IN VIVO CHRONIC INTERMITTENT ETHANOL EXPOSURE REVERSES THE
POLARITY OF SYNAPTIC PLASTICITY IN THE NUCLEUS ACCUMBENS SHELL

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d) Abbreviations
NAc: nucleus accumbens
MSNs: medium spiny neurons
AMPA: α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid
NMDA: N-methyl-D-aspartate
GABA: gamma-aminobutyric acid
aCSF: artificial cerebrospinal fluid
EPSC: excitatory postsynaptic current
LTD: long term depression
EtOH: ethanol
CIE: chronic intermittent ethanol
DARPP-32: dopamine and cAMP regulated phosphoprotein of 32 kDa
DNQX: 6,7-Dinitroquinoxaline-2,3-dione
DL-APV: DL-2-Amino-5-phosphonovaleric acid
Ro 25-6981: (R-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol
(±)SKF38393: (±)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzaazepine-7,8-diol hydrochloride
SCH23390: R-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benazaepine-7-ol

e) Recommended section assignment: Neuropharmacology
ABSTRACT

Glutamatergic synaptic plasticity in the nucleus accumbens (NAc) is implicated in response to sensitization to psychomotor stimulating agents yet ethanol effects here are undefined. We studied the acute \textit{in vitro} and \textit{in vivo} effects of ethanol in medium spiny neurons from the shell NAc subregion of slices of C57BL/6 mice using whole-cell voltage clamp recordings of AMPA EPSCs. Synaptic conditioning (low frequency stimulation with concurrent postsynaptic depolarization) reliably depressed AMPA EPSCs by nearly 30%; this accumbal long-term depression (NAc LTD) was blocked by a non-selective NMDA receptor antagonist (DL-APV) and a selective NMDAR2B antagonist (Ro 25-6981). Acute ethanol exposure inhibited the depression of AMPA EPSCs differentially with increasing concentrations, but this inhibitory action of ethanol was occluded by a D1-selective dopamine receptor agonist. Ethanol dependence was elicited in C57BL/6 mice by two separate four day bouts of chronic intermittent ethanol (CIE) vapor exposure. When assessed 24 hrs following a single bout of \textit{in vivo} CIE vapor exposure, NAc LTD was absent and instead NMDAR receptor-dependent synaptic potentiation (LTP) was reliably observed. Interestingly, both LTP and LTD were completely absent following an extended withdrawal (72 hours) after a single 3 day CIE vapor bout. These observations demonstrate that accumbal synaptic depression 1) is mediated by NR2B receptors, 2) is highly sensitive to both acute and chronic ethanol exposure, and 3) alterations in this synaptic process may constitute a neural adaptation that contributes to the induction and/or expression of ethanol dependence.
INTRODUCTION

GABAergic medium spiny neurons (MSNs) of the nucleus accumbens (NAc) in the ventral striatum are principal neurons in the mesocorticolimbic system that process information concerning reward behavior (Nestler, 2001). These neurons receive a dopaminergic projection from the ventral tegmental area (VTA) and glutamatergic projections from prefrontal cortex and other limbic structures. It is generally thought that neuroadaptations in response to chronic drug abuse underlie development of craving and other drug-seeking behaviors associated with dependence. Much evidence indicates that NAc MSNs are very likely involved in such aberrant neuroadaptive responses. While neuroadaptations underlying chronic ethanol abuse remain undefined, interactions between dopaminergic, glutamatergic and GABAergic systems are likely crucial in this regard (for reviews see (Gonzales et al., 2004; Zhang et al., 2006).

Indeed, a large literature indicates that ethanol reinforcement involves the activation of the VTA-accumbal dopamine system (Gonzales et al., 2004). Ethanol has unique pharmacological actions to excite VTA dopamine neurons, and withdrawal from chronic ethanol exposure reduces their firing (Brodie et al., 1990; Shen and Chiodo, 1993; Brodie et al., 1999; Shen, 2003). Dopamine release increases in the NAc during operant self-administration of ethanol (Weiss et al., 1993; Gonzales and Weiss, 1998; Yim et al., 1998) and D1-dopamine receptor antagonists reduce operant ethanol responding (Rassnick et al., 1992; Hodge et al., 1993; Samson et al., 1993). Additionally, ethanol self-administration is reduced in animals lacking D1 receptors (El-Ghundi et al., 1998) or one of its intracellular partners, DARPP-32 (Risinger et al.,...
Neuroadaptations which contribute to ethanol abuse likely share common mechanisms with those seen in other abused reinforcers – especially the psychomotor stimulating agents such as cocaine and amphetamine. Recent evidence indicates that adaptations in accumbal glutamatergic plasticity constitute a mechanism encoding repetitive drug experience to psychomotor stimulants. In control NAc MSNs, low frequency conditioning stimulation (LFS) paired with postsynaptic depolarization, which mimics the upstate of about -50 mV common in bistable MSNs, produces long-term depression (LTD) of AMPA EPSCs (Thomas et al., 2000; Thomas et al., 2001). Like hippocampal NMDA LTD (Dudek and Bear, 1992; Man et al., 2000), NAc LTD is induced by a moderate increase in intracellular Ca\(^{2+}\) through NMDA receptor activation (Thomas et al., 2000).

Thomas and colleagues first reported marked differences in basal AMPA EPSCs in NAc MSNs from cocaine sensitized animals (Thomas et al., 2001). Furthermore, NAc LTD expression was completely occluded in these sensitized animals thus suggesting that repetitive cocaine experience directly induced LTD. In a very elegant series of experiments, Wang and colleagues also reported LTD occlusion in NAc MSNs following amphetamine sensitization and dissected the mechanisms underlying neuroadaptation to psychomotor stimulants (Brebner et al., 2005). Since expression of hippocampal LTD had previously been demonstrated to be dependent upon GluR2 subunit internalization (Luscher et al., 1999), these investigators also proposed that endocytotic process as a prime mechanism whereby amphetamine experience modulated glutamatergic plasticity. The role of GluR2 internalization in expression of NAc LTD and
sensitization to amphetamine was directly tested using a peptide which disrupted internalization of GluR2-containing AMPA receptors. Active, but not inactive, peptides completely occluded NAc LTD, and the most critical observations further came from \textit{in vivo} studies. Intravenous or intra-accumbal, but not intra-VTA, injection of active, but not inactive, peptides completely occluded expression of amphetamine sensitization in previously sensitized rats (Brebner et al., 2005).

No studies investigating neuroadaptive changes in glutamatergic synaptic plasticity in the nucleus accumbens following ethanol exposure exist. This is particularly significant since, in contrast with other drugs, ethanol has a unique action to inhibit NMDA receptors and to disrupt NMDA receptor-dependent plasticity in hippocampal and other structures (Sinclair and Lo, 1986; Lovinger et al., 1989; Lovinger et al., 1990; Morrisett and Swartzwelder, 1993; Nie et al., 1993; Nie et al., 1994; Maldve et al., 2002; Zhang et al., 2005). Reports of plasticity changes in MSNs of the dorsal striatum in response to ethanol exposure do exist (Yamamoto et al., 1999; Xia et al., 2006; Wang et al., 2007); however, those involve different forms of plasticity from that involved herein. Additionally, the MSNs of the dorsal striatum are involved in habit formation and not thought to be involved in ethanol reinforcement and reward such as processed in the NAc (Everitt and Robbins, 2005). Taken together, these findings prompted us to investigate neuroadaptive changes in glutamatergic transmission in NAc medium spiny neurons following \textit{in vivo} ethanol exposure. Since induction of NAc LTD is NMDA receptor-dependent, we analyzed the direct effects of \textit{in vitro} ethanol exposure on LTD in the NAc as well.
METHODS

Brain slice preparation. Parasagittal slices (210-250 µm thick) containing the NAc were prepared from the brains of 4-8 week-old male C57BL/6J mice (Jackson Labs, Bar Harbor, ME). Mice were lightly anesthetized by inhalation of halothane, and the brains were rapidly removed and placed in ice-cold (4ºC) oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 110 choline, 25 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 7 MgCl2, 0.5 CaCl2, 25 dextrose, 11.6 Na+-ascorbate, and 3.1 Na+-pyruvate, bubbled with 95% O2/5% CO2. Slices were then transferred to an incubation ACSF for a minimum of 45-60 minutes prior to recording that contained the following (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 2.4 MgCl2, 1.8 CaCl2, 10 dextrose, bubbled with 95% O2/5% CO2; pH 7.4, 32ºC. Unless otherwise noted, all drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Patch clamp electrophysiology. We conducted all recordings at 31-33ºC in ACSF containing (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 0.9 MgCl2, 2 CaCl2, 10 dextrose, bubbled with 95% O2/5% CO2. The GABA_A receptor antagonist, picrotoxin (50 µM), was added to the external recording solution throughout all recordings to inhibit GABA_A receptor-mediated synaptic currents; this improves the reliability of synaptic plasticity in the dorsal and ventral striatum by favoring postsynaptic depolarization during conditioning stimuli (Berretta et al., 2008). Whole-cell voltage clamp recordings were obtained from NAc shell MSNs visually identified using the MRK200 Modular Imaging system (Siskiyou Corporation, Grants Pass, OR) mounted on a vibration isolation table under IR-Dodt optics. MSNs represent ~95% of the neurons in the NAc and have distinctly smaller cell bodies (about 10 µm in diameter). MSNs were also
identified by their highly negative resting membrane potential (less than -75 mV). MSNs from the most rostral and ventral areas of the NAc were chosen to make sure all recordings arose from the NAc shell subregion. Only one neuron per slice was used for recording. ACSF continuously perfused the recording chamber at 2.0-2.5 mL/min. Recording electrodes (thin-wall glass, WPI Instruments) were made using a Brown-Flaming model P-88 electrode puller (Sutter Instruments, San Rafael, CA) to yield resistances between 3-5 M\(\Omega\) and contained (in mM): 135 KMeSO\(_4\), 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Tris-GTP, (pH 7.3 with KOH). Input and access resistances were monitored throughout all experiments, and the recording was terminated if either resistance varied by more than 20%. These parameters were measured by application of a -10 mV, 100 ms voltage step at 5-10 min intervals. Synaptic currents were monitored at a holding potential of -80 mV. Changes in the holding current were observed to detect any resealing or other instability of the patch.

**Data acquisition and analysis.** Excitatory afferents, the majority of which arise from the prefrontal cortex, were stimulated with a stainless steel bipolar stimulating electrode (FHC, Inc., Bowdoin, ME) placed between the recorded MSN and prefrontal cortex, typically 150-300 \(\mu\)m from the MSN cell body. EPSCs were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, and digitized at 10-20 kHz via a Digidata 1440A interface board using pClamp 10.2 (Axon Instruments, Foster City, CA). Standard evoked EPSCs elicited by local stimulation were established in NAc shell MSNs for at least 10 minutes (at 0.1 Hz) to ensure stable recordings. LTD induction was assessed by delivering conditioning stimuli (500 pulses at 1 Hz at baseline stimulation intensity) while continuously and simultaneously depolarizing the
postsynaptic cell to -50 mV (referred to below as conditioning stimulation). EPSCs were then monitored for 30-45 minutes post-pairing (at 0.1 Hz).

Peak EPSC values were determined using Clampfit 10.2 software (Axon Instruments). For each recording, peak EPSC amplitude values were normalized to the average EPSC amplitude of the final 10 minutes of baseline (60 sweeps) for that single recording. The mean normalized EPSC amplitudes for 12 consecutive sweeps were condensed into 2 minute bins and represented as a single data point in scatter plots for each treatment group. Each data point represents the average of 12 consecutive EPSC amplitudes at that time point from each neuron within its respective treatment group.

We used two parameters to determine whether plasticity of EPSC amplitudes (either depression or potentiation) occurred. An unpaired Student's t test (p value < 0.5) was used to compare the five normalized EPSC values from 20 to 30 minutes (minutes 40-50 on figures) after the pairing protocol to the five normalized EPSC values during the last 10 minutes of baseline. In addition, the change in average EPSC amplitude after conditioning needed to be greater than 2 standard deviations from baseline. If both of these criteria were met, that treatment group was determined to exhibit plasticity.

For each experiment, the 40-50 minute time period was used to compare the magnitude of plasticity after different drug exposures. The five normalized EPSC values between min 40-50 were compared between groups using a single factor ANOVA with Bonferroni post-hoc analyses. Statistical significance for between treatment group comparisons was defined as p value < 0.05. Thus, LTD was considered the control outcome to which all drug (either in vitro or in vivo) exposures were compared. LTD was determined to be reduced and not completely blocked in situations where the post-
pairing average EPSC amplitude (min 40-50) was significantly increased from control LTD (ANOVA) and significantly decreased from its respective baseline (Student’s t test). Experiments testing different antagonists were interleaved with control experiments using slices prepared from the same animals where possible.

**Chronic Intermittent Ethanol Exposure.** Ethanol dependence was induced by exposing mice to chronic intermittent ethanol vapor (Becker and Hale, 1993; Becker and Lopez, 2004; Lopez and Becker, 2005). Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 0.2-0.3 L/min. The resulting ethanol vapor then combined with a separate air stream to give a total flow rate of around 4 L/min which was delivered to mice in special mouse chamber units (Allentown Inc., Allentown, NJ). These chambers resembled normal acrylic cages but contain an additional air-tight seal top, a vapor inlet, and an exhaust outlet. Food and water was available ad libitum on the wire cage tops. The ethanol flow rate was determined empirically to yield target blood ethanol concentrations (35-45 mM, or 150-200 mg/dL) measured from a 10 µL tail blood sample using an Analox AM1 alcohol analyzer (Analox, Lunenberg, MA). Two identical cages of mice were always run simultaneously; one cage for exposure to ethanol vapor and the second cage for an air only control. The ethanol group received a single, daily intraperitoneal injection containing both ethanol (20% v/v, 1.5 g/kg) and pyrazole (68 mg/kg) in sterile PBS. Mice were then immediately chambered and exposed to ethanol vapor or air (from 1700 to 0900 hrs daily under a reverse light/dark cycle- lights off at 1200 hrs) for three consecutive days. Air control mice received only the pyrazole injection but were otherwise handled exactly as the ethanol group. On the fourth day, animals were returned to home cages for 24 or 72 hours (depending on
experiment). On the fifth or seventh day, electrophysiological experiments were performed as described above.

**Two bottle choice drinking.** Eight-week old C57BL/6J mice (Jackson Labs) were acclimated to a 12:12 hour reverse light environment (lights off at 1200 hrs) for two weeks. The animals had free access to food and water throughout the experiment. Subjects were tested for baseline ethanol consumption (g/kg) using a two-hour, two bottle choice limited access drinking paradigm (15% ethanol/water or water choice) for 21 days, administered 30 minutes prior to the beginning of the dark cycle. Ethanol consumption was recorded for the last five days of the baseline period and used to divide subjects equally into control and experimental groups. Mice were subjected to ethanol vapor or control air chamber exposure as described above for four consecutive days followed by 74 hours of rest. Post chamber ethanol consumption was recorded for five days as before. A second round of chamber exposure was administered for four consecutive days followed by 74 hours of rest. Post chamber ethanol consumption was again recorded for five days. Statistical significance for average ethanol consumption over each five day period was defined as p < 0.05 using a student's t test with Bonferroni post-hoc analyses.
RESULTS

NMDAR-mediated LTD in the NAc shell of ethanol-naïve mice

All data in this study were gathered using whole-cell voltage clamp of MSNs solely in the mouse NAc shell region in the presence of the GABA_A receptor antagonist, picrotoxin (50 µM). Low frequency stimulation (500 pulses @ 1Hz) paired with postsynaptic depolarization to -50 mV has been reported to induce NMDA receptor-dependent LTD in the NAc core and shell (Thomas et al., 2000). Following a similar protocol involving coincident depolarization and low frequency afferent stimulation (referred to below as conditioning stimulation), we consistently observed reliable LTD of EPSCs from all ethanol-naïve MSNs examined (71.4 ± 0.7% of baseline, n=21 neurons from 15 animals, p<0.001, post-conditioning vs baseline, Fig. 1). Baseline and post-conditioning EPSCs recorded at -80 mV were solely AMPA receptor-mediated since they were completely abolished by the AMPA/kainate receptor antagonist, DNQX (6,7-dinitroquinoxaline-2,3-dione, 10 µM, supplemental data).

We measured the paired-pulse ratio (PPR) of EPSCs (2 pulses, 50ms apart) before and after conditioning stimulation to determine whether LTD expression was related to presynaptic and/or postsynaptic changes in glutamatergic transmission. In six neurons from different mice, we observed LTD using paired test stimuli identical to that of LTD using single stimuli (72.9 ± 1.4% of baseline, n=6 neurons from 6 animals, p>0.05, paired stimuli LTD vs single stimuli LTD, Fig. 1). Additionally, we observed no significant difference (p>0.05, student’s t test) in paired-pulse facilitation (PPF) between
baseline EPSCs (1.73 ± 0.18) and following induction of LTD (1.85 ± 0.18) indicating that changes in neurotransmitter release do not contribute to 1Hz-LTD in the NAc (Fig. 1C).

Non-specific or subunit-specific NMDA receptor inhibition blocks NAc LTD

Thomas and colleagues first described low frequency NAc LTD and showed its induction depended upon NMDA receptor activation (Thomas et al., 2000); therefore, we determined whether the plasticity we observed in response to conditioning was likewise dependent upon NMDA receptors. In the presence of the non-selective NMDA receptor antagonist, DL-APV (100 µM), LTD was completely blocked (96.6 ± 1.0% of baseline, n=4 neurons from 4 animals, p>0.05, vs baseline; p<0.05, vs control LTD, Fig. 2).

We also tested whether NMDA receptors containing the NR2B subunit are required for induction of NAc LTD. In presence of the specific NR2B antagonist, Ro 25-6981 (0.5 µM), LTD in ethanol-naïve mice was completely abolished (105.6 ± 1.2% of baseline, n=5 neurons from 4 animals, p<0.05, vs baseline; p<0.05, vs control LTD, vs DL-APV, Fig. 2). The small potentiation observed in the presence of Ro 25-6981 was significant from baseline; however, the magnitude of potentiation was not greater than two standard deviations from baseline, which does not meet our criteria for plasticity expression.
Acute in vitro ethanol exposure of increasing concentrations differentially inhibits NAc LTD

Ethanol is well known to inhibit NMDA receptors; therefore, we next tested whether ethanol inhibits NAc LTD expression. Bath application of a low, intoxicating concentration of ethanol (20 mM) partially but significantly reduced NAc LTD (79.7 ± 2.0% of baseline, n=6 neurons from 5 animals, p<0.05, vs baseline; p<0.05, vs control LTD, Fig. 3). LTD expression was completely inhibited by a moderately to strongly intoxicating concentration of ethanol (40 mM) equivalent to the target concentration used in the in vivo vapor model described below (104.3 ± 1.3% of baseline, n=7 neurons from 6 animals, p>0.05, vs baseline; p<0.05, vs 20 mM EtOH, vs control LTD, Fig. 3). Interestingly, when conditioning was performed in a highly intoxicating concentration of ethanol (60 mM), NAc LTD appeared similar in magnitude to that observed in the presence of the lowest concentration of ethanol (20 mM) tested (81.5 ± 0.7% of baseline, n=7 neurons from 6 animals, p<0.05, vs baseline, vs 40 mM EtOH, vs control LTD; p>0.05, vs 20 mM EtOH, Fig. 3).

Activation of dopamine D1 receptors restores LTD expression in the presence of ethanol

Previous work in our lab demonstrated that activation of D1-dopamine receptors decreased the ethanol sensitivity of NMDA receptors on MSNs in the NAc (Maldve et al., 2002; Zhang et al., 2005). Therefore, we next tested whether D1-dopamine receptor activation might antagonize the inhibitory effects of ethanol on NAc LTD. Bath application of the selective D1 receptor agonist (±SKF38393, 50 µM) did not affect the
magnitude of LTD expression in control slices (65.9 ± 1.7% of baseline, n=7 neurons from 6 animals, p<0.001, vs baseline, Fig. 4). However, pre-treatment of slices with SKF38393 for 30 minutes prior to ethanol (40 mM) application rescued LTD from acute ethanol inhibition (66.8 ± 1.5% of baseline, n=6 neurons from 5 animals, p<0.001, vs baseline, Fig. 4). When LTD magnitude was compared between the SKF only, SKF+EtOH, and control NAc LTD groups, there were no significant differences apparent (p>0.05, one-way ANOVA, Bonferroni post-hoc). Likewise, a specific D1 receptor antagonist, SCH23390 (10 µM), had no effect on LTD expression when applied alone (63.9 ± 1.8% of baseline, n=4 neurons from 3 animals, p<0.001, vs baseline, Fig. 5).

Co-application of both the D1 receptor agonist and antagonist 30 minutes prior to ethanol reversed the ability of the D1 agonist to occlude ethanol inhibition of NAc LTD. Thus, in the presence of both D1 agonist and antagonist and ethanol (40 mM), we observed NAc LTD similar to that from the ethanol alone treatment group (97.8 ± 1.7% of baseline, n=5 neurons from 4 animals, p>0.05, vs baseline, Fig. 5).

NAc LTD is completely occluded in the presence of ethanol (60mM) when D1 receptors are inhibited

We next investigated the possibility that the highest concentration of ethanol (60 mM) did not fully inhibit NAc LTD because of a subsequent release of dopamine upon ethanol application. When pre-treated for 20 minutes with the specific D1 receptor antagonist, SCH23390 (10 µM), the application of ethanol (60 mM) completely blocked NAc LTD (99.1 ± 2.6% of baseline, n=6 from 4 animals, p>0.05 vs baseline; p<0.05 vs control LTD, vs 60mM EtOH alone; Fig. 6).
Chronic intermittent ethanol exposure increases voluntary ethanol consumption in C57BL/6J mice

Repeated cycles of chronic intermittent ethanol (CIE) exposure to C57BL/6 mice will significantly increase voluntary ethanol consumption (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009). In order to determine whether NAc LTD may represent an important synaptic process that may be altered in ethanol dependence, we first developed this mouse model in our lab. We examined ethanol intake in two hour drinking bouts initiated 30 minutes prior to the dark cycle before and after two successive periods of four days of intermittent 16 hour ethanol exposure. The average baseline ethanol consumption for air vapor control and ethanol vapor groups prior to CIE exposure was not significantly different (1.51 ± 0.08 g/kg/day vs. 1.51 ± 0.05 g/kg/day, n=8 animals per group, p>0.05, student’s t test, Bonferroni post-hoc, Fig. 7). Following the first bout of CIE exposure (four days, 16 hour vapor exposure), the average ethanol intake of the ethanol vapor group was not significantly increased compared to the air vapor control group (2.90 ± 0.33 g/kg/day vs. 2.19 ± 0.23 g/kg/day, n=8 animals per group, p>0.05, student’s t test, Bonferroni post-hoc, Fig. 7). However, only the ethanol vapor group showed a significantly increased ethanol intake compared to baseline (p>0.05 for air baseline vs. air post-CIE 1; p<0.05 for ethanol baseline vs. ethanol post-CIE 1). Following the second bout of CIE exposure, the average ethanol intake of the ethanol vapor group was significantly increased compared to the air vapor control group (2.77 ± 0.13 g/kg/day vs. 1.75 ± 0.23 g/kg/day, n=8 animals per group, p<0.01, student’s t test, Bonferroni post-hoc, Fig. 7), and neither group differed
significantly between their respective post-CIE 1 average ethanol consumptions (p>0.05).

Synaptic conditioning elicits long-term potentiation following in vivo chronic intermittent ethanol vapor exposure

Repetitive exposure to psychomotor stimulating agents occludes NAc LTD (Thomas et al., 2001; Brebner et al., 2005); therefore, we next investigated whether a similar neuroadaptation occurs following intermittent ethanol exposure. The standard CIE protocol involved two periods of ethanol exposure; however, since ethanol intake was significantly increased following the first ethanol exposure period, we chose to investigate the immediate effects following the first few 16 hour bouts of intermittent ethanol exposure for three consecutive days. NAc MSNs prepared from mice 24 hours following brief CIE exposure displayed marked differences in excitatory transmission in response to the standard conditioning stimulation (Fig. 8). Normal synaptic depression was present in NAc MSNs from the air control mouse group (65.3 ± 1.1% of baseline, n=4 from 3 animals, p<0.001 vs baseline; p>0.05 vs control LTD). Instead, synaptic conditioning elicited a striking and highly significant synaptic potentiation of EPSC amplitudes in NAc MSNs prepared 24 hours following CIE treatment (124.9 ± 1.3% of baseline, n=8 neurons from 8 animals, p<0.05, vs baseline; p<0.001, vs air control). Furthermore, DL-APV (100 µM) completely abolished this synaptic potentiation observed in slices from CIE-exposed mice (91.2 ± 1.6% of baseline, n=7 neurons from 4 animals, p<0.05, vs baseline; p<0.05, vs air control, vs 24hrs post-CIE, Fig. 9). This small depression did satisfy our criteria for plasticity, yet it was still significantly different from
air control LTD. Finally, we assessed NAc plasticity in slices from mice that had been allowed 72 hours of recovery after the conclusion of their CIE exposure to investigate the endurance of these synaptic changes (Fig. 9). In these mice, conditioning elicited a slight, yet significantly less depression of synaptic transmission than air control LTD (91.1 ± 0.9% of baseline, n=5 neurons from 3 animals, p<0.05, vs baseline, vs 24hr post CIE, vs air control; p>0.05 vs CIE-APV).
DISCUSSION

This report includes the following novel observations. First, that synaptic depression of glutamatergic excitatory transmission onto medium spiny neurons of the shell of the nucleus accumbens requires activation of NR2B-containing NMDA receptors. Second, acute ethanol exposure blocks NAc LTD in a differential manner depending on the concentration. Third, activation of D1-dopamine receptors completely occludes the ability of ethanol to inhibit NAc LTD. Fourth, a repeated regimen of intermittent ethanol exposure enhances voluntary ethanol intake in C57BL/6J mice, and such intermittent exposure induces NAc metaplasticity from LTD to LTP. Occlusion of both NAc LTD and LTP is retained for at least 72 hours following intermittent ethanol exposure.

NAc LTD, in vitro ethanol exposure and the role of NMDA and dopamine D1 receptors

Medium spiny neurons rest at more hyperpolarized membrane potentials (a “down-state” ~ -80 mV) and display transitions to a depolarized potential (the “up-state ~ -50 mV) coupled with an ensemble of action potentials. When differing patterns of conditioning stimulation are delivered to NAc MSNs at least three forms of synaptic depression may result (Berretta et al., 2008). The focus of this report is the only form expressed via NMDA receptor activation and decreased postsynaptic AMPA receptor function (Brebner et al., 2005). Two forms of presynaptic LTD, independent of NMDA receptor activation, have been demonstrated – these are induced by endocannabinoid or metabotropic glutamate receptor activation, are due to decreased glutamate release,
and occur following delivery of higher frequency synaptic stimuli than that used during NMDA-LTD (Robbe et al., 2002a; Robbe et al., 2002b).

In the present study, we also observed that the low frequency form of postsynaptic NAc LTD is NMDA receptor-dependent and additionally showed that this LTD is mediated by NR2B subunit-containing NMDA receptors. This is a novel result not previously documented concerning NAc LTD, yet it is not surprising since NR2B-containing receptors are highly expressed and targeted on NAc MSNs (Chapman et al., 2003; Dunah and Standaert, 2003). We did not investigate the involvement of NR2A-containing NMDARs in LTD expression given the lack of a specific pharmacological tool (Neyton and Paoletti, 2006). There is limited involvement of NR2C or NR2D-containing NMDA receptors since their NAc expression is nearly non-existent at this age (Standaert et al., 1994; Landwehrmeyer et al., 1995; Ghasemzadeh et al., 1996). The NR2A and NR2B subunits have been implicated to differentially contribute to LTP and LTD respectively in several brain regions (Liu et al., 2004; Massey et al., 2004; Zhao et al., 2005); however, these conclusions are controversial and specific contribution of one particular NMDA receptor subunit to exclusively LTD or LTP may not hold true in every instance (Berberich et al., 2005; Weitlauf et al., 2005; Morishita et al., 2007). Thus, we cannot derive any conclusions about the contributions of other subunits besides NR2B to LTD expression in the NAc and future studies will have to address the possibility.

Our observations demonstrate that pharmacologically-relevant concentrations (40 mM) of ethanol completely inhibit the expression of LTD when applied in vitro to a slice preparation from ethanol-naïve mice. Evidence exists for both promoting and inhibiting effects of ethanol on LTD expression in other brain regions including the
hippocampus, dorsal striatum, and cerebellum (Hendricson et al., 2002; Izumi et al., 2005; Yin et al., 2007; Belmeguenai et al., 2008). These reports certainly support the likelihood that ethanol modulates NAc LTD as we observe, but the different underlying mechanisms of plasticity expression interject a degree of complexity that tempers direct comparisons between these different reports.

Given the extensive literature that demonstrates ethanol inhibition of NMDA receptors, it is likely that a similar inhibition of NMDA receptors is the primary mechanism through which ethanol occludes the expression of NAc LTD. Excitatory transmission onto NMDA receptors is reduced by acute ethanol application in slice preparations of the hippocampus (Lovinger et al., 1989; Lovinger et al., 1990) and the nucleus accumbens (Nie et al., 1993; Nie et al., 1994; Maldve et al., 2002; Zhang et al., 2005; Wang et al., 2007). Our results indicate that both NR2B inhibition and application of ethanol prevent the expression of LTD, and it is tempting to speculate that ethanol blocks LTD expression by predominantly interacting with NR2B-containing NMDA receptors.

Our lab and others previously observed that activation of D1 receptors strongly suppressed the inhibitory effects of ethanol on NMDA receptor-mediated synaptic transmission dependent upon activation of DARPP-32 and phosphorylation of serine 897 of the NR1 subunit (Maldve et al., 2002; Zhang et al., 2005; Lin et al., 2006) but also see (Xu and Woodward, 2006). In the present report, we again observed that prior activation of D1-dopamine receptors rescues the expression of LTD in the NAc in the presence of ethanol. Thus, NAc LTD is ethanol-sensitive and displays characteristics consistent with NMDA receptor-mediated processes documented previously. One
major goal is to determine how pharmacological manipulation of these processes can mitigate the long term effects of ethanol exposure and therefore may be therapeutic in ethanol dependence; hence, modulation of D1-dopamine signaling may give insights to novel therapeutic targets. Furthermore, the observation that a high concentration of ethanol (60 mM) did not occlude NAc LTD is consistent with the idea that such concentrations of ethanol can induce dopamine release (Brodie et al., 1990; Brodie et al., 1999) and DARPP-32 phosphorylation directly (Maldve et al., 2002). Both of these effects therefore increase serine 897-NR1 phosphorylation and thereby decrease ethanol sensitivity of NMDA receptors (Maldve et al., 2002; Zhang et al., 2005; Lin et al., 2006). Therefore, the observation of NAc LTD in the presence of a high concentration of ethanol, which is occluded by D1 receptor inhibition, is consistent with prior observations.

*In vivo chronic ethanol exposure increases intake and significantly alters NAc LTD*

One major hindrance for basic research into the neurobiological mechanisms of alcoholism has been the difficulties associated with development of a convenient animal model. Recently, a model based upon two 3-4 day regimens of passive ethanol administration via peripheral injection and subsequent 16 hour vapor inhalation (termed chronic intermittent exposure) to C57BL/6 mice has become widely adopted (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009). In this study, we observed significant increases in voluntary ethanol consumption in our own cohort of animals following two bouts of CIE exposure. To test our hypothesis—as seen with chronic exposure to psychomotor stimulants—that occlusion of NAc LTD will result
following CIE, we assessed the response of NAc MSNs to conditioning following a single bout of CIE as a baseline to determine the minimal level of ethanol exposure which might occlude LTD. Indeed, we were surprised that such a profound metaplasitcity—a switch from LTD to LTP—occurred following such a brief ethanol exposure bout. We interpret such robust changes in NAc plasticity as an indicator of the potential importance of this process in ethanol neuroadaptation and therefore chose to first characterize this initial change in response to short-term CIE. Indeed, after a single 3-day cycle of this CIE model, we contend that significant alterations in glutamatergic synaptic plasticity in the NAc have already begun.

While we observed increases in drinking at these short exposure durations, Becker and colleagues demonstrated that the maximum increases in voluntary ethanol consumption in mice are not observed until three days after withdrawal from CIE exposure. Thus, considerable study will be required to determine the temporal characteristics of the consolidation of this neuroadaptive response. Our results indicate that synaptic potentiation has subsided, while LTD remains occluded in the NAc shell at this same time point. Just as multiple CIE exposures are necessary to observe a sustained increase in ethanol consumption, we believe synaptic potentiation also persists after multiple CIE exposures. The current study indicates that synaptic activity is disrupted in the NAc shell at an early time point in a model of ethanol dependence. Future studies will address whether alterations in synaptic plasticity in the NAc shell directly contribute to the behaviors that lead toward increased voluntary ethanol consumption. The matter is made more complex since psychostimulants and ethanol have fundamentally distinct effects on NAc LTD. Chronic exposure to either drug
appears to artificially induce an LTD-like state via occlusion of the response to in vitro conditioning stimulation. However, the unique NMDA receptor blocking actions of ethanol result in an additional potentiating action; thus, we contend that alterations in NAc shell glutamatergic synaptic function due to CIE exposure are fundamentally distinct from those of psychostimulants. Nonetheless, disruption of GluR2 internalization suppressed the sensitized response to psychostimulants (Brebner et al., 2005), and therefore, the likelihood remains that LTD occlusion is critical to ethanol-seeking behavior. However, the ethanol-dependent state may conceivably be further and uniquely driven by the resultant LTP and subsequent enhancement of glutamatergic transmission. Could blockade of synaptic potentiation in the NAc after CIE exposure prevent an increase in voluntary ethanol consumption? Such possibilities deserve investigation. It is hoped that these results provide novel insights into the relevant contributions of NAc synaptic plasticity in the complex array of neuroadaptations that re-wire the motivational circuitry in the ethanol-dependent animal.
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LEGENDS FOR FIGURES

Figure 1. Low frequency stimulation induces LTD in the NAc shell. A, conditioning stimulation (500 pulses @ 1Hz with concurrent depolarization to -50 mV, denoted as “pairing”) induces long-term depression of evoked AMPA receptor-mediated EPSCs in NAc shell MSNs of ethanol-naïve mice (71.4 ± 0.7% of baseline, n=21 neurons from 15 animals, ***p<0.001, post-pairing vs baseline). Each data point represents an average of 12 consecutive normalized EPSC amplitudes condensed into 2 minute bins (% Baseline ± SEM) from each neuron studied at that time point. Pairing stimulation also induced LTD when paired EPSCs (50 ms apart) were evoked during baseline and post-pairing (72.9 ± 1.4% of baseline, n=6 neurons from 6 animals, p<0.001, vs baseline; p>0.05, vs control LTD). B, sample EPSC traces from a single neuron. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). Bar graph representing the percentage change ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50). C, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from single and paired-pulse groups. Scale bars represent 20 ms (horizontal) and 50 pA (vertical). Bar graph represents the paired-pulse ratio (PPR) ± SEM between baseline and post-pairing. PPR determined by dividing the amplitude of EPSC 2 by EPSC1 for each sweep. Average PPR during baseline and post-pairing were not significantly different (1.73 ± 0.18 and 1.85 ± 0.18 respectively, p>0.05).
Figure 2. **NR2B-containing NMDA receptors required for NAc LTD expression.** A, pairing stimulation does not induce LTD expression in the presence of the non-selective NMDA receptor antagonist, DL-APV (100 µM), (96.6 ± 1.0% of baseline, n=4 neurons from 4 animals, p>0.05, vs baseline) or the NR2B subunit-selective NMDA receptor antagonist, Ro 25-6981 (0.5 µM), (105.6 ± 1.2% of baseline, n=5 neurons from 4 animals, p<0.05 vs baseline, vs DL-APV). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug exposure group.

Figure 3. **In vitro ethanol exposure, at increasing concentrations, differentially alters NAc LTD expression.** A, escalating concentrations of ethanol applied to the recording bath have varying effects on the magnitude of LTD. Maximum inhibition of LTD expression is achieved through application of ethanol (40 mM) (104.3 ± 1.3% of baseline, n=7 neurons from 6 animals, p>0.05, vs baseline). In the presence of ethanol (20 mM) and (60 mM), pairing stimulation induces an LTD magnitude that is decreased from control LTD but not completely occluded (79.7 ± 2.0% of baseline, n=6 neurons from 5 animals, *p<0.05, vs baseline, † p<0.05 vs 40mM) and (81.5 ± 0.7% of baseline, n=7 neurons from 6 animals, *p<0.05, vs baseline; † p<0.05 vs 40 mM; p>0.05, vs 20 mM) respectively. B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each ethanol exposure group.
exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each ethanol exposure group.

Figure 4. D1 receptor activation occludes ethanol (40 mM) inhibition of NAc LTD. A, pre-treatment (30 min) of NAc slices with the D1 receptor agonist, (±)SKF38393 (50 µM), reverses ethanol inhibition of LTD expression (66.8 ± 1.5% of baseline, n=6 neurons from 5 animals, ***p<0.001, vs baseline; p<0.05, vs 40mM EtOH alone). Application of SKF38393 alone did not have any effect on LTD expression (65.9 ± 1.7% of baseline, n=7 neurons from 6 animals, ***p<0.001, vs baseline; p>0.05, vs SKF+EtOH). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug exposure group.

Figure 5. Ethanol recovers inhibition of NAc LTD when D1 activation is occluded. A, bath application of the D1 receptor antagonist, SCH23390 (10 µM), prior to SKF38393 pre-treatment, permits inhibition of LTD expression by ethanol (40 mM) similar to that observed with ethanol alone (97.8 ± 1.7% of baseline, n=5 neurons from 4 animals, p>0.05, vs baseline; † p<0.05 vs SCH23390 alone). SCH23390 alone does not alter the magnitude of LTD compared to control (63.9 ± 1.8% of baseline, n=4 neurons from 4 animals, p>0.05, vs baseline).
neurons from 3 animals, ***p<0.001, vs baseline). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline, mean ± SEM, for average normalized EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug exposure group.

Figure 6. NAc LTD is completely inhibited by highest ethanol concentration (60mM) when D1 receptor activation is blocked. A, bath application of the D1 receptor antagonist, SCH23390 (10 µM), 20 min prior to ethanol (60 mM) completely occludes NAc LTD (99.1 ± 2.6% of baseline, n=6 neurons from 4 animals, p>0.05, vs baseline; p<0.05 vs 60mM EtOH alone). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative recording from all neurons recorded. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for this drug exposure group.

Figure 7. C57BL/6J mice increase voluntary ethanol consumption following two CIE exposures. A, average daily EtOH intake (g/kg/day) in a 2 hour drinking bout begun 30 min prior to start of dark cycle over 5 days in a baseline (pre-vapor exposure) and following 2 successive periods of 4 days of 16 hr EtOH or air vapor exposure. Baseline EtOH intake was measured during the last five days of the 21-day baseline period. The CIE vapor exposure periods were delivered over days 6-9 and 18-21.
Animals rested 74 hrs after the final vapor exposure before resuming drinking; *p<0.05 ethanol vs air group. B, bar graph representing average EtOH intake (g/kg/day), shown as mean ± SEM, for air and EtOH vapor treatment groups measured over each 5-day drinking period. Baseline ethanol intake did not differ between the air (1.51 ± 0.08) and EtOH vapor (1.51 ± 0.05) groups (p>0.05, student’s t test, Bonferroni post-hoc). Only the ethanol vapor group significantly increased ethanol consumption after the first CIE vapor treatment compared to baseline (air, 2.19 ± 0.23, EtOH, 2.90 ± 0.33; *p<0.05). Following the second CIE treatment, the air group (1.75 ± 0.23) showed significantly lower ethanol intake than the EtOH group (2.77 ± 0.13) (†† p<0.01) (**p<0.01, ethanol post-CIE 2 vs baseline). N=8 animals per group.

Figure 8. CIE vapor exposure converts NAc LTD to synaptic potentiation. A, 24 hours following three consecutive days in vivo CIE vapor exposure, pairing stimulation induces synaptic potentiation rather than LTD of EPSCs (124.9 ± 1.3% of baseline, n=8 neurons from 8 animals, *p<0.05, vs baseline; † p<0.05 vs air vapor control). In the air vapor control group, pairing stimulation induces a similar LTD to that observed in the ethanol-naïve control group (65.3 ± 1.1% of baseline, n=4 neurons from 3 animals, ***p<0.001 vs baseline). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each vapor (air or ethanol) exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each vapor (air or ethanol) exposure group.
Figure 9. Synaptic potentiation following CIE exposure is NMDAR-dependent and dissipates after 72 hours withdrawal. A, 24 hours following three consecutive days in vivo CIE vapor exposure, synaptic potentiation is blocked by the NMDAR antagonist, DL-APV (100 µM), (91.2 ± 1.6% of baseline, n=7 neurons from 4 animals, *p<0.05, vs baseline, vs air vapor control, vs CIE EtOH 24hrs). Following 72 hours of withdrawal from the identical CIE vapor exposure, pairing stimulation does not induce either synaptic potentiation or LTD (91.1 ± 0.9% of baseline, n=5 neurons from 3 animals, *p<0.05, vs baseline, vs air vapor control, vs CIE EtOH 24hrs; p>0.05, vs CIE+APV). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug/vapor exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug/vapor exposure group.
Figure 1

(A) Graph showing the EPSC Amplitude (% Baseline) over time for EtOH-naïve control (n=21 neurons) and Control w/ Paired Pulse (n=6 neurons).

(B) Control graph showing EPSC Amplitude (% Baseline) with baseline and post-pairing data.

(C) Paired Pulse graph showing baseline and post-pairing data for paired pulse ratio.
Figure 2

A

EPSC Amplitude (% Baseline)

0 10 20 30 40 50

DL-APV (100μM) (n=4 neurons)
Ro 25-6981 (0.5μM) (n=6 neurons)

pairing

Time (minutes)

B

DL-APV
Ro 25-6981

C

EPSC Amplitude (% Baseline)

25 50 75 100 125

DL-APV
Ro 25-6981
Post-Pairing (min 40-50)
Figure 3

A

EPSC Amplitude (% Baseline)

Δ EtOH (20mM) (n=6 neurons)
■ EtOH (40mM) (n=7 neurons)
○ EtOH (60mM) (n=7 neurons)

pairing

1

2

Time (minutes)

B

20mM
40mM
60mM

1

2

C

EPSC Amplitude (% Baseline)

20mM
40mM
60mM

Post-Pairing (min 40-50)

*↑
Figure 4
Figure 5
Figure 6
Figure 7
Figure 9
Ethanol-naïve cells @ -80mV; EPSCs recorded are AMPA receptor-mediated; Ethanol does not alter baseline AMPA EPSCs