Operant alcohol self-administration targets GluA2-containing AMPAR expression and synaptic activity in the nucleus accumbens in a manner that drives the reinforcing properties of the drug

Sara Faccidomo [1,2], Briana L Saunders [1], Ashley M. May [1], Vallari R. Eastman[1], Michelle Kim [1], Seth M. Taylor [1], Jessica L. Hoffman [1], Zoé A McElligott [1,2,3], and Clyde W Hodge [1,2,3]

[1] Bowles Center for Alcohol Studies
[2] Department of Psychiatry
[3]Department of Pharmacology

School of Medicine
The University of North Carolina at Chapel Hill
Chapel Hill, NC 27599

Correspondence:
Dr. Clyde W Hodge
Bowles Center for Alcohol Studies
Department of Psychiatry
University of North Carolina at Chapel Hill
CB #7178, Thurston Bowles Building
Chapel Hill, NC 27599
Voice: 919-843-4823
Fax: 919-966-5679
e-mail: chodge@med.unc.edu

Support*:* This research was supported by grants from the National Institute on Alcohol Abuse and Alcoholism R01 AA028782 and P60 AA011605 (CWH); K01AA023555 (ZAM), and by funds from the UNC Bowles Center for Alcohol Studies.

Conflict of Interest Statement: The authors declare that they have no conflicts of interest.

ABSTRACT

***Rationale*:** The positive reinforcing effects of alcohol (ethanol) drive its repetitive use and contribute to alcohol use disorder (AUD). Ethanol alters the expression of glutamate AMPA receptor (AMPAR) subunits in reward-related brain regions, but the extent to which this molecular effect regulates ethanol's reinforcing properties is unclear. ***Objective*:** This study investigates whether ethanol self-administration changes AMPAR subunit expression and synaptic activity in the nucleus accumbens core (AcbC) to regulate ethanol's reinforcing effects in male C57BL/6J mice. ***Results*:** Sucrose-sweetened ethanol self-administration (0.81±0.11 g/kg/day) increased AMPAR GluA2 protein expression in the AcbC, without effect on GluA1, compared to sucrose-only controls. Infusion of myristoylated Pep2m in the AcbC, which blocks GluA2 binding to N-ethylmaleimide-sensitive fusion protein (NSF) and reduces GluA2-containing AMPAR activity, reduced ethanol-reinforced responding without affecting sucrose-only self-administration or motor activity. Antagonizing GluA2-lacking AMPARs, through AcbC infusion of NASPM, had no effect on ethanol self-administration. AcbC neurons receiving projections from the basolateral amygdala (BLA) showed increased sEPSC frequency and GluA2-like decay kinetics in ethanol self-administering mice as compared to sucrose. Optogenetic activation of these neurons revealed an ethanol-enhanced AMPA/NMDA ratio and reduced paired-pulse ratio, indicating elevated AMPAR activity and glutamate release specifically at AcbC terminals of BLA projecting neurons. ***Conclusions*:** Ethanol use upregulates GluA2 protein expression in the AcbC and AMPAR synaptic activity in AcbC neurons receiving BLA projections. GluA2-containing AMPAR activity in the AcbC regulates the positive reinforcing effects of ethanol through an NSF-dependent mechanism. This highlights a potential target for therapeutic interventions in AUD.

KEYWORDS: AMPA, GluA2, NSF, alcohol, self-administration, nucleus accumbens, basolateral amygdala

INTRODUCTION

The development of alcohol use disorder (AUD) represents maladaptive neuroplasticity, where alcohol hijacks neural mechanisms that regulate adaptive reward-seeking behavior, leading to excessive use (Hyman 2005; Kelley 2004a). Viewing AUD as aberrant plasticity in reward-associated neural circuits provides a compelling model for understanding how the memory of a drug's rewarding properties is stored long-term, offering insights into the persistent nature of addiction-related behaviors (Nestler 2001). Understanding how drugs target specific plasticity mechanisms to produce and maintain harmful alcohol use can guide the development of novel medications (Anton et al. 1995; Gass and Olive 2008; Heilig and Egli 2006; Holmes et al. 2013; Hopf and Mangieri 2018; Kalivas and Volkow 2011).

Experience-dependent plasticity involves long-term increases in synaptic strength, largely due to upregulation of glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) expression and activity at excitatory synapses (Malenka 2003; Malinow and Malenka 2002; Nicoll and Schulman 2023). AMPARs, formed by combinations of GluA1-4 subunits, are glutamate-gated ion channels. Their activity and role in plasticity are influenced by post-translational modifications such as phosphorylation, membrane trafficking, and subunit composition, with the presence or absence of GluA2 determining key functional properties related to memory induction, maintenance, and loss (Bredt and Nicoll 2003; Jiang et al. 2006; Malenka 2003).

Evidence suggests that experiences inducing plasticity, including exposure to ethanol and other drugs, promote an activity-dependent increase in synaptic expression of GluA2-lacking Ca^{2+}-permeable AMPARs (CP-AMPARs) in brain regions regulating addiction-related behaviors, such as the nucleus accumbens (Acb) and amygdala (Bellone and Luscher 2006; Conrad et al. 2008; Faccidomo et al. 2021; Liu and Cull-Candy 2000; Mameli et al. 2011; Marty and Spigelman 2012; Wolf and Tseng 2012). Following plasticity induction, glutamate synapses stabilize and maintain memories via expression of GluA2-containing Ca^{2+}-impermeable AMPARs (CI-

AMPARs), which constitute about 95% of total AMPAR expression in the Acb, hippocampus (HPC), and prefrontal cortex (PFC) (Lu et al. 2009; Reimers et al. 2011). Memory loss occurs with the activity-dependent removal of GluA2-containing AMPARs from the synapse (Migues et al. 2016). Thus, the dynamic expression and activity of specific AMPAR subunits may reflect the development, progression, and maintenance of addiction-linked maladaptive plasticity in brain function and behavior.

Growing evidence indicates that AMPAR activity in specific reward-related brain regions plays a crucial role in the positive reinforcing properties of ethanol (Acosta et al. 2011; Agoglia et al. 2015; Ary et al. 2012; Backstrom and Hyytia 2004; Bauer et al. 2022; Cannady et al. 2013; Cannady et al. 2016; Czachowski et al. 2012; Faccidomo et al. 2020; Hoffman et al. 2021; Hoffman et al. 2022; Hopf and Mangieri 2018; Montagud-Romero et al. 2021; Neasta et al. 2010; Pickering et al. 2007; Salling et al. 2016; Sciascia et al. 2015; Stephens and Brown 1999; Stuber et al. 2008). This activity establishes the neurobehavioral conditions for repetitive use and promotes the development and maintenance of AUD (Stolerman 1992). However, the role of specific AMPAR subunits in ethanol's reinforcing effects is not fully understood. Our previous research investigated GluA2-lacking CP-AMPAR activity as a potential target of ethanol that regulates its positive reinforcing effects. Long-term ethanol self-administration by C57BL/6J mice, modeling the initial positive reinforcing effects in non-dependent subjects, increased phosphorylation of AMPAR pGluA1-S831, enhanced spontaneous excitatory postsynaptic currents (sEPSC), and promoted synaptic insertion of CP-AMPARs in basolateral amygdala (BLA) neurons projecting to the Acb (BLA→Acb) (Faccidomo et al. 2021). We also found that CP-AMPAR activity and synaptic trafficking of GluA1-containing AMPARs in the BLA are required for ethanol's reinforcing effects (Faccidomo et al. 2021). These findings suggest that the initial development of ethanol's reinforcing properties is mediated by plasticity at GluA1-containing AMPAR synapses in BLA neurons projecting to the Acb.

Despite these discoveries, the involvement of specific AMPAR subtypes in the Acb in ethanol's reinforcing effects has not been evaluated. To address this gap in knowledge, this study evaluates the hypothesis that non-dependent ethanol self-administration targets AMPAR subunit expression and synaptic function in the Acb, regulating the drug's reinforcing properties. The Acb, a central component of the brain's reward circuitry, plays a pivotal role in mediating the reinforcing effects of drugs, including ethanol (Besheer et al. 2010; Hodge et al. 1995; Hodge et al. 1997; Hodge et al. 1992; Salling et al. 2017; Samson et al. 1999; Samson and Hodge 1993; Samson et al. 1992; Schroeder et al. 2008). Within the Acb, excitatory neurotransmission through ionotropic and metabotropic glutamate receptors is required for ethanol's reinforcing and subjective stimulus properties (Besheer et al. 2003; Besheer et al. 2010; Besheer et al. 2009; Rassnick et al. 1992a; Rassnick et al. 1992b). The Acb receives glutamatergic projections from brain regions, including the BLA, where glutamate AMPA receptor activity is required for ethanol's reinforcing effects (Agoglia et al. 2015; Cannady et al. 2016; Faccidomo et al. 2021; McCool et al. 2010; Salling et al. 2016), and evidence indicates that optogenetically enhanced excitatory transmission from the BLA to the Acb is rewarding (Stuber et al. 2011). Therefore, we also evaluated the impact of ethanol self-administration on AMPAR synaptic activity in Acb neurons receiving CaMKII-dependent excitatory projections from the BLA. Our findings indicate that the expression and synaptic activity of GluA2-containing CI-AMPARs in the Acb are influenced by ethanol and are necessary for its reinforcing properties.

Materials and methods

Mice

Adult (8-week-old) male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine, US) were group-housed (n=4/cage) in Plexiglas cages with food and water available ad libitum except during initial lever press training. The mouse housing room and testing facility were maintained on a 12:12 light–dark cycle with lights off at 08:00 AM. Behavioral experiments were conducted

during the dark to avoid light-associated disruption in mice (Roedel et al. 2006). Body weights were measured daily to calculate ethanol (EtOH) dosage consumed (g/kg). All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al. 2011).

Operant Ethanol Self-Administration Training and Baseline

Ethanol and sucrose-only self-administration was conducted in computer-controlled two-lever operant conditioning chambers (Med Associates) as previously described (Faccidomo et al. 2009; Faccidomo et al. 2020; Faccidomo et al. 2016; Faccidomo et al. 2015; Salling et al. 2008; Salling et al. 2016; Salling et al. 2017). Lever-press responding was trained and maintained by sucrose-only (2% w/v; parallel non-drug control) or sweetened ethanol (9% v/v ethanol + 2% w/v sucrose) on a fixed-ratio 4 (FR4) schedule of reinforcement. Each chamber was equipped with two levers; responses on an “active” lever (randomized across subjects) produced a delivery of 0.014 ml of the reinforcing solution. Responses on an “inactive” lever were recorded but produced no programmed consequences. After daily 1-hr baseline sessions (40-45 sessions), separate cohorts of mice were used for 1) measurement of AMPAR subunit expression via immunoblot; 2) whole-cell patch-clamp recordings of BLA→AcbC projection neurons; or 3) site-specific infusion of AMPAR inhibitor compounds into the Acb. Mice that did not exhibit baseline levels of operant behavior ≥10 reinforcements/hr were excluded.

Gel Electrophoresis and Immunoblot

Immediately after the 40th self-administration session, mice (n=8/group) were rapidly decapitated, and brains were flash frozen in -20°C isopentane and stored at -80°C until use. Bilateral tissue punches (1 x 1 mm circle; **Fig 1C**) taken from Acb, primarily AcbC possible inclusion of minimal dorsal Acb shell, collected from frozen tissue on a cryostat (Leica Biosystems,

Deer Park, IL USA) and homogenized in an SDS homogenization buffer containing protease/phosphatase inhibitors. Protein concentration was quantified using a Bradford colorimetric assay kit (BCA Assay; Pierce Protein Biology, ThermoFisher Sci) and gel electrophoresis and immunoblots were conducted as previously reported (Agoglia et al. 2015; Faccidomo et al. 2018; Wilkie et al. 2007). Briefly, sample protein (5-8 µg) and molecular weight ladders were loaded onto a 4–15% Criterion TGX precast gels (Bio-Rad) for gel electrophoresis separation. Proteins were transferred to PVDF membranes using an iBlot® Semi-Dry blotting system (Thermo Fisher Sci).

For protein measurement with immunoblots, a two-day protocol was used. Membranes were rocked in a blocking buffer (5% Normal Goat Serum [NGS]; Vector Labs) for 2 hrs at room temperature and then incubated with the following primary antibodies overnight at 4°C on separate blots: monoclonal mouse anti-GluA2 (1:2000, NeuroMab; #75-002), monoclonal rabbit anti-GluA1 (1:1000, Millipore; #04-855), polyclonal rabbit anti NSF (N-ethylmaleimide-sensitive fusion protein (NSF); 1:5000, Cell Signaling; #3924). The next day, membranes were washed and incubated with an HRP-conjugated secondary antibody (goat anti-rabbit and goat anti-mouse, 1:10,000; Jackson Immuno Research) for 1 hr. The chemiluminescent signal was visualized with ECL Select or Prime (GE Healthcare) and digitally captured using a digital imager (ImageQuant LAS 4000, GE Healthcare). On a different day, membranes were probed with an anti-mouse GAPDH antibody (1:10,000, Advanced Immunochemical) which served as a non-specific loading control. The optical density of each band was visualized and quantified using ImageQuant TL software. The optical density of target bands was normalized to the optical density of GAPDH and expressed as a ratio (target/GAPDH). Within each individual blot, data ratios were expressed as a percentage relative to sucrose mice.

Stereotaxic Surgery

Operant trained mice underwent stereotaxic surgery for bilateral guide cannula implantation into the AcbC (AP: +1.6, ML: +/-.75, DV: -3.8 mm from skull; 26 gauge, 6mm length,

1.5 C-C distance; Plastics One/Protech International) or bilateral AAV injections with AAV2-CaMKIIa-hChR2(H134R)-EYFP (Deisseroth Stock Vectors from UNC Gene Therapy Vector Core) into the BLA (AP: -1.3, ML: +/-3.4, DV: -4.4 mm from dura). Microinjection cannula surgeries occurred after 40 sessions of acquisition so the cannulae stayed patent throughout the microinjection study. Surgeries for the AAV infusion occurred before acquisition of operant responding to give time to the vector to express and travel from BLA to AcbC such that electrophysical recordings could be occur after 40-45 days of operant ethanol or sucrose self-administration sessions. Briefly, mice were anesthetized with 2-4% isoflurane throughout the duration of the aseptic surgery. The skull was visualized, Bregma was determined and (1) guide cannula was stereotaxically placed targeting the AcbC and affixed with dental cement or (2) the ChR2 AAV was bilaterally infused to the BLA at a rate of 0.1 ul/min (0.3 ul/side). Next, the surgical opening was closed, and mice were given a single bolus saline (1ml, s.c.) and ibuprofen (15mg/kg, 3days) to aid in recovery from surgery.

Intra-AcbC Microinjections

For the microinjection experiments, mice were returned to daily operant sessions 1 week after recovery from surgery. Baseline responding was re-established, and sham injections were conducted to habituate the mice to the microinjection procedure. The injectors (bilateral, 7mm length, 1mm past cannula guide) were inserted and then NASPM (0, 1, or 10 ug/0.5ul/side) or Pep2m (0, 1, or 10 ug/0.5ul/side) was infused (1.25 ul/min; separate groups of mice) into the targeted AcbC immediately prior to the self-administration session.

To address the possibility that changes in ethanol self-administration induced by intra-AcbC NASPM or Pep2m infusion were associated with nonspecific behavioral effects, spontaneous open-field locomotor activity (general motor function) and thigmotaxis (anxiety-like behavior) were assessed in separate 1-hr tests after completion of the self-administration dose-

effect curves as reported previously (Agoglia et al. 2016; Faccidomo et al. 2015; Hodge et al. 2004; Hodge et al. 1999; Hodge et al. 2006; Hodge et al. 2002).

Whole-cell patch-clamp recordings

Slices (300 µm) containing the Acb were prepared as previously described (Faccidomo et al. 2021). Briefly, mice were anesthetized with isoflurane, and brains were extracted and sliced on a Leica VT1200 or VT1000 in ice cold high-sucrose low Na^+ artificial cerebral spinal fluid (sucrose ACSF, in mm: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$) that had been perfused for at least 15 minutes in carbogen (95% O_2, 5% CO_2). Subsequent to slicing, sections containing the nucleus accumbens equilibrated in the same ACSF that was used for recordings (in mm: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$, 95% O_2, 5% $CO_2$34°C) with picrotoxin (25 µM) for 30 minutes prior to being transferred to the recording chamber where they were perfused (2 ml/min) with ACSF for an additional 30 minutes prior to recording. All recordings were made in Cs-gluconate intracellular solution (in mM: 135 Cs-gluconate, NaCl 5, $MgCl_2$ 2, HEPES 10, EGTA 0.6, Na_2ATP 4, Na_2GTP 0.4). All recordings were made in ClampEx 10 and were analyzed with ClampFit 10 (Molecular Devices), or MiniAnalysis (Synaptosoft). Spontaneous EPSCS were recorded at -80 mV, paired-pulse ratios were recorded at -70 mV, and AMPA/NMDA ratios were recorded at -70/+40 mV. Optogenetic stimulation of ChR2 was performed with a 470 nm LED (CoolLabs). Evoked excitatory post-synaptic currents (eEPSCs) were evoked by local stimulation with Ni-chrome bipolar electrodes while neurons were voltage-clamped at −65 mV as previously reported (Faccidomo et al. 2021). Drugs were bath applied at final concentrations.

Drugs

The CP-AMPAR antagonist NASPM [N-[3-[[4-[(3-Aminopropyl)amino]butyl]amino]propyl]-1-naphthaleneacetamide trihydrochloride; Bio-Techne, Tocris, USA) was dissolved in phosphate-buffered saline for bath application in patch-clamp electrophysiological experiments or artificial

cerebrospinal fluid (ACSF) for site-specific infusion in the AcbC in behavioral procedures. Myristoylated Pep2m (Pep2m; Formula: $C_{63}H_{118}N_{18}O_{14}S$; Sequence: KRMKVAKNAQ; Bio-Techne, Tocris, USA) was dissolved in aCSF for site-specific inhibition of GluA2-containing AMPARs in the AcbC of behaving mice.

Statistical Analysis

Data are presented as MEAN±SEM. All statistical analyses and graphical representations of data were performed using GraphPad Prism software (version 10.1, GraphPad Software, San Diego, CA). Group comparisons were conducted via unpaired t-test, one-way RM ANOVA, or two-way RM ANOVA. Post hoc comparisons were conducted with Dunnett's or Šídák's multiple comparison procedures, where appropriate. P values of < 0.05 were statistically significant.

Results

Self-administered ethanol increases GluA2 protein expression in the Acb

We first sought to determine if non-dependent operant ethanol self-administration is associated with altered AMPAR subunit expression in the AcbC. Parallel groups of male C57BL/6J mice were trained to self-administer sweetened ethanol ($\bar{X}$ = 0.81 g/kg/1hr) or sucrose only (sucrose 2% w/v) during 40 daily 1-hr sessions (**Fig 1A & B**). Importantly, analysis of operant self-administration behavior showed no statistically significant group differences on measures of response rate (resp / hr) or the percentage of responses on the active lever (**Fig 1B**) indicating that differences observed in protein expression are not attributable to differential reinforcer efficacy, motor activity, learning and memory, or general reward processing between sucrose and ethanol trained mice.

Western blot analysis was conducted on whole-cell lysates that targeted the AcbC core where *Gria2* gene expression is higher than the adjacent ventral shell subregion (**Fig 1C; Allen Brain Atlas**). Immunoblot analysis of protein expression found that ethanol self-administration produced no change in AMPAR GluA1 subunit expression (**Fig 1D, left panel**). However, there

was a statistically significant increase in AMPAR GluA2 subunit protein expression in ethanol self-administering mice as compared to sucrose administering controls (**Fig 1C, middle panel**). We also showed that ethanol had no immediate effect on NSF expression in the AcbC as compared to sucrose (**Fig 1C, right panel**) indicating that the potential for NSF-dependent anchoring of GluA2-containing CI-AMPARs to AcbC excitatory synapses remained intact. This suggests that a history of non-dependent ethanol self-administration is only associated with an increase in GluA2-containing AMPARs in the AcbC.

GluA2-containing AMPAR activity in the AcbC is required for the positive reinforcing effects of ethanol

This experiment sought to determine if GluA2-containing AMPARs in the AcbC mechanistically regulate the positive reinforcing effects of ethanol. To address this question, C57BL/6J mice received site-specific injections of myristoylated Pep2m targeting the AcbC prior to operant ethanol or sucrose self-administration sessions (**Fig 2A)**. Pep2m dose-dependently decreased the reinforcing effects of ethanol as measured by reduced response rate (Time x Pep2m interaction $F(24, 168)=2.7$, $P=0.0001$) and reinforcers (Main effect $F(2,20)=3.9$, $P = 0.037$) (**Fig 2B & C**). Pep2m had no effect on general consummatory behaviors since headpokes into the reinforcer delivery cup per reinforcer remained constant (**Fig 2D**). Self-administration of sucrose in a parallel behavior-matched control group was not changed (**Fig 2E-G**). Moreover, infusion of the effective dosage of Pep2m (10 μg/0.5μl) to reduce operant ethanol self-administration had no effect on locomotor activity by either ethanol or sucrose mice (**Fig 2H & I**), indicating the absence of nonspecific effect on motor ability. These results show that that GluA2-containing AMPAR activity in the AcbC is required for the full expression of the positive reinforcing effects of ethanol.

To test the specificity of GluA2-containing AMPARs in the functional regulation of ethanol reinforcement, we injected NASPM (0, 1 or 10 μg/0.5μl) into the AcbC of a separate group of ethanol self-administering mice to inhibit GluA1-containing CP-AMPARs (**Fig 3A**). NASPM had

no effect on the rate of ethanol-reinforced lever presses, reinforcers earned, or number of headpokes per reinforcer as compared to vehicle control (**Fig 3B**). In addition, there was no effect on locomotor activity in the open-field test following NASPM (0 or 10 µg/0.5µl) infusion in the AcbC (**Fig 3C**). This finding suggests that CP-AMPARs in the AcbC do not regulate the reinforcing effects of ethanol and suggest specificity of control by GluA2-containing AMPARs.

Self-administered ethanol increases synaptic activity of CI-AMPARs in AcbC neurons receiving BLA projections

We discovered previously that operant ethanol self-administration selectively increased synaptic activity and insertion of GluA2-lacking CP-AMPARs in the BLA of neurons that project to the Acb, as compared to behavior-matched sucrose controls (Faccidomo et al. 2021). However, that study did not evaluate the activity of Acb neurons. Thus, to determine if the ethanol-induced upregulation of AMPAR GluA2 protein expression is associated with altered synaptic activity in AcbC neurons receiving BLA projections, male C57BL/6J mice received site-specific infusion of AAV2-CaMKIIa-hChR2(H134R)-EYFP in the BLA to probe neurons in the AcbC receiving BLA synaptic input. After a 7-day recovery, mice were separated into two groups and trained to self-administer sweetened ethanol or sucrose-only. After a 43d baseline and 1-d of ethanol clearance, brains were removed for patch-clamp electrophysiological recordings of electrical- or light-evoked ChR2-positive neurons in the AcbC (**Fig 4A)**. Infusion of ChR2 in the BLA under the CaMKII promoter resulted in significant expression in AcbC / shell subregions (**Fig 4B**). Behavioral results showed that mice self-administered ethanol and sucrose (control) solutions at an equivalent rate indicating no potential impact of differential behavior between the groups (**Fig 4C**).

Ethanol self-administration had no effect on sEPSC amplitude but significantly increased frequency (**Fig 4D**), which is classically interpreted as an increase in glutamate release but may reflect an increase in the number of synapses on a neuron. In addition, we found that ethanol self-administration significantly slowed sEPSC decay kinetics as measured by decay time (ms) and

area under the curve (**Fig 4D**), which is consistent with upregulated GluA2-containing AMPARs (Chater and Goda 2014; Pampaloni and Plested 2022).

Optically evoked EPSC (oEPSC) in AcbC neurons showed an increase in AMPA/NMDA ratio (**Fig 4E**), indicating that a history of ethanol self-administration is associated with an increase in AMPAR synaptic activity in AcbC neurons receiving BLA projections. In addition, there was a significant reduction in the paired-pulse ratio (**Fig 4E**) suggesting that ethanol use is associated with an enhancement of glutamate release in this neuronal population. However, there was no ethanol-associated change in the AMPA/NMDA ratio when measured after local electrical stimulation in the AcbC (MEAN±SEM: sucrose 6.09±1.6; ethanol 5.3±1.07), suggesting specificity of the BLA projection to the AcbC.

Although the present results are consistent with upregulated GluA2-containing CI-AMPAR activity (protein expression and decay kinetics), our previous findings indicate that ethanol self-administration increased synaptic insertion of CP-AMPARs in the BLA (Faccidomo et al. 2021). Thus, we assessed the potential contribution of GluA2-lacking CP-AMPARs to synaptic transmission in AcbC neurons receiving BLA projections by bath application of the CP-AMPAR blocker NASPM. Bath application of (NASPM, 100 μM) had no effect on oEPSC amplitude in ChR2-positive neurons from ethanol self-administering mice or in sucrose controls (**Fig 4F**). This suggests that changes in excitatory transmission are not a function of synaptic insertion of CP-AMPARs but may reflect ethanol-induced upregulated GluA2-containing CI-AMPAR activity.

Discussion

Through a series of experiments combining operant ethanol self-administration with molecular, physiological, optogenetic, and pharmacological approaches, we found that GluA2-containing CI-AMPARs in the AcbC are targeted by self-administered ethanol. This mechanism underlies the drug's positive reinforcing properties, which are essential for the experience-dependent development and progression of AUD (Wise and Koob 2013). This discovery deepens

our understanding of alcohol addiction's molecular mechanisms and neural circuitry. Given GluA2's role in memory maintenance, it also unveils a potential therapeutic target for medications aimed at promoting the extinction of behavioral pathologies associated with AUD, including chronic alcohol drinking.

Self-administered ethanol increases GluA2 protein expression in the AcbC

A novel finding of this study is that chronic operant self-administration of sweetened ethanol significantly increased AMPAR GluA2 subunit protein expression by 67% in the AcbC relative behavior-matched sucrose controls. Importantly, no group differences were seen in AMPAR GluA1 protein expression in the AcbC. We measured NSF expression due to its crucial role in recruiting and inserting GluA2-containing AMPARs into the synaptic membrane by binding to the GluA2 C-terminus (Araki et al. 2010; Huang et al. 2005) (Nishimune et al. 1998; Shi et al. 2001). NSF expression was unchanged indicating that a key mechanism required for synaptic delivery and activity of GluA2-containing AMPARs was intact in the AcbC following a history of ethanol self-administration. The upregulation of GluA2 and intact NSF protein expression is significant, as we previously observed decreased NSF expression in the amygdala of mice trained to self-administer sweetened ethanol (Faccidomo et al. 2021). This suggests that distinct and dynamic glutamatergic mechanisms in the amygdala and AcbC regulate reward-driven behaviors.

In this context, it is important to underscore the importance of comparison to the behavior-matched sucrose controls, which is critical to the interpretation that the observed change in GluA2 expression was a specific function of ethanol and not related to alternative factors such as general reward processing. This control also rules out nonspecific effects of variables such as learning and memory associated with the complex operant task, motor activity (lever presses), and general consummatory behavior, all of which could be regulated by glutamate activity in the AcbC (e.g., (Grueter et al. 2012; Kelley 2004b)). Thus, these results support the conclusion that chronic ethanol self-administration produces a reinforcer-specific, AMPAR subunit-selective, increase in GluA2 expression in the AcbC.

These results complement and extend preclinical research showing that home-cage voluntary ethanol drinking alters protein expression of AMPAR GluA2 in the Acb and connected forebrain nuclei. For example, home-cage ethanol drinking increased both GluA1 and GluA2 expression in the synaptic fraction of cells from the dorsal medial striatum of rats at 24-hrs post exposure (Wang et al. 2012) and was associated with an increase in AMPAR GluA1 expression in C57BL/6J mouse Acb at 24-hrs post intake (Ary et al. 2012). Evidence is mixed, however, regarding how long AMPAR subunit adaptations persist in withdrawal and may be brain-region specific. GluA1 expression was not changed in the Acb of rats following extended withdrawal of 40 – 60 days from chronic intermittent ethanol drinking (Marty and Spigelman 2012); however, both GluA1 and GluA2 expression were increased in the prefrontal cortex of male and female C57BL/6J mice following a 30-day history of home-cage ethanol drinking and 28-days of withdrawal (Szumlinski et al. 2023). Together with the results from the present study, these findings present consistent evidence that voluntary ethanol intake is associated with an increase in expression of GluA2-containing AMPARs in reward-related brain regions.

GluA2-containing AMPAR activity in the AcbC is required for the positive reinforcing effects of ethanol

To address the GluA2 specificity of the AMPAR behavioral regulation of ethanol self-administration via the AcbC, two microinjection studies were conducted. The first used Pep2m which is a cell-permeable inhibitor of GluA2-containing AMPARs that inhibits the interaction between the GluA2 C-terminus and NSF. Disruption of this interaction decreases AMPAR mediated synaptic activity (Nishimune et al. 1998) and surface expression of GluA2-containing AMPARs (Noel et al. 1999). The second used NASPM to inhibit GluA1-containing AMPARs as a test of AMPAR subtype specificity in regulation of ethanol self-administration. Both infusions targeted the AcbC; however, it should be noted that drug solutions may have diffused to adjacent loci.

Intra-AcbC infusion of Pep2m produced a dose-dependent decrease in ethanol -reinforced lever presses, indicating reduced reinforcing effects of ethanol. This led to fewer ethanol reinforcers earned at the highest dose without affecting headpokes in the delivery cup, showing no impact on consummatory behavior. Notably, 10 µg Pep2m reduced response rate during the 20-60 min period of 1-hour sessions without altering the onset of responding. This indicates that Pep2m infusion in the AcbC did not nonspecifically inhibit ethanol operant conditioning or motor ability, as confirmed by an independent open field assessment. Sucrose self-administering mice, used as behavior-matched controls, showed similar responses to the ethanol-trained group under aCSF conditions (~150 responses/hr). Importantly, however, Pep2m had no effect on sucrose-reinforced responding or spontaneous open field activity, supporting an ethanol-specific effect. These findings highlight the GluA2 and NSF interaction in the AcbC as a novel mechanism of ethanol self-administration.

Prior studies have shown that GluA1-containing AMPAR activity in the BLA is required for the reinforcing effects of ethanol (Faccidomo et al. 2021) but it was not known whether GluA1-containing AMPAR activity in the Acb also regulates ethanol's reinforcing properties. Results of the present study showed that intra-AcbC NASPM had no effect on ethanol self-administration or locomotor activity. This suggests that GluA2-lacking CP-AMPAR activity in the AcbC does not mediate the reinforcing properties of sweetened ethanol as tested under the conditions of the present study. It is important to consider that others have found that NASPM (20 µg/side) infused in the Acb (shell) decreased home-cage ethanol drinking by both female and male C57BL/6J mice with no effect on concurrent water intake (Kwok et al. 2021). However, that study used a different ethanol drinking method and higher NASPM dosage suggesting that CP-AMPAR regulation of ethanol operant self-administration and home-cage intake may be Acb-subregion specific and influenced by behavioral factors, such as motivation. Together, these studies underscore the importance of considering neuroanatomical specificity, the nature of the reinforcing or consumed

solution, antagonist dosage, and ethanol self-administration method in evaluating AMPAR subtype regulation of ethanol reinforcement.

Self-administered ethanol increases synaptic activity of CI-AMPARs in AcbC neurons receiving BLA projections

We previously found that ethanol self-administration selectively increased excitatory synaptic activity and membrane insertion of GluA2-lacking CP-AMPARs in BLA neurons projecting to the Acb, and that GluA1-containing AMPARs are required for ethanol's reinforcing effects (Faccidomo et al. 2021). Here, we extended these findings by examining if operant ethanol self-administration alters synaptic activity of AcbC neurons receiving excitatory BLA projections. Whole-cell patch-clamp recordings from ChR2-positive neurons in the AcbC showed that chronic ethanol self-administration ($\bar{X}$ = 0.79 g/kg/day) significantly increased sEPSC frequency without changing sEPSC amplitude. This suggests increased glutamate release probability from presynaptic BLA neurons in ethanol drinking mice compared to sucrose controls, aligning with our prior observation of upregulated AMPAR activity in the BLA (Faccidomo et al. 2021). Additionally, sEPSC currents in AcbC neurons from ethanol drinking mice showed increased decay time and area under the curve, indicating a shift to increased synaptic representation of GluA2-containing AMPARs (Chater and Goda 2014; Pampaloni and Plested 2022). These data suggest that ethanol self-administration is associated with increased spontaneous glutamate release on AcbC neurons receiving excitatory BLA projections and enhanced representation of GluA2-containing AMPARs, consistent with upregulated GluA2 protein expression in the AcbC and microinjection results. Alternatively, there may be an increase in the number of synapses in ethanol self-administering mice compared to controls.

To determine the impact of ethanol self-administration on synaptic activity, we recorded glutamatergic transmission with whole-cell patch clamp recordings in the BLA→AcbC circuit and found that optical stimulation of ChR2-positive BLA terminals in the AcbC exhibited a significant increase in the oEPSC AMPA/NMDA ratio in ethanol self-administering mice as compared to

sucrose controls suggesting an ethanol-induced increase in synaptic strength or plasticity, or a potential increase in the number or function of AMPARs, which is also consistent with our observation of upregulated GluA2 protein expression. Interestingly, the AMPA/NMDA ratio was not altered in ChR2-positive cells following local electrical stimulation in the AcbC suggesting an ethanol-specific effect on BLA terminals. In addition, we observed a significant decrease in the paired-pulse ratio (PPR) in ethanol self-administering mice as compared to sucrose controls following optical stimulation of ChR2-positive BLA terminals in the AcbC. Paired-pulse depression (PPD) of oEPSCs is also indicative of an increase in the probability of glutamate release, which is consistent with the increase in sEPSC frequency described above. Although the specific mechanism of PPD was not pursued in this study, it is likely influenced by enhanced synaptic activity GluA1-containing CP-AMPARs in the BLA projection neurons as we discovered previously (Faccidomo et al. 2021), and may be modified by a variety of related cellular processes including increased CaMKII expression or activity in the BLA, or upregulation of AMPAR accessory proteins such as TARP ɣ-8 in the postsynaptic membrane, both of which enhance AMPAR activity (Hayashi et al. 2000; Tomita et al. 2003) and regulate ethanol self-administration (Agoglia et al. 2015; Cannady et al. 2016; Faccidomo et al. 2016; Hoffman et al. 2021; Hoffman et al. 2023; Salling et al. 2016).

Finally, bath application of the CP-AMPAR inhibitor NASPM confirmed the specificity of GluA2-containing AMPAR activity on synaptic function. NASPM had no effect on oEPSC amplitude in either ethanol or sucrose self-administering mice, suggesting the absence of a significant population of CP-AMPARs in the BLA → AcbC synapses following the operant behavioral protocol used in this study. This contrasts with our previous discovery in BLA neurons projecting to the Acb, where NASPM selectively decreased eEPSC amplitude in ethanol self-administering mice but not in sucrose controls, indicating increased membrane insertion of CP-AMPARs in the BLA (Faccidomo et al. 2021). Together, these studies support the conclusion that ethanol self-administration produces differential, brain-region-dependent effects on AMPAR

subunit composition and synaptic function, with upregulation of GluA2-containing CI-AMPAR activity in the AcbC and GluA1-containing AMPAR activity in the BLA. These data also suggest increased plasticity in both pre- and postsynaptic compartments in the BLA → AcbC circuit following ethanol self-administration.

Overall Conclusions

The implications of these experiments are significant for understanding how neural plasticity within the BLA → AcbC excitatory circuit regulates the positive reinforcing properties of ethanol. Numerous reports indicate that GluA2-lacking CP-AMPARs, which can be GluA1 homomers, are rapidly inserted in synapses undergoing specific forms of synaptic plasticity, such as long-term potentiation, and following tests of behavioral plasticity, including fear conditioning and exposure to drugs of abuse (reviewed by (Diering and Huganir 2018)). However, the synaptic expression of CP-AMPARs is transient and followed by replacement with GluA2-containing CI-AMPARs (Plant et al. 2006), stabilizing long-term changes in synaptic efficacy (Shi et al. 2001). Our prior results showing upregulated CP-AMPAR activity in BLA neurons projecting to the Acb following chronic ethanol self-administration (Faccidomo et al. 2021) suggest ongoing plasticity in BLA projection neurons with repeated ethanol exposure. Alternatively, AcbC neurons receiving BLA projections exhibit increased GluA2-containing CI-AMPAR expression and activity following chronic ethanol self-administration, consistent with long-term stabilization of ethanol's impact on neural function in this key reward system nucleus (Plant et al. 2006). These observations raise the hypothesis that GluA1-containing CP-AMPAR activity in the BLA may drive ongoing changes in ethanol's reinforcing effects, whereas GluA2-containing CI-AMPAR activity in the AcbC may underlie the long-term maintenance, or memory, of this maladaptive stimulus property of the drug.

In conclusion, harmful alcohol use is a significant public health concern worldwide (WorldHealthOrganization 2018), and understanding the neural mechanisms underlying its positive reinforcing effects is crucial for developing effective treatments (Stolerman 1992). Here we show evidence that ethanol targets AMPAR GluA2 expression and activity in the Acb, a key

nucleus in the brain's reward pathway, regulating the drug's reinforcing effects. Based on these results and observations that long-term memory consolidation requires GluA2-containing AMPARs (Pereyra and Medina 2021), we propose that ethanol's ability to drive repetitive self-administration in AUD is mediated through hijacking this fundamental process in the Acb. Furthermore, pharmacological tools like Pep2m, which mediate AMPAR GluA2 endocytosis and downregulation, have potential to promote forgetting of specific long-term memories (Hardt et al. 2014; Migues et al. 2016), such as the reinforcing effects of alcohol. Future studies should test this hypothesis using models of cue-induced reinstatement and other techniques that specifically test the regulation of alcohol memories via AMPAR activity.

Figure Captions

Figure 1. Operant ethanol self-administration increases AMPAR GluA2 subunit protein expression in the AcbC. (A) Schematic of operant conditioning chamber showing active and inactive lever with timeline. (**B**) Measures of operant ethanol (EtOH) and sucrose self-administration plotted as MEAN±SEM for n=8 mice per group. (**C**) Mouse brain atlas image juxtaposed with photomicrograph showing GluA2 gene expression in mouse brain (Allen Brain Atlas) with the dissection region of the AcbC highlighted by the dashed-line circle. (**D**) Ethanol self-administration was associated with a significant increase in GluA2 protein expression in the AcbC as compared to sucrose-only controls [$t(9) = 2.6$, $P = 0.029$]. Data represent MEAN±SEM, n=5 and n=6 / group, respectively. * - indicates $P<0.05$ relative to sucrose (S) control. No changes were observed in GluA1 or NSF protein expression.

Figure 2. GluA2-containing AMPAR activity in the AcbC is required for the positive reinforcing effects of ethanol. (**A**) Schematic showing timeline of baseline behavioral training with ethanol (EtOH) and sucrose reinforcement, surgery, and Pep2m infusion targeting the AcbC. (**B**) Line graph shows ethanol reinforced response rate (resp / 5-min) plotted as a function of time (min). Two-way ANOVA - significant Time x Pep2m interaction $F (24, 168) = 2.708$, $P=0.0001$. *$P < 0.05$ Pep2m (10 µg, n=8) significantly different from aCSF (n=8) at corresponding time point; Dunnett's multiple comparison test. (**C**) Bar graph shows number of ethanol reinforcers earned per hr. One-way ANOVA – significant main effect of Pep2m: $F (2, 20) = 3.909$, $P=0.036$. *$P<0.05$ Pep2m (10 µg, n=8) significantly different from aCSF (n=8); Dunnett's multiple comparison test. (**D**) Head pokes in the ethanol delivery cup were unchanged by Pep2m infusion. (**E - G**) Parameters of sucrose-only self-administration were not changed by infusion of Pep2m in the AcbC. (**H**) Schematic of open-field locomotor activity monitor (**I**) No effect of Pep2m infusion in the AcbC on locomotor activity in the ethanol or sucrose-only groups. All data are MEAN±SEM.

Figure 3. **GluA2-lacking CP-AMPAR activity in the AcbC does not regulate the positive reinforcing effects of ethanol**. (**A**) Schematic showing timeline of baseline behavioral training with ethanol (EtOH) and sucrose reinforcement, surgery, and NASPM infusion targeting the AcbC. (**B**) NASPM had no effect on ethanol reinforced response rate, number of reinforcers earned, or headpokes in the delivery cup. (**C**) Schematic of open-field locomotor test chamber. (D) Distance traveled in the open field after NASPM administration showing no effect. All data are MEAN±SEM.

Figure 4. **Ethanol self-administration increases CI-AMPAR but not CP-AMPAR activity in AcbC neurons receiving input from the BLA.** (**A**) Experimental timeline and schematic showing ChR2 infusion in BLA by 43-days of ethanol (EtOH) or sucrose (Suc) self-administration. BLA recordings were conducted 24-h after the last self-administration session. (**B**) Schematic of ChR2 infusion in the BLA and photograph of expression in the AcbC. (**C**) Parameters of ethanol (red bars) and sucrose (gray bars) self-administration show behavior-matched performance (n=6-8 per group). (**D**) Average plots showing no change in sEPSC amplitude [$t(18)=0.885$, $p = 0.39$] but significantly increased sEPSC frequency [$t(16)=1.8$, $P = 0.04$] and increased decay time (ms) [$t(18)=2.7$, $P = 0.014$] and area under the curve [$t(18)=2.6$, $P = 0.015$]. (**E**) Average plots showing an increase in optical evoked AMPA/NMDA ratio [$t(22)=2.1$, $P = 0.03$] and a reduction in the paired-pulse ratio (PPR) [$t(28)=2.4$, $P = 0.02$] in AcbC neurons receiving BLA projections. (**D-E**) Representative traces illustrating EtOH-induced increases are shown above and to the right of average plots. (**F**) Time course of optical eEPSC amplitude (MEAN±SEM) during baseline (min 1 – 4) and bath application of NASPM (100 μM; min 5 - 16) from sucrose vs. ethanol self-administering mice. Data are normalized to minute 4 of the baseline. Bar graph in F shows summary of eEPSC during NASPM bath.

References

Acosta G, Freidman DP, Grant KA, Hemby SE (2011) Alternative splicing of AMPA subunits in prefrontal cortical fields of cynomolgus monkeys following chronic ethanol self-administration. Front Psychiatry 2: 72.

Agoglia AE, Holstein SE, Eastman VR, Hodge CW (2016) Cannabinoid CB1 receptor inhibition blunts adolescent-typical increased binge alcohol and sucrose consumption in male C57BL/6J mice. Pharmacology, biochemistry, and behavior 143: 11-7.

Agoglia AE, Holstein SE, Reid G, Hodge CW (2015) CaMKIIalpha-GluA1 Activity Underlies Vulnerability to Adolescent Binge Alcohol Drinking. Alcoholism, clinical and experimental research 39: 1680-90.

Anton RF, Kranzler HR, Meyer RE (1995) Neurobehavioral aspects of the pharmacotherapy of alcohol dependence. Clin Neurosci 3: 145-54.

Araki Y, Lin DT, Huganir RL (2010) Plasma membrane insertion of the AMPA receptor GluA2 subunit is regulated by NSF binding and Q/R editing of the ion pore. Proc Natl Acad Sci U S A 107: 11080-5.

Ary AW, Cozzoli DK, Finn DA, Crabbe JC, Dehoff MH, Worley PF, Szumlinski KK (2012) Ethanol up-regulates nucleus accumbens neuronal activity dependent pentraxin (Narp): implications for alcohol-induced behavioral plasticity. Alcohol 46: 377-87.

Backstrom P, Hyytia P (2004) Ionotropic glutamate receptor antagonists modulate cue-induced reinstatement of ethanol-seeking behavior. Alcohol Clin Exp Res 28: 558-65.

Bauer MR, McVey MM, Germano DM, Zhang Y, Boehm SL, 2nd (2022) Intra-dorsolateral striatal AMPA receptor antagonism reduces binge-like alcohol drinking in male and female C57BL/6J mice. Behav Brain Res 418: 113631.

Bellone C, Luscher C (2006) Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression. Nat Neurosci 9: 636-41.

Besheer J, Cox AA, Hodge CW (2003) Coregulation of ethanol discrimination by the nucleus accumbens and amygdala. Alcoholism, clinical and experimental research 27: 450-6.

Besheer J, Grondin JJ, Cannady R, Sharko AC, Faccidomo S, Hodge CW (2010) Metabotropic glutamate receptor 5 activity in the nucleus accumbens is required for the maintenance of ethanol self-administration in a rat genetic model of high alcohol intake. Biol Psychiatry 67: 812-22.

Besheer J, Grondin JJ, Salling MC, Spanos M, Stevenson RA, Hodge CW (2009) Interoceptive effects of alcohol require mGlu5 receptor activity in the nucleus accumbens. J Neurosci 29: 9582-91.

Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. Neuron 40: 361-79.

Cannady R, Fisher KR, Durant B, Besheer J, Hodge CW (2013) Enhanced AMPA receptor activity increases operant alcohol self-administration and cue-induced reinstatement. Addiction biology 18: 54-65.

Cannady R, Fisher KR, Graham C, Crayle J, Besheer J, Hodge CW (2016) Potentiation of amygdala AMPA receptor activity selectively promotes escalated alcohol self-administration in a CaMKII-dependent manner. Addiction biology 22: 652-664.

Chater TE, Goda Y (2014) The role of AMPA receptors in postsynaptic mechanisms of synaptic plasticity. Front Cell Neurosci 8: 401.

Conrad KL, Tseng KY, Uejima JL, Reimers JM, Heng LJ, Shaham Y, Marinelli M, Wolf ME (2008) Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature 454: 118-21.

Czachowski CL, Delory MJ, Pope JD (2012) Behavioral and neurotransmitter specific roles for the ventral tegmental area in reinforcer-seeking and intake. Alcohol Clin Exp Res 36: 1659-68.

Diering GH, Huganir RL (2018) The AMPA Receptor Code of Synaptic Plasticity. Neuron 100: 314-329.

Faccidomo S, Besheer J, Stanford PC, Hodge CW (2009) Increased operant responding for ethanol in male C57BL/6J mice: specific regulation by the ERK1/2, but not JNK, MAP kinase pathway. Psychopharmacology (Berl) 204: 135-47.

Faccidomo S, Cogan ES, Hon OJ, Hoffman JL, Saunders BL, Eastman VR, Kim M, Taylor SM, McElligott ZA, Hodge CW (2021) Calcium-permeable AMPA receptor activity and GluA1 trafficking in the basolateral amygdala regulate operant alcohol self-administration. Addict Biol 26: e13049.

Faccidomo S, Holstein SE, Santanam TS, Saunders BL, Swaim KS, Reid GT, O'Neill C, Eastman VR, Hodge CW (2020) Pharmacological inhibition of glycogen synthase kinase 3 increases operant alcohol self-administration in a manner associated with altered pGSK-3beta, protein interacting with C kinase and GluA2 protein expression in the reward pathway of male C57BL/6J mice. Behav Pharmacol 31: 15-26.

Faccidomo S, Reid GT, Agoglia AE, Ademola SA, Hodge CW (2016) CaMKII inhibition in the prefrontal cortex specifically increases the positive reinforcing effects of sweetened alcohol in C57BL/6J mice. Behavioural brain research 298: 286-90.

Faccidomo S, Salling MC, Galunas C, Hodge CW (2015) Operant ethanol self-administration increases extracellular-signal regulated protein kinase (ERK) phosphorylation in reward-related brain regions: selective regulation of positive reinforcement in the prefrontal cortex of C57BL/6J mice. Psychopharmacology 232: 3417-30.

Faccidomo S, Swaim KS, Saunders BL, Santanam TS, Taylor SM, Kim M, Reid GT, Eastman VR, Hodge CW (2018) Mining the nucleus accumbens proteome for novel targets of alcohol self-administration in male C57BL/6J mice. Psychopharmacology (Berl) 235: 1681-1696.

Gass JT, Olive MF (2008) Glutamatergic substrates of drug addiction and alcoholism. Biochem Pharmacol 75: 218-65.

Grueter BA, Rothwell PE, Malenka RC (2012) Integrating synaptic plasticity and striatal circuit function in addiction. Current opinion in neurobiology 22: 545-51.

Hardt O, Nader K, Wang YT (2014) GluA2-dependent AMPA receptor endocytosis and the decay of early and late long-term potentiation: possible mechanisms for forgetting of short- and long-term memories. Philos Trans R Soc Lond B Biol Sci 369: 20130141.

Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 287: 2262-7.

Heilig M, Egli M (2006) Pharmacological treatment of alcohol dependence: target symptoms and target mechanisms. Pharmacol Ther 111: 855-76.

Hodge CW, Chappelle AM, Samson HH (1995) GABAergic transmission in the nucleus accumbens is involved in the termination of ethanol self-administration in rats. Alcohol Clin Exp Res 19: 1486-93.

Hodge CW, Kelley SP, Bratt AM, Iller K, Schroeder JP, Besheer J (2004) 5-HT(3A) receptor subunit is required for 5-HT3 antagonist-induced reductions in alcohol drinking. Neuropsychopharmacology 29: 1807-13.

Hodge CW, Mehmert KK, Kelley SP, McMahon T, Haywood A, Olive MF, Wang D, Sanchez-Perez AM, Messing RO (1999) Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. Nat Neurosci 2: 997-1002.

Hodge CW, Miles MF, Sharko AC, Stevenson RA, Hillmann JR, Lepoutre V, Besheer J, Schroeder JP (2006) The mGluR5 antagonist MPEP selectively inhibits the onset and maintenance of ethanol self-administration in C57BL/6J mice. Psychopharmacology (Berl) 183: 429-38.

Hodge CW, Raber J, McMahon T, Walter H, Sanchez-Perez AM, Olive MF, Mehmert K, Morrow AL, Messing RO (2002) Decreased anxiety-like behavior, reduced stress hormones, and

neurosteroid supersensitivity in mice lacking protein kinase Cepsilon. The Journal of clinical investigation 110: 1003-10.

Hodge CW, Samson HH, Chappelle AM (1997) Alcohol self-administration: further examination of the role of dopamine receptors in the nucleus accumbens. Alcohol Clin Exp Res 21: 1083-91.

Hodge CW, Samson HH, Haraguchi M (1992) Microinjections of dopamine agonists in the nucleus accumbens increase ethanol-reinforced responding. Pharmacol Biochem Behav 43: 249-54.

Hoffman JL, Faccidomo S, Saunders BL, Taylor SM, Kim M, Hodge CW (2021) Inhibition of AMPA receptors (AMPARs) containing transmembrane AMPAR regulatory protein gamma-8 with JNJ-55511118 shows preclinical efficacy in reducing chronic repetitive alcohol self-administration. Alcohol Clin Exp Res 45: 1424-1435.

Hoffman JL, Faccidomo SP, Taylor SM, DeMiceli KG, May AM, Smith EN, Whindleton CM, Hodge CW (2022) Inhibition of AMPA receptors bound to transmembrane AMPA receptor regulatory protein γ-8 (TARP γ-8) blunts the positive reinforcing properties of alcohol and sucrose in a brain region-dependent manner. bioRxiv: 2022.12.14.520457.

Hoffman JL, Faccidomo SP, Taylor SM, DeMiceli KG, May AM, Smith EN, Whindleton CM, Hodge CW (2023) Negative modulation of AMPA receptors bound to transmembrane AMPA receptor regulatory protein gamma-8 blunts the positive reinforcing properties of alcohol and sucrose in a brain region-dependent manner in male mice. Psychopharmacology (Berl) 240: 1261-1273.

Holmes A, Spanagel R, Krystal JH (2013) Glutamatergic targets for new alcohol medications. Psychopharmacology 229: 539-54.

Hopf FW, Mangieri RA (2018) Do Alcohol-Related AMPA-Type Glutamate Receptor Adaptations Promote Intake? Handb Exp Pharmacol 248: 157-186.

Huang Y, Man HY, Sekine-Aizawa Y, Han Y, Juluri K, Luo H, Cheah J, Lowenstein C, Huganir RL, Snyder SH (2005) S-nitrosylation of N-ethylmaleimide sensitive factor mediates surface expression of AMPA receptors. Neuron 46: 533-40.

Hyman SE (2005) Addiction: a disease of learning and memory. Am J Psychiatry 162: 1414-22.

Jiang J, Suppiramaniam V, Wooten MW (2006) Posttranslational modifications and receptor-associated proteins in AMPA receptor trafficking and synaptic plasticity. Neurosignals 15: 266-82.

Kalivas PW, Volkow ND (2011) New medications for drug addiction hiding in glutamatergic neuroplasticity. Mol Psychiatry 16: 974-986.

Kelley AE (2004a) Memory and addiction: shared neural circuitry and molecular mechanisms. Neuron 44: 161-79.

Kelley AE (2004b) Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27: 765-76.

Kwok C, Lei K, Pedrozo V, Anderson L, Ghotra S, Walsh M, Li L, Yu J, Hopf FW (2021) Differential importance of nucleus accumbens Ox1Rs and AMPARs for female and male mouse binge alcohol drinking. Sci Rep 11: 231.

Liu SQ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. Nature 405: 454-8.

Lu W, Shi Y, Jackson AC, Bjorgan K, During MJ, Sprengel R, Seeburg PH, Nicoll RA (2009) Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. Neuron 62: 254-68.

Malenka RC (2003) Synaptic plasticity and AMPA receptor trafficking. Ann N Y Acad Sci 1003: 1-11.

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25: 103-26.

Mameli M, Bellone C, Brown MT, Luscher C (2011) Cocaine inverts rules for synaptic plasticity of glutamate transmission in the ventral tegmental area. Nature neuroscience 14: 414-6.
Marty VN, Spigelman I (2012) Long-lasting alterations in membrane properties, k(+) currents, and glutamatergic synaptic currents of nucleus accumbens medium spiny neurons in a rat model of alcohol dependence. Frontiers in neuroscience 6: 86.
McCool BA, Christian DT, Diaz MR, Lack AK (2010) Glutamate plasticity in the drunken amygdala: the making of an anxious synapse. Int Rev Neurobiol 91: 205-33.
Migues PV, Liu L, Archbold GE, Einarsson EO, Wong J, Bonasia K, Ko SH, Wang YT, Hardt O (2016) Blocking Synaptic Removal of GluA2-Containing AMPA Receptors Prevents the Natural Forgetting of Long-Term Memories. J Neurosci 36: 3481-94.
Montagud-Romero S, Cantacorps L, Fernandez-Gomez FJ, Nunez C, Minarro J, Rodriguez-Arias M, Milanes MV, Valverde O (2021) Unraveling the molecular mechanisms involved in alcohol intake and withdrawal in adolescent mice exposed to alcohol during early life stages. Prog Neuropsychopharmacol Biol Psychiatry 104: 110025.
National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals., Institute for Laboratory Animal Research (U.S.), National Academies Press (U.S.) (2011) Guide for the care and use of laboratory animals, 8th edn. National Academies Press, Washington, D.C.
Neasta J, Ben Hamida S, Yowell Q, Carnicella S, Ron D (2010) Role for mammalian target of rapamycin complex 1 signaling in neuroadaptations underlying alcohol-related disorders. Proc Natl Acad Sci U S A 107: 20093-8.
Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2: 119-28.
Nicoll RA, Schulman H (2023) Synaptic memory and CaMKII. Physiol Rev 103: 2877-2925.
Nishimune A, Isaac JT, Molnar E, Noel J, Nash SR, Tagaya M, Collingridge GL, Nakanishi S, Henley JM (1998) NSF binding to GluR2 regulates synaptic transmission. Neuron 21: 87-97.
Noel J, Ralph GS, Pickard L, Williams J, Molnar E, Uney JB, Collingridge GL, Henley JM (1999) Surface expression of AMPA receptors in hippocampal neurons is regulated by an NSF-dependent mechanism. Neuron 23: 365-76.
Pampaloni NP, Plested AJR (2022) Slow excitatory synaptic currents generated by AMPA receptors. J Physiol 600: 217-232.
Pereyra M, Medina JH (2021) AMPA Receptors: A Key Piece in the Puzzle of Memory Retrieval. Front Hum Neurosci 15: 729051.
Pickering C, Avesson L, Lindblom J, Liljequist S, Schioth HB (2007) Identification of neurotransmitter receptor genes involved in alcohol self-administration in the rat prefrontal cortex, hippocampus and amygdala. Prog Neuropsychopharmacol Biol Psychiatry 31: 53-64.
Plant K, Pelkey KA, Bortolotto ZA, Morita D, Terashima A, McBain CJ, Collingridge GL, Isaac JT (2006) Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. Nature neuroscience 9: 602-4.
Rassnick S, D'Amico E, Riley E, Pulvirenti L, Zieglgansberger W, Koob GF (1992a) GABA and nucleus accumbens glutamate neurotransmission modulate ethanol self-administration in rats. Ann N Y Acad Sci 654: 502-5.
Rassnick S, Pulvirenti L, Koob GF (1992b) Oral ethanol self-administration in rats is reduced by the administration of dopamine and glutamate receptor antagonists into the nucleus accumbens. Psychopharmacology (Berl) 109: 92-8.
Reimers JM, Milovanovic M, Wolf ME (2011) Quantitative analysis of AMPA receptor subunit composition in addiction-related brain regions. Brain Res 1367: 223-33.
Roedel A, Storch C, Holsboer F, Ohl F (2006) Effects of light or dark phase testing on behavioural and cognitive performance in DBA mice. Lab Anim 40: 371-81.

Salling MC, Faccidomo S, Hodge CW (2008) Nonselective suppression of operant ethanol and sucrose self-administration by the mGluR7 positive allosteric modulator AMN082. Pharmacol Biochem Behav 91: 14-20.

Salling MC, Faccidomo SP, Li C, Psilos K, Galunas C, Spanos M, Agoglia AE, Kash TL, Hodge CW (2016) Moderate Alcohol Drinking and the Amygdala Proteome: Identification and Validation of Calcium/Calmodulin Dependent Kinase II and AMPA Receptor Activity as Novel Molecular Mechanisms of the Positive Reinforcing Effects of Alcohol. Biological psychiatry 79: 430-42.

Salling MC, Hodge CJ, Psilos KE, Eastman VR, Faccidomo SP, Hodge CW (2017) Cue-induced reinstatement of alcohol-seeking behavior is associated with increased CaMKII T286 phosphorylation in the reward pathway of mice. Pharmacology, biochemistry, and behavior 163: 20-29.

Samson HH, Chappell A, Slawecki C, Hodge C (1999) The effects of microinjection of d-amphetamine into the n. accumbens during the late maintenance phase of an ethanol consumption bout. Pharmacol Biochem Behav 63: 159-65.

Samson HH, Hodge CW (1993) The role of the mesoaccumbens dopamine system in ethanol reinforcement: studies using the techniques of microinjection and voltammetry. Alcohol and alcoholism 2: 469-74.

Samson HH, Tolliver GA, Haraguchi M, Hodge CW (1992) Alcohol self-administration: role of mesolimbic dopamine. Ann N Y Acad Sci 654: 242-53.

Schroeder JP, Spanos M, Stevenson JR, Besheer J, Salling M, Hodge CW (2008) Cue-induced reinstatement of alcohol-seeking behavior is associated with increased ERK1/2 phosphorylation in specific limbic brain regions: blockade by the mGluR5 antagonist MPEP. Neuropharmacology 55: 546-54.

Sciascia JM, Reese RM, Janak PH, Chaudhri N (2015) Alcohol-Seeking Triggered by Discrete Pavlovian Cues is Invigorated by Alcohol Contexts and Mediated by Glutamate Signaling in the Basolateral Amygdala. Neuropsychopharmacology 40: 2801-12.

Shi S, Hayashi Y, Esteban JA, Malinow R (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell 105: 331-43.

Stephens DN, Brown G (1999) Disruption of operant oral self-administration of ethanol, sucrose, and saccharin by the AMPA/kainate antagonist, NBQX, but not the AMPA antagonist, GYKI 52466. Alcohol Clin Exp Res 23: 1914-20.

Stolerman I (1992) Drugs of abuse: behavioural principles, methods and terms. Trends Pharmacol Sci 13: 170-6.

Stuber GD, Hopf FW, Hahn J, Cho SL, Guillory A, Bonci A (2008) Voluntary ethanol intake enhances excitatory synaptic strength in the ventral tegmental area. Alcohol Clin Exp Res 32: 1714-20.

Stuber GD, Sparta DR, Stamatakis AM, van Leeuwen WA, Hardjoprajitno JE, Cho S, Tye KM, Kempadoo KA, Zhang F, Deisseroth K, Bonci A (2011) Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. Nature 475: 377-80.

Szumlinski KK, Herbert JN, Mejia Espinoza B, Madory LE, Scudder SL (2023) Alcohol-drinking during later life by C57BL/6J mice induces sex- and age-dependent changes in hippocampal and prefrontal cortex expression of glutamate receptors and neuropathology markers. Addict Neurosci 7.

Tomita S, Chen L, Kawasaki Y, Petralia RS, Wenthold RJ, Nicoll RA, Bredt DS (2003) Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. J Cell Biol 161: 805-16.

Wang J, Ben Hamida S, Darcq E, Zhu W, Gibb SL, Lanfranco MF, Carnicella S, Ron D (2012) Ethanol-mediated facilitation of AMPA receptor function in the dorsomedial striatum: implications for alcohol drinking behavior. The Journal of neuroscience : the official journal of the Society for Neuroscience 32: 15124-32.

Wilkie MB, Besheer J, Kelley SP, Kumar S, O'Buckley TK, Morrow AL, Hodge CW (2007) Acute ethanol administration rapidly increases phosphorylation of conventional protein kinase C in specific mammalian brain regions in vivo. Alcoholism, clinical and experimental research 31: 1259-67.

Wise RA, Koob GF (2013) The Development and Maintenance of Drug Addiction. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39: 254-62.

Wolf ME, Tseng KY (2012) Calcium-permeable AMPA receptors in the VTA and nucleus accumbens after cocaine exposure: when, how, and why? Front Mol Neurosci 5: 72.

WorldHealthOrganization (2018) Global status report on alcohol and health. WHO Press, Geneva

Figure 1

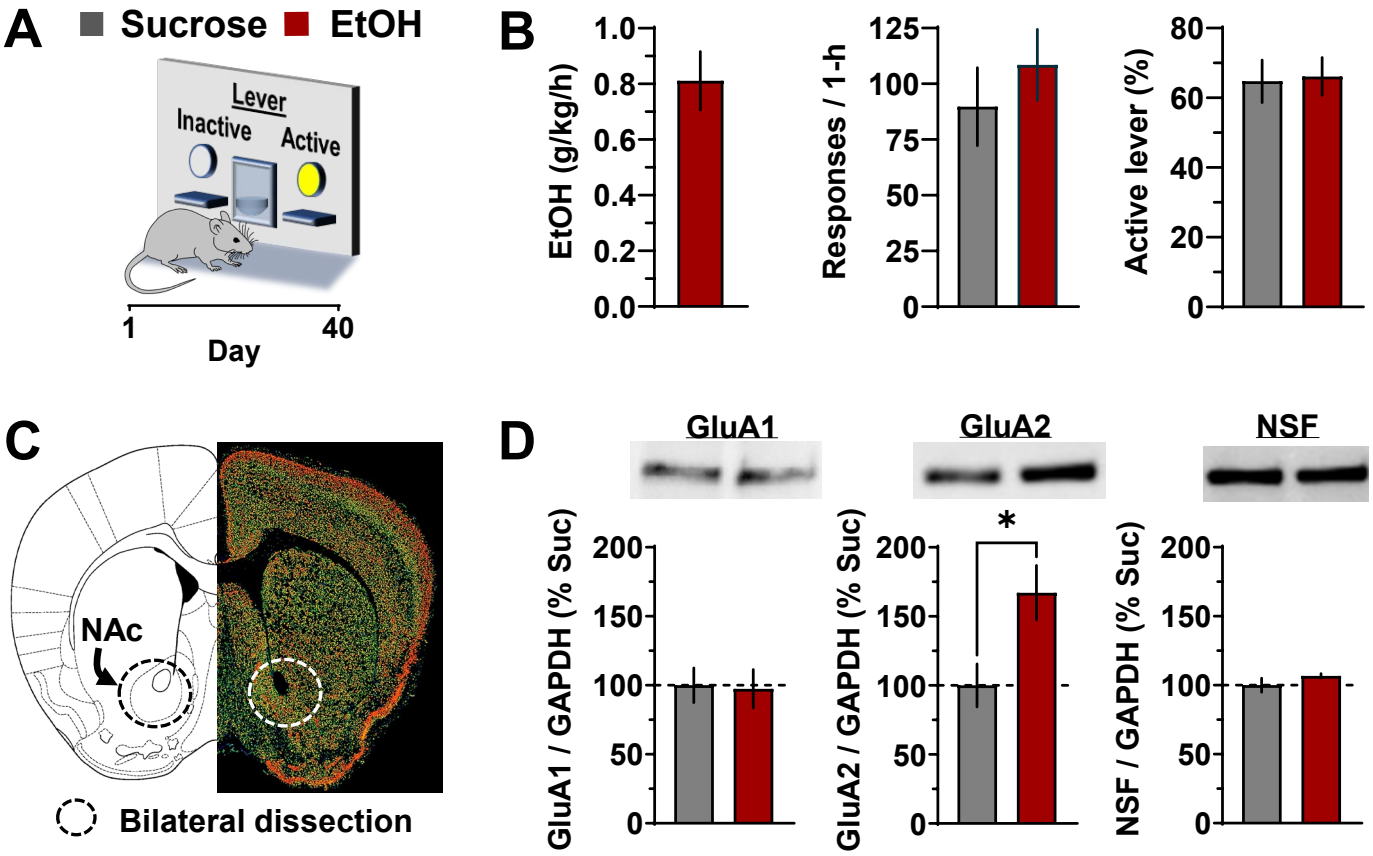

A
Sucrose
EtOH
Lever
Inactive
Active
1
40
Day
B
EtOH (g/kg/h)
1.0
0.8
0.6
0.4
0.2
0.0
Responses / 1-h
125
100
75
50
25
0
Active lever (%)
80
60
40
20
0
C
NAc
Bilateral dissection
D
GluA1
GluA2
NSF
GluA1 / GAPDH (% Suc)
GluA2 / GAPDH (% Suc)
NSF / GAPDH (% Suc)
200
150
100
50
0
*

Figure 2

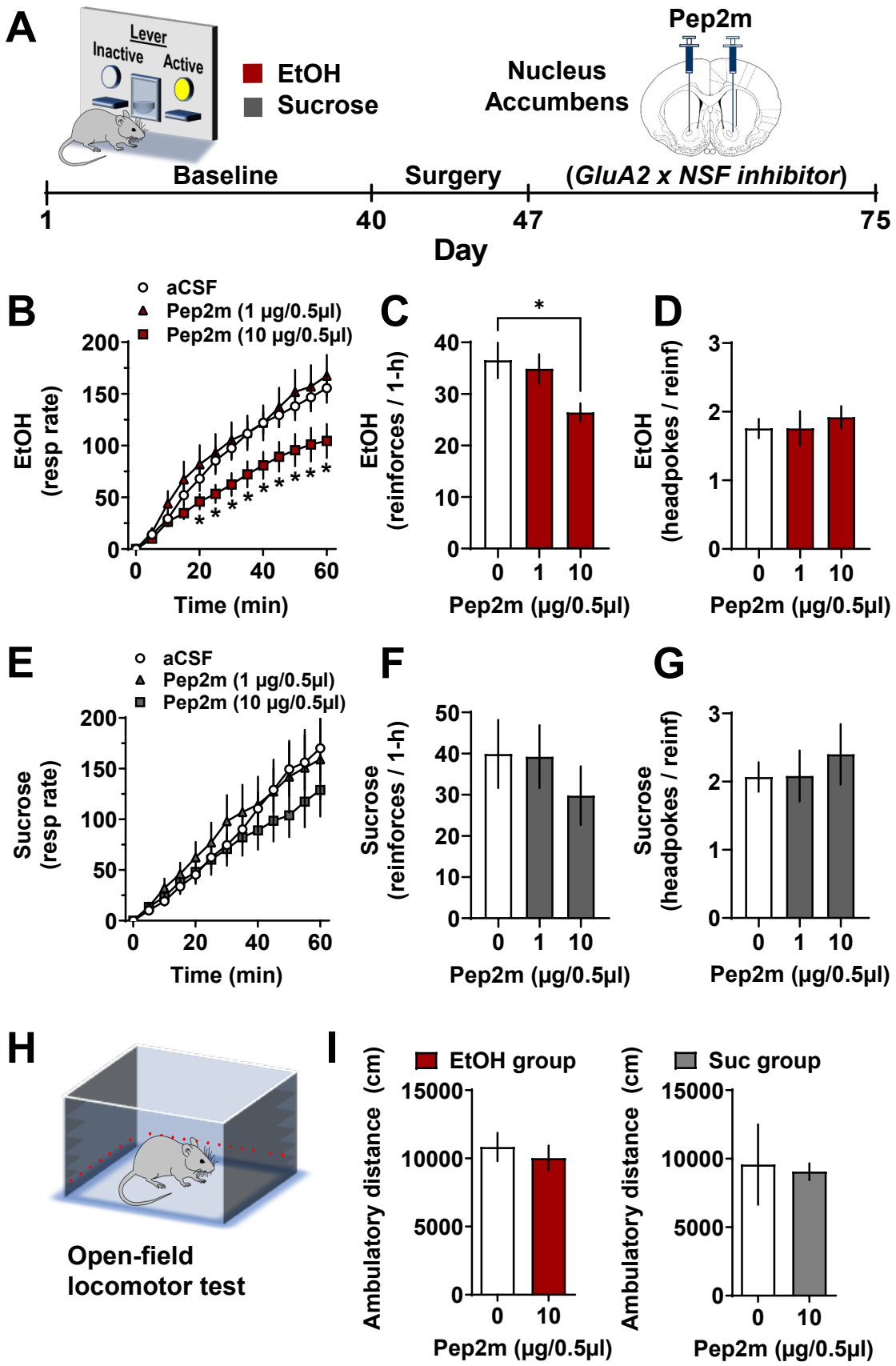

A
Lever
Inactive
Active
EtOH
Sucrose
Nucleus Accumbens
Pep2m
Baseline
Surgery
(GluA2 x NSF inhibitor)
1
40
47
75
Day
B
aCSF
Pep2m (1 µg/0.5µl)
Pep2m (10 µg/0.5µl)
EtOH (resp rate)
Time (min)
C
EtOH (reinforces / 1-h)
Pep2m (µg/0.5µl)
D
EtOH (headpokes / reinf)
Pep2m (µg/0.5µl)
E
aCSF
Pep2m (1 µg/0.5µl)
Pep2m (10 µg/0.5µl)
Sucrose (resp rate)
Time (min)
F
Sucrose (reinforces / 1-h)
Pep2m (µg/0.5µl)
G
Sucrose (headpokes / reinf)
Pep2m (µg/0.5µl)
H
Open-field locomotor test
I
EtOH group
Suc group
Ambulatory distance (cm)
Pep2m (µg/0.5µl)

Figure 3

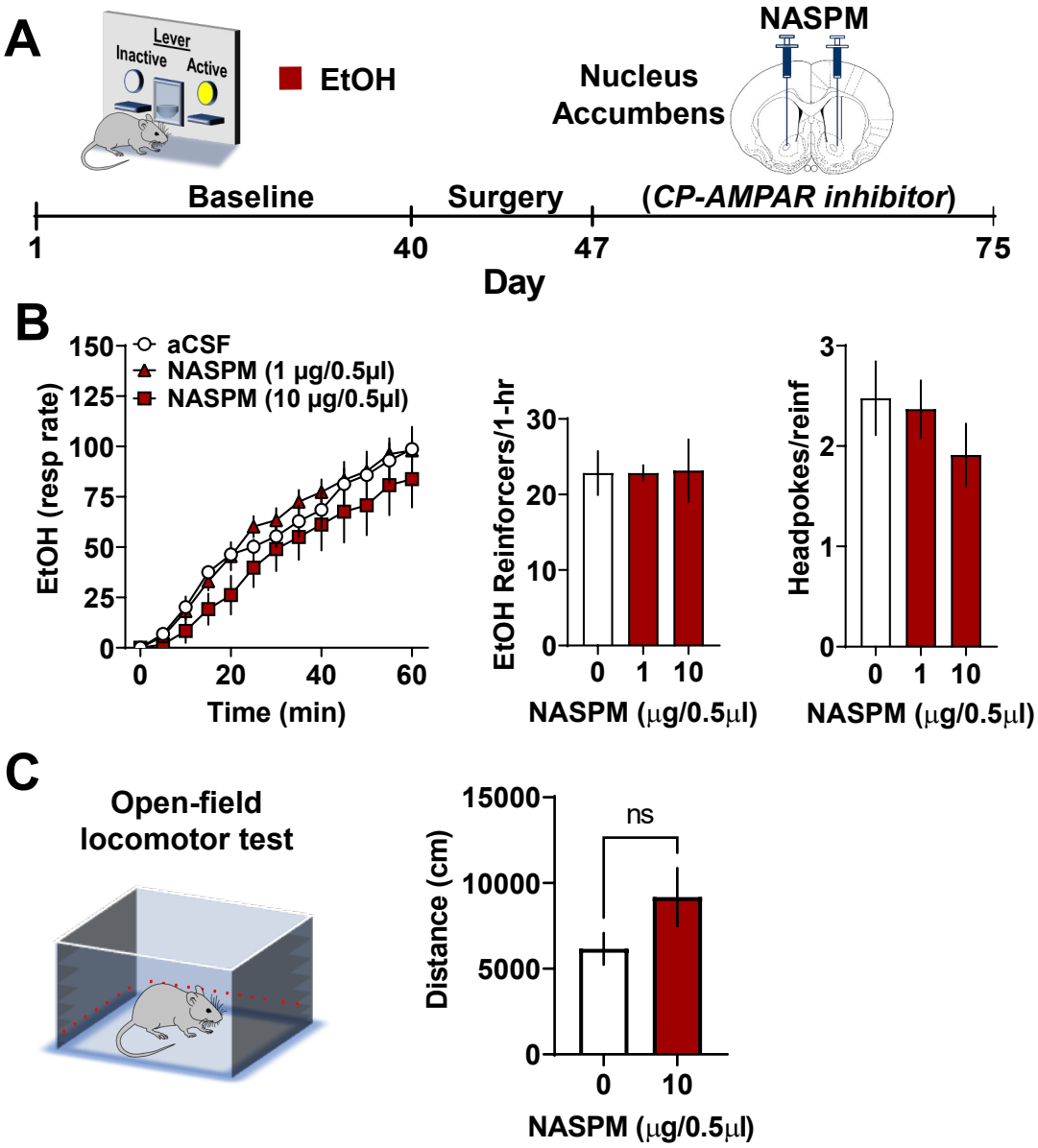
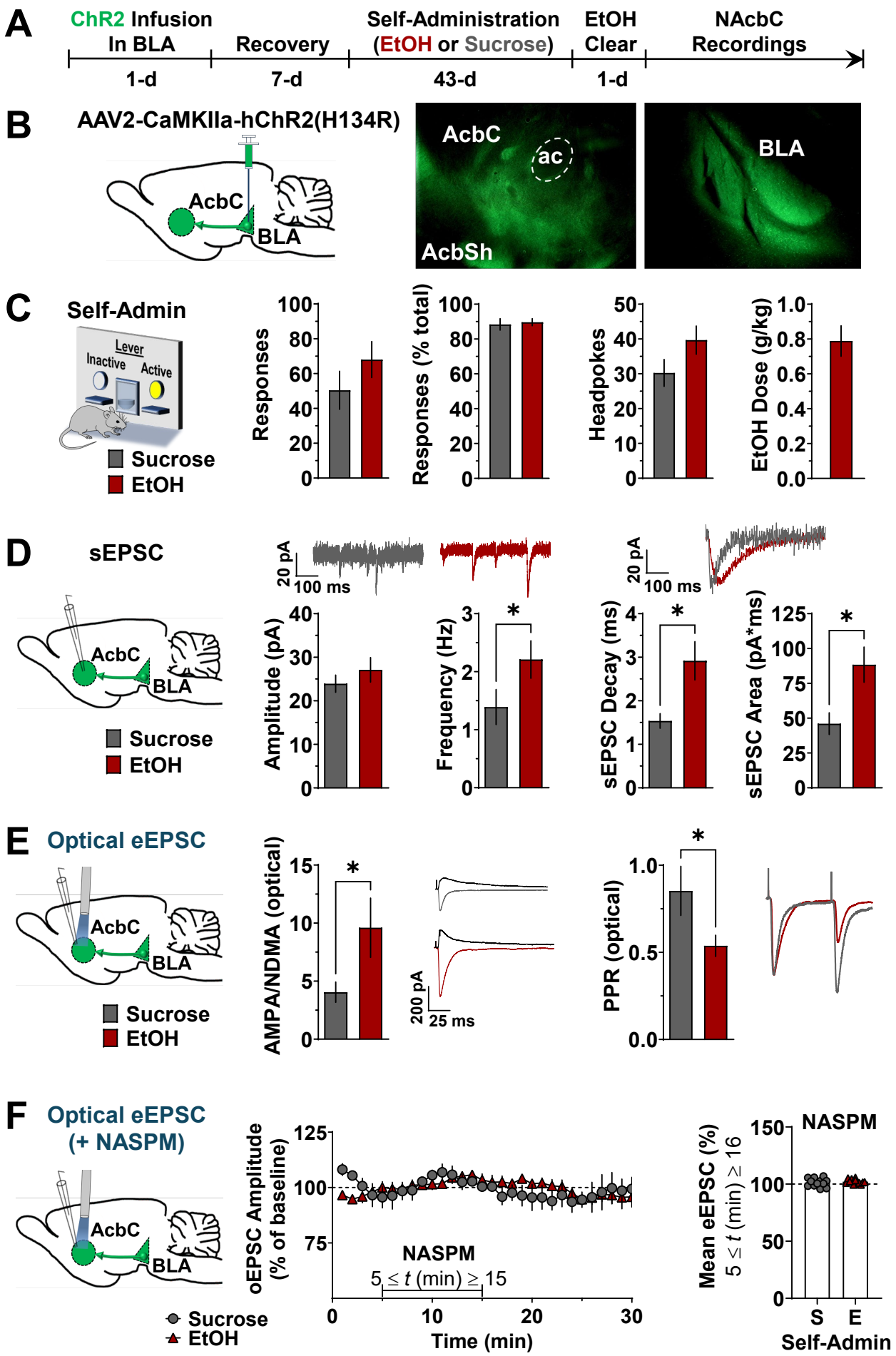

A
Lever
Inactive
Active
EtOH
NASPM
Nucleus
Accumbens
Baseline
Surgery
(CP-AMPAR inhibitor)
1
40
47
75
Day
B
aCSF
NASPM (1 µg/0.5µl)
NASPM (10 µg/0.5µl)
EtOH (resp rate)
Time (min)
EtOH Reinforcers/1-hr
NASPM (µg/0.5µl)
Headpokes/reinf
NASPM (µg/0.5µl)
C
Open-field
locomotor test
Distance (cm)
ns
NASPM (µg/0.5µl)

Figure 4


A
ChR2 Infusion In BLA
1-d
Recovery
7-d
Self-Administration (EtOH or Sucrose)
43-d
EtOH Clear
1-d
NAcbC Recordings
B
AAV2-CaMKIIa-hChR2(H134R)
AcbC
BLA
AcbC
ac
AcbSh
BLA
C
Self-Admin
Lever
Inactive
Active
Sucrose
EtOH
Responses
Responses (% total)
Headpokes
EtOH Dose (g/kg)
D
sEPSC
20 pA
100 ms
AcbC
BLA
Sucrose
EtOH
Amplitude (pA)
Frequency (Hz)
sEPSC Decay (ms)
sEPSC Area (pA*ms)
E
Optical eEPSC
AcbC
BLA
Sucrose
EtOH
AMPA/NDMA (optical)
200 pA
25 ms
PPR (optical)
F
Optical eEPSC (+ NASPM)
AcbC
BLA
Sucrose
EtOH
oEPSC Amplitude (% of baseline)
NASPM
5 ≤ t (min) ≥ 15
Time (min)
Mean eEPSC (%) 5 ≤ t (min) ≥ 16
NASPM
S
E
Self-Admin