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Authors

Seif, Taban
Chang, Shao-Ju
Simms, Jeffrey A
et al.

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Cortical activation of accumbens hyperpolarization-active NMDARs mediates aversion-resistant alcohol intake

Taban Seif[1], **Shao-Ju Chang**[1], **Jeffrey A Simms**[1], **Stuart L Gibb**[1], **Jahan Dadgar**[1], **Billy T Chen**[2], **Brandon K Harvey**[2], **Dorit Ron**[1,3], **Robert O Messing**[1,3,5], **Antonello Bonci**[2,3,4], and **F Woodward Hopf**[1,3]

[1]Ernest Gallo Clinic and Research Center, University of California at San Francisco, San Francisco, California, USA

[2]Intramural Research Program, National Institute on Drug Abuse, Baltimore, Maryland, USA

[3]Department of Neurology, University of California at San Francisco, San Francisco, California, USA

[4]Solomon H. Snyder Department of Neuroscience, The Johns Hopkins University, School of Medicine, Baltimore, Maryland, USA

Abstract

Compulsive drinking despite serious adverse medical, social and economic consequences is a characteristic of alcohol use disorders in humans. Although frontal cortical areas have been implicated in alcohol use disorders, little is known about the molecular mechanisms and pathways that sustain aversion-resistant intake. Here, we show that nucleus accumbens core (NAcore) NMDA-type glutamate receptors and medial prefrontal (mPFC) and insula glutamatergic inputs to the NAcore are necessary for aversion-resistant alcohol consumption in rats. Aversion-resistant intake was associated with a new type of NMDA receptor adaptation, in which hyperpolarization-active NMDA receptors were present at mPFC and insula but not amygdalar inputs in the NAcore. Accordingly, inhibition of Grin2c NMDA receptor subunits in the NAcore reduced aversion-resistant alcohol intake. None of these manipulations altered intake when alcohol was not paired with an aversive consequence. Our results identify a mechanism by which hyperpolarization-active NMDA receptors under mPFC- and insula-to-NAcore inputs sustain aversion-resistant alcohol intake.

Compulsive intake of alcoholic beverages despite negative economic, legal and physical consequences is a major obstacle to treatment of alcohol use disorders in humans[1–4]. To study aversion-resistant aspects of alcohol addiction, procedures have been developed whereby animals voluntary self-administer alcohol despite the presence of aversive stimuli, such as adulteration of alcohol with the bitter tastant quinine[4–6]. Although the compulsion to

Correspondence should be addressed to F.W.H. (woody@gallo.ucsf.edu) or A.B. (antonello.bonci@nih.gov).
[5]Present address: College of Pharmacy, University of Texas at Austin, Austin, Texas, USA

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drink excessively is a major clinical problem[1–4], very little is known about the neural or molecular underpinnings of this behavior.

Prefrontal cortical areas may mediate compulsive behavior in humans, as activity in these regions can correlate with craving and relapse[1,3,4,7] and encode conflict in rats and humans[8,9]. Several groups[2,3] have theorized that these cortical areas promote compulsive intake because of the presence of a conflict (that is, in the face of aversive consequences); this is in contrast to habitual intake, which is proposed to preferentially recruit striatal but not cortical areas in the absence of such challenges[2,3]. Thus, we predicted a priori that cortical areas would be more prominent in the regulation of aversion-resistant alcohol intake and would contribute much less to regulating alcohol intake not overtly paired with an aversive challenge (quinine or footshock). In rodents, the mPFC encodes aversiveness[10] and regulates anxiety and fear[8,11], in addition to regulating behavioral control, decision-making[8,9] and drug seeking[12–14] including aversion-resistant cocaine intake[15]. Aversive stimuli are also processed by the insula (INS), which encodes interoceptive cues that can promote addiction- and aversion-related behavior, including compulsive aspects of addiction[1–3,7,16,17].

To identify neural circuits that regulate alcohol intake despite aversive consequences, we focused on glutamatergic mPFC and INS inputs to the nucleus accumbens (NAc) core, which integrates information about motivational drives and adaptive behavior[9,12,15,17–21]. The NAcore also encodes aversiveness[10,21] and mediates some aversion-related behaviors[21,22] (but see ref. 23). The NAcore receives inputs from mPFC and INS[20], and glutamate release from mPFC-to-NAcore terminals promotes cocaine seeking[24]. In addition, repeated alcohol exposure produces a hyperglutamatergic state, including upregulation of NMDA receptor (NMDAR) function[4]. Also, NAc NMDARs contribute to operant alcohol self-administration[25], alcohol conditioned place preference[26] and the discriminative stimulus properties of alcohol[27].

Here, we examined the hypothesis that excitatory cortical inputs that activate NAcore NMDARs drive aversion-resistant alcohol consumption. We used an intermittent alcohol drinking model that produces aversion-resistant intake[6] and probed circuits and NMDARs using *in vitro* and *in vivo* optogenetics, pharmacology and RNA interference. Our findings suggest that excitatory mPFC and INS inputs onto Grin2c-containing NMDARs in the NAcore mediate aversion-resistant intake of alcohol.

Results

NAcore NMDARs promoted quinine-resistant alcohol intake

Pairing alcohol drinking with an aversive stimulus, the bitter tastant quinine, has been used to model aversion-resistant intake in rodents[4–6]. After 3–4 months of intermittent access to 20% alcohol, rats continue drinking despite adulteration of alcohol with quinine (10 or 30 mg l^{-1})[6]. We used this procedure to determine whether NAcore NMDARs mediate alcohol intake with or without quinine adulteration, using a within-subjects design. Rats were allowed ∼2.5 months of intermittent overnight access to alcohol, then ∼4 weeks of 20 min d^{-1}, 5 d $week^{-1}$ intake before beginning intake experiments. Histology examples for these and all other experiments are shown in Supplementary Figure 1.

Injecting the NMDAR blocker D(−)-2-amino-5-phosphonovaleric acid (AP5; 1 μg μl^{-1}, 0.5 μl per side) into the NAcore significantly reduced alcohol intake in the 20-min intake session when alcohol was adulterated with 30 mg l^{-1} quinine (Fig. 1a), with no effect on quinine-free alcohol consumption (n = 11; $F_{quinine}(1,10) = 9.803$, $P = 0.011$; $F_{AP5}(1,10) = 20.086$, $P = 0.001$; $F_{interaction}(1,10) = 5.682$, $P = 0.038$). The selective effect of AP5 on

quinine-adulterated alcohol intake was also observed during the first 30 min of an intermittent overnight drinking session in a separate set of rats (Fig. 1b; $n = 6$; $F_{quinine}(1,5) = 1.541$, $P = 0.270$; $F_{AP5}(1,5) = 18.257$, $P = 0.008$; $F_{interaction}(1,5) = 9.831$, $P = 0.026$). These results suggest that NAcore NMDARs mediated aversion-resistant alcohol intake.

Cortical-NAcore inputs promoted aversion-resistant intake

To identify glutamatergic inputs to the NAcore that regulate aversion-resistant alcohol intake, we used halorhodopsin (eNpHR3.0)[28–31] to selectively inhibit inputs from the mPFC or INS. We targeted a chronically implanted fiber-optic cable at the NAcore to activate halorhodopsin and reduce glutamate release. Laser stimulation to activate halorhodopsin in mPFC-to-NAcore inputs reduced intake of alcohol adulterated with 10 or 30 mg l^{-1} quinine but not of quinine-free alcohol (Fig. 2a; $n = 11$; $F_{quinine}(1,10) = 11.233$, $P = 0.007$; $F_{laser\text{-}stimulation}(1,10) = 10.864$, $P < 0.001$; $F_{interaction}(1,10) = 5.576$, $P = 0.012$). Laser stimulation did not alter intake in control rats expressing EYFP in mPFC-to-NAcore inputs (Fig. 2b; $n = 9$; $F_{quinine}(1,8) = 1.622$, $P = 0.228$; $F_{laser\text{-}stimulation}(1,8) = 0.266$, $P = 0.620$; $F_{interaction}(1,8) = 0.062$, $P = 0.940$). To compare drinking in rats with mPFC halorhodopsin versus EYFP, we measured the difference in intake between laser stimulation and no stimulation under six conditions: halorhodopsin or EYFP, each with 0, 10 or 30 mg l^{-1} quinine. Laser stimulation of halorhodopsin, but not EYFP, decreased alcohol intake in the presence of quinine and had no effect without quinine (Fig. 2c; $F_{quinine}(2,18) = 2.785$, $P = 0.075$; $F_{halorhodopsin/EYFP}(1,18) = 8.470$, $P = 0.009$; $F_{interaction}(2,18) = 3.727$, $P = 0.034$). In addition, inhibiting mPFC-to-NAcore inputs had no effect on saccharin intake with or without quinine (Fig. 2d; $n = 9$; $F_{quinine}(1,8) = 6.368$, $P = 0.036$; $F_{laser\text{-}stimulation}(1,8) = 0.230$, $P = 0.644$; $F_{interaction}(1,8) = 3.486$, $P = 0.099$), suggesting that halorhodopsin inhibition of quinine-adulterated alcohol intake did not reflect changes in taste reactivity.

Optogenetic inhibition of INS-to-NAcore terminals also reduced consumption of quinine-adulterated (30 mg l^{-1}) alcohol but not quinine-free alcohol (Fig. 2e; $n = 11$; $F_{quinine}(1,10) = 11.233$, $P = 0.007$; $F_{laser\ stimulation}(1,10) = 10.864$, $P < 0.001$; $F_{interaction}(1,10) = 5.576$, $P = 0.012$). Laser stimulation of EYFP in INS-to-NAcore terminals did not alter intake (Fig. 2f; $n = 8$; $F_{quinine}(1,7) = 0.866$, $P = 0.383$; $F_{laser\text{-}stimulation}(1,7) = 0.088$, $P = 0.775$; $F_{interaction}(1,7) = 0.018$, $P = 0.897$). We also analyzed the difference between intake with and without laser stimulation. Laser stimulation of halorhodopsin but not EYFP decreased alcohol intake in the presence of quinine, with no effect in the absence of quinine (Fig. 2g; $F_{quinine}(1,17) = 5.026$, $P = 0.039$; $F_{halorhodopsin/EYFP}(1,17) = 4.949$, $P = 0.040$; $F_{interaction}(1,17) = 4.478$, $P = 0.049$). Halorhodopsin activation reduced glutamate release from INS- and mPFC-to-NAcore terminals *in vitro* (Supplementary Fig. 2), as seen in studies of mPFC[32] and other terminals[29,30]. Thus, both mPFC and INS inputs to the NAcore promote aversion-resistant alcohol intake. Simultaneous stimulation of halorhodopsin in both INS- and mPFC-to-NAcore terminals also inhibited quinine-adulterated alcohol intake (Supplementary Fig. 3).

To investigate whether cortical inputs to the NAcore promote alcohol consumption paired with a different aversive stimulus, we examined footshock–resistant intake. After ∼2.5 months of two-bottle choice and 7–8 weeks of operant training, rats received intermittent footshocks during daily operant sessions. On the seventh or eighth footshock-paired session, we examined whether halorhodopsin inhibition of mPFC- or INS-to-NAcore terminals would reduce operant responding.

Inhibition of mPFC-to-NAcore inputs significantly reduced footshock-resistant responding for alcohol, with no effect during the baseline pre-shock period before the footshock-paired sessions began ($n = 10$; Fig. 3a; $F_{pre\text{-}shock/shock}(1,9) = 11.941$, $P = 0.007$; $F_{laser\text{-}stimulation}(1,9) = 9.815$, $P = 0.012$, $F_{interaction}(1,9) = 5.849$, $P = 0.039$), and reduced

alcohol intake during footshock but not pre-shock sessions (Fig. 3b; $F_{\text{pre-shock/shock}}(1,9) = 10.078$, $P = 0.011$; $F_{\text{laser-stimulation}}(1,9) = 16.975$, $P = 0.003$, $F_{\text{interaction}}(1,9) = 6.774$, $P = 0.029$). Inhibiting INS-to-NAcore terminals also reduced footshock-resistant responding but not baseline responding ($n = 10$; Fig. 3c; $F_{\text{pre-shock/shock}}(1,9) = 5.668$, $P = 0.041$; $F_{\text{laser-stimulation}}(1,9) = 6.701$, $P = 0.029$, $F_{\text{interaction}}(1,9) = 10.027$, $P = 0.011$), with a similar pattern—effect on footshock-resistant but not basal responding—for alcohol intake (Fig. 3d; $F_{\text{pre-shock/shock}}(1,9) = 5.735$, $P = 0.040$; $F_{\text{laser-stimulation}}(1,9) = 7.444$, $P = 0.023$, $F_{\text{interaction}}(1,9) = 9.645$, $P = 0.013$). We observed individual differences in responding under footshock: some rats responded near baseline, while others greatly reduced responding, and halorhodopsin reduced footshock-resistant intake in the shock-resistant rats (Supplementary Fig. 4). Also, when examined across all mPFC-injected rats, inhibiting mPFC-to-NAcore inputs produced a small but significant reduction in baseline responding (no halorhodopsin, 83.9 ± 12.8 presses; halorhodopsin, 67.0 ± 9.7 presses; $n = 17$, $t = 3.176$, $P = 0.006$, paired t-test), which was not observed for INS-to-NAcore inputs (no halorhodopsin, 78.9 ± 12.2 presses; halorhodopsin, 76.1 ± 9.2 presses; $n = 12$, $t = 0.251$, $P = 0.807$, paired t-test). Thus, mPFC-and INS-to-NAcore inputs regulated alcohol intake paired with aversive stimuli involving different sensory modalities (somatosensory versus gustatory) and different manners of intake (operant versus two-bottle choice drinking).

Hyperpolarization-active NAcore NMDARs in alcohol drinkers

Because NAcore NMDARs mediated aversion-resistant alcohol intake (Fig. 1) and repeated alcohol exposure can upregulate NMDARs[4], we hypothesized that NMDAR function in the NAcore was elevated in rats with aversion-resistant intake[6]. We thus activated NMDARs using channelrhodopsin (ChR2) expressed in mPFC-to-NAcore inputs, with 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM) to block AMPA-type glutamate receptors. As expected, we were able to evoke NMDAR currents at potentials greater than 0 mV (ref. 33). In addition, however, we detected excitatory currents in neurons from alcohol-drinking rats at hyperpolarized potentials (Fig. 4a, $n = 11$ naive, 12 alcohol), where NMDARs are normally blocked by intracellular Mg^{2+} (ref. 33). AP5 (50 μM) inhibited currents evoked at −50 mV (Fig. 4b, 92.5 ± 0.5% inhibition, $n = 5$, paired t-test $t = 14.35$, $P < 0.001$), indicating that the currents were mediated by NMDARs. We next compared the magnitude of mPFC-evoked currents at −50 mV and +40 mV. At the hyperpolarized potential, NMDAR currents were significantly greater in alcohol-drinking rats than in alcohol-naive, age-matched control rats; in contrast, evoked currents at +40 mV were not different between control and alcohol-drinking rats ($n = 11$ each naive and alcohol; Fig. 4c,d; $F_{\text{holding-potential}}(1,20) = 253.61$, $P < 0.001$; $F_{\text{alcohol/naive}}(1,20) = 0.327$, $P = 0.574$; $F_{\text{interaction}}(1,20) = 6.664$, $P = 0.018$). Thus, alcohol intake was associated with the appearance of NMDARs active at hyperpolarized potentials (HA-NMDARs) under mPFC-to-NAcore synaptic terminals, without changes in input resistance or cell capacitance (Supplementary Table 1).

To use an independent method to look for HA-NMDARs, we measured whether AP5 could reduce excitatory postsynaptic currents (EPSCs) generated at −70 mV in the absence of DNQX. We predicted that AP5 would have no effect in control neurons because NMDARs are largely blocked by intracellular Mg^{2+} at −70 mV, and the AMPA receptor contribution to the EPSC predominates[33]. AP5 (50 μM) had no effect on mPFC-evoked EPSCs at −70 mV in control rats, but significantly reduced these currents in alcohol-drinking rats (Fig. 5a,b; $n = 5$ alcohol, 5 naive; $t = 2.418$, $P = 0.042$), confirming that NMDARs were active at hyperpolarized membrane potentials under mPFC-to-NAcore inputs in alcohol-drinking rats (Fig. 4).

We next examined whether HA-NMDARs were present in other excitatory inputs to the NAcore. When ChR2 was expressed in INS-to-NAcore terminals, AP5 significantly reduced

light-evoked EPSCs at −70 mV in alcohol-drinking but not naive control rats (Fig. 5c; $n = 8$ alcohol, 9 naive; $t = 2.466$, $P = 0.026$). In contrast, AP5 did not alter light-evoked EPSCs when ChR2 was expressed in terminals from basolateral amygdala (BLA) neurons (Fig. 5d; $n = 5$ alcohol, 5 naive; $t = 0.499$, $P = 0.631$). AP5 also had no effect on EPSCs evoked by field electrical stimulation, which activates all glutamatergic synapses[18,20] (Fig. 5e; $n = 6$ alcohol, 7 naive; $t = 0.791$, $P = 0.445$). Thus, alcohol-drinking rats showed HA-NMDARs under glutamatergic inputs from the mPFC and INS but not BLA.

Having more HA-NMDARs (Figs. 4 and 5a–c) might enhance action potential generation, as HA-NMDARs increase firing in the reticular thalamus despite the small magnitude of currents[34]. Thus, we stimulated ChR2 in mPFC-to-NAcore terminals at 20 Hz (ten pulses) to evoke EPSP trains every 10 s. Neurons were depolarized to approximately −55 mV to allow EPSP-evoked firing. AP5 significantly reduced action potential generation by mPFC-ChR2-evoked EPSP trains in NAcore neurons from alcohol-drinking but not naive rats (Fig. 6; $n = 7$ alcohol, 8 naive; $F_{\text{alcohol/naive}}(1,13) = 0.991$, $P = 0.338$; $F_{\text{pre/post-AP5}}(1,13) = 45.529$, $P < 0.001$; $F_{\text{interaction}}(1,13) = 18.498$, $P < 0.001$). Thus, HA-NMDARs can promote firing in the NAcore of alcohol-drinking rats.

Grin2c NMDARs mediated aversion-resistant alcohol intake

HA-NMDARs might reflect the presence of Grin2c, Grin2d or Grin3 subunits (also called NR2C, NR2D and NR3), all of which show reduced Mg^{2+} inhibition and thus are active at hyperpolarized potentials[33–35]. Because NMDARs containing Grin1 and Grin3 are not blocked by AP5 (ref. 35), we generated short hairpin RNAs targeting *Grin2c* and *Grin2d* subunits (Supplementary Fig. 5) and used AAVs to infect NAcore cells with one of these shRNAs or a control shRNA not targeting any known coding sequence in the genome[36]. Expression of *Grin2c* shRNA but not control or *Grin2d* shRNA in NAcore neurons prevented AP5 from reducing mPFC-evoked EPSCs at −70 mV in neurons from alcohol-drinking rats (Fig. 7a; $n = 6$ control and Grin2d, 7 Grin2c; one-way ANOVA, $F(2,16) = 5.504$, $P = 0.015$), suggesting a role for Grin2c subunits. There are also considerable amounts of Grin2b in the NAcore[37], although these subunits are relatively inactive at hyperpolarized resting potentials[33]. As expected, the Grin2b inhibitor ifenprodil (2 μM) had no effect on mPFC-evoked EPSCs in alcohol-drinking rats (Fig. 7b; $n = 5$; $t = 3.840$, $P = 0.005$, effect of ifenprodil versus AP5 in Fig. 5b). Thus, Grin2c subunits rather than Grin2d or Grin2b mediated HA-NMDARs under mPFC-to-NAcore inputs in alcohol-drinking rats.

We next used the NMDAR shRNAs to determine whether NAcore HA-NMDARs promote aversion-resistant alcohol intake. Only *Grin2c* shRNA significantly reduced intake of quinine-adulterated alcohol relative to quinine-free alcohol (Fig. 7c; $n = 7$ control, 6 Grin2c, 8 Grin2d; $F_{\text{quinine}}(1,18) = 23.351$, $P < 0.001$; $F_{\text{shRNA}}(2,18) = 0.621$, $P = 0.548$; $F_{\text{interaction}}(2,18) = 17.698$, $P < 0.001$). In addition, intra- NAcore injection of the Grin2b inhibitor ifenprodil (1 μg μl^{-1}, 0.5 μl per side, which reduces operant intake in dorsal striatum but not NAc[38]) did not alter consumption of quinine-adulterated alcohol (Fig. 7d, $n = 11$; $F_{\text{quinine}}(1,10) = 1.184$, $P = 0.302$; $F_{\text{ifenprodil}}(1,10) = 0.004$, $P = 0.952$; $F_{\text{interaction}}(1,10) = 0.054$, $P = 0.821$). Thus, Grin2c but not Grin2d or Grin2b in the NAcore mediated quinine-resistant alcohol intake.

Discussion

Here, we found that NMDARs under inputs to the NAcore from the INS and mPFC mediated aversion-resistant alcohol intake. Both mPFC- and INS-to-NAcore inputs promoted quinine-resistant two-bottle-choice alcohol intake, as well as footshock-resistant operant alcohol intake, with little effect on intake not paired with a specific aversive consequence (quinine or footshock). In addition, alcohol-drinking rats showed an

unexpected NMDAR adaptation in the NAcore, with NMDARs active at hyperpolarized membrane potentials under INS and mPFC but not BLA inputs. HA-NMDARs in alcohol-drinking rats promoted action potential firing. RNA interference studies demonstrated that Grin2c subunits contributed to HA-NMDARs and mediated quinine-resistant alcohol intake. Thus, our studies reveal an alcohol-related adaptation in NAcore NMDARs, whereby the appearance of Grin2c subunits under several cortical inputs promotes aversion-resistant alcohol intake.

Several studies implicate NAc NMDARs in alcohol reward[25–27], but we found that blocking NAcore NMDARs with AP5 did not reduce quinine-free alcohol intake. There are several possible explanations for our seemingly paradoxical result. A noncompetitive NMDAR blocker substitutes for the discriminative stimulus properties of alcohol, whereas a competitive NMDAR blocker does not[27], and AP5 is a competitive NMDAR antagonist[33]. Furthermore, AP5 alters alcohol place preference when infused simultaneously into both the NAcore and NAc shell[26], but it can have divergent effects when administered separately into these two subregions[19,39,40]. In addition, AP5 concentrations that reduce operant alcohol self-administration[26] are higher (3–6 μg) than those used here and can suppress locomotor activity[39–41]. Given these caveats, we conclude that NAcore NMDARs supported quinine-resistant but not quinine-free alcohol intake.

Little is known about the molecular and circuit mechanisms that mediate compulsive, aversion-resistant alcohol intake. Here we used optogenetic inhibition of specific projections to demonstrate that both mPFC- and INS-to-NAcore inputs promote aversion-resistant intake with aversive stimuli of different sensory modalities (somatosensory versus gustatory) and different manners of intake (operant versus two-bottle-choice drinking). Thus, our results suggest that a common neural circuit involving mPFC and INS inputs to the NAcore is a critical mechanism that promotes aversion-resistant intake and that this circuit is related more to the choice to self-administer despite aversive consequences rather than to sensory or motivational aspects of each aversive consequence. In addition, this circuit had little impact on quinine- or footshock-free alcohol intake. In this regard, cortical regions have been proposed to predominate in the aversion-resistant, compulsive aspects of human addiction, where intake is accompanied by conflict or challenge[1–3,7]; in contrast, intake in the absence of conflict is considered to use more habit-based striatal systems, with much less of a role for cortical areas[2,3]. Ours is, to our knowledge, the first direct evidence supporting the hypothesis that cortical regions can act more prominently in aversion-resistant intake.

The mPFC mediates decision making and conflict processing[8,9] and promotes expression of fear and aversion[8,10,11]. In this case, inhibiting mPFC-to-NAcore inputs did not alter consumption of quinine-free alcohol, saccharin or saccharin + quinine, suggesting that reduced intake of quinine-adulterated alcohol was not secondary to locomotor effects or changes in taste reactivity. However, the taste of alcohol + quinine may differ from these other substances, and effects on taste reactivity cannot be completely ruled out. Also, the broader mPFC role in behavioral control may explain the small decrease in basal operant responding after inhibiting mPFC-to-NAcore inputs. In addition, inhibiting mPFC cell bodies with halorhodopsin enhances compulsive cocaine seeking, whereas activating the mPFC with ChR2 reduces compulsive cocaine seeking[15], perhaps suggesting differential regulation of aversion-resistant alcohol and cocaine intake by the mPFC. Our results also implicate the INS, a regulator of interoceptive stimuli[2,16] and aversive and taste stimuli[2,16,17,42] that can drive addictive behaviors[2,16]. The INS is activated by alcohol cues in alcoholics[1,7,17], which could promote compulsive aspects of intake[1–3,8]. However, ours is, to our knowledge, the first study directly implicating the INS in aversion-resistant alcohol intake. The NAc also receives inputs from the hippocampus and amygdala, which can

regulate many goal-directed behaviors, including alcohol intake, as does the NAc shell[1,15,16,43–46]. Future experiments will be able to examine the contribution of subcortical NAc inputs and the NAc shell to aversion-resistant intake.

One possible concern with optogenetics is a nonspecific effect during prolonged laser stimulation. Other groups use longer laser exposure without adverse side effects[29,30,32], with some observing slower recovery after halorhodopsin activation[29,32] (Supplementary Fig. 2). Here, laser stimulation did not change alcohol drinking in control EYFP-infected rats and did not reduce quinine- or footshock-free alcohol intake or saccharin ± quinine consumption, suggesting that halorhodopsin inhibition of aversion-resistant alcohol intake did not reflect nonspecific motor or motivational effects.

Alcohol exposure can induce a hyperglutamatergic state, including increased NMDAR function[4]. In this study, alcohol-drinking rats showed NMDARs active at hyperpolarized potentials under mPFC-and INS- but not BLA-to-NAcore inputs, which might reflect activity of Grin2c, Grin2d or Grin3 subunits[33–35]. Grin2c but not Grin2d or Grin2b subunits mediated both mPFC-evoked HA-NMDAR currents *in vitro* and quinine-resistant alcohol intake. Little is otherwise known about the behavioral functions of Grin2c and Grin2d, although alcohol intake decreases *Grin2c* striatal mRNA[47] and Grin2d is implicated in acute drug responses[48] and spatial memory[49]. Adult striatum has very little Grin2c and Grin2d[50], and these subunits have not been detectable in total brain tissue (data not shown). This would be predicted if only a small subset of glutamatergic terminals contained the alcohol-related adaptation. HA-NMDARs were not observed under BLA-to-NAcore inputs or in EPSCs evoked with field electrical stimulation, which activates all glutamate inputs onto a NAcore neuron[20], and thus mPFC and INS synapses may represent a minority of glutamatergic synapses impinging on NAcore neurons. Although the magnitude of HA-NMDAR currents was relatively small, NAcore HA-NMDARs regulated action potential firing evoked by ChR2 stimulation of mPFC-to-NAcore inputs in alcohol-drinking but not naive rats. Together, these results support the possibility that HA-NMDARs regulate physiological activity in the NAcore in alcohol-drinking rats.

In conclusion, mPFC- and INS-to-NAcore inputs and NAcore HA-NMDARs all supported aversion-resistant alcohol intake, with little contribution to alcohol intake not paired with the aversive stimuli footshock or quinine. Our results are the first experimental demonstration of the proposed[2,3] critical role for frontal cortical regions in regulating aversion-resistant intake. mPFC- and INS-to-NAcore inputs promote both quinine- and footshock-resistant intake. In addition, Grin2c subunits mediated a newly identified HA-NMDAR neuroadaptation under cortical inputs to the NAcore in alcohol-drinking rats, and this NMDAR change promoted aversion-resistant intake, the first identified behavioral role for Grin2c. Thus, NMDAR blockers or inhibitory allosteric modulators might decrease compulsive aspects of alcohol use disorders[4].

Online Methods

Animal handling

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted by the NIH, and approval of an Institutional Animal Care and Use Committee of the Ernest Gallo Clinic and Research Center. Adult male Wistar rats (∼450–550 g, ∼5–6 months) at the time of the surgery were individually housed and maintained on a 12-h light cycle with *ad libitum* access to food and water except for experiments in the operant chamber. Experiments were performed in the light cycle.

Two-bottle choice alcohol intake

Rats were trained to voluntarily consume alcohol using a two-bottle, home-cage intermittent access to 20% alcohol procedure (IAA)[51]. Rats had 24 h of continuous access to two bottles, one containing alcohol (20% vol/vol with water) and the other water, each Monday, Wednesday and Friday evening. The amount of intake was calculated by bottle weight. For some *in vitro* experiments, patch-clamp in NAcore neurons was performed after 3–4 months IAA (Fig. 5). For other *in vitro* experiments (Figs. 4 and 7) and all *in vivo* experiments, rats were allowed ∼2.5 months of overnight IAA intake, then were switched to two-bottle choice access to alcohol or water for 20 min d^{-1}, 5 d $week^{-1}$ for at least 4 weeks. For two-bottle choice halorhodopsin or EYFP experiments, rats were then switched to drink alcohol for 2 min d^{-1}, 5 d $week^{-1}$ in an optogenetic chamber (an operant chamber modified to allow two-bottle choice intake). Intakes of alcohol in 2 min and 20 min were similar (for example, compare Figs. 1a and 7c with Fig. 2a,e), indicating that most alcohol was consumed in the first 2 min, representing what has been previously described as the initial “loading-up” period[52]. Rats had at least 2 weeks of daily intake with dummy cables attached to their chronically implanted fiber optic cables (see below for details) before beginning intake experiments. On each experimental day, rats were placed in the optogenetic chamber with dummy cables attached for 5 min before we provided the bottles. Blood ethanol concentrations at the end of a 20 min two-bottle intake session (determined as in ref. 51) were 42.3 ± 10.7 mg/dl for $n = 16$ rats that drank 1.01 ± 0.10 g alcohol per kilogram body weight, in agreement with a previous study of drinking in intermittent-access rats[51]. Although this does not reach what is considered binge drinking as defined by BAC in humans, intake under the intermittent access model used here is associated with several factors considered to model aspects of human alcohol use disorders, including escalation of intake[51], sensitivity to compounds that reduce intake in human alcoholics[51] and moderate signs of dependence[53], as well as continued intake despite pairing with aversive consequences.

Alcohol intake with and without quinine

Rats first had 2–3 d of habituation to alcohol + quinine. Alcohol intake was tested using a within-rat design for drinking alcohol ± quinine and with treatment versus control (for example, for AP5 experiments, the four groups were AP5 + quinine, AP5 + no quinine, saline + quinine, saline + no quinine). The different conditions were randomized across and within rats using a Latin-square design. Rats were given at least 1 d of drinking alcohol without quinine between each treatment day. In addition, for all experiments except AP5 and ifenprodil, rats were subject to each of the four experimental conditions twice. The two sessions were then averaged, giving a single intake value for each rat for each of the four experimental conditions.

For shRNA experiments, we could not examine alcohol intake with and without shRNA in the same randomized way. Thus, alcohol intakes with or without quinine were each averaged from two intake sessions ∼6–7 weeks after shRNA infection.

Saccharin intake with and without quinine

Two-bottle choice saccharin ± quinine intake was measured using methods similar to those described for alcohol intake with and without quinine, except examining drinking of 300 mg l^{-1} saccharin or 1,300 mg l^{-1} saccharin plus 30 mg l^{-1} quinine. Rats for these studies were previously used to examine mPFC-to-NAcore inhibition and two-bottle-choice alcohol intake. Preliminary experiments identified two concentrations that gave relatively low volumes of intake that were relatively similar to the alcohol volume consumed. Rats were then allowed to drink one of these two solutions in the late morning, 20 min d^{-1}, 5 d $week^{-1}$, under two-bottle choice conditions. The other bottle contained only water. Rats also drank

the usual 20% alcohol under two-bottle choice in the late afternoon each day, 20 min d^{-1}, 5 d $week^{-1}$. After 1–2 weeks of saccharin ± quinine intake, rats began drinking the saccharin for 8 min d^{-1}, 5 d $week^{-1}$ in the optogenetic chamber each late morning. Laser stimulation experiments began after 1–2 weeks habituation to having dummy cables attached. On each day, rats were placed in the optogenetic chamber for 5 min before we provided the bottles. Exposure to the two saccharin solutions, with or without laser stimulation in the NAcore, was randomized using a within-rat, Latin-squares design. Unlike for alcohol, rats did not show a rapid load-up period of intake of saccharin ± quinine; thus, we allowed rats 8 min for intake during saccharin optogenetic experiments.

One concern is that laser stimulation of halorhodopsin could have nonspecific locomotor effects, but that giving rats 8 min to drink saccharin ± quinine but 2 min to drink alcohol might allow rats to overcome the locomotor deficit in 8 min. This would give a false negative result for saccharin ± quinine intake. However, we believe this not to be the case. First, halorhodopsin stimulation did not alter intake of quinine-free alcohol with 2 min access, suggesting no locomotor effects *per se*. Second, rats were allowed 20 min to drink alcohol in the intra-NAcore AP5 and shRNA experiments and operant footshock experiments. In these cases, manipulations had no effect on quinine- or footshock-free alcohol intake, even though there would have plenty of time to overcome locomotor deficits.

Operant self-administration methods

After ∼2.5 months of overnight IAA intake, rats were trained to operantly respond for alcohol[54]. After two overnight sessions, rats were switched to respond for alcohol 20 min d^{-1}, 5 d $week^{-1}$, all under a fixed ratio 3 (FR3) schedule. Operant sessions were initiated by presentation of a tone and light (which were also paired with each FR3 response) and raising of the dipper containing 100 μl alcohol; licking the dipper resulted in lowering the dipper and extension of two levers. Thereafter, each FR3 response on the active lever resulted in raising the alcohol-containing dipper for 5 s and presenting the light/tone cue for the first 2 s access to alcohol. Surgery was performed after 2 weeks of operant training. After a further 7–8 weeks, rats were tested for the impact of halorhodopsin activation on baseline operant responding for alcohol. We then introduced daily intermittent footshock, where 1 in 8 FR3 responses paired with a footshock (0.25 mA, 0.5 s) just before raising the dipper containing alcohol.

We observed shock-resistant and shock-sensitive subpopulations of rats, as observed in some studies[55,56] of aversion-resistant cocaine intake. We should note that aversive challenges of different qualities could have different impact on alcohol intake, for example, where the majority of rats tolerated quinine while only ∼50% tolerated footshock. In fact, studies of cocaine self-administration have found that all rats will give up responding for cocaine when the shock intensity is high enough[57]. In addition, compulsive-like cocaine self-administration is observed in a subset of rats responding for cocaine with footshock[55,56] but is seen all rats responding for cocaine when paired with a cue predicting footshock[58]. Thus, rats may differ in the threshold of adversity that would be tolerated to receive a given reward.

In vivo optogenetics

Surgery was performed 6–8 weeks before onset of experiments. Rats were infected in the mPFC (AP +3.20, ML ±1.20, DV −3.00, 10 degrees tilt) or INS (AP +2.80, ML ±4.80 DV −4.70) with 0.6 μl per side of an adeno-associated virus (AAV) encoding halorhodopsin (eNpHR3.0), a chloride pump that silences neurons when activated by a 532-nm green laser. Halorhodopsin was under control of a *Camk2a* promoter and tethered to EYFP to allow visualization for histology. Control rats were infected with an AAV encoding EYFP alone

under control of a *Camk2a* promoter. During the same surgery, rats were implanted bilaterally with chronically implanted fiber-optic ferrules (CFOs) targeting the medial NAcore (AP +2.20, ML ±2.70, DV −7.18, 8 degrees tilt). These were custom made by inserting a 200 μm optical fiber into a ferrule (a hollow metal cylinder 12 mm long, 2 mm OD, 260 μm ID, Fiber Optics for Sale Co., part F1-0064F-25) and gluing it, so that the fiber extended beyond the ferrule ∼7.5 mm to reach the NAcore. For some INS-virally-injection rats, viral infection spread into the adjacent somatosensory or orbito-frontal cortices which do not project to the medial NAcore[59]; however, the majority did not exhibit such spread. ChR2 distribution in terminals (Supplementary Fig. 1c,d) was consistent with that reported for mPFC and INS[59].

During *in vivo* optogenetic experiments, a 532 nm laser light (8–12 mW) was turned on during the entire intake session (2 min for two-bottle choice alcohol, 8 min for saccharin, 20 min for operant). The laser was attached by a single cable to a rotating uni-to-bilateral optical commutator (Doric Lenses, custom-made part FRJ-ID(50:50)_FC-sma(2)_v01); light then emerged into two fiber-optic cables with mini-SMA plugs (custom made by Doric) which attached to the bottom of the commutator. At the other end of each cable, the fiber-optic was mounted through a metal ferrule to which a zirconium sleeve (Fiber Instrument Sales, part F18300SSC25) was attached. This was then attached to the CFO in the rat's head during behavioral experiments.

In vitro optogenetics

Surgery was performed 6–8 weeks before onset of experiments. Rats were infected in the mPFC, INS, or BLA (AP: −2.80, ML: ±5.55, DV: −8.70, 10 degrees tilt) with 0.6 μl per side of an AAV encoding ChR2, a cation channel that excites neurons when activated by blue light. ChR2 was under control of a *Camk2a* promoter and tethered to a yellow fluorescent protein (EYFP) to allow visualization for histology. To generate ChR2-evoked EPSCs *in vitro*, we adjusted the LED intensity (Prizmatrix, 480 nm, 5ms) to get an EPSC of 130–160 pA at −70 mV or the maximum current that could be generated.

The reversal potential (E_{Rev}) for NMDARs in Figure 4c was somewhat positive to 0 mV without junction null correction, which would shift the E_{Rev} ∼10–15 mV hyperpolarized (based on the junction null calculator in Clampex), in line with observations in the adult mouse NAc shell. We should note that the NMDAR currents in these cells are small compared to the background noise except at +40 mV, making accurate measurement of E_{Rev} somewhat challenging.

For histology, the anterior cortex or BLA was sliced in 500 μm sections, and these living slices were placed into 4% paraformaldehyde just after cutting for assessment of viral injection placement.

shRnAs

Sequences for shRNA were identified using the iRNAi program (www.mekentosj.com). Identified sequences were additionally analyzed using BLAST to ensure subunit specificity. The shRNA targeting sequence for *Grin2c* was AACCCCCTACACCAAGCTCTGTT and for *Grin2d* was AAACGCCCTGGTGGCTCTACCTT. Assessment of shRNA knockdown of Grin2c or Grin2d was performed using plasmids for rat *Grin2c* or *Grin2d* receptors (a kind gift from Dr. John Woodward) (Supplementary Fig. 5), which were transfected into HEK293 cells along with the *Grin2c*, *Grin2d* or control shRNA, using described methods[60]. 24 h later, protein levels were analyzed with western blot[60] (polyclonal anti-Grin2c, sc-1470 Santa Cruz, 1:2,000; polyclonal anti-Grin2d, sc-1471 Santa Cruz, 1:2000, anti-GAPDH, sc-25778 Santa Cruz, 1:50,000) The NMDAR subunit shRNAs were cloned into a pAAV

modified to have multiple cloning sites, and with a U6 promoter to drive the shRNA expression and CMV to drive GFP expression. Infected neurons for *in vitro* electrophysiology could be identified by co-expression of EYFP from the same AAV vector containing the shRNA.

Surgery

Bilateral implantation of cannulas and fiber optic implants targeting the NAcore and other surgical details were as described[54]. The coordinates used for cannulas and fiber optic implants were previously used[54] to show that injection of potassium channel modulators within the NAcore but not NAc shell altered alcohol intake; thus, these coordinates have been validated as targeting the NAcore. For viral infection, injection occurred over 10 min followed by an additional 10 min to allow diffusion of viral particles away from the injection site.

Patch-clamp electrophysiology

Slice preparation and electrophysiology methods were generally as described[54], except for *in vitro* optogenetics, for which the methods are described above. All experiments were performed using whole-cell recording and visualized using infrared-DIC with 3.0 to 4.5 MW electrodes. The internal solution for EPSCs contained (in millimolar): 120 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA chloride, 2.5 Mg-ATP and 0.25 Na-GTP, pH 7.2 to 7.3, 270 to 285 mOsm. Electrical signals were recorded using Clampex 9.2 or 10.1 and an Axon 700A or 700B patch amplifier (Axon Instruments, Foster City, CA). During recordings, EPSCs were filtered at 2 kHz, digitized at 10 kHz and collected online using Igor Pro software (Wavemetrics, Lake Oswego, OR). Series resistance (10–30 MΩ) and input resistance were monitored on-line with a 4-mV depolarizing step (50 ms) given after every EPSC. Electrically evoked currents were elicited using a bipolar stimulating electrode placed dorsal to the anterior commissure.

For *in vitro* experiments examining changes in EPSCs at −70 mV caused by NMDAR blockers, we first normalized the baseline EPSC values during the 4 min before drug exposure to 100%, then calculated the percent change in EPSC relative to baseline during the last 3 min of blocker exposure. We then compared blocker-induced changes in EPSCs between neurons from alcohol-drinking and control rats. For firing experiments, EPSPs were evoked by a train of 10 laser pulses at 20 Hz to excite ChR2. Neurons were brought to ∼ −55–60 mV with the patch amplifier to allow EPSPs to evoke action potentials. Measurements of eNpHR3.0 inhibition *in vitro* in Supplementary Figure 2 used sagittal slices which may enrich for prefrontal inputs to the NAcore.

Statistics

Unless otherwise indicated, results were analyzed with SigmaStat 3.1 using two-way repeated-measures ANOVA with a Tukey *post hoc* test for multiple comparisons, since the majority of results involve within-rat measurements of intake with and without aversive consequences. All *t*-tests were unpaired unless otherwise indicated and were two-sided. All data conform to the requirements for each statistical test (for example, normal distribution), and the variances were not different between groups that were compared statistically. Sample sizes were taken from similar previous studies where statistical differences in alcohol intake or *in vitro* parameters after alcohol intake were observed[54]. For behavior experiments, *n* values represent number of individual rats. For *in vitro* experiments, *n* values represent number of individual cells, with no more than two cells for a given condition from a particular rat. All data in manuscript and figures are shown as mean ± s.e.m.

For *in vitro* and *in vivo* experiments, rats were randomized before surgery so that the mean and s.e.m. of alcohol intake was approximately equal in each group (for example, see Supplementary Fig. 5d). Rats with intake less than 0.2 g alcohol kg^{-1} under two-bottle choice were not included for analysis. Researchers were not blind to the experimental condition; however, *in vitro e*xperiments were typically conducted by two researchers working at the same time on different electrophysiology rigs.

Reagents

AAV-ChR2, AAV-eNpHR3.0 and AAV-EYFP were from the University of North Carolina, Chapel Hill Vector Core. AAVs containing shRNAs for *Grin2c* and *Grin2d* were packaged at the NIDA Optogenetics and Transgenic Technology Core facility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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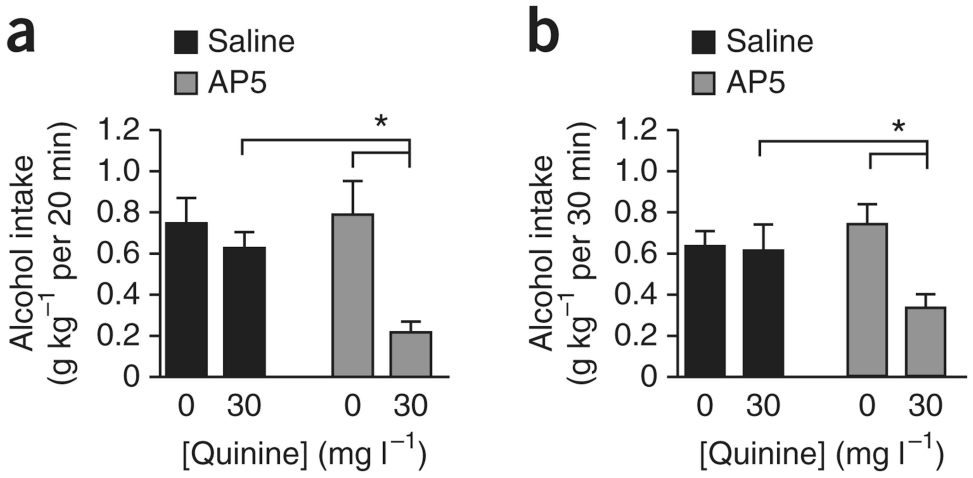

Figure 1.

Inhibition of NMDARs in the NAcore reduced aversion-resistant alcohol intake. (**a**) Intra-NAcore infusion of AP5 (0.5 μl per side of 1 μg μl^{-1}) reduced intake, relative to that in saline-infused rats, of quinine-adulterated alcohol but not quinine-free alcohol in rats drinking alcohol 20 min d^{-1}, 5 d $week^{-1}$. (**b**) Similar results were observed when examining the impact of intra-NAcore AP5 on alcohol intake during the first 30 min of an intermittent overnight alcohol intake session (see Online Methods). Tukey *post hoc* $*P < 0.05$. Error bars indicate s.e.m.

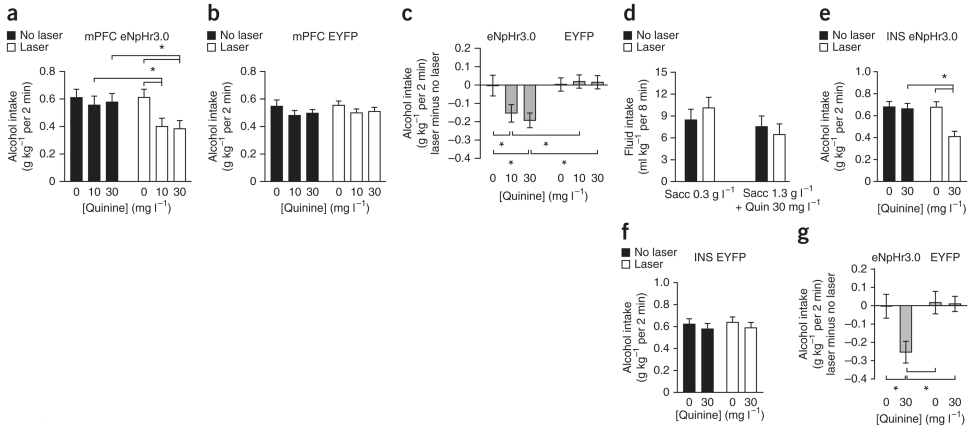

Figure 2.

Halorhodopsin inhibition of mPFC- and INS-to-NAcore inputs reduced quinine-resistant alcohol intake. (**a**) Halorhodopsin (eNpHr3.0) activation in mPFC-to-NAcore terminals significantly reduced intake, relative to that in the absence of laser stimulation, of alcohol adulterated with 10 or 30 mg l^{-1} quinine but not quinine-free alcohol. (**b**) Laser stimulation had no effect on alcohol intake in control rats infected with EYFP in mPFC-to-NAcore terminals. (**c**) To compare laser stimulation of halorhodopsin versus EYFP in mPFC terminals, we calculated the difference in intake between laser stimulation and no stimulation under six conditions: halorhodopsin + 10 or 30 mg l^{-1} quinine, halorhodopsin + no quinine, EYFP + 10 or 30 mg l^{-1} quinine, EYFP + no quinine. Laser stimulation only decreased alcohol intake with halorhodopsin + quinine. (**d**) Inhibiting mPFC-to-NAcore terminals did not alter intake of saccharin (sacc) ± quinine (quin). (**e**) Halorhodopsin inhibition of INS-to-NAcore terminals significantly reduced intake of quinine-adulterated alcohol (30 mg l^{-1} quinine) but not quinine-free alcohol. (**f**) Laser stimulation of INS-to-NAcore terminals had no effect in EYFP-infected rats. (**g**) Difference scores. Laser stimulation of INS-to-NAcore terminals decreased alcohol intake only with halorhodopsin + quinine. Tukey *post hoc* $*P < 0.05$. Error bars indicate s.e.m.

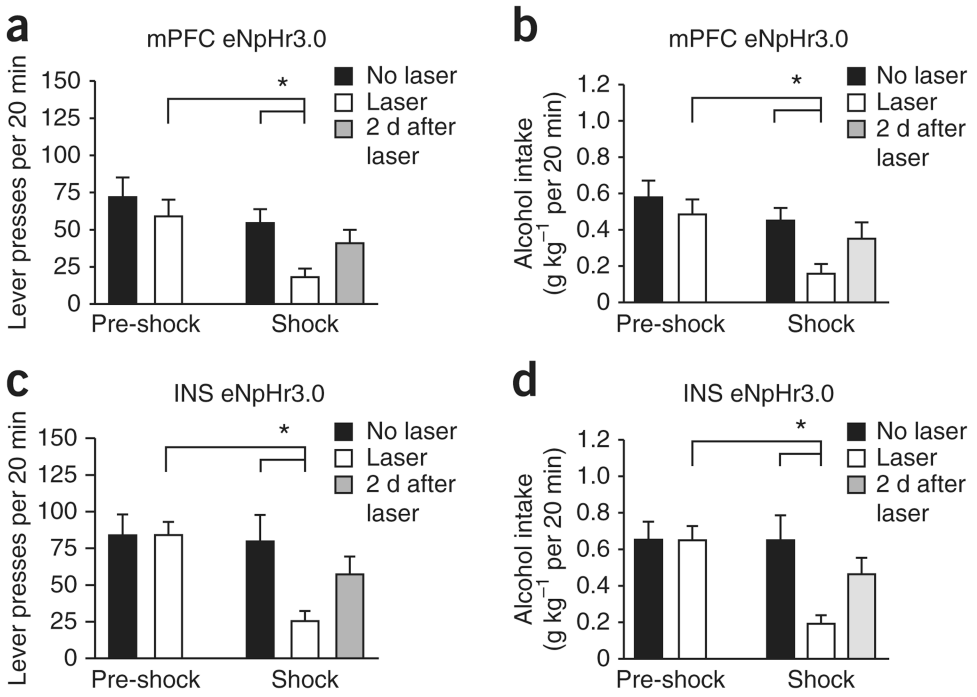

Figure 3.

Halorhodopsin inhibition of mPFC- and INS-to-NAcore inputs reduced footshock-resistant alcohol intake. Rats underwent 7–8 weeks of fixed ratio 3 operant responding for alcohol, 20 min d^{-1}, 5 d $week^{-1}$. Thereafter, one in eight FR3 responses were paired with a footshock (0.25 mA, 0.5 s). By the sixth footshock session, alcohol intake could be considered shock-resistant in some rats (Supplementary Fig. 4a–c). We examined only those rats that gave more than 15 lever presses by the sixth footshock-paired session (10 of 17 rats for mPFC, 10 of 12 rats for INS). (**a,b**) Inhibiting mPFC-to-NAcore inputs significantly reduced responding for alcohol (**a**) and alcohol intake paired with intermittent footshock (**b**). Halorhodopsin (eNpHr3.0) had no effect on baseline, pre-shock sessions in this data set; however, when tested across all mPFC-injected rats, there was a small but significant reduction in baseline responding. (**c,d**) Inhibiting INS-to-NAcore inputs significantly reduced responding for alcohol (**c**) and alcohol intake paired with intermittent footshock (**d**). Halorhodopsin had no effect during baseline, pre-shock sessions. In addition, responding under footshock recovered in the 2 d after halorhodopsin exposure, suggesting no lasting impairments. “No laser” indicates average responding in the 2 d before halorhodopsin exposure under footshock. Tukey *post hoc* $*P < 0.05$. Error bars indicate s.e.m.

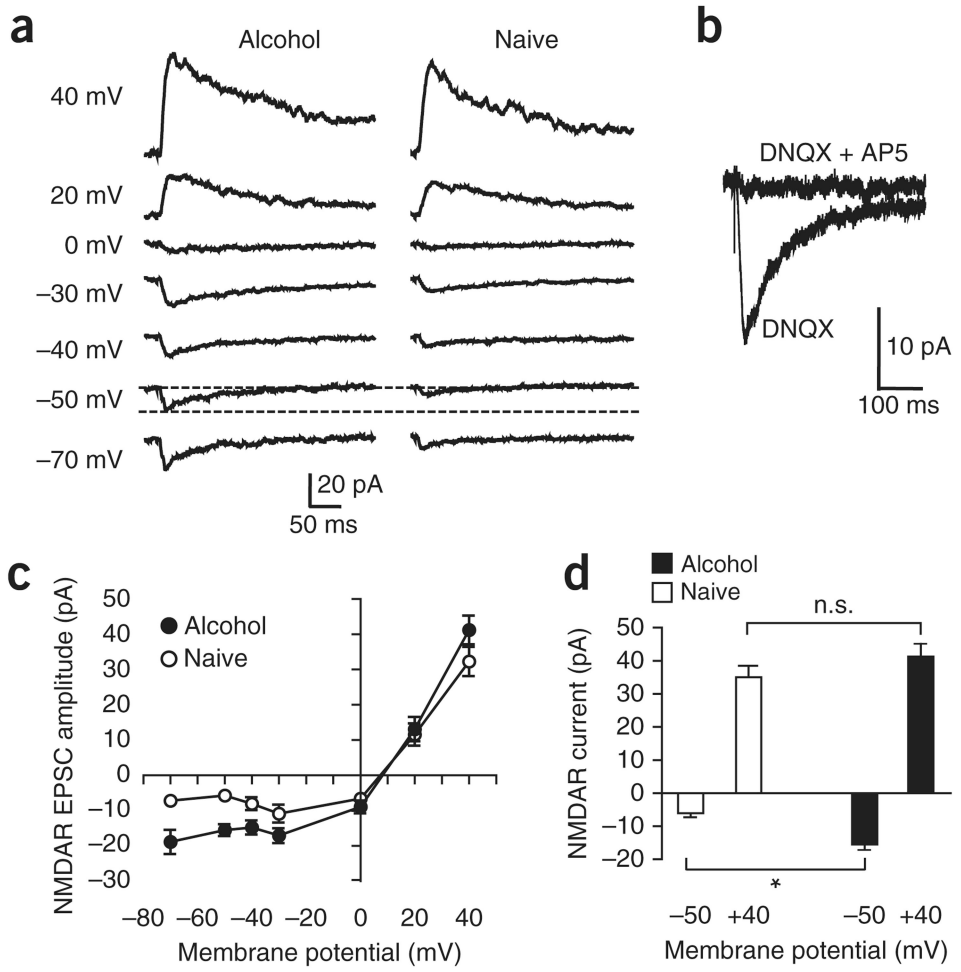

Figure 4.

NAcore neurons from alcohol-drinking rats showed hyperpolarization-active NMDARs under mPFC-to-NAcore inputs. Currents were evoked by ChR2 stimulation of mPFC-to-NAcore terminals in alcohol-drinking and naive rats. (**a**) Example traces of NMDAR currents at different holding potentials. Dashed lines highlight the greater NMDAR current at −50 mV in a neuron from an alcohol-drinking rat. Not all voltages were determined for all neurons. (**b**) Example trace of AP5-sensitive NMDAR currents at −50 mV. (**c**) Grouped data for NMDAR currents under mPFC-to-NAcore inputs at different holding membrane potentials. For some data points the error bars fall within the symbol. The reversal potential for NMDARs was somewhat positive to zero mV without junction null correction, in line with adult mouse NAc shell[18]. (**d**) Alcohol-drinking rats showed significantly greater NMDAR currents at −50 mV than alcohol-naive control rats, with no difference in NMDAR currents evoked at +40 mV holding potential. Tukey *post hoc* $*P < 0.05$; n.s., not significant. Error bars indicate s.e.m.

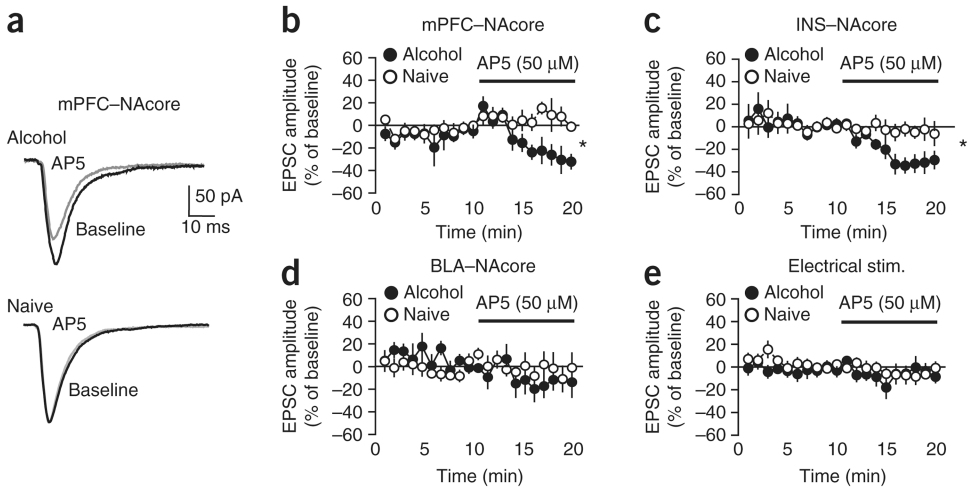

Figure 5.

NAcore neurons from alcohol-drinking rats showed hyperpolarization-active NMDARs under mPFC and INS but not BLA inputs to the NAcore. (**a,b**) AP5 (50 μM) significantly reduced mPFC-ChR2-evoked EPSCs at a −70 mV membrane potential in alcohol-drinking but not control naive rats. (**c**) AP5-sensitive NMDAR currents were apparent in INS-ChR2-evoked EPSCs at −70 mV from alcohol-drinking but not control rats. (**d,e**) AP5 had no effect on EPSCs evoked at −70 mV by (**d**) ChR2 stimulation of BLA-NAcore terminals or (**e**) electrical field stimulation of glutamatergic terminals within the NAcore. **P* < 0.05. Error bars indicate s.e.m.

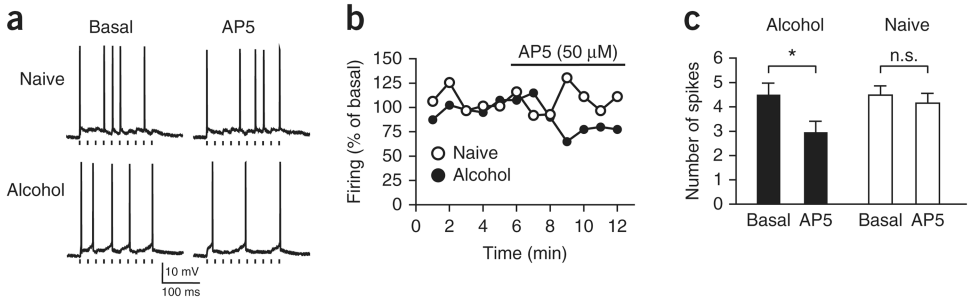

Figure 6.

NMDARs regulate evoked action potential firing under mPFC-to-NAcore inputs from alcohol-drinking but not naive rats. A ten-pulse train (20 Hz) of mPFC-ChR2-evoked EPSPs was stimulated every 10 s to evoke action potential firing; neurons were depolarized to about −55 mV (alcohol, −56.7 ± 2.5 mV; naive, −54.1 ± 2.9 mV; $P = 0.491$) with the patch amplifier to allow EPSPs to evoke ∼5 action potentials. (**a,b**) Traces (**a**) and time courses (**b**) from individual neurons showing AP5 inhibition of firing evoked by mPFC-to-NAcore ChR2 stimulation in neurons from alcohol-drinking but not naive rats. Each point represents the average of the six sweeps in that minute. Tick marks under firing traces in **a** indicate ChR2 stimulation. Firing does not completely track the timing of ChR2 stimulation, which likely reflects the strong potassium channel–mediated after hyperpolarization in NAcore neurons. (**c**) AP5 decreased firing in neurons from alcohol-drinking but not naive rats. Because of variability in firing across time, we averaged the firing across 4 min before AP5 administration (“basal”) and averaged the firing across the minutes 4–7 of AP5 exposure (“AP5”), as firing tended to run down in all cells after this point. For **b**, firing was normalized to the basal firing value. Tukey *post hoc* $^*P < 0.05$. Error bars indicate s.e.m.

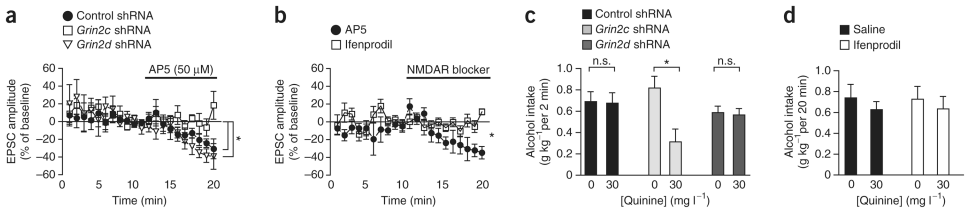

Figure 7.

Grin2c but not Grin2d or Grin2b subunits mediate hyperpolarization-active NMDARs under mPFC-to-NAcore terminals and promote quinine-resistant alcohol intake. (**a**) Infection of NAcore neurons with *Grin2c* but not *Grin2d* or control[36] shRNA prevented AP5 from inhibiting mPFC-evoked EPSCs at −70 mV. (**b**) The Grin2b inhibitor ifenprodil (2 μM) did not reduce mPFC-evoked EPSCs. Data for AP5 in **b** are the same as shown in Figure 5b. (**c**) Infecting NAcore neurons with *Grin2c* but not *Grin2d* or control[34] shRNA reduced intake of quinine-adulterated alcohol. (**d**) Injecting the Grin2b inhibitor ifenprodil in the NAcore (1 μg μl^{-1}) did not alter alcohol intake. *$P < 0.05$. Error bars indicate s.e.m.