci 26:1749–1758.

Liang J, Suryanarayanan A, Chandra D, Homanics GE, Olsen RW, Spigelman I (2008) Functional consequences of GABAA receptor alpha 4 subunit deletion on synaptic and extrasynaptic currents in mouse dentate granule cells. Alcohol Clin Exp Res 32:19–26.

Lobo IA, Harris RA (2008) GABA(A) receptors and alcohol. Pharmacol Biochem Behav 90:90–94.

Lovinger DM, Homanics GE (2007) Tonic for what ails us? High-affinity GABAA receptors and alcohol. Alcohol 41:139–143.

McBride WJ, Murphy JM, Ikemoto S (1999) Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. Behav Brain Res 101:129–152.

Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL, Homanics GE (2001) GABA(A)-receptor delta subunit knockout mice have multiple defects in behavioral responses to ethanol. Alcohol Clin Exp Res 25:1708–1718.

Morrow AL, VanDoren MJ, Penland SN, Matthews DB (2001) The role of GABAergic neuroactive steroids in ethanol action, tolerance and dependence. Brain Res Brain Res Rev 37:98–109.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815–850.

Robbins TW, Everitt BJ (2002) Limbic-striatal memory systems and drug addiction. Neurobiol Learn Mem 78:625–636.

Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR (2003)

Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. Proc Natl Acad Sci U S A 100:2053–2058.

Rodd-Henricks ZA, McKinzie DL, Li TK, Murphy JM, McBride WJ (2002) Cocaine is self-administered into the shell but not the core of the nucleus accumbens of Wistar rats. J Pharmacol Exp Ther 303:1216–1226.

Schwarzer C, Berresheim U, Pirker S, Wieselthaler A, Fuchs K, Sieghart W, Sperk G (2001) Distribution of the major gamma-aminobutyric acid(A) receptor subunits in the basal ganglia and associated limbic brain areas of the adult rat. J Comp Neurol 433:526–549.

Sinnott RS, Phillips TJ, Finn DA (2002) Alteration of voluntary ethanol and saccharin consumption by the neurosteroid allopregnanolone in mice. Psychopharmacology 162:438–447.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, Smith SS (2002) Hormonally regulated alpha(4)beta(2)delta GABA(A) receptors are a target for alcohol. Nat Neurosci 5:721–722.

Wallner M, Hanchar HJ, Olsen RW (2003) Ethanol enhances alpha 4 beta 3 delta and alpha 6 beta 3 delta gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. Proc Natl Acad Sci U S A 100:15218–15223.

Wei W, Faria LC, Mody I (2004) Low ethanol concentrations selectively augment the tonic inhibition mediated by delta subunit-containing $GABA_A$ receptors in hippocampal neurons. J Neurosci 24:8379–8382.

Weiss F, Porrino LJ (2002) Behavioral neurobiology of alcohol addiction: recent advances and challenges. J Neurosci 22:3332–3337.

Yamashita M, Marszalec W, Yeh JZ, Narahashi T (2006) Effects of ethanol on tonic GABA currents in cerebellar granule cells and mammalian cells recombinantly expressing GABA(A) receptors. J Pharmacol Exp Ther 319:431–438.

Zhu PJ, Lovinger DM (2006) Ethanol potentiates GABAergic synaptic transmission in a postsynaptic neuron/synaptic bouton preparation from basolateral amygdala. J Neurophysiol 96:433–441.