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Increased Alcohol-Drinking Induced by Manipulations of mGlu5 Phosphorylation within the Bed Nucleus of the Stria Terminalis

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The bed nucleus of the stria terminalis (BNST) is part of the limbic-hypothalamic system important for behavioral responses to stress, and glutamate transmission within this region has been implicated in the neurobiology of alcoholism. Herein, we used a combination of immunoblotting, neuropharmacological and transgenic procedures to investigate the role for metabotropic glutamate receptor 5 (mGlu5) signaling within the BNST in excessive drinking. We discovered that mGlu5 signaling in the BNST is linked to excessive alcohol consumption in a manner distinct from behavioral or neuropharmacological endophenotypes that have been previously implicated as triggers for heavy drinking. Our studies demonstrate that, in male mice, a history of chronic binge alcohol-drinking elevates BNST levels of the mGlu5-scaffolding protein Homer2 and activated extracellular signal-regulated kinase (ERK) in an adaptive response to limit alcohol consumption. Male and female transgenic mice expressing a point mutation of mGlu5 that cannot be phosphorylated by ERK exhibit excessive alcohol-drinking, despite greater behavioral signs of alcohol intoxication and reduced anxiety, and are insensitive to local manipulations of signaling in the BNST. These transgenic mice also show selective insensitivity to alcohol-aversion and increased novelty-seeking, which may be relevant to excessive drinking. Further, the insensitivity to alcohol-aversion exhibited by male mice can be mimicked by the local inhibition of ERK signaling within the BNST. Our findings elucidate a novel mGluR5-linked signaling state within BNST that plays a central and unanticipated role in excessive alcohol consumption.

Key words: anxiety; bed nucleus of the stria terminalis; binge-drinking; extracellular signal-regulated kinase; Homer protein; mGlu5 receptor

Significance Statement

The bed nucleus of the stria terminalis (BNST) is part of the limbic-hypothalamic system important for behavioral responses to stress and alcohol, and glutamate transmission within BNST is implicated in the neurobiology of alcoholism. The present study provides evidence that a history of excessive alcohol drinking increases signaling through the metabotropic glutamate receptor 5 (mGlu5) receptor within the BNST in an adaptive response to limit alcohol consumption. In particular, disruption of mGlu5 phosphorylation by extracellular signal-regulated kinase within this brain region induces excessive alcohol-drinking, which reflects a selective insensitivity to the aversive properties of alcohol intoxication. These data indicate that a specific signaling state of mGlu5 within BNST plays a central and unanticipated role in excessive alcohol consumption.

Introduction

Individual variation in alcohol sensitivity is a contributing factor to alcoholism vulnerability (Krystal et al., 2003; Trim et al., 2009). The inverse relation between alcohol-sensitivity and addiction vulnerability is well exemplified in pharmacogenetics studies, in

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which alcoholism vulnerability has been associated with dysregulated excitatory neurotransmission, particularly through ionotropic NMDA glutamate receptors (NMDARs; Krystal et al., 2003). In murine models, binge alcohol-drinking elicits robust, behaviorally relevant, increases in both NMDAR and Group1 metabotropic glutamate receptor (mGluRs; subtypes mGlu1 and mGlu5) signaling within structures embedded within the extended amygdala circuit, including the shell subregion of the nucleus accumbens (NAsh) and the central nucleus of the amygdala (CeA). Intact receptor scaffolding by Homer2 is required for the capacity of mGlu1/5 to regulate alcohol sensitivity and intake (Szumlinski et al., 2005, 2008b; Cozzoli et al., 2009, 2012, 2014, 2016); Lum et al., 2014. Homer2 scaffolds mGlu1/5 by interacting with a TPPSPF motif on the C-terminus of the receptor that is targeted by proline-directed kinases, including extracellular signal-regulated kinase (ERK). ERK-dependent phosphorylation of mGlu5(T1123/S1126) increases receptor affinity for Homer EVH1 binding to facilitate mGlu5-dependent gating of slow inward currents through NMDARs (Beneken et al., 2000; Orlando et al., 2009; Hu et al., 2012; Park et al., 2013). The importance of ERK-dependent receptor cross talk for cocaine-induced neuroplasticity within the NAsh was highlighted previously (Park et al., 2013). Here we identified a novel role for ERK signaling in the bed nucleus of the stria terminalis (BNST) regulating alcohol intake.

The BNST is an important hub within the extended amygdala, interconnecting stress and reward subcircuits, which is implicated in alcoholism-related behaviors (cf. Erb et al., 2001; Kash, 2012; Zamora-Martinez and Edwards, 2014; Lovinger and Kash, 2015). Alcohol exposure increases ERK activation throughout the extended amygdala, including the BNST, and systemic treatment with ERK inhibitors increases alcohol intake in mice (Faccidomo et al., 2009). Neurons in the BNST receive glutamatergic innervation from several limbic regions and exhibit mGlu5- and NMDAR-dependent synaptic plasticity in response to noncontingent alcohol (cf. Erb et al., 2001; Kash, 2012; Wills et al., 2012; Lovinger and Kash, 2015). Although relatively little is known regarding how voluntary alcohol intake influences BNST excitatory neurotransmission, binge alcohol-drinking increases immunohistochemical indices of cellular activity within this region, which is associated with anxiety during alcohol withdrawal (Lee et al., 2015).

The present study first tested the hypothesis that a history of binge alcohol-drinking augments BNST indices of glutamate signaling by conducting immunoblotting procedures on BNST tissue from C57BL/6J (B6) mice that underwent our 30 d, single-bottle (20% ethanol v/v) drinking-in-the-dark (DID) procedure (same as the mice used by Cozzoli et al., 2012, 2014, 2016). We next used an adeno-associated virus (AAV) strategy to examine the functional relevance of Homer scaffolding within the BNST for alcohol intake and related the effects to sensitivity to alcohol's sedative-hypnotic properties. We also used neuropharmacological procedures to confirm a necessary role for ERK within the BNST for binge-drinking and the negative affective valence of high-dose alcohol. Last and critically, we then characterized the alcohol phenotype of, and conducted BNST-targeted neuropharmacological studies in, male and female mice heterozygous ($GRM5^{TS/AA}$) or homozygous ($GRM5^{AA/AA}$) for alanine substitutions at T1123 and S1126 of mGluR5 that prevent phosphorylation of the receptor by proline-directed kinases, including ERK (Park et al., 2013). This was done to determine whether mGlu5(T1123/S1126) within the BNST might be a target of ERK regulating the propensity to binge-drink. Additional behavioral experiments related the alcohol-drinking phenotype of the mGlu5 mutants to behavioral endophenotypes associated with alcoholism, notably reactivity to stressors, as well as, examined the generalization of our observed effects to other drugs of abuse or non-drug reinforcers/rewards. Through this study, we identify the BNST as a likely neural locus in which the conditional potentiation of mGlu5-Homer scaffolding by ERK-dependent phosphorylation of mGlu5(T1123/S1126) functions to curb excessive alcohol-drinking by gating the aversiveness of high-dose alcohol intoxication.

Materials and Methods

Animals

Adult (8 weeks of age) male inbred C57BL/6J (B6) mice were purchased from Jackson Laboratories, single-housed and adapted to a temperature-controlled (25°C) and humidity-controlled (71%) colony room under a 12 h reverse light cycle (lights off at 11:00 h) for at least 2 d before surgical procedures. Heterozygous breeder pairs of transgenic mice with alanine substitution mutations at T1123 and S1126 sites on mGlu5 were obtained from the laboratory of Dr. P. F. Worley (Johns Hopkins University School of Medicine; Hu et al., 2012; Park et al., 2013) and used to generate wild-type ($GRM5^{TS/TS}$), heterozygous mutant ($GRM5^{TS/AA}$) and homozygous mutant ($GRM5^{AA/AA}$) offspring. Heterozygous breeder pairs of *Homer1-null* mutant (*Homer1*$^{-/-}$ or KO) mice (Yuan et al., 2003) were also obtained from the Worley laboratory. Mice bred in-house remained multi-housed with same-sex littermates throughout experimentation unless slated (i.e., for home-cage alcohol consumption at which time they were single-housed). All studies of mutant mice used littermates from no less than three different litters and the mice were tested between 7 and 15 weeks of age. Food and water were available *ad libitum*, with the exception of the 2 h binge-drinking period as described in the next subsection. All studies were approved by the IACUC of the University of California Santa Barbara.

Home-cage alcohol-drinking procedures

The procedures used to determine alcohol intake under limited-access (Cozzoli et al., 2009, 2012, 2014, 2016; Lum et al., 2014), as well as both alcohol and saccharin intake under continuous-access conditions (Szumlinski et al., 2008b; Cozzoli et al., 2009; Goulding et al., 2011; Haider et al., 2015), in the home-cage were similar to those described by our group previously. Continuous-access procedures involved simultaneous presentation of four 50 ml sipper tubes containing 0, 3, 6, and 12% (v/v) alcohol or 0, 0.0625, 0.125, 0.25% (w/v) saccharin for 7 consecutive days. In both cases, the volume consumed over a 24 h period was determined by the difference in bottle weight, measured once daily (Szumlinski et al., 2005, 2008b).

For the majority of our limited-access, alcohol-drinking studies, we used modified DID binge-drinking procedures that involved the 2 h presentation of a single 50 ml sipper tube containing 20% (v/v) alcohol. This paradigm consistently generates high alcohol intakes across days and typically results in blood alcohol concentrations (BACs) ≥80% mg (Cozzoli et al., 2012, 2014, 2016; Lum et al., 2014), which satisfies the NIAAA criterion for binge-drinking. Based on prior results indicating that the effects of AAV-mediated manipulations of CeA Homer2 expression manifest only at high alcohol concentrations (Cozzoli et al., 2014), we used a similar multi-bottle DID procedure for the present AAV study in B6 mice, which involved the 2 h presentation of four 50 ml sipper tubes containing 5, 10, 20 and 40% (v/v) alcohol (Cozzoli et al., 2014; Lee et al., 2016). For both DID procedures, at 3 h after lights out, the home-cage

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water bottles were temporarily substituted with alcohol-containing sipper tubes for a 2 h period. As indicated above, the mice used in the immunoblotting study were presented with 20% alcohol for 30 consecutive days. In the AAV study, 5, 10, 20 and 40% alcohol was presented for 7 consecutive days. In the neuropharmacological studies, microinjections did not commence until 20% alcohol consumption stabilized (<10% variability across 3 consecutive presentations), which occurred typically within 3–5 d. All alcohol solutions were reformulated weekly and bottles refreshed as required. Sipper tubes were weighed before and immediately after each 2 h drinking session to ascertain the volume of alcohol consumed by each animal.

For all home-cage drinking experiments, subjects were weighed once a week to calculate alcohol or saccharin intake as a function of the animal's body weight (in kg).

Immunoblotting

B6 mice were killed 24 h following 30 consecutive days of alcohol or water drinking under single-bottle DID procedures (same mice as in Cozzoli et al., 2012) and the BNST was dissected out over ice. The BNST tissue was homogenized in a medium consisting of 0.32 M sucrose, 2 mM EDTA, 1% w/v SDS, 50 μM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, pH 7.2, and 50 mM sodium fluoride, 50 mM sodium pyrophosphate, 20 mM 2-glycerol phosphate, 1 mM *p*-nitrophenyl phosphate, and 2 μM microcystin-LR were included to inhibit phosphatases. Samples were then subjected to low-speed centrifugation at 10,000 × *g* for 20 min. Protein determinations were performed on the supernatant using the Bio-Rad DC protein assay according to the manufacturer's instructions, and homogenates were stored at −80°C until immunoblotting was completed. In this study, protein samples (5–30 μg) were exposed to SDS-PAGE and Tris-acetate gradient gels (3–8%; Invitrogen) were used for separation of all our proteins. Proteins were transferred to PVDF membranes and pre-blocked with PBS containing 0.1% (v/v) Tween 20 and either 5% (w/v) nonfat dried milk powder (for non-phosphorylated proteins) or 5% (w/v) bovine serum albumin (for all phosphorylated proteins) for at least 1 h before overnight incubation with primary antibodies. The following polyclonal antibodies were used: rabbit anti-Homer 2a/b (1:250 dilution; Abcam) and anti-Homer 1b/c (1:1000 dilution; Santa Cruz Biotechnology), mouse anti-mGlu5 (1:500 dilution; BD Transduction Laboratories), rabbit anti-GluN2a and anti-GluN2b (1:500 dilution; Calbiochem), rabbit anti-PI3K antibody (1:4000 dilution; Millipore) and rabbit anti-p(Tyr)p85 PI3K binding motif (1:250; Cell Signaling Technology), rabbit Akt (1:1000; Cell Signaling Technology) and p(Thr308)-Akt (1:500; Cell Signaling Technology), rabbit anti-ERK (1:4000) and mouse p(Tyr204)-ERK (1:2000; both from Santa Cruz Biotechnology), rabbit anti-PKCε and p(Ser729)-PKCε (1:1000; Santa Cruz Biotechnology). Membranes were washed, incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:20,000 to 1:70,000 dilution; Millipore) or anti-mouse secondary antibody (1:20,000 to 1:70,000 dilution; Jackson ImmunoResearch) for 90 min. Immunoreactive bands were sensed by enhanced chemiluminescence using either ECL Plus (GE Healthcare) or Pierce SuperSignal West Femto (ThermoFisher Scientific). A rabbit anti-calnexin polyclonal primary antibody (Stressgen) was also used to index protein loading and transfer. The levels of immunoreactivity for all proteins were quantified with NIH ImageJ, and the immunoreactivity for each protein was first normalized to that of its appropriate calnexin signal to control for uneven protein loading/transfer and the protein/calnexin ratios of each of the samples on a gel were then normalized to the mean of the control samples for each individual gel (n = 3–4 per gel).

Surgical procedures

The surgical procedures for implanting bilateral guide cannulae (20-gauge, 10 mm long; Small Parts) into the BNST of either B6 mice or *GRM5*$^{TS/TS}$ and *GRM5*$^{AA/AA}$ mice were similar to those used in prior neuropharmacological studies by our group (Cozzoli et al., 2009, 2012, 2014, 2016; Lum et al., 2014). Mice were anesthetized by inhalation of 1–2% isoflurane with oxygen as the carrier gas and fixed to a stereotaxic device with tooth and ear bars adapted for mice. The animal's skull was exposed and leveled, and holes were drilled based on a set of coordinates starting from the bregma for the BNST with the arms at a 20° angle (AP: 0 mm; ML: + 3.00 mm; DV: −2.1 mm), according to the mouse brain atlas of Paxinos and Franklin (2004). The skull was prepared for polymer resin application, and then guide cannulae were lowered such that the tips of the cannulae were 2 mm above the intended region. A resin mound was light-cured around the guide cannulae for stabilization. The incision was closed with a tissue adhesive and dummy cannulae (24-gauge, 10 mm long) prevented cannulae blockage or contamination. After surgery, the animals are allowed to recover for a minimum of 3 d before commencing drinking procedures. The location of the microinjections was verified using standard histological approaches on Nissl-stained coronal sections. Only mice with correct placement of microinjector tips within the more dorsal aspects of the BNST were used in the statistical analyses of the results.

Intra-BNST drug infusion

The procedures to infuse pharmacological agents into the BNST of B6 mice or *GRM5*$^{TS/TS}$/*GRM5*$^{AA/AA}$ mice were identical to those used in our previous neuropharmacological studies of binge-drinking (Cozzoli et al., 2009, 2012, 2014, 2016; Lum et al., 2014). Drugs were infused at a rate of 0.25 μl/min for 1 min (vol = 0.25 μl/side). In brief, mice were lightly restrained for removal of the dummy cannulae and sterilized microinjectors (33-gauge, threaded through a 24-gauge wire for stability, 12 mm long) were lowered bilaterally. Drugs were infused at a rate of 0.25 μl/min for 1 min (vol = 0.25 μl/side) and microinjectors left in place for an additional minute to allow for spread. Upon completion of the microinjection, injectors were removed, and the dummy cannulae replaced. For drinking studies, the mice were returned to their home cage for immediate alcohol bottle presentation. For other behavioral studies, the mice were placed immediately into the testing apparatus.

The following drugs and drug doses were infused and were selected based on their ability to inhibit binge alcohol intake or to interrupt some measure of drug reward when infused intracranially: the MEK inhibitor U0126 [1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene; 0, 0.095, 0.95 or 9.5 ng/side, Tocris Bioscience; Koya et al., 2009], the mGluR5-negative allosteric modulator MTEP [3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride; 30 μg/side, Tocris Bioscience; Cozzoli et al., 2012, 2014], the GluN2b-containing NMDAR antagonist ifenprodil [(1*R**,2*S**)-*erythro*-2-(4-benzylpiperidino)-1-(4-hydroxyphenyl)-1-propanol hemi-tartrate; 11 ng/side, Tocris Bioscience; Wang et al., 2010], the mGlu1-negative modulator JNJ-16259685 [(3,4-dihydro-2*H*-pyrano[2,3-*b*]quinolin-7-yl)-(*cis*-4-methoxycyclohexyl)-methanone; 30 pg/side, Tocris Biosciences; Lum et al., 2014], the Class 1 PI3K inhibitor GDC-0941 [(2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1ylmethyl)-4-morpholin-4-yl-thieno(3,2-d)pyrimidine bismesylate; 0.58, 5.8, and 58 ng/side, Axon Medchem; Cozzoli et al., 2014], and the nonselective PI3K inhibitor wortmannin [(1*S*,6b*R*,9a*S*,11*R*,11b*R*)11-(acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3*H*-furo(4,3,2-*de*)indeno(4,5,-*h*)-2-*h*)-2-benzopyran-3,6,9-trione, 50 μg/side; Tocris Bioscience; Cozzoli et al., 2009, 2012, 2014]. To conserve animal numbers, the neuropharmacological studies used within-subjects designs, with no animal receiving more than four microinjections and with the doses counterbalanced across subjects within each treatment/genotype as detailed in the Experimental Design subsection below. No carryover effects of any manipulation were observed on intervening drinking days.

Following behavioral testing, the brains from mice tested under our neuropharmacological procedures were drop-fixed in 4% paraformaldehyde for a minimum of 7 d at room temperature. Then, the brains were sectioned (50–75 μm thick) on a vibratome at the level of the BNST and the sections mounted onto gel-coated slides. The slides were stained with cresyl violet and examined under a light microscope to determine the location of the microinjector tip. Any animals exhibiting one or both tip placements outside of the anterior-dorsal BNST were excluded from data analyses.

Intra-BNST AAV infusion

The specific details related to the construction of the AAVs carrying *Homer2b* and *Homer1a* cDNAs, as well as the AAV carrying an shRNA

against Homer2b, used in this study are detailed in prior reports (Szumlinski et al., 2004, 2005, 2008b; Cozzoli et al., 2009, 2014; Goulding et al., 2011), as is the *in vivo* validation of the efficacy of our AAVs for altering Homer protein expression in brain (Klugmann and Szumlinski, 2008; Ary et al., 2013; Cozzoli et al., 2014; Haider et al., 2015).

Homer2b and Homer1a were expressed as an N-terminal fusion protein with the hemagglutinin (HA) tag in a recombinant AAV backbone containing the 1.1 kb cytomegalovirus immediate early enhancer/chicken β-actin (CBA) promoter (AAV–Homer2b). The same backbone encoding *Renilla* green fluorescent protein (hrGFP) was used as control (AAV–GFP). AAV pseudotyped vectors (virions containing a 1:1 ratio of AAV1 and AAV2 capsid proteins with AAV2 inter-trigeminal regions) were generated as described previously (Klugmann et al., 2005). Briefly, human embryonic kidney 293 cells were transfected with the AAV *cis*-plasmid, the AAV1 (pH21) and AAV2 (pRV1) helper plasmids (pF6), and the adenovirus helper plasmid by standard calcium phosphate transfection methods. For generation of *Homer2b*-specific small hairpin RNAs (shRNAs), we searched the *Homer2b* mRNA using an shRNA design algorithm (Ambion) and identified the targets sequences H2b#1 (5-GUGUGAAUAUGUCUCUGAGTT-3) and H2b#2 (5-CACAGAG UGCUGCCAAUGUTT-3). We generated sense and antisense oligonucleotides corresponding to these targets, annealed them, and cloned them into an AAV plasmid that simultaneously drives the expression of the shRNAs and hrGFP as described previously (Franich et al., 2008). In this cassette, shRNAs for knockdown of Homer2b were driven by the human U6 promoter inserted upstream of the CBA promoter in the AAV–GFP vector (Franich et al., 2008). This bicistronic strategy allowed for easy identification of cells transduced by the shRNA-expressing vector. Genomic titers ranged from 3.6 to 4.9 $\times$ 10^{11} and were determined using a Prism 7700 sequence detector system (Applied Biosystems) with primers designed to woodchuck post-transcriptional regulatory element (During et al., 2003).

Infusion of AAVs occurred in B6 mice under 1% isoflurane anesthesia at a rate of 0.05 μl/min over 5 min (total volume = 0.25 μl/side) and microinjectors were left in place for an additional 5 min postinfusion to allow for spread. Behavioral testing commenced at 3 weeks postinfusion to allow sufficient time for maximal neuronal transduction and localization of neuronal transduction by our AAVs carrying cDNA for Homer2b (cDNA-H2b) or cDNA for Homer2a (cDNA-H1a) was determined by immunohistochemical staining for the HA tag. For AAVs carrying the GFP reporter (cDNA-GFP) or an shRNA against Homer2b (shRNA-H2b), neuronal transduction and localization was examined by GFP immunofluorescence. Neuronal transduction/localization was conducted on coronal sections (50 μm) through the BNST as described previously for other brain regions (Szumlinski et al., 2004, 2005, 2008b; Cozzoli et al., 2009, 2014; Ary et al., 2013) and only animals exhibiting bilateral neuronal transduction within the anterior-dorsal BNST were included in the data analyses.

Operant responding for sucrose and alcohol

A distinct cohort of freely-fed $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice were assayed for sucrose and alcohol reinforcement under operant-conditioning procedures. The mice were trained in 30 min operant conditioning procedures in standard, two-lever, operant chambers, equipped with a reservoir located on the wall between the two levers into which the reinforcer solution was delivered (Med Associates). Depression of the active lever resulted in the activation of the infusion pump, delivery of 20 μl of the reinforcer solution into the reservoir and a 20 s activation of an amber stimulus light above the active lever and tone. During the 20 s cue presentation, depression of the active lever had no further consequences and throughout the session responding on the inactive lever had no programmed consequences. Mice were initially trained to lever-press for a 10% (w/v) sucrose solution under an FI20 (FR1; 20 s timeout) schedule of reinforcement until mice earned a minimum of 10 reinforcers/session with $<$10% variability in responding across 3 consecutive days. All subjects met this qualification within 14–21 d of training. The response requirement was then increased to FR2 and then FR5 (with 20 s timeout), with advancement requiring stable responding across 3 consecutive days (Szumlinski et al., 2005). Following lever-response training for sucrose reinforcement, alcohol was substituted for sucrose and the dose–response function for alcohol reinforcement (5, 10, 20, and 30% v/v) was determined under the FR5 reinforcement schedule with responding for each alcohol dose assessed across 5–7 d and the average of the last 3 d of responding for each dose was calculated for each animal and used in the statistical analyses of the data.

Blood alcohol concentrations

BACs were determined in $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice following their fifth alcohol presentation under DID procedures to determine the correlation between intake and BAC (Cozzoli et al., 2016). For this, blood (75–100 μl) was sampled by submandibular vein puncture immediately upon bottle removal. To relate the excessive alcohol-drinking phenotype exhibited by $GRM5^{AA/AA}$ mice to alcohol metabolism, $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice were injected intraperitoneally with 3.0 g/kg alcohol. As in our prior study of *Homer2* knock-out mice (Szumlinski et al., 2005), blood was sampled from the infraorbital sinus at 5, 15, and 30 min after the alcohol injection. In both studies, plasma was assayed using an Analox Analyzer according to the manufacturer's instructions.

Assays of the sedative-hypnotic effects of alcohol

To determine the effects of the mGlu5 phospho-mutation upon indices of the sedative-hypnotic effects of alcohol, the intoxication expressed by $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice were compared using a fixed-speed (10 rpm) rotarod assay (IIT Life Science). An identical rotarod procedure was used in male B6 mice to determine the effects of locally inhibiting ERK within the BNST upon alcohol-induced motor incoordination, with the exception that mice were infused with different doses of the MEK1/2 inhibitor U0126 before testing. The rotarod procedures used were identical to those described previously by our group (Quadir et al., 2016, 2017). $GRM5^{TS/TS}$, $GRM5^{AA/AA}$ or B6 mice were initially habituated to walking on the apparatus in a 2 min session during which mice were placed immediately back onto the rotarod if they fell. Following habituation, mice underwent a series of 3 min training trials, during which mice remained on the floor of the apparatus if they fell until the next trial began. When an animal could remain on the rotarod for three 3 min trials, it was considered "trained" and this was observed within 3–4 training sessions with no genotypic or sex differences. In the study of the phospho-mutant mice, male and female animals were injected intraperitoneally with either 1 or 2 g/kg alcohol and ~15 min postinjection, each mouse was subjected to three 3 min rotarod testing trials (30 s apart), during which a mouse was left on the floor of the apparatus if it fell until the next trial began and the latency of each fall was recorded. In the neuropharmacological study, male B6 mice were microinjected with either 0, 0.95, or 9.50 ng/side of U0126, before an intraperitoneal injection of 2 g/kg alcohol. Then ~15 min postinjection, the mice were tested in the same manner as the mutant animals. The average latency to fall from the three test sessions was used as an index of alcohol intoxication.

We also used a regain of righting reflex procedure to examine for genotypic differences in the sedative effect of high-dose alcohol, as well as the effects of ERK inhibition upon alcohol-induced sedation. As conducted in prior studies of *Homer2* knock-out mice (Szumlinski et al., 2005), a separate cohort of male and female $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice were injected intraperitoneally with 5 g/kg alcohol and immediately placed in empty home cages. Once immobile (~2–3 min), the mice were placed onto their backs for the duration of the study. The time taken for the animals to right themselves and place all four paws on the floor of the testing cage was determined by behavioral observation using a stopwatch. To probe a working model in which the facilitation of mGlu5-Homer cross-linking functions to reduce sensitivity to the sedative-hypnotic effects of alcohol (Szumlinski et al., 2005, 2008a), we compared also male $Homer1^{+/+}$ and $Homer1^{-/-}$ mice for their ability to regain their righting reflex using identical procedures. Finally, to test the hypothesis that ERK-dependent signaling within the BNST regulates the sedative hypnotic effects of alcohol, we also assayed the effects of infusing 0, 0.95, or 9.5 ng/side U0126 upon the ability of B6 mice to regain their righting reflex. For this latter experiment, mice were microinjected with U0126 before intraperitoneal alcohol injection and behavioral testing.

Place-conditioning and locomotor activity

To determine genotypic differences in the perception of alcohol's interoceptive effects as appetitive or aversive, we conducted an alcohol-induced place-conditioning study in $GRM5^{TS/TS}$, $GRM5^{TS/AA}$, and $GRM5^{AA/AA}$ male and female mice. For alcohol-induced place-conditioning, distinct groups of animals received intraperitoneal injections of alcohol (1, 2, or 3 g/kg; vol = 20 ml/kg; Szumlinski et al., 2008b). To determine whether or not the effects of the mGlu5 mutation on alcohol-conditioning generalized to a different drug of abuse, a distinct cohort of male and female $GRM5^{TS/TS}$, $GRM5^{TS/AA}$ and $GRM5^{AA/AA}$ mice underwent cocaine place-conditioning procedures (3, 10, or 30 mg/kg; vol = 10 ml/kg; a generous gift from National Institute on Drug Abuse, Bethesda, MD; Ary et al., 2013). To localize the effects of interrupting ERK signaling upon alcohol aversion to the BNST, male B6 mice were conditioned with 3 g/kg alcohol using procedures identical to those used in the study of the phosphomutant mice. However, before testing for the conditioned response, mice were microinjected with either 0 or 9.5 ng/side U016.

The apparatus and procedures to induce place-conditioning were similar to those described in published work (Szumlinski et al., 2008a; Ary et al., 2013). To elicit place-conditioning, mice were conditioned in a two-compartment apparatus where the compartments were tactilely (floor texture) and visually (wall pattern) distinct and the time spent in each compartment was recorded using a digital video-tracking system (ANY Maze, Stoelting). Conditioning commenced with a 15 min habituation session to familiarize the animals to the entire apparatus. For each conditioning session, mice were confined to one compartment for 15 min. On alternating days, the mice were injected with an equivalent volume of saline and confined for 15 min to the other compartment. The day following the final pairing (8 pairings for alcohol; 4 pairings for cocaine), animals again had *ad libitum* access to the entire apparatus in a drug-free state during a 15 min post-conditioning test. The amount of time spent on the drug-paired versus saline-paired compartment during the post-conditioning test served to index the direction and magnitude of the conditioned response.

The locomotor activity exhibited by the mice during the alcohol-conditioning sessions was also recorded and the distance traveled during the first conditioning session (i.e., acute locomotor response to alcohol) and the change in locomotor activity with repeated alcohol-pairing (i.e., locomotor sensitization) were determined. The data pertaining to genotypic differences in cocaine-induced locomotion were published by Park et al. (2013) and thus, not presented herein.

PCP-induced locomotion

To determine whether or not the genotypic difference in alcohol-induced locomotion generalized to another nonselective NMDA receptor antagonist, we determined the dose–response function for phencyclidine (PCP)-induced locomotion using a within-subjects design. For this, a separate cohort of $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice were injected intraperitoneally, once daily, with increasing doses of PCP (0, 0.25, 0.5, and 1.0 mg/kg) and mice were placed in locomotor activity chambers where their distance traveled (in meters) was recorded for 1 h using digital video-tracking (ANY Maze, Stoelting).

Behavioral screen for negative affect

Negative affect is an endophenotype associated with the propensity to consume excessive amounts of alcohol (Blaine and Sinha, 2017; cf. Becker, 2017). As $GRM5^{AA/AA}$ mice exhibited an excessive alcohol-drinking phenotype (see Results), we assayed the behavior of male and female $GRM5^{TS/TS}$, $GRM5^{TA/AA}$, and $GRM5^{AA/AA}$ mice in forced swim, reactivity to novelty, and elevated plus-maze tests using procedures identical to those published previously by our group (Szumlinski et al., 2004, 2005; Ary et al., 2013; Guo et al., 2016). Mice were assayed in all of the above tests, with the order of testing randomized across cohorts of animals to avoid carryover effects. To localize the effects of interrupting ERK signaling on negative affect to the BNST, a cohort of male B6 mice were microinjected with 0, 0.95, or 9.5 ng/side U0126 and then assayed, in random order, for behavior in the reactivity to novelty and elevated plus-maze tests, before testing in the forced swim test. The specific details of each procedure is described below.

Porsolt/forced swim test. "Behavioral despair" was assessed via a Porsolt swim test, where floating serves as a measure of learned helplessness (Porsolt et al., 2001). As previously described by our group (Ary et al., 2013), mice were placed into a pool (30 cm in diameter; 45 cm high) filled with room-temperature water up to 35 cm and allowed to swim for a total of 15 min. In 30 s intervals, behavior was scored by an experimenter as either swimming (front and/or back paws mobile), floating (both front and back paws immobile), or climbing (swimming with forepaws making contact with the walls of the pool). Latency to first float was also determined using a stopwatch.

Reactivity to a novel object. To assess for effects of our manipulations on neophobia-related anxiety, a novel-object test was conducted. Mice were placed into an activity monitor containing one small, inedible object for 2 min. Animals were allowed to explore the object in the tub and both the number of contacts the mice made with the object and the amount of time the mice spent investigating the object (as defined by forepaw or snout contact with an object) were recorded by an experimenter.

Elevated plus maze. Animals were placed on the center intersection of a four-arm radial plus maze with two white open arms and two black walled arms 24 cm high. Each arm measured 123 cm long × 5 cm wide. Latency to first open-arm entry, number of open-arm entries, and total time spent in an open arm were monitored for the 4 min trial by a trained observer who was blind to the drinking history of the mice. Differences in the amount of time spent in an open versus enclosed arm were also used to assess anxiety.

Experimental design and statistical analysis

Immunoblotting. For the initial immunoblotting studies, the effects of binge-drinking on protein expression and indices of kinase activation were determined using two-tailed *t* tests conducted on the calnexin-normalized data between male water-drinking B6 mice and male B6 mice with a 30 d history of drinking under DID procedures.

AAV-mediated changes in Homer2-scaffolding within BNST. To determine the effects of mimicking the alcohol-induced increase in BNST Homer2 expression (using cDNA-H2b) and the effects of disrupting Homer2 scaffolding within the BNST (using shRNA-H2b or cDNA-Homer1a), the data from male B6 mice infused with these AAV constructs were compared with those from mice infused with a GFP control AAV. This comparison was evaluated using ANOVAs, with the between-subjects factor of AAV (GFP, cDNA-H2b, shRNA-H2b, cDNA-H1a) and the within-subjects factor of Injection (for locomotion; 2 levels: 0 vs 3 g/kg) or Concentration (for drinking; 4 levels: 5, 10, 20, and 40% v/v). This latter analysis was used to examine AAV effects on Day 1 of binge-drinking to index the initiation of binge-drinking, as well as on the average alcohol intake from each concentration over the course of binge-drinking. One-way ANOVAs across the between-subjects factor of AAV were used to examine the effects of altering Homer2 scaffolding upon alcohol intake under continuous-access conditions and an AAV by Saccharin Concentration ANOVA was used to examine the effects of our AAV manipulations upon the dose-intake function on Day 1 of saccharin availability, as well as for the average saccharin intake. The AAV experiments commenced with $n = 10–12$/AAV and the final sample sizes ranged from $n = 9–11$ as a result of subject attrition because of transduction failure, inability to localize microinjector tip in BNST tissue or the development of illness that did not respond to nursing care.

Transgenic studies of the behavioral effect of blocking ERK-dependent phosphorylation of mGluR5 using $GRM5^{TS/TS}$, $GRM5^{TS/AA}$, and $GRM5^{AA/AA}$ mice. To determine the role played by ERK-dependent phosphorylation of mGlu5 in regulating alcohol intake and alcoholism-related behavior, a series of experiments compared the phenotypes of $GRM5^{TS/TS}$, $GRM5^{TS/AA}$, and $GRM5^{AA/AA}$ mice. Additional experiments examined for the generalization of the $GRM5^{AA/AA}$ phenotype to either other drugs of abuse or to non-drug rewards/reinforcers. All of these experiments used approximately equal numbers of male and female mice (±2) from each genotype and all initial analyses included Sex as a between-subjects factor. However, with the exception of the elevated plus maze, analyses did not reveal any significant interactions between the Sex and Genotype factors. Thus, with the exception of the data for the elevated plus maze, the data were

collapsed across Sex and analyzed for Genotype effects and interactions as detailed in the paragraph below.

We first assayed mGlu5 mutant mice for alcohol and saccharin intake using simple home-cage drinking procedures. The data generated from these experiments were analyzed using a mixed-model ANOVA, with the between-subjects factor of Genotype (2 or 3 levels, depending upon the specific experiment) and the within-subjects factors of Concentration (3–4 levels, depending on the specific experiment). We next compared for genotypic differences in alcohol and saccharin self-administration under operant-conditioning procedures to index motivation for alcohol and a non-alcohol reinforcer, respectively. These data were analyzed also by a mixed model ANOVA, with the between-subjects factor of Genotype ($GRM5^{TS/TS}$, $GRM5^{TS/AA}$, $GRM5^{AA/AA}$) and the within-subjects factors of Concentration (3–4 levels, depending on the reinforcer) or Lever (active vs inactive). We then examined for genotypic differences in alcohol metabolism using a Genotype ($GRM5^{TS/TS}$ vs $GRM5^{AA/AA}$) by Time (5, 15, 30 min) ANOVA, with repeated measures of the Time factor. We related the alcohol-drinking phenotype of $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice to their sensitivity to alcohol intoxication using a rotarod assay. Mice were injected with one of two alcohol doses in this test and the data were analyzed using a between-subjects Genotype ($GRM5^{TS/TS}$ vs $GRM5^{AA/AA}$) × Dose (1 vs 2 g/kg alcohol) ANOVA. The role for mGlu5-Homer cross-linking in alcohol-induced sedation was tested by comparing the amount of time taken by $GRM5^{TS/TS}$ versus $GRM5^{AA/AA}$ and by $Homer1^{+/+}$ and $Homer1^{-/-}$ mice to regain their righting reflex and the data were analyzed separately for each genotype using two-tailed *t* tests. To relate the differences between $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice in alcohol-induced sedation to their perception of alcohol's effects as appetitive or aversive, the dose–response function for alcohol-induced place-conditioning was determined. To examine for the generalization of alcohol-conditioning effects to a different drug of abuse, a separate cohort of mice were tested for cocaine-induced place-conditioning. In both experiments, the time spent in the drug-paired versus -unpaired compartment was analyzed using a mixed-model Genotype (2 or 3 levels, depending on drug) × Dose (3 levels) × Side (paired vs unpaired) ANOVA, with repeated measures on the Side factor, followed by *t* test comparisons of time spent in each compartment to determine the presence/absence of a conditioned response.

To examine for genotypic differences in the locomotor response to the acute versus repeated injection with the various alcohol doses, mixed model Genotype × Dose × Injection ANOVA was conducted, with repeated measures on the Injection factor (8 pairings/injections). As this analysis failed to indicate any injection-dependent change in locomotion, the data for Injection 1 was reanalyzed alone to index genotypic differences in the acute locomotor response to alcohol. The locomotor response to cocaine was published previously by Park et al. (2013). To determine whether or not the genotypic difference in alcohol-induced locomotion generalized to another nonselective NMDA receptor antagonist, a within-subjects design was used to generate a dose–response function for PCP-induced locomotion. Thus, these data were analyzed using a Genotype ($GRM5^{TS/TS}$ vs $GRM5^{AA/AA}$) by Dose (0, 0.25, 0.5, 1.0 mg/kg) ANOVA, with repeated measures on the Dose factor.

To relate the alcohol-drinking phenotype of $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice to endophenotypes associated with alcoholism, mice were subjected to a behavioral test battery that included various tests of negative affect (e.g., Porsolt forced swim test, elevated plus maze, novel object encounter). The data from these assays were analyzed using univariate ANOVAs along the Genotype factor ($GRM5^{TS/TS}$, $GRM5^{TS/AA}$, and $GRM5^{AA/AA}$).

Neuropharmacological studies in B6 and mGlu5 phospho-mutant mice. To test the functional relevance of alcohol-induced changes in BNST ERK and PI3K activity for binge-drinking by male B6 mice and to examine the BNST as a locus underpinning the potentiating effects of mutations of T1123 and S1126 on mGlu5 upon alcohol intake, a series of distinct neuropharmacological studies were conducted. The effects of our neuropharmacological manipulations on the binge-alcohol intake of B6 mice were analyzed separately for each drug using repeated-measures ANOVAs across the dose factor (2–4 levels, depending upon the specific experiment), whereas those conducted on *GRM5* mutant mice included the additional between-subjects factor of Genotype ($GRM^{TS/TS}$ vs $GRM5^{AA/AA}$). The initial sample sizes for the neuropharmacological studies of the mGlu5 wild-type and mutant mice were Finally, to confirm ERK-dependent signaling within the BNST in regulating basal anxiety-like behavior, as well as the sedative-hypnotic and rewarding properties of alcohol, a series of neuropharmacological studies were conducted in male B6 mice and the effects of our neuropharmacological manipulations were analyzed using ANOVA with the between-subjects factors of U0126 dose (2–3 levels, depending upon the experiment).

For all ANOVAs, significant interactions were deconstructed along the relevant factor to examine for main effects and group differences followed-up using LSD *post hoc* tests or *t* tests, when appropriate. All analyses were two-tailed and $\alpha = 0.05$.

Results

Alcohol increases Homer2 expression in the BNST

We first immunoblotted for changes in glutamate receptor-related proteins in BNST tissue obtained from B6 mice with a history of binge-drinking under DID procedures (average daily intake = 4.4 g/kg; range = 3.0–6.5 g/kg; same as the mice used by Cozzoli et al., 2012, 2014, 2016). Homer2a/b expression was elevated by ~50% within the BNST of alcohol-binge drinking mice versus water-drinking controls (Fig. 1*a*; $t_{(20)} = 3.11$, $p = 0.005$). However, in contrast to prior results for the NAsh and CeA (Cozzoli et al., 2012, 2014; Lum et al., 2014), we detected no binge-induced change in BNST levels of Homer1b/c, mGlu1/5 or GluN2a/b (Fig. 1*a*; *t* tests, all *p* values >0.15).

Interrupting homer crosslinking in the BNST promotes alcohol sensitivity and binge-intake

Homer2 expression within NAsh and CeA is required for the manifestation of binge-drinking (Cozzoli et al., 2009, 2012, 2014), and Homer2 in NAsh regulates alcohol intoxication (Szumlinski et al., 2005, 2008a). Thus, we tested the functional relevance of BNST Homer2 for alcohol-related behaviors using AAV-mediated gene delivery approaches. Despite evidence for robust neuronal transduction (Fig. 1*b*), intra-BNST infusion of an AAV carrying *Homer2b* cDNA (cDNA-H2b) did not influence alcohol-related behavior in any of our assays (Fig. 1*c–e*). In contrast, disrupting Homer2 expression via infusion of an AAV carrying an shRNA against *Homer2b* (shRNA-H2b) augmented behavioral sensitivity to high-dose alcohol, as evidenced by greater alcohol-induced inhibition of locomotor activity upon bolus administration (Fig. 1*c*; AAV × Injection: $F_{(3,35)} = 2.92$, $p = 0.04$; one-way ANOVA for 0 g/kg alcohol, $p = 0.79$; one-way ANOVA for 3.0 g/kg; $F_{(3,33)} = 5.34$, $p = 0.01$, LSD *post hoc* tests, *p* values < 0.05). Further, examination of the dose–response function for binge-alcohol intake indicated that disrupting Homer2 expression elevated the binge-intake of high-dose alcohol (Fig. 1*d*; AAV × Concentration: $F_{(9,90)} = 2.66$, $p = 0.009$), and this effect was observed even on the first day of binge-drinking (data not shown; AAV effect: $F_{(3,30)} = 3.50$, $p = 0.03$; Dose effect: $F_{(3,90)} = 94.77$, $p < 0.0001$; AAV × Dose, $p = 0.36$). Deconstruction of the interaction for the average alcohol intake along with Concentration factor revealed that both shRNA-H2b and cDNA-H1a elevated the intake of 20% alcohol (Fig. 1*d*; one-way ANOVA: $F_{(3,33)} = 2.91$, $p = 0.05$; LSD *post hoc* tests, *p* values <0.05) and shRNA-H2b elevated the intake of 40% alcohol (Fig. 1*d*; one-way ANOVA: $F_{(3,33)} = 4.16$, $p = 0.02$; LSD *post hoc* tests, $p = 0.04$). As such, the average total alcohol intake under 2 h DID procedures was greater in shRNA-H2b and cDNA-H1a mice, versus GFP controls and cDNA-H2b mice (Fig. 1*e*; one-way ANOVA: $F_{(3,30)} = 5.49$, $p = 0.004$; LSD *post hoc* tests, *p* values <0.05).

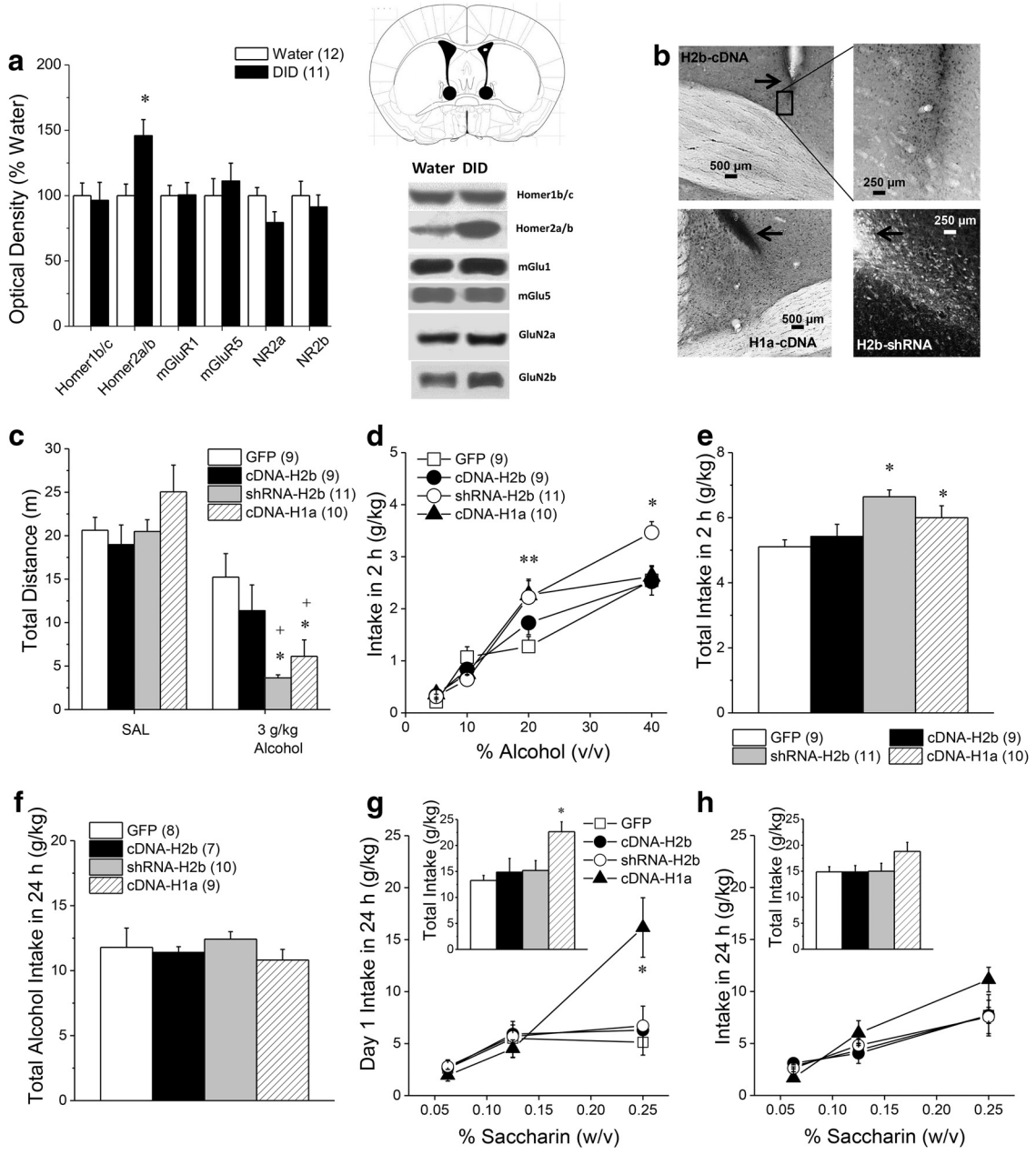

Figure 1. Disruption of Homer2 scaffolding within the BNST promotes behavioral sensitivity to high-dose alcohol. ***a***, Relative to water-drinking controls (Water), mice with a 30 d history of 20% (v/v) alcohol intake under 2 h DID procedures exhibited elevated protein expression of Homer2a/b within BNST, but no change in Homer1b/c, mGlu1/5, or GluN2a/b levels. *$p < 0.05$ versus water (*t* tests). Representative immunoblots are also shown for the proteins examined and a diagram depicting the tissue dissection within the anterior-dorsal BNST. ***b***, Representative micrographs of immunostaining of the HA tag within the BNST of B6 mice infused with an AAV carrying *Homer2b* cDNA (cDNA-H2b) or with an AAV carrying *Homer1a* cDNA (cDNA-H1a), as well as immunofluorescence of the GFP tag in mice infused with an AAV carrying an shRNA against *Homer2b* (shRNA-H2b). Arrows in ***b*** depict the tip of the microinjector. ***c***, Intra-BNST infusion of shRNA-H2b or cDNA-H1a augmented the motor-impairing effects of an acute intraperitoneal injection of 3 g/kg alcohol, whereas no saline-alcohol differences in locomotion were observed in GFP or cDNA-H2b mice. ***d***, Examination of the alcohol dose-intake function under 2 h DID procedures revealed a shift upward in shRNA-H2b and cDNA-H1a mice, relative to GFP controls, with both shRNA-H2b and cDNA-H1a elevating the intake of 20% alcohol and shRNA-H2b elevating the intake of 40% alcohol. ***e***, Thus, the average total alcohol intake under 2 h DID procedures was greater in shRNA-H2b and cDNA-H1a mice versus controls. ***f***, In contrast, intra-BNST AAV infusion did not influence the average total alcohol intake when alcohol was made continuously available in the home cage. Sample sizes are for ***a***–***f*** are indicated in parentheses in their corresponding panels. ***g***, However, when saccharin was substituted for alcohol, the cDNA-H1a mice exhibited a shift upward in the saccharin dose-intake function (0.0625, 0.125, 0.25%), as well as greater total saccharin intake (inset), when assessed on Day 1 of continuous access procedures. ***h***, However, intra-BNST AAV infusion did not influence significantly the dose–response function for the average saccharin intake when the data from the entire 7 d drinking period was considered, nor did AAV infusion alter total saccharin intake (inset). The sample sizes for ***g*** and ***h*** are indicated in parentheses in ***f*** because the same animals were tested for alcohol and saccharin consumption under continuous-access procedures. *$p < 0.05$ versus GFP; **both groups $p < 0.05$ versus GFP (LSD *post hoc* tests), +$p < 0.05$ versus saline (*t* tests).

Constitutively expressed Homer proteins, including Homer2, possess two functional domains: (1) an N-terminal EVH1 domain mediates binding to proline-rich sequences in Group 1 mGluRs, as well as intracellular ion channels, TrpC channels, and potassium channels; and (2) a C-terminal coiled coil/leucine zipper domain that mediates oligomerization (cf. Shiraishi-Yamaguchi and Furuichi, 2007; Constantin, 2016). Accordingly, Homer2 can bind and crosslink signaling proteins to enhance

transduction (Kammermeier et al., 2000). The immediate early gene form of Homer1 (Homer1a) possesses only the N-terminal EVH1 domain and functions as a natural dominant-negative to disrupt crosslinking. AAV expression of Homer1a cDNA in the BNST mimicked Homer2 knockdown (Fig. 1*c–e*). Thus, interrupting Homer2 crosslinking within the BNST enhances both the initiation and maintenance of binge-drinking, as well as sensitivity to alcohol's sedative effects.

The effects of interrupting Homer2 crosslinking in the BNST are selective for binge alcohol-drinking procedures

AAV-mediated disruption of Homer2 scaffolding within the BNST did not influence alcohol intake when alcohol became continuously available concurrently with water (Fig. 1*f*; one-way ANOVA, $p = 0.79$), nor did it influence water intake (data not shown). When saccharin was substituted for alcohol, fluid intake increased, but only in cDNA-H1a mice and this effect was only apparent on the first day of saccharin availability (Fig. 1*g*,*h*; for Day 1: Saccharin Concentration effect: $F_{(2,60)} = 11.95$, $p < 0.0001$; AAV effect: $F_{(3,30)} = 4.92$, $p = 0.007$; AAV × Saccharin Concentration: $F_{(6,60)} = 3.52$, $p = 0.005$; 7 d average: AAV × Saccharin Concentration ANOVA, all p values >0.15). This saccharin effect contrasts with the cDNA-H1a effect upon binge-alcohol intake, which remained elevated in cDNA-H1a mice throughout study (Fig. 1*d*).

These results demonstrate the behavioral specificity of the effect of disrupting BNST Homer2 crosslinking for binge alcohol-drinking and argue that the potentiation of binge-drinking does not reflect general changes in alcohol taste-reactivity or a dysregulation of factors influencing the oral consumption of natural reinforcers (e.g., thirst/osmotic balance).

Inhibiting ERK activity within the BNST promotes binge-drinking

ERK activity in the BNST is induced by acute oral alcohol consumption (cf. Zamora-Martinez and Edwards, 2014), and systemic ERK inhibitors increase oral alcohol self-administration (Faccidomo et al., 2009). Immunoblotting BNST tissue for ERK(pY204) revealed increased ERK activity in B6 mice with a month-long history of binge-drinking [Fig. 2*a*; phospho-(Tyr204)ERK (p-ERK): $t_{(21)} = 2.60$, $p = 0.02$; p-ERK–ERK ratio: $t_{(21)} = 2.38$, $p = 0.04$], with no change in total ERK (t test, $p > 0.35$). Examining the potential relevance of ERK activity in the BNST for binge-drinking, the localized infusion of the selective MEK1/2 inhibitor U0126 dose-dependently augmented binge-alcohol intake in B6 mice (Fig. 2*b*; $F_{(3,18)} = 5.68$, $p = 0.006$; $n = 7$; *post hoc t* tests).

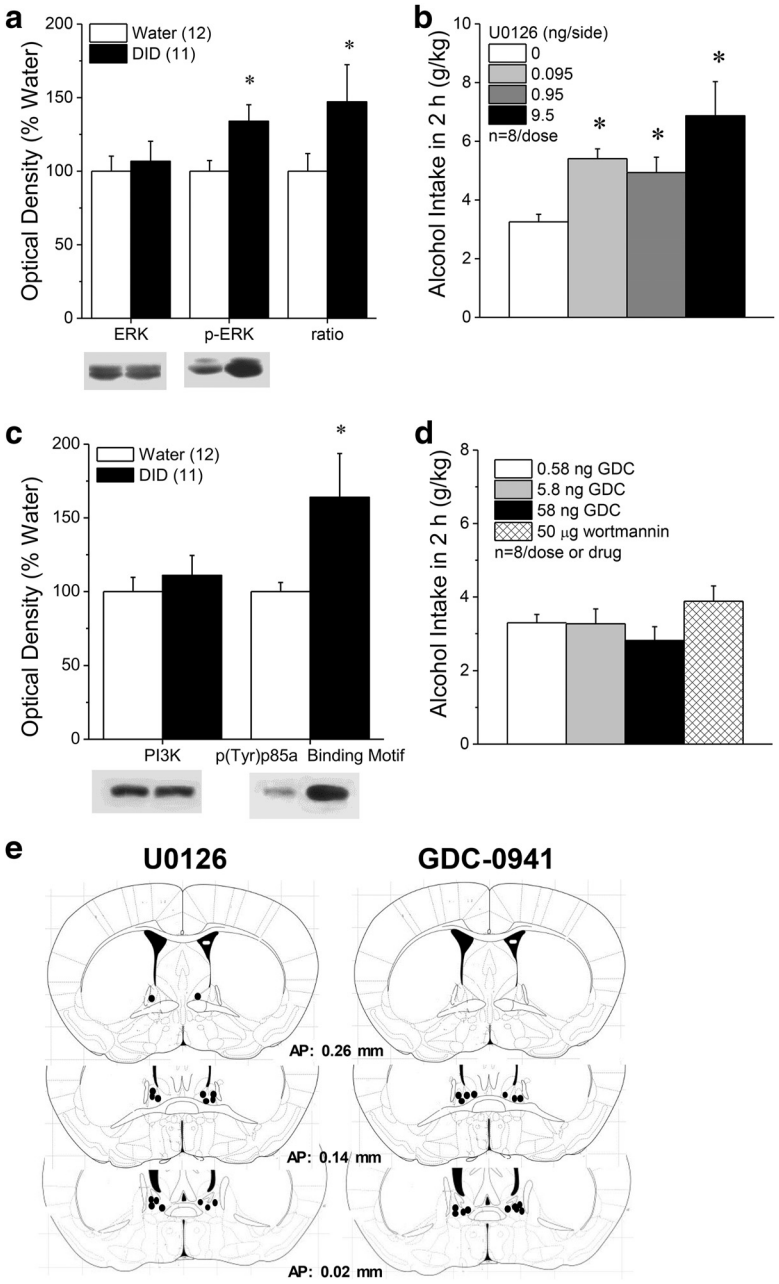

Figure 2. Inhibition of ERK within the BNST promotes binge-drinking. ***a***, Relative to water-drinking controls (Water), mice with a 30 d history of binge-drinking under modified DID procedures exhibited higher total and relative expression of p-ERK within the BNST, with no change in total ERK. ***b***, Intra-BNST infusions (0.25 μl/side) of the ERK inhibitor, U0126, dose-dependently elevated binge-alcohol intake under single-bottle DID procedures, with significant U0126 effects observed at all doses tested (*$p < 0.05$ vs 0 ng/side, *post hoc t* tests). ***c***, Chronic binge-drinking also increased the expression of the PI3K activation marker p(Tyr)p85α PI3K binding motif within BNST tissue, without influencing total PI3K expression (t test, $p > 0.60$). ***d***, However, intra-BNST fusions of the PI3K antagonists GDC-0941 or wortmannin did not impact binge-drinking under our single-bottle DID procedures in B6 mice. ***e***, Diagram summarizing locations of the microinjector tips within the BNST of the B6 mice infused with U0126 versus those infused with GDC-0941. Sample sizes used in the statistical analyses of the immunoblotting data are indicated in parentheses in their respective panels.

To probe the selectivity of the ERK effect, we indexed the relative activity of several other kinases implicated in the neurobiology of binge-drinking, including PI3K, Akt and PKCε in this BNST tissue (Cozzoli et al., 2009, 2012, 2014, 2016; Lum et al., 2014). We detected increased expression of p(Y)p85α PI3K binding motif (Fig. 2*c*; $t_{(19)} = 2.18$, $p = 0.04$), with no effect on total

Table 1. Summary of the immunoblotting results for the expression and activational state of Akt and PKCε within the BNST

	Water (12)	DID (11)	Water DID
Akt	100 ± 8.2	93.5 ± 8.7	
p(Ser473)-Akt	100 ± 11.1	95.0 ± 8.6	
p-Akt:Akt ratio	100 ± 12.4	109.4 ± 19.7	
PKCε	100 ± 8.4	108.3 ± 13.2	
p(Ser729)-PKCε	100 ± 14.0	114.7 ± 15.1	
p-PKCε:PKCε ratio	100 ± 13.4	117.5 ± 20.4	

Analysis failed to indicate any significant group differences. Sample sizes are indicated in parentheses with representative immunoblots presented in each of the corresponding cells.

PI3K expression (t test, $p > 0.60$), suggesting increased PI3K activation (Zhang et al., 2006). However, BNST infusion of the PI3K inhibitors GDC-0941 and wortmannin did not alter binge-alcohol intake in B6 mice (Fig. 2*d*), despite localization of the microinjector tips within the BNST (Fig. 2*e*). Finally, in contrast to ERK and PI3K, we detected no significant changes in the activational state of Akt or PKCε within the BNST (Table 1; t tests, all p values >0.35). From these data, we conclude that ERK activation within BNST is part of a natural adaptation that selectively suppresses binge-drinking.

Interrupting mGluR5 phosphorylation increases alcohol consumption

ERK phosphorylates many proteins including mGlu5(T1123/S1126) (Beneken et al., 2000; Orlando et al., 2009; Hu et al., 2012; Park et al., 2013), which increases the affinity of Homer EVH1 binding by as much as 40-fold (Park et al., 2013). Accordingly, we determined whether mGlu5(T1123/S1126) might be a target of ERK involved in suppressing binge-drinking by characterizing the alcohol phenotype of mice heterozygous ($GRM5^{TS/AA}$) or homozygous ($GRM5^{AA/AA}$) for alanine substitutions at T1123 and S1126 of mGlu5 (Park et al., 2013). We first examined alcohol preference and intake under simple, continuous-access procedures and observed a genotype-dependent increase in alcohol consumption (Fig. 3*a*; Genotype effect: $F_{(1,33)} = 7.70$, $p = 0.002$; Genotype × Concentration: $p = 0.16$), without effects on water intake (data not shown; Genotype effect: $p = 0.24$). The elevated alcohol intake of $GRM5^{AA/AA}$ mice extended to alcohol reinforcement as indicated by greater average alcohol intake (Fig. 3*b*; Genotype effect: $F_{(1,16)}) = 4.53$, $p = 0.04$; Genotype × Concentration: $p = 0.08$), and a greater number of alcohol reinforcers earned (Fig. 3*c*; Genotype effect: $F_{(1,16)} = 6.77$, $p = 0.02$; Genotype × Lever: $F_{(1,48)} = 5.17$, $p = 0.04$; all other p values >0.05), by $GRM5^{AA/AA}$ versus $GRM5^{TS/TS}$ mice when tested under operant-conditioning procedures. These data provide initial evidence that ERK-dependent phosphorylation of mGlu5(T1123/S1126) normally functions to curb alcohol intake.

We next determined whether the BNST as a neural locus of underpinning the excessive alcohol-drinking of $GRM5^{AA/AA}$ mice, we performed a series of neuropharmacological experiments under DID procedures. Consistent with their B6.129 hybrid genetic background, $GRM5^{TS/TS}$ mice consumed less alcohol under single-bottle DID procedures than did isogenic B6 mice (Figs. 4*a–d* vs 1*e*). When sampled in a subset of $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice, BACs were positively correlated with intake ($r = 0.77$, $p < 0.0001$; $N = 16$); however, the BACs attained were well below the 80 mg% criterion for binge-drinking ($GRM5^{TS/TS}$: 15.9 ± 2.7 mg%, $n = 7$; $GRM5^{AA/AA}$: 54.7 ± 18.1 mg%, $n = 9$). However, vehicle-infused $GRM5^{AA/AA}$ mice consistently exhibited higher alcohol intake under DID procedures than their $GRM5^{TS/TS}$ counterparts (Fig. 4*a–d*), indicating that the higher propensity of $GRM5^{AA/AA}$ mice to drink alcohol (Fig. 3*a*,*b*) extends across models. Intra-BNST infusion of the ERK inhibitor U0126 potentiated alcohol intake in $GRM5^{TS/TS}$ but not $GRM5^{AA/AA}$ mice (Fig. 4*a*; Genotype × Infusion: $F_{(1,27)} = 4.41$, $p = 0.04$; *post hoc t* tests, for TS/TS: $t_{(17)} = 2.85$, $p = 0.01$; for AA/AA: $p > 0.25$). ERK is typically activated by NMDA receptor signaling (cf. Zamora-Martinez and Edwards, 2014), which is implicated in alcohol-induced neuroplasticity within BNST (Kash, 2012; Wills et al., 2012; Zamora-Martinez and Edwards 2014; Lovinger and Kash, 2015). BNST infusion of GluN2B selective NMDAR antagonist ifenprodil also increased alcohol consumption in $GRM5^{TS/TS}$, but not $GRM5^{AA/AA}$ mice (Fig. 4*b*; Genotype × Infusion: $F_{(1,18)} = 5.46$, $p = 0.03$; *post hoc t* tests, for TS/TS: $t_{(10)} = 2.26$, $p = 0.05$; for AA/AA: $p > 0.25$). In contrast, an intra-BNST infusion of 30 μg/side of the mGlu5-negative allosteric modulator MTEP (Fig. 4*c*; Genotype effect: $F_{(1,23)} = 4.63$, $p = 0.04$; no Infusion effect or interaction, p values > 0.40) or of 30 pg/side of the mGlu1-negative modular JNJ 16259685 (Fig. 4*d*; Genotype effect: $F_{(1,18)} = 6.77$, $p = 0.02$; no Infusion effect or interaction, $p > 0.10$) were without effect in either genotype.

The insensitivity to inhibitors of ERK and GluN2B-containing NMDA receptors exhibited by $GRM5^{AA/AA}$ mice supports the notion that the effects of ERK and NMDAR signaling are mediated by phosphorylation of mGlu5, such that ERK antagonist effects are already maximized in $GRM5^{AA/AA}$ mice and therefore "occluded". An alternative explanation is that $GRM5^{AA/AA}$ mice simply cannot consume more alcohol in response to antagonists (i.e., a "ceiling effect"); however, this is unlikely as B6.129 mice are reported to consume upward of 4 g/kg alcohol within a 2 h session (Cozzoli et al., 2012, 2014, 2016; Lum et al., 2014), whereas consumption in these neuropharmacological studies was much <3.0 g/kg.

Constitutive disruption of mGlu5 phosphorylation or Homer1 scaffolding increases alcohol intoxication

An increased capacity of $GRM5^{AA/AA}$ mice to consume alcohol might reflect an effect of the mGlu5 mutation upon alcohol pharmacokinetics. However, when injected intraperitoneally with 3 g/kg alcohol, no genotypic difference was apparent in BACs for up to 30 min postinjection (Fig. 5*a*; Time effect: $F_{(2,28)} = 87.66$, $p < 0.0001$; no Genotype effect or interaction, p values >0.40). Thus, the mGlu5 mutation does not appear to impact alcohol metabolism to account for its marked effects on alcohol intake.

The propensity to drink alcohol excessively can be inversely related to sensitivity to alcohol's sedative-hypnotic effects (Krystal et al., 2003; Trimm et al., 2009). However, AAV-mediated disruption of Homer2 scaffolding within the BNST promoted both alcohol-induced sedation and consumption (Fig. 1). This latter finding prompted us to compare $GRM5^{TS/TS}$ and $GRM5^{AA/AA}$ mice for differences in alcohol intoxication using a rotarod assay. Acute alcohol injection (1 or 2 g/kg; 30 min prior) dose-dependently impaired rotarod performance in both genotypes; however, $GRM5^{AA/AA}$ mice were more sensitive to this effect (Fig. 5*b*; Dose effect: $F_{(1,45)} = 59.55$, $p < 0.0001$; Genotype × Dose: $F_{(1,45)} = 16.06$, $p < 0.0001$), most notably at the 2 g/kg dose (t tests: 1 g/kg, $p = 0.19$; 2 g/kg, $t_{(22)} = 4.79$, $p < 0.0001$). Thus, $GRM5^{AA/AA}$ mice exhibit *increased* sensitivity to alcohol intoxication.

One model that rationalizes this finding and those following intra-BNST shRNA-H2b infusion is that alcohol consumption normally induces a rapid increase of ERK activity, which increases mGlu5 phosphorylation and Homer crosslinking to re-

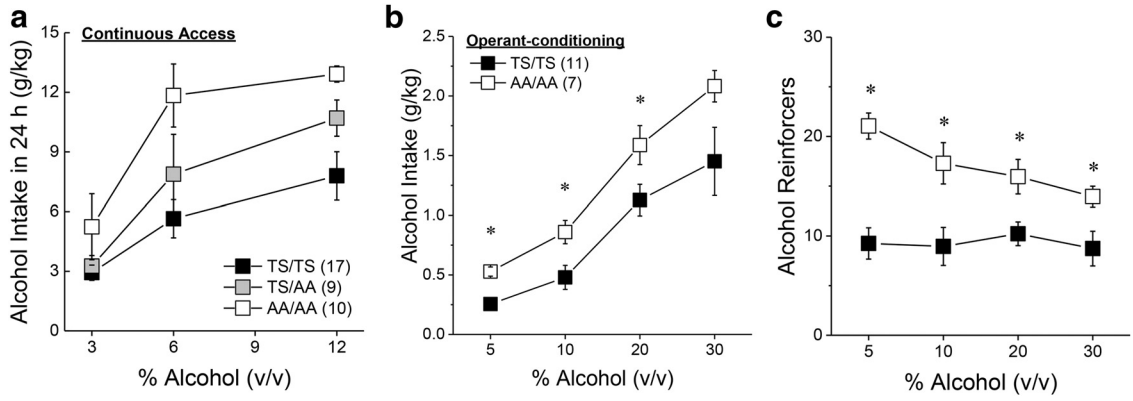

Figure 3. Alcohol drinking phenotype of GRM5 $^{AA/AA}$ mice. ***a***, Relative to wild-type controls (TS/TS), the dose–response function for the average alcohol intake under continuous-access conditions in the home cage was shifted upward in a genotype-dependent manner in male and female mice heterozygous (TS/AA) or homozygous (AA/AA) for point mutations at T1123 and S1126 on mGlu5. ***b***, When required to lever-press for delivery of alcohol under an FR5 reinforcement schedule, male AA/AA mice exhibited higher alcohol intake than TS/TS controls. ***c***, AA/AA mice consistently earned a greater number of alcohol reinforcers than TS/TS controls, regardless of the dose of alcohol available. Sample sizes are indicated in parentheses. *$p <$ 0.05 versus TS/TS.

duce sensitivity to alcohol's motor-impairing effects. Consistent with this model, *Homer2*$^{-/-}$ mice are supersensitive to alcohol's sedative-hypnotic effects (Szumlinski et al., 2005). To further probe this model, we compared *GRM5*$^{TS/TS}$ versus *GRM5*$^{AA/AA}$ mice, as well as *Homer1*$^{+/+}$ versus *Homer1*$^{-/-}$ mice, for their ability to right following injection with a sedative alcohol dose (5 mg/kg). As per *Homer2*$^{-/-}$ mice (Szumlinski et al., 2005), *GRM5*$^{AA/AA}$ mice (Fig. 5*c*; $t_{(26)} = 9.83$, $p < 0.0001$) and *Homer1*$^{-/-}$ mice (Fig. 5*d*; $t_{(13)} = 4.21$, $p = 0.001$) exhibited a significantly longer latency to right than their wild-type controls. However, the local inhibition of ERK activity within the BNST failed to alter the intoxicating effect of 2 g/kg alcohol on the rotarod (Fig. 5*e*; $F_{(1,23)} = 0.94$, $p = 0.41$) or the time taken by B6 mice to regain their righting reflex, following injection with 5 g/kg alcohol (Fig. 5*f*; $F_{(1,23)} = 0.42$, $p = 0.66$), despite microinjector localization within the BNST (Fig. 5*g*). Thus, although the genotypic differences in the sedative-hypnotic effects of alcohol further the idea that ERK-dependent mGlu5 phosphorylation and subsequent Homer crosslinking are important negative regulators of alcohol behavioral sensitivity, the neuropharmacological data argue against the BNST as the neural locus of this regulation.

Interestingly, while *GRM5*$^{AA/AA}$ mice clearly exhibit greater sensitivity to the sedative-hypnotic properties of alcohol (Fig. 5*b*,*c*), an examination of the dose–response function for the distance traveled early postinjection indicated greater locomotor stimulation overall in *GRM5*$^{AA/AA}$ versus *GRM5*$^{TS/TS}$ mice when treated acutely with alcohol (Fig. 5*h*; Genotype effect: $F_{(1,57)} = 5.70$, $p = 0.02$; Genotype × Dose: $p = 0.12$). However, we failed to detect any genotypic differences in the change in alcohol-induced locomotor stimulation during repeated alcohol treatment (data not shown; Dose × Injection: $F_{(14,364)} = 2.55$, $p = 0.002$; Genotype × Injection: $p = 0.58$, Genotype × Dose × Injection: $p = 0.14$). These data for acute alcohol-induced locomotion are consistent with, in part, with the Differentiator model, which poses that individuals at risk for, or suffering from, alcohol use disorder, tend to be more sensitive to the stimulating effects of low-dose alcohol (Morean et al., 2015).

To determine whether the effects of disrupting mGlu5 phosphorylation on alcohol-induced locomotor activity extended to another nonselective, and addictive, NMDA receptor antagonist, the locomotor response to increasing doses of PCP were compared between *GRM5*$^{TS/TS}$ and *GRM5*$^{AA/AA}$ mice. PCP dose-dependently increased locomotor hyperactivity; however, there were no genotypic differences observed in this regard (Fig. 5*i*; PCP Dose: $F_{(3,75)} = 5.92$, $p = 0.001$; Genotype effect and interaction, p values >0.40). These data argue further that the potentiation of behavioral sensitivity to alcohol produced by disruption of mGlu5 phosphorylation is selective for alcohol.

Interrupting mGluR5 phosphorylation reduces the aversive properties of high-dose alcohol, but not cocaine

To relate the differences in alcohol intake/reinforcement and behavioral sensitivity observed between *GRM5*$^{TS/TS}$ and *GRM5*$^{AA/AA}$ mice to their perception of alcohol's interoceptive effects, *GRM5*$^{TS/TS}$ and *GRM5*$^{AA/AA}$ mice were tested for conditioned approach/avoidance using alcohol place-conditioning procedures. The dose–response function for alcohol-conditioning exhibited by *GRM5*$^{AA/AA}$ mice was shifted markedly to the right of *GRM*$^{TS/TS}$ controls (Fig. 6*a*; Genotype × Dose × Side interaction: $F_{(2,52)} = 10.96$, $p < 0.0001$). At the 1 g/kg dose, the conditioned response exhibited by *GRM5*$^{AA/AA}$ mice was less than controls (Genotype × Side: $F_{(1,19)} = 5.64$, $p = 0.03$), with only *GRM5*$^{TS/TS}$ mice exhibiting a significant place-preference (Paired vs Unpaired, for *GRM5*$^{TS/TS}$: $t_{(10)} = 17.65$, $p = 0.002$; for *GRM5*$^{AA/AA}$: $p = 0.52$). A genotypic difference in place-conditioning was also observed at the 3 g/kg dose (Genotype × Side: $F_{(1,17)} = 18.81$, $p < 0.0001$), which reflected a place-aversion in *GRM5*$^{TS/TS}$ mice (Paired vs Unpaired, $t_{(8)} = 5.97$, $p = 0.04$), but a place-preference in *GRM5*$^{AA/AA}$ animals ($t_{(9)} = 14.67$, $p = 0.004$). A priori comparisons of side differences at the 2 g/kg dose, indicated a place-preference in *GRM5*$^{AA/AA}$ mice only (for TS/TS, $p > 0.35$; for AA/AA, $t_{(8)} = 15.92$, $p = 0.004$). These data indicate that *GRM5*$^{AA/AA}$ mice are less sensitive to both low-dose alcohol reward and high-dose alcohol aversion. To probe whether or not the BNST is a neural locus in which ERK-dependent phosphorylation gates the motivational valence of high-dose alcohol, we examined the effects of an intra-BNST infusion of 9.5 ng/side U0126 upon the expression of alcohol-induced place-aversion in B6 mice. Akin to the data for *GRM5*$^{AA/AA}$ mice (Fig. 6*a*), inhibiting ERK activity within the BNST completely reversed the place-aversion induced by conditioning with 3 g/kg alcohol (Fig. 6*b*; Dose × Side: $F_{(1,12)} = 12.56$, $p = 0.004$), without affecting the distance traveled

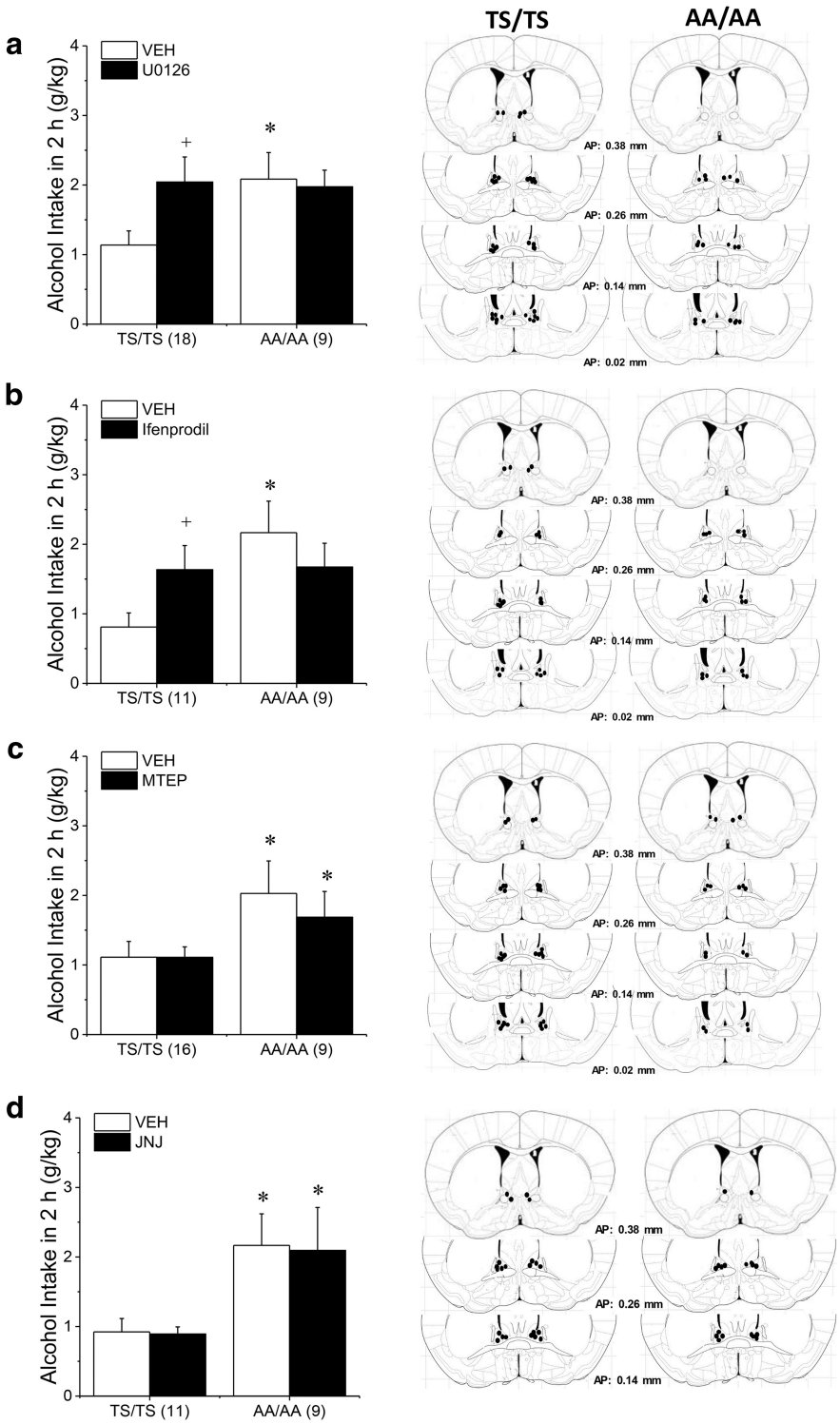

Figure 4. The GRM5$^{AA/AA}$ mutation occludes the effects of intra-BNST ERK and GluN2b inhibition upon binge-drinking. ***a***, Vehicle (VEH)-infused AA/AA mice exhibited higher alcohol intake under single-bottle DID procedures than TS/TS controls and were insensitive to the increase in alcohol intake produced by an intra-BNST infusion of 9.5 ng/side of the ERK inhibitor U-0126. ***b***, A similar genotypic difference was also noted regarding the potentiation of drinking produced by an intra-BNST infusion of 11 ng/side of the GluN2B-containing NMDAR antagonist ifenprodil. ***c***, In contrast, an intra-BNST infusion of 30 μg/side of the mGlu5-negative allosteric modulator, MTEP, did not influence alcohol drinking in either genotype nor did (***d***) an intra-BNST infusion of 30 pg/side of the mGlu1-negative modular, JNJ 16259685. Diagrams summarizing the locations of the microinjector tips for each experiment are presented in the right column and sample sizes are indicated in parentheses along the *x*-axis. $*p < 0.05$ versus TS/TS, $+p < 0.05$ versus VEH.

during the after conditioning test (data not shown; $t_{(14)} = 0.04$, $p = 0.85$). Thus, ERK phosphorylation of mGlu5(T1123/S1126) functions to gate the motivational valence of the interoceptive effects of alcohol and its disruption by mutation of the receptor or pharmacological inhibition of kinase activity within the BNST, renders animals less sensitive to the aversive properties of high-dose alcohol.

Cocaine also increases ERK activation throughout the extended amygdala and interconnected reward circuitry (Valjent et al., 2004) and *GRM5*$^{AA/AA}$ mice exhibit blunted cocaine-induced locomotor sensitization (Park et al., 2013). Thus, we determined whether disrupting mGlu5 phosphorylation might also alter the perception of cocaine's interoceptive effects. Opposite alcohol (Fig. 6*a*), the dose–response function for cocaine-induced place-conditioning was shifted to the left of wild-type controls in mice homozygous and heterozygous for the mGlu5 mutation versus wild-type controls (Fig. 6*c*; Genotype × Dose × Side: $F_{(4,158)} = 4.66$, $p = 0.001$). A priori comparisons of the time spent on the cocaine-paired versus -unpaired compartment indicated place-ambivalence in *GRM5*$^{TS/TS}$ mice conditioned with 3 mg/kg cocaine (*t* test, $p = 0.07$), but a significant place-preference was detected in both *GRM5*$^{TS/AA}$ ($t_{(17)} = 6.88$, $p < 0.0001$) and *GRM5*$^{AA/AA}$ animals ($t_{(16)} = 4.24$, $p = 0.001$). At 10 mg/kg and 30 mg/kg cocaine, marked genotypic differences in cocaine-conditioning were also apparent (for 10 mg/kg, Genotype × Side: $F_{(2,48)} = 6.08$, $p = 0.004$; for 30 mg/kg, Genotype × Side: $F_{(2,61)} = 4.87$, $p = 0.01$), with *GRM5*$^{TS/TS}$ mice exhibiting a place-preference at both cocaine doses (for 10 mg/kg, $t_{(16)} = 2.95$, $p = 0.009$; for 30 mg/kg, $t_{(23)} = 12.23$, $p < 0.0001$). In contrast, the conditioning exhibited by both *GRM5*$^{TS/AA}$ and *GRM5*$^{AA/AA}$ mice trended toward aversion at 10 mg/kg cocaine (for both genotypes, *t* tests: *p* values >0.15) and both mutant genotypes exhibited a significant place-aversion at the 30 mg/kg cocaine dose (*GRM5*$^{TS/AA}$: $t_{(11)} = 3.02$, $p = 0.01$; *GRM5*$^{AA/AA}$: $t_{(27)} = 2.26$, $p = 0.03$). Combined, these cocaine-conditioning data suggest an important role for intact mGlu5(pT1123/S1126) in the development of tolerance to cocaine's aversive properties with repeated cocaine experience, a finding in line with a prior report of blunted cocaine-induced neuroplasticity in *GRM5*$^{AA/AA}$ mice (Park et al., 2013). Importantly, these cocaine data indicate that the insensitivity to alcohol-

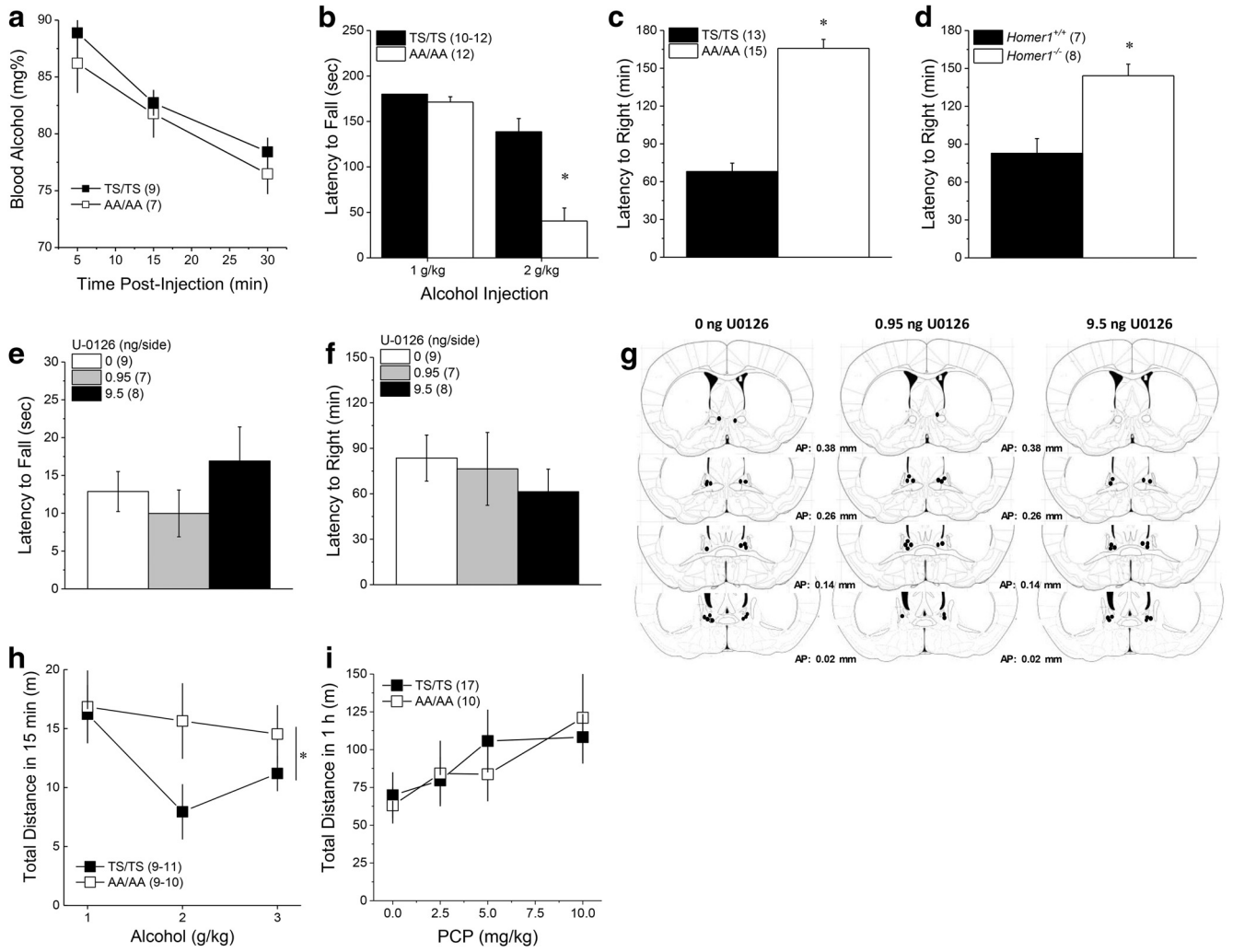

Figure 5. GRM5 $^{AA/AA}$ and Homer1 $^{-/-}$ mice are supersensitive to the sedative-hypnotic effects of alcohol. ***a***, When injected with 3 g/kg alcohol, no genotypic difference was apparent in blood alcohol concentrations for up to 30 min postinjection, indicating no obvious genotypic differences in alcohol metabolism. ***b***, Acute alcohol injection (1 or 2 g/kg; 30 min prior) dose-dependently impaired rotarod performance, but *GRM5*$^{AA/AA}$ (AA/AA) mice were more sensitive to this effect than their wild-type controls (TS/TS) at the 2 g/kg dose. When injected acutely with 5 g/kg alcohol, both *GRM5*$^{AA/AA}$ (***c***) and *Homer1*$^{-/-}$ (***d***) mice exhibited greater sleep time than their wild-type controls. ***e***, Intra-BNST infusion of U0126 did not alter the latency of B6 mice to fall from the rotarod when injected with 2 g/kg alcohol. ***f***, U0126 infusion also did not alter the latency taken by B6 mice to regain their righting reflex following injection with 5 g/kg alcohol. ***g***, Diagram of the microinjector placements within the BNST of U0126-infused mice. ***h***, When injected acutely with alcohol, the dose–response function for locomotor activity was shifted upward in *GRM5*$^{AA/AA}$ versus *GRM5*$^{TS/TS}$ mice, although this effect appeared to be driven primarily by genotypic differences at the 2 g/kg dose. ***i***, No genotypic difference was observed for the dose–response function of PCP-induced locomotion. *$p < 0.05$ versus TS/TS or Homer1+/+ (*t* tests). Samples sizes are indicated in parentheses.

aversion exhibited by *GRM5*$^{AA/AA}$ mice does not generalize across addictive substances.

Interrupting mGlu5 phosphorylation reduces anxiety and increases novelty-seeking

Alcohol is an anxiolytic and this property can contribute to its abuse liability, particularly in hyper-anxious individuals (Becker, 2017; Blaine and Sinha, 2017). Thus, we tested the hypothesis that the excessive drinking phenotype exhibited by *GRM5*$^{AA/AA}$ mice might relate to a hyper-anxious state. However, when screened in a variety of tests for emotionality, *GRM5*$^{AA/AA}$ mice exhibited less negative affect than *GRM5*$^{TS/TS}$ controls. This was evidenced by reduced floating behavior in a forced swim test (Fig. 7*a*; $F_{(2,71)} = 3.51$, $p = 0.04$) and a doubling of the time spent in contact with a novel object in a reactivity test (Fig. 7*b*; $F_{(2,70)} = 5.41$, $p = 0.007$). Overall, females made fewer open arm entries than males when tested in the elevated plus maze (Sex effect: $F_{(1,72)} = 4.26$, $p = 0.04$). Despite this sex difference, we observed a gene dose-dependent increase in the number of open arm entries by mutant mice (Fig. 7*c*; Genotype effect: $F_{(2,72)} = 4.93$, $p = 0.01$; Sex × Genotype interaction: $p > 0.25$). Likewise, *GRM5*$^{AA/AA}$ mice also spent more time in the open arm of an elevated plus maze than *GRM5*$^{TS/TS}$ and *GRM5*$^{TS/AA}$ mice (Fig. 7*d*; Genotype effect: $F_{(2,72)} = 5.37$, $p = 0.007$). These observations indicate that the excessive alcohol-drinking phenotype of *GRM5*$^{AA/AA}$ mice is not attributable to increased anxiety or depressive endophenotypes. However, enhanced novelty-seeking is potentially relevant to excessive drinking behavior as this endophenotype is highly correlated with the propensity to consume drug (Lee et al., 2014).

In contrast to the *GRM5*$^{AA/AA}$ mutation, the local inhibition of ERK signaling within the BNST did not alter behavior in the novel object encounter (data not shown; for all variables, one-way ANOVAs, *p* values > 0.40) or the forced swim tests (Fig. 7*e*; one-way ANOVA, $p = 0.67$). Although mice infused with 9.5 ng/side U0126 tended to make fewer entries into, and spend less time in, the open arm of the elevated plus maze, relative to the other doses (Fig. 7*f*), no significant U0126 effect was observed in

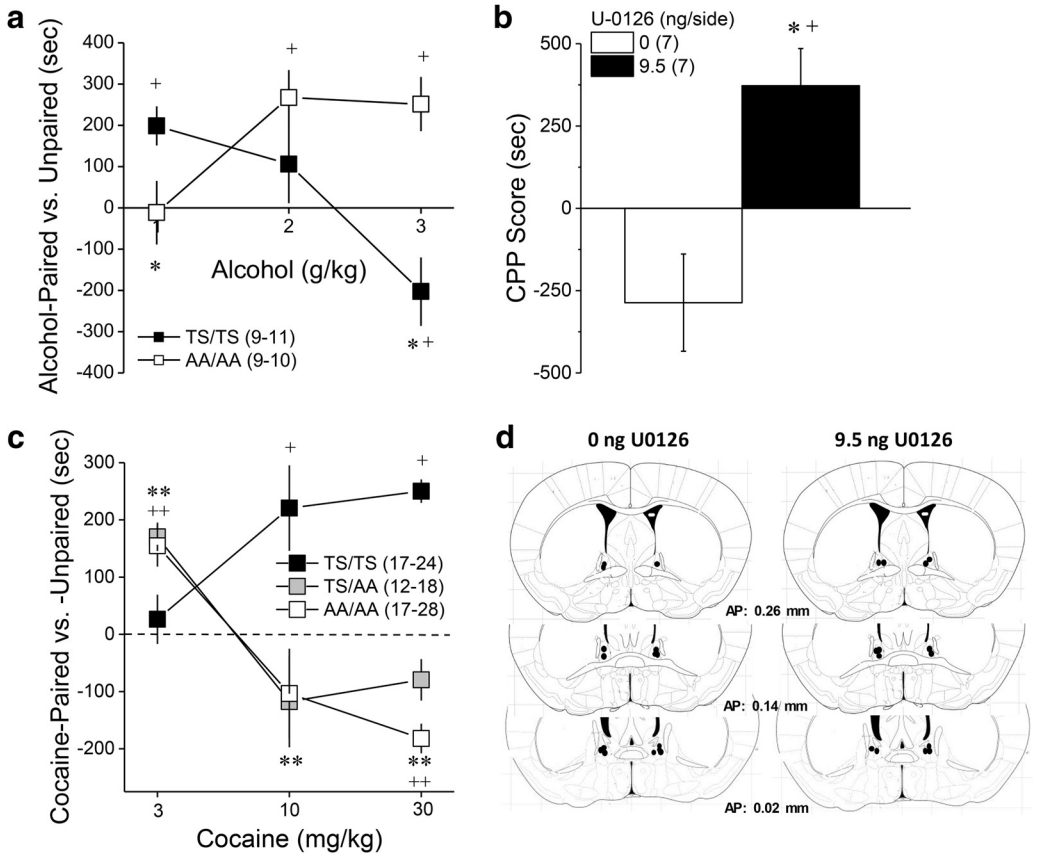

Figure 6. GRM5$^{AA/AA}$ mice are insensitive to the aversive properties of high-dose alcohol, but not cocaine. ***a***, The dose–response function for alcohol-induced place-conditioning was shifted markedly to the right in *GRM5*$^{AA/AA}$ mice (AA/AA), relative to their *GRM5*$^{TS/TS}$ controls (TS/TS). ***b***, An intra-BNST infusion of 9.5 ng/side U0126 mimicked the effect of the *GRM5*$^{AA/AA}$ mutation upon the conditioned response to 3 g/kg alcohol. ***c***, In contrast, the dose–response function for cocaine-induced place-conditioning was shifted to the left in both GRM5AA/AA and GRM5AA/TS mice, relative to GRM5TS/TS controls. ***d***, Diagram depicting the placements of the microinjectors within the BNST of the mice in ***b***. $*p < 0.05$ versus TS/TS (*t* tests or one-way ANOVAs, followed by LSD *post hoc* tests), $**p < 0.05$ both genotypes versus TS/TS, $+p < 0.05$, Paired versus Unpaired (a priori *t* tests), $++p < 0.05$ both genotypes versus Paired. Samples sizes are indicated in parentheses.

this assay (one-way ANOVAs for time in open arm, time in closed arm and time spent in open arm, all p values > 0.11). From these data, it would not appear that the BNST is the neural locus mediating the anxiolytic effects of disrupting ERK-dependent phosphorylation of mGlu5.

Discussion

The present data support a model in which alcohol-induced changes in mGlu5 scaffolding and signaling within the BNST act to limit alcohol consumption. Alcohol augments Homer2 expression, which presumably increases Homer-crosslinking of mGlu5. This appears to be an adaptation that normally limits alcohol consumption as interrupting Homer2-crosslinking in BNST increases alcohol consumption and reinforcement. Binge-drinking also increases BNST ERK activity and ERK phosphorylates mGlu5 at the Homer binding site to facilitate Homer-crosslinking through increased Homer-EVH1 binding affinity (Beneken et al., 2000; Orlando et al., 2009; Hu et al., 2012; Park et al., 2013). Increases in BNST ERK activity appear to be a further adaptation that normally limits alcohol consumption because local ERK inhibition increases alcohol consumption in wild-type mice. The effect of ERK upregulation on mGluR5 is selectively disrupted in *GRM5*$^{AA/AA}$ mice (Park et al., 2013), which also show increased alcohol consumption. Moreover, local ERK inhibition does not increase alcohol consumption in *GRM5*$^{AA/AA}$ mice, suggesting that mGlu5 is the important site of action of ERK within the BNST to limit alcohol consumption.

Alternatively, the mGlu5(T1123A/S1126A) mutation may reduce the Homer-EVH1 binding affinity, independent of phosphorylation. However, prior studies of mice with an mGlu5 (F1128R) mutation revealed either normal or reduced alcohol intake (Cozzoli et al., 2009, 2012); the mGlu5(F1129R) mutation markedly reduces the Homer-EVH1 binding affinity, but does not disrupt phosphorylation of mGlu5(T1123/S1126) or the consequent increases in Homer EVH1 binding affinity (Park et al., 2013). Accordingly, our data suggest that it is the alcohol-induced increase of Homer-mGlu5 crosslinking in the BNST that is most relevant to limiting alcohol consumption. Notably, although constitutive deletion of *Homer2* does not result in excessive alcohol drinking (Szumlinski et al., 2005; Cozzoli et al., 2009, 2012), Homer2 knockdown within the NAsh and CeA, which are both highly interconnected with the BNST, blunts alcohol intake in mice (Szumlinski et al., 2008b; Cozzoli et al., 2012, 2014) indicating regionally localized roles of Homer2 in regulation of alcohol consumption.

The ability to dynamically regulate mGlu5-Homer crosslinking may be important to change the nature of signaling between the basal versus alcohol-intoxicated state. Homer2-crosslinking could increase mGlu5-coupling to multiple targets, including IP3 or ryanodine receptors to enhance glutamate-induced release of intracellular Ca^{2+}, TRPC channels to increase Ca^{2+} influx, or Shank to increase association with postsynaptic density proteins, including NMDA recep-

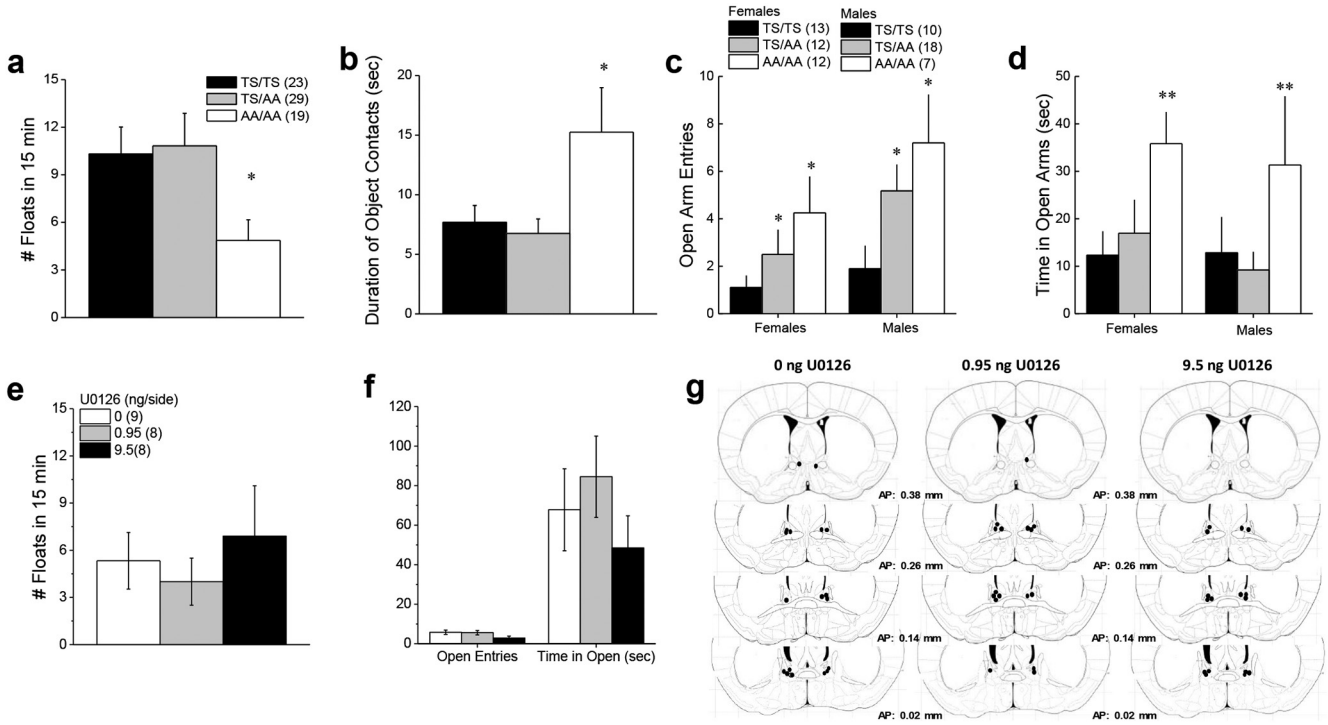

Figure 7. Emotionality in GRM5 $^{AA/AA}$ mice. ***a***, An examination of the average number of floats exhibited by male and female mice during a 15 min forced swim test revealed less floating behavior in *GRM5*$^{AA/AA}$ (AA/AA) mice versus the other two genotypes. ***b***, Likewise, *GRM5*$^{AA/AA}$ mice spent more time interacting with a novel object during a 2 min test. ***c***, Although females made fewer open arm entries than males when tested in the elevated plus maze, the number of open arm entries was gene dose-dependent. ***d***, *GRM5*$^{AA/AA}$ mice also spent more time in the open arm of an elevated plus maze than *GRM5*$^{TS/TS}$ (TS/TS) and *GRM5*$^{TS/AA}$ (TS/AA) mice. ***e***, In contrast, an intra-BNST infusion of U0126 did not alter floating behavior in the forced swim test. ***f***, Likewise, no effect of U0126 infusion was observed in the elevated plus maze. ***g***, Cartoon depicting the locations of the microinjector tips within the BNST of mice treated with the different doses of U0126. Sample sizes are indicated in parentheses. *$p < 0.05$ versus TS/TS (*t* tests or LSD *post hoc* tests); **$p < 0.05$ versus TS/TS and TS/AA.

tors. Homer-crosslinking can also reduce coupling to membrane K^+ channels (Kammermeier et al., 2000). Reciprocally, interrupting Homer-crosslinking increases agonist-independent mGlu5 activity and this process underlies homeostatic scaling-down of synaptic strength consequent to increased activity that induces Homer1a (Hu et al., 2010). Which of these various intracellular outputs is most critical for control of alcohol consumption remains a compelling question.

Alcohol-induced synaptic plasticity within the BNST

Alcohol-induced glutamatergic plasticity within the BNST is purported to regulate affective states to promote excessive drinking (McElligott and Winder, 2009; Kash, 2012; Wills et al., 2012; Lovinger and Kash, 2015; Vranjkovic et al., 2017). Although binge-drinking history did not alter the total glutamate receptor expression herein, chronic, intermittent alcohol can increase BNST glutamate receptor expression, notably that of the alcohol-sensitive GluN2B-containing NMDA receptor (Kash et al., 2008, 2009; Wills et al., 2012; Wills and Winder, 2013) as well as, GluN2B-dependent long-term potentiation within BNST (Wills et al., 2012). The robust upregulation of BNST Homer2 expression in binge-drinking mice form a possible molecular substrate for NMDA receptor-dependent synaptic plasticity as Homer2 targets GluN2B-containing NMDA receptors to synapses (Shiraishi et al., 2003) and is required for intact NMDA receptor function (Smothers et al., 2016).

The BNST also exhibits a postsynaptically mediated, mGlu5-dependent, long-term depression (LTD) of excitatory transmission that requires ERK activation (Grueter et al., 2006, 2008). In cocaine-experienced animals, this form of LTD increases excitatory drive within BNST-mesolimbic projections to promote cocaine-seeking/taking behavior (Grueter et al., 2006, 2008). The precise mechanism(s) through which ERK activation leads to mGlu5-dependent LTD within BNST, and its disruption by cocaine, are not clear. Nor is it known whether alcohol experience promotes or prevents this form of synaptic plasticity. However, increased mGluR5-Homer-crosslinking facilitates the cytosolic retention of mGlu5- a mechanism implicated in cocaine's ability to disrupt mGlu5-dependent LTD within the NAsh (Fourgeaud et al., 2004). ERK-mediated phosphorylation of mGlu5(T1223/S1126) also facilitates slow inward currents through NMDA receptors (Park et al., 2013), the activation of which is reported to inhibit mGlu5-dependent LTD within the NAsh via the activation of Ca^{2+}-dependent signaling pathways and Homer phosphorylation (Huang and Hsu, 2012).

The induction and maintenance mechanisms for mGlu5-dependent LTD can vary across brain regions (cf. Grueter et al., 2007) and different environmental stimuli (McElligott et al., 2010). Indeed, the glutamatergic consequences of binge-drinking are not only brain region-specific (Cozzoli et al., 2012, 2014), but quite distinct from those observed in cocaine-experienced animals (Ary and Szumlinski, 2007; Knackstedt et al., 2010; Ghasemzadeh et al., 2011). Further studies are required to assess how ERK-dependent changes in mGlu5 scaffolding/signaling may contribute to alcohol-induced synaptic plasticity within the BNST to limit alcohol intake.

ERK-mGlu5 interactions in the affective/motivational properties of high-dose alcohol

The increase in alcohol intake, reinforcement, and conditioned reward produced by inhibiting ERK signaling/mGlu5 scaffolding within the BNST coincide with either no change in, or increased sensitivity to, the stimulant properties of low-dose alcohol, as well as the sedative-hypnotic properties of higher alcohol doses. The observation that *GRM5*$^{AA/AA}$ mice and B6 mice infused intra-BNST with U0126 perceive the interoceptive effects of high-dose alcohol as appetitive, rather than aversive, aligns well with the results of human and animal studies consistent with the differentiator model of alcoholism vulnerability (Newlin and Thomson, 1990, 1999; Holdstock et al., 2000; King et al., 2002; Fritz et al., 2013; Morean et al., 2015). This model posits that predisposition to excessive drinking reflects accentuated sensitivity to alcohol's psychomotor effects during the ascending limb of the blood alcohol curve, combined with attenuated sensitivity to the aversive negative affective state produced during the descending limb of the blood alcohol curve. The perception of high-dose alcohol's effects as appetitive by *GRM5*$^{AA/AA}$ mice does not reflect an inability to perceive interoceptive drug effects as aversive, as clearly indicated by the results of our cocaine place-conditioning studies. The observation that pharmacological inhibition of ERK within BNST mimicked the effects of global disruption of ERK-dependent mGlu5 phosphorylation points to this brain region as a neural locus contributing to the motivational valence of high-dose alcohol.

An alternative explanation that the excessive alcohol consumption and heightened reward/reinforcement produced by disrupting ERK signaling/mGlu5 scaffolding reflects an increase in the drug's negative reinforcing (i.e., anxiolytic) properties (Conger, 1956) is not supported by the following observations: (1) *GRM5*$^{AA/AA}$ mice were hypo-anxious on several behavioral measures, (2) intra-BNST cDNA-H1a infusion produced a transient increase in saccharin consumption, and (3) pharmacological inhibition of ERK signaling within the BNST did not significantly impact behavioral measures of anxiety- or depressive-like behavior. Thus, we propose that abhorrent ERK-mGlu5 interactions within BNST may contribute to individual differences in the affective/motivational valence of high-dose alcohol that bears directly upon the capacity to drink excessively.

BNST-related circuitry inhibiting the propensity to binge-drink

The BNST is neuroanatomically and cytoarchitecturally complex (Ju and Swanson, 1989; Bota and Swanson, 2010). The majority of BNST neurons are GABAergic (Cullinan et al., 1993; Esclapez et al., 1993), with modest expression of glutamate cells in the posterior, dorsomedial, and fusiform nuclei (Poulin et al., 2009). BNST efferents project to many mesolimbic structures, including the ventral tegmental area (VTA; Jennings et al., 2013; Kim et al., 2013), the latter of which receives dense GABAergic and more sparse glutamatergic efferents from the more ventral aspects of the BNST, as well as the anterior-dorsal BNST targeted herein (Jennings et al., 2013). BNST efferents to the VTA have been the major foci of investigation regarding the neurocircuitry of addiction because of their role in regulating motivated behavior (cf. Vranjkovic et al., 2017). Stimulation of both GABAergic and glutamatergic efferents from the BNST excite VTA neurons (Georges and Aston-Jones, 2001; Jennings et al., 2013) to drive drug/alcohol-conditioned reward and drug/alcohol-seeking behavior (Sartor and Aston-Jones, 2012; Jennings et al., 2013; Pina et al., 2015; Pina and Cunningham, 2017; Vranjkovic et al., 2017). Further, chemogenetically inhibiting BNST-VTA projections that contain corticotropin-releasing factor (CRF) attenuates binge-alcohol intake (Pleil et al., 2015; Rinker et al., 2017). The opposite effects of chemogenetic inhibition of CRF BNST-VTA projections (Pina et al., 2015; Pleil et al., 2015; Pina and Cunningham, 2017; Rinker et al., 2017) versus the local inhibition of mGlu5 crosslinking and ERK signaling within BNST (present study) upon alcohol reward and reinforcement argues a neurocircuitry model in which alcohol induces dynamic changes in mGlu5 scaffolding/signaling either within non-CRF BNST-VTA projections or within other BNST efferents to counteract the drive to drink/alcohol-seek exerted by alcohol's activation of the CRF projections to the VTA (Pleil et al., 2015; Rinker et al., 2017).

With respect to the latter, the GABAergic BNST projection to the magnocellular region of the periventricular nucleus of the hypothalamus (PVN) is a particularly intriguing candidate. The PVN is comprised of both oxytocinergic and vasopressinergic cells (Sawchenko and Swanson, 1983) that exert opposing effects upon the manifestation of negative affect, the propensity to drink, and the sedative-hypnotic effects of alcohol (Bowen et al., 2015; MacFadyen et al., 2016; King et al., 2017; Ryan et al., 2017); endophenotypes all affected herein by disrupting ERK-dependent mGlu5 crosslinking. It remains to be determined whether a binge-drinking history impacts neuroplasticity within BNST-PVN projections (or any other major target region, for that matter) and how this neuroplasticity influences neurohormone release to regulate alcoholism-related behaviors and affective states.

Conclusions

The present data support a model in which alcohol-induced changes in mGlu5 scaffolding and signaling within the BNST act to limit alcohol consumption and appear to do so by gating the motivational valence of the interoceptive effects of high-dose alcohol, rather than by affecting other factors typically associated with problem drinking. Additional study is necessary to understand more precisely how ERK-dependent mGlu5 scaffolding and signaling impacts alcohol-induced synaptic plasticity within the BNST to influence neuronal activity within its diverse projections. Nevertheless, the present study provides novel insight into the role for ERK-dependent phosphorylation of mGlu5, and for ERK signaling within the BNST, in regulating excessive alcohol consumption of relevance to our neurobiological understanding of alcohol abuse and alcoholism.

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