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Long-term subregion-specific encoding of enhanced ethanol intake by D1DR medium spiny neurons of the nucleus accumbens

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Abstract

The nucleus accumbens (NAc) is a critical component of the mesocorticolimbic system and is involved in mediating the motivational and reinforcing aspects of ethanol consumption. Chronic intermittent ethanol (CIE) exposure is a reliable model to induce ethanol dependence and increase volitional ethanol consumption in mice. Following a CIE-induced escalation of ethanol consumption, NMDAR-dependent long-term depression (LTD) in D1 dopamine receptor (D1DR) expressing medium spiny neurons (MSNs) of the NAc shell was markedly altered with no changes in plasticity in D1DR MSNs from the NAc core. This disruption of plasticity persisted for up to two weeks after cessation of ethanol access. To determine if changes in AMPA receptor (AMPA) composition contribute to this ethanol-induced neuroadaptation, we monitored the rectification of AMPAR excitatory postsynaptic currents (EPSCs). We observed a marked decrease in the rectification index in the NAc shell, suggesting the presence of GluA2-lacking AMPARs. There was no change in the amplitude of spontaneous EPSCs (sEPSCs) but there was a transient increase in sEPSC frequency in the NAc shell. Using the paired pulse ratio we detected a similar transient increase in the probability of neurotransmitter release. With no change in sEPSC amplitude, the change in the rectification index suggests that GluA2-containing AMPARs are removed and replaced with GluA2-lacking AMPARs in the NAc shell. This CIE-induced alteration in AMPAR subunit composition may contribute to the loss of NMDAR-dependent LTD in the NAc shell and therefore may constitute a critical neuroadaptive response underlying the escalation of ethanol intake in the CIE model.

Keywords

alcohol; CIE; LTD; plasticity; striatum

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Author contributions

RR contributed to the study design, conducted all experiments, analyzed and interpreted data and prepared the manuscript. TRB genotyped and maintained the *Drd1a*-tdTomato mouse colony. RAM contributed to the study design and preparation of the manuscript. All authors have critically reviewed content and approved final version submitted for publication.

Introduction

Shen and Kalivas, 2012; Thomas *et al*, 2001). The most prevalent and widely studied form of accumbal synaptic plasticity, postsynaptic NMDAR-dependent long-term depression (LTD) which involves internalization of AMPA receptors (AMPA), has been strongly implicated in dependence to psychostimulants, and direct disruption of this process has been demonstrated to occlude the expression of behavioral sensitization to amphetamine (Brebner *et al*, 2005). Similarly, chronic ethanol exposure has been shown to result in altered glutamatergic signaling and impaired expression of NMDAR-dependent LTD in the NAc (Abraham *et al*, 2013; Jeanes *et al*, 2011, 2014; Renteria *et al*, 2017; Spiga *et al*, 2014). However, no reports to date have investigated the long-term effects of ethanol exposure on such neuroadaptations in NAc signaling associated with ethanol dependence as seen in excessive ethanol drinking. The chronic intermittent ethanol (CIE) vapor procedure currently used in our lab is a proven and widely adopted model of ethanol dependence that results in an escalation of voluntary ethanol consumption (Becker and Lopez, 2004; Griffin 3rd *et al*, 2009; Griffin, 2014; Lopez and Becker, 2005). Previous work in our lab using the CIE model of vapor exposure focused initially upon identifying changes in excitatory signaling in the NAc induced by passive ethanol vapor exposure prior to measuring changes associated with escalation of ethanol drinking (Jeanes *et al*, 2011, 2014; Renteria *et al*, 2017). In this report, we present the first demonstration of enduring neuroadaptive synaptic plasticity changes in the CIE model that may contribute to the escalation of ethanol drinking and dependence.

The NAc, which is composed of the core and shell subregions, has been shown to be involved in the acute reinforcing effects of ethanol (Di Chiara and Imperato, 1988; Weiss *et al*, 1993) but it is unclear whether long-term changes in NAc signaling contribute to ethanol dependence or whether it is differentially encoded between the core and the shell subregions. The core, which is directly ventral and continuous with the dorsal striatum, and the shell, which makes up the most ventral and medial portions of the NAc, have distinct connectivity, cellular morphology, neurochemistry and functions (Humphries and Prescott, 2010). In addition, the two subregions have been shown to be differentially involved in cue-induced and context-mediated ethanol seeking (Chaudhri *et al*, 2010; Gremel and Cunningham, 2008). Given the functional differences between the core and shell subregions, the question remains whether the long-term effects of CIE exposure and excessive ethanol drinking are differentially encoded between the two subregions.

In both the shell and core of the NAc, AMPAR currents are mediated primarily by GluA2-containing AMPARs (Conrad *et al*, 2008; Cull-Candy *et al*, 2006). NMDAR-dependent LTD in the NAc has been shown to require the internalization of GluA2-containing AMPARs. Preventing the internalization of GluA2-containing AMPARs with a peptide that mimics the carboxyl tail of the GluA2 subunit blocks the expression of NMDAR-dependent LTD (Brebner *et al*, 2005; Jeanes *et al*, 2014). Given the importance of GluA2-containing AMPARs for the expression of NMDAR-dependent LTD, the ethanol induced disruption of plasticity may be due to alterations in the number of AMPARs or changes in AMPAR subunit composition. The accumulation of GluA2-lacking AMPARs has been shown to be critical for cue induced cocaine craving (Loweth *et al*, 2014), and has been detected with both contingent and non-contingent cocaine exposure (Terrier *et al*, 2015). In

addition, GluA2-lacking AMPARs have been observed in the NAc after chronic opioid exposure (Hearing *et al*, 2016). In the present study we show long-term changes in AMPAR subunit composition and NMDAR-dependent plasticity that correspond with an escalation of ethanol drinking in ethanol dependent mice.

Materials and methods

Mice

Male *Drd1a*-tdTomato mice generated on a C57Bl/6J background were obtained from Jackson Laboratory (B6.Cg-Tg(Drd1a-tdTomato)6Calak/J) and bred in house. Mice were at least 8 weeks of age for behavioral experiments and 16 weeks of age for electrophysiological experiments. Mice were housed at 22°C with a 12:12 light:dark cycle (lights on at 12 AM). Water and chow were available *ad libitum*. All of the following experimental procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

Chronic intermittent ethanol

Multiple cohorts (n=6) of mice were exposed to 3 rounds of ethanol vapor using the CIE exposure model, a proven method to increase voluntary ethanol consumption in mice (Becker *et. al.*, 2004). Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 0.2 to 0.3 liter/min. The resulting ethanol vapor was then combined with a separate air stream to give a total flow rate of approximately 4 liters/min, which was delivered to mice in special mouse chamber units that contain an airtight top, a vapor inlet and an exhaust outlet (Allentown Inc., Allentown, NJ). Each round consisted of 16 hours of ethanol vapor exposure followed by an 8 hour withdrawal, repeated for 4 days (Figure 1a.). Mice were injected (i.p.) with a loading dose of ethanol (20% v/v, 1.5 g/kg) and pyrazole (68.1 mg/kg) in 0.1M PBS prior to vapor exposure. Blood ethanol concentration (BEC) was measured daily upon removal from the chambers and were within range of 150–200 mg/dl. Air control mice were handled the same but were injected with a solution of only pyrazole (68.1 mg/kg) in PBS.

Two bottle choice drinking

Two bottle choice (TBC) drinking is a limited access paradigm used to measure changes in voluntary ethanol consumption (Griffin, 2014). Thirty minutes prior to the onset of the dark cycle mice are given access to two bottles containing either an ethanol solution (15%) or tap water. Access is limited to 2 hours. Bottles are weighed before and after each drinking session and mice are weighed after each drinking session. Twenty-one consecutive days of TBC drinking was conducted prior to the first round of ethanol vapor exposure. The last 5 days were measured as the baseline. Seventy-two hours after each round of vapor exposure TBC drinking was conducted for 5 days to measure CIE induced changes in ethanol consumption (Figure 1a). Drinking data were analyzed using a repeated measures two-way analysis of variance (ANOVA) with EtOH and Air groups as the between subject variable and the drinking session as the repeated measure. Further analysis using Bonferroni post hoc test was used to determine differences in drinking between groups at each drinking session and changes in drinking session vs baseline drinking within each group.

Blood ethanol concentrations

Tail blood samples of 5–10 μL were collected upon removal from the chambers each day and BECs were measured using gas chromatography with a Bruker 430-GC (Bruker Corporation, Fremont, CA) equipped with a flame ionization detector and CombiPAL autosampler. Two samples of whole blood were added to 10 mL vials containing supersaturated sodium chloride solution in which the blood sample was 10% of total volume. Samples were warmed to 65°C and the solid-phase micro extraction fiber (SPME; 75 μm CAR/PDMS, fused silica; Supelco, Bellefonte, PA) was used to absorb ethanol vapor from the samples. The stationary phase was a capillary column (30 m x 0.53 mm x 1 μm film thickness; Agilent Technologies, Santa Clara, CA) and helium, at a flow rate of 8.5 mL/min was used in the mobile phase. Resultant ethanol peaks were analyzed using CompassCDS Workstation software (Bruker Corporation, Fremont, CA) and calibration was achieved using freshly prepared ethanol standards (100–400mM).

Brain slice preparation

Mice were at least 16 weeks of age at the time of slice preparation. They were anesthetized by inhalation of isoflurane and the brains were rapidly removed and placed in 4°C oxygenated ACSF containing the following (in mM): 210 sucrose, 26.2 NaHCO_3 , 1 NaH_2PO_4 , 2.5 KCl, 11 dextrose, bubbled with 95% O_2 / 5% CO_2 . Parasagittal slices (240 μm thick) containing the NAc were obtained using a Leica vibrating microtome. Slices were transferred to an ACSF solution for incubation containing the following (in mM): 120 NaCl, 25 NaHCO_3 , 1.23 NaH_2PO_4 , 3.3 KCl, 2.4 MgCl_2 , 1.8 CaCl_2 , 10 dextrose, were continuously bubbled with 95% O_2 / 5% CO_2 ; pH 7.4, 32°C, and were maintained in this solution for 60 minutes prior to recording.

Patch clamp electrophysiology

In ethanol naïve mice, NMDAR-dependent LTD is expressed only in D1DR MSNs (Jeanes *et al*, 2014; Renteria *et al*, 2017) therefore we selectively recorded from D1DR expressing MSNs using *Drd1a*-tdTomato mice on a C57Bl6 background. Whole-cell voltage clamp recordings were made in D1DR MSNs of the NAc shell and core. Cells were identified using the MRK200 Modular Imaging system (Siskiyou Corporation) mounted on a vibration isolation table. Recordings were made in ACSF containing (in mM): 120 NaCl, 25 NaHCO_3 , 1.23 NaH_2PO_4 , 3.3 KCl, 0.9 MgCl_2 , 2.0 CaCl_2 , and 10 dextrose, bubbled with 95% O_2 / 5% CO_2 . ACSF was continuously perfused at a rate of 2.0 mL/min and maintained at a temperature of 32°C. Picrotoxin (50 μM) was included in the recording ACSF to block GABA_A receptor-mediated synaptic currents. Recording electrodes (thin-wall glass, WPI Instruments, Sarasota, FL) were made using a Brown-Flaming model P-88 electrode puller (Sutter Instruments, San Rafael, CA) to yield resistances between 36 M Ω . Electrodes were filled with (in mM): 120 CsMeSO₄, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4 Mg-ATP, 0.3 Na-GTP, 0.1 spermine, and 5 QX-314-Cl. Input resistance and access resistance were monitored throughout the experiments. Cells in which input and access resistance varied more than 20% were not included for analysis.

Data acquisition and analysis

Glutamatergic afferents were stimulated with a stainless steel bipolar stimulating electrode (FHC, Inc.) placed dorsal and anterior to the recording electrode, about 150–300µm from the cell body. Excitatory postsynaptic currents (EPSCs) were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, and digitized at 10–20 kHz with a Digidata 1440A interface board using pClamp 10.2 (Axon Instruments). In experiments for long term plasticity, EPSCs were evoked by local stimulation for at least 10 minutes at 0.1 Hz, to ensure stable recordings. To induce LTD, a conditioning stimulus of 500 pulses at 1 Hz was paired with continuous postsynaptic depolarization to –50 mV. EPSCs were then monitored for 30 minutes after pairing at a rate of 0.1 Hz. The magnitude of LTD was calculated by averaging normalized EPSC values from 20 to 30 minutes after the pairing protocol and comparing that value to the average normalized EPSCs during the 10 minute baseline. Plasticity was determined if the average EPSCs between 20 to 30 minutes post-pairing was greater than 2 standard deviations away from the 10 minute baseline. A current-voltage plot was generated by measuring AMPAR mediated currents at the following holding potentials (in mV): –80, –40, 0, +20, and +40. The rectification index was determined as the ratio of EPSC amplitude at +40 mV and –80 mV. Data from each neuron within a treatment group was combined and represented as percent baseline values. Summary data are presented as mean ± SEM. Data were analyzed using a one-way ANOVA and Bonferroni post hoc test in which statistical significance from baseline for within each group is defined as $p < 0.05$.

Results

CIE induced escalation of volitional ethanol intake

Ethanol consumption was measured using the two-bottle choice paradigm. Baseline drinking was stable in both the air and ethanol vapor exposed mice, with no significant difference in baseline drinking between groups (Figure 1b, repeated measure two-way ANOVA, baseline daily drinking, $F_{(1, 38)} = 1.180$, $p=0.284$). In the ethanol vapor exposed mice, ethanol consumption escalated across drinking sessions but not in the air exposed mice. This is supported by a repeated measure two-way ANOVA that indicated a significant main effect of EtOH exposure ($F_{(1, 38)} = 7.58$, $p = 0.009$) and drinking session ($F_{(3, 114)} = 3.606$, $p = 0.0156$) as well as a EtOH exposure x drinking session interaction ($F_{(3, 114)} = 10.56$, $p < 0.0001$). Post hoc analysis indicated that ethanol vapor exposed mice significantly increased drinking vs. air exposed mice in drinking sessions 2 and 3 ($++ p < 0.01$, $+++ p < 0.001$). Additionally, ethanol vapor exposed mice significantly increased drinking vs. baseline across all drinking sessions ($** p < 0.01$ vs. baseline, $**** p < 0.0001$ vs. baseline), while ethanol drinking in air exposed mice remained stable.

Long term disruption of NMDAR-dependent LTD in the NAc shell

Several reports have observed a disruption in NMDAR-dependent plasticity after drug or ethanol exposure that is directly related to a drug induced behavior (Abraham *et al*, 2013; Brebner *et al*, 2005; Kasanetz *et al*, 2010; Pascoli *et al*, 2011). To determine whether a CIE induced increase in ethanol drinking disrupts NMDAR-dependent plasticity we used an induction protocol that has been shown to reliably induce NMDAR-dependent LTD in the

NAc (Brebner *et al*, 2005; Jeanes *et al*, 2011, 2014; Renteria *et al*, 2017; Thomas *et al*, 2000). We have previously shown that ethanol-naïve mice express NMDAR-dependent LTD only in D1DR expressing MSNs (Jeanes *et al*, 2014; Renteria *et al*, 2017). To selectively record only from D1DR MSNs in the both the shell and core of the NAc, we used *Drd1a*-tdTomato mice. NMDAR-dependent LTD was expressed in D1DR MSNs of both the shell and core of air treated control mice (Figure 2a, shell: 55.10 ± 14.11 % of baseline, $n=5$, 4 animals; core: 47.80 ± 13.59 % baseline, $n=4$, 3 animals). In the EtOH vapor exposed mice, 24 hours after the last drinking session, the expression of NMDAR-dependent LTD was blocked in the shell (Figure 2b, e, 24 hrs: 96.62 ± 7.76 % baseline, $n=6$, 5 animals) but not the core (Figure 2b, f, 24 hrs: 59.65 ± 14.68 % baseline, $n=4$, 3 animals). The ethanol induced disruption of plasticity in the shell persisted for up to two weeks after the last drinking session (Figure 2c, e, 1–2 wks: 94.87 ± 4.95 % baseline, $n=7$, 5 animals; one-way ANOVA: $F_{(3,18)} = 8.874$, $p = 0.0008$, Bonferroni post hoc test $** p < 0.01$ air vs 24 hrs/1–2wks) with normal LTD in the core (48.31 ± 7.96 % baseline, $n = 7$, 7 animals). Three weeks after drinking, the expression of NMDAR-dependent LTD was recovered in the shell (Figure 2d, e, 3 wks: 53.88 ± 8.64 % baseline, $n=4$, 3 animals) and remained intact in the core (Figure 2d, f, 3 wks: 64.5 ± 7.55 % baseline, $n=3$, 3 animals, one-way ANOVA: $F_{(3,14)} = 0.8682$, $p = 0.4807$).

Decreased rectification index in the NAc shell up to two weeks after drinking

In the NAc, the majority of AMPAR signaling is mediated by GluA2-containing AMPA receptors (Conrad *et al*, 2008). GluA2-containing AMPARs have Ohmic properties and are impermeable to Ca^{2+} while GluA2-lacking AMPARs have an inward rectifying current voltage (IV) relationship and are Ca^{2+} permeable (Cull-Candy *et al*, 2006). The distinct IV curves of GluA2containing and GluA2-lacking AMPARs make them detectable using electrophysiological methods. The rectification index is the ratio of AMPAR mediated current at +40 mV and –80 mV. To isolate AMPAR mediated currents we used DL-APV (50 μM). In the air control mice, 24 hours after ethanol drinking, D1DR MSNs of the NAc shell (Figure 3a, Air: 0.49 ± 0.02 , $n=9$, 4 animals) and core (Figure 3c, Air: 0.44 ± 0.02 , $n = 7$, 3 animals) had a linear IV curve. Twenty-four hours after drinking, ethanol vapor exposed mice had a significant decrease in the rectification index in the shell, suggesting the presence of GluA2-lacking AMPARs (Figure 3a, b, 24 hrs: 0.19 ± 0.04 , $n = 7$, 3 animals; one-way ANOVA: $F_{(3, 24)} = 16.74$, $p < 0.0001$; Bonferroni post hoc test, Air vs. 24 hrs $**** p < 0.0001$). Similar to the disruption of NMDAR-dependent LTD, this ethanol induced alteration in AMPAR signaling persisted for up to 2 weeks after drinking (1–2 wks: 0.27 ± 0.08 , $n = 5$, 3 animals; Bonferroni post hoc test, Air vs. 1–2 wks $** p < 0.01$) in the shell and recovered at 3 wks (0.44 ± 0.03 , $n = 7$, 4 animals) while the rectification index remained unchanged in the core (Figure 3c, d, 24 hrs: 0.53 ± 0.06 , $n = 7$, 3 animals; 1–2 wks: 0.49 ± 0.05 , $n = 6$, 3 animals; 3 wks: 0.42 ± 0.03 , $n = 3$, 2 animals; one-way ANOVA, $F_{(3,19)} = 1.21$, $p = 0.36$).

Transient increase in excitatory neurotransmitter release in the NAc shell

In an ethanol naïve mouse, the expression of NMDAR-dependent LTD in the NAc requires the removal of GluA2-containing AMPA receptors from the post-synaptic membrane (Brebner *et al*, 2005; Jeanes *et al*, 2014). The long term disruption of NMDAR-dependent

LTD observed after excessive ethanol drinking (Figure 2) may be due to a CIE induced internalization of AMPARs. A similar occlusion of NMDAR-dependent LTD in the NAc has been observed with cocaine experience (Kourrich *et al*, 2007; Thomas *et al*, 2001). We observed that EtOH vapor exposed mice had a significant increase in sEPSC frequency in the NAc shell 24 hours after drinking (Figure 4a, c, Air: 3.70 ± 0.91 , $n = 12$, 8 animals; 24 hrs: 7.49 ± 0.89 , $n = 17$, 5 animals; one-way ANOVA: $F_{(3, 41)} = 4.137$, $p = 0.0119$, Bonferroni post hoc test $** p < 0.01$ versus air controls) with no change in frequency in the core (Figure 4b, d, Air: 4.40 ± 0.82 , $n = 15$, 10 animals; 24 hrs: 3.64 ± 0.66 , $n = 7$, 4 animals, one-way ANOVA, $F_{(3, 34)} = 0.60$, $p = 0.62$). There was no effect of CIE on the amplitude of sEPSCs in the NAc shell (Figure 4e, one-way ANOVA, $F_{(3, 41)} = 0.134$, $p = 0.94$) or core (Figure 4f, one-way ANOVA, $F_{(3, 35)} = 0.189$, $p = 0.90$) over the three week period following ethanol drinking. With no change in sEPSC amplitude, the CIE-induced disruption of plasticity does not appear to be due to the occlusion of AMPAR internalization. To test for changes in the probability of presynaptic neurotransmitter release, we used the paired pulse ratio (PPR) with a 50 ms interstimulus interval (ISI). Twenty-four hours after drinking we observed a decrease in the PPR in the shell (Figure 4g, Air: 1.60 ± 0.05 , $n = 7$, 3 animals; 24 hrs: 1.16 ± 0.10 , $n = 10$, 3 animals; one-way ANOVA: $F_{(3, 21)} = 4.402$, Bonferroni post hoc test $** p < 0.01$ versus air controls), indicating an increase in the probability of neurotransmitter release. The PPR remained unchanged in the core of ethanol exposed mice compared to air controls (Figure 4h, one-way ANOVA, $F_{(3, 14)} = 0.574$, $p = 0.64$). The transient increase in sEPSC frequency may be due to an increase in the probability of neurotransmitter release but this presynaptic alteration may not contribute to the long lasting disruption of NMDAR-dependent LTD as it is only present 24 hours after excessive ethanol drinking (Figure 4c).

Discussion

In the present study we observed a long-term disruption of NMDAR-dependent LTD in the NAc shell of mice that corresponds with CIE-induced enhanced ethanol intake. We believe these neuroadaptations we observed may encode the CIE-induced increase in voluntary ethanol consumption. Previous work using the CIE vapor model has shown that the increase in ethanol intake can last up to 4 weeks after the ethanol vapor exposure (Lopez and Becker, 2005). When mice are exposed to two rounds of ethanol vapor, they will increase drinking for up to one week after vapor exposure but with 4 rounds of ethanol vapor exposure the increase in drinking will persist for up to 4 weeks. The long lasting disruption of NMDAR-dependent LTD that we have observed in the D1DR MSNs in the NAc shell corresponds well with the time course of increased ethanol drinking. In the present study, 3 rounds of ethanol vapor exposure induced a disruption in NMDAR-dependent LTD that was first detected 24 hours after drinking (8 days after last ethanol vapor exposure) and persisted for up to 2 weeks after drinking (22 days after last ethanol vapor exposure).

Ethanol induced alterations of NMDAR function may contribute to the loss of NMDAR-dependent LTD as well as excessive alcohol intake as changes in NMDAR function have been linked to addictive behaviors (Hopf, 2016). In a study of ethanol induced locomotor sensitization, sensitized mice had an attenuation of NMDAR-dependent LTD due to a decrease in NMDAR function measured as a loss of the GluN1 subunit (Abraham *et al*,

2013). A similar result was observed in rats that were fed an ethanol containing diet in which the authors observed a decrease in NMDAR function and impaired expression of NMDAR-dependent LTD (Spiga *et al*, 2014). We have also observed alterations in NMDAR function after one round of ethanol vapor exposure although we did not characterize the time course of this change specifically after multiple rounds of CIE exposure (Renteria *et al*, 2017).

Our previous work has shown that the removal of GluA2-containing AMPARs is necessary for the expression of NMDAR-dependent LTD in the NAc (Jeanes *et al*, 2014). In addition to the disruption of plasticity we found electrophysiological evidence that GluA2-containing AMPARs were replaced with GluA2-lacking AMPARs. The loss of GluA2-containing AMPARs could account for the disruption of NMDAR-dependent LTD as both had a similar time course of up to two weeks after drinking and recovery by the third week. Although, the rectification index is only an indirect measure of changes to AMPAR subunit composition. The observed decrease in the rectification index could reflect a change in the GluA2 subunit Q/R editing. GluA2 transcripts undergo editing at the Q/R site primarily by the enzyme adenosine deaminase acting on RNA 2b (ADAR2b). Editing of the GluA2 subunit at the Q/R site makes them impermeable to Ca^{2+} and contributes to the linear IV curve (Cull-Candy *et al*, 2006). A recent study found that abstinence from cocaine self-administration is associated with decreased GluA2 Q/R site editing and reduced ADAR2 expression specifically in the NAc shell but not core (Schmidt *et al*, 2014).

GluA2-lacking AMPARs have a prominent role in mediating the incubation of cocaine craving (Loweth *et al*, 2014). GluA2-lacking AMPARs accumulate in the NAc during extended withdrawal from cocaine self-administration and the use of NASPM, a GluA2-lacking AMPAR antagonist, significantly decreased the expression of cue induced cocaine seeking (Conrad *et al*, 2008). The incubation of drug craving is not specific to cocaine self-administration as it is seen with other drugs of abuse (Pickens *et al*, 2011) as well as ethanol (Li *et al*, 2014). The replacement of GluA2-containing AMPARs with GluA2-lacking AMPARs could serve as a mediator of excessive ethanol intake. Further studies that include the use of NASPM could uncover a role for GluA2-lacking AMPARs in maladaptive ethanol behaviors.

The incorporation of GluA2-lacking AMPARs could alter the mechanisms for the induction and expression of plasticity and may account for the CIE-induced disruption in NMDAR-dependent LTD we have observed in the present study. The Ca^{2+} permeability of GluA2-lacking AMPARs make them candidates as mediators of synaptic plasticity. In the basolateral amygdala, activation of GluA2-lacking AMPA receptors has been shown to lead to LTP that is independent of NMDAR activity (Mahanty and Sah, 1998). GluA2-lacking AMPA receptors have also been attributed to the consolidation of NMDAR-dependent LTP in the hippocampus (Plant *et al*, 2006). The induction of NMDAR-dependent LTP results in the transient insertion of GluA2-lacking AMPARs and subsequent Ca^{2+} influx from these receptors is necessary for the insertion of GluA2-containing AMPARs and the consolidation of LTP. A similar mechanism has been observed in the striatum in which activation of GluA2-lacking AMPARs leads to the synaptic insertion of GluA1 (Tukey and Ziff, 2013).

We observed a transient increase in sEPSC frequency as well as a decrease in PPR, suggesting an increase in the probability of neurotransmitter release. The increase in presynaptic glutamate release that we measured in the present study was observed only at the 24 hour time point and returned to control levels 1 week after drinking. Also, we observed this increase to be subregion specific as no change was found in the NAc core. Recent quantitative microdialysis experiments have detected a two-fold increase in extracellular glutamate from CIE treated mice (Griffin III et al., 2014). This is consistent with previous studies that have found chronic ethanol exposure to result in a decrease of glutamate reuptake (Melendez *et al*, 2005). Ethanol is an inhibitor of excitatory signaling (Lovinger and Roberto, 2013). Thus, the increase in glutamate release may be a compensatory mechanism necessary to adapt to the chronic inhibitory effects of ethanol exposure. The CIE induced increase in glutamate release that we detected in the NAc shell may represent a form of synaptic scaling of presynaptic neurotransmission. We also observed evidence of the replacement of GluA2-containing AMPARs with GluA2-lacking AMPARs which may be the result of synaptic scaling on the postsynaptic membrane as GluA2lacking AMPARs are Ca²⁺ permeable and have a higher conductance than GluA2-containing AMPARs (Cull-Candy *et al*, 2006).

The NAc shell may be the initial site of drug encoding as we observed these changes in excitatory signaling exclusively in the shell with no detected changes in the core. The subregion-specific effect of CIE exposure and ethanol drinking may reflect the functional connectivity of the striatum and the midbrain. The NAc shell sends projections to the VTA to directly influence dopamine neurons that project to the NAc core which then projects to the substantia nigra to directly influence cells that project to more dorsal and lateral regions of the striatum (Haber *et al*, 2000; Sesack and Grace, 2010). This ventromedial to dorsolateral spiraling loop of connectivity is believed to underlie the progression of drug induced neuroadaptations from early drug experience to more compulsive drug taking (Belin and Everitt, 2008; Keramati and Gutkin, 2013). GluA2-lacking AMPARs have been observed in the NAc core of rats 40–60 days after ethanol exposure using a CIE procedure in which rats were administered ethanol via oral intubation (Marty and Spigelman, 2012). In the current study, changes in excitatory signaling and plasticity were observed up to 22 days after ethanol vapor exposure. It is possible that the observed changes in the NAc shell precede those in the core and more dorsal regions of the striatum with prolonged ethanol exposure.

In order to further advance our understanding of ethanol induced neuroadaptations we must look beyond cell-type specificity and begin to uncover changes in isolated excitatory inputs. Using an optogenetic approach, it has been shown that acute ethanol exposure differentially modulates isolated excitatory inputs to the NAc (Ji *et al*, 2015). Further studies need to be conducted in order to understand the precise mechanisms in which chronic drug and ethanol experience is encoded in the brain in a cell-type and input specific manner. Such an understanding may uncover new avenues for the development of novel and more effective pharmacotherapies for the treatment of ethanol craving and dependence.

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Figure 1.

Chronic intermittent ethanol (CIE) vapor exposure induced an escalation of ethanol intake.

(a.) The CIE procedure consisted of 3 rounds of ethanol vapor exposure separated by a 3 day withdrawal and 5 day periods of TBC drinking (15% ethanol). All electrophysiological recordings were conducted after the last ethanol drinking period. (b.) The daily ethanol intake of ethanol treated mice and air controls during each drinking period. (c.) Average ($\pm$ SEM) of 5 days of TBC drinking (in g/kg) during each drinking period.

Data points and bar graphs represent the average $\pm$ SEM. ** $p < 0.01$ versus baseline, **** $p < 0.0001$ versus baseline; ++ $p < 0.01$ versus Air, +++ $p < 0.001$ versus Air.

Figure 2.

Long-term disruption of NMDAR-dependent LTD in D1DR MSNs of the NAc shell. **(a.)** The induction protocol which consists of 1 Hz stimulation paired with postsynaptic depolarization to -50 mV for 500 s, resulted in LTD in both the shell and core of Air treated mice. **(b.–d.)** Expression of NMDAR-dependent LTD in the shell and core 24 hrs – 3 wks after the last drinking session. **(e., f.)** Summary bar graphs of alterations in plasticity over time representing the percentage change $\pm$ S.E.M. for average EPSC amplitude between baseline (min 0–10) and post-pairing (min 40–50) in the shell and core. Data points and bar graphs represent the average $\pm$ SEM. ** $p < 0.01$ versus air controls. Scale bars represent 50 pA (vertical) and 10 ms (horizontal).

Figure 3.

Decrease in the rectification index in the NAc shell persist for up to two weeks after ethanol drinking. **(a.)** Current-voltage relationship of AMPAR mediated EPSCs (normalized to EPSC amplitude at -80 mV) in the shell in Air and EtOH exposed mice 24 hours after drinking. **(b.)** The rectification index in the shell of Air and EtOH exposed mice 24 hrs – 3 wks after the last drinking session. **(c.)** Current-voltage relationship of AMPAR mediated EPSCs (normalized to EPSC amplitude at -80 mV) in the core of Air and EtOH exposed mice 24 hours after drinking **(d.)** The rectification index in the shell of Air and EtOH exposed mice 24 hrs – 3 wks after the last drinking session.

Data points and bar graphs represent the average $\pm$ SEM. **** $p < 0.0001$ versus Air, ** $p < 0.01$ versus Air.

Figure 4.

CIE induced increase in sEPSC frequency and decrease in PPR in D1DR MSNs of the NAC shell but not the core. **(a., b.)** Sample traces of sEPSCs from Air and EtOH exposed mice in the shell and core. Scale bars represent 15 pA (vertical) and 1 s (horizontal). **(c., d.)** sEPSC frequency in the shell and core of Air and EtOH exposed mice 24 hrs – 3 wks after the last drinking session. **(e., f.)** sEPSC amplitude in the shell and core of Air and EtOH exposed mice 24 hrs – 3 wks after the last drinking session. **(g., h.)** The paired-pulse ratio (PPR) in

the shell and core of Air and EtOH exposed mice 24 hrs – 3 wks after the last drinking session. Scale bars represent 50 pA (vertical) and 25 ms (horizontal).

Bar graphs represent the average $\pm$ SEM. ** $p < 0.01$ versus Air.

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