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CELL TYPE-SPECIFIC SYNAPTIC ENCODING OF ETHANOL EXPOSURE IN THE NUCLEUS ACCUMBENS SHELL

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Abstract

Synaptic alterations in the nucleus accumbens (NAc) are crucial for the aberrant reward-associated learning that forms the foundation of drug dependence. Altered glutamatergic synaptic plasticity, in particular, is thought to be a vital component of the neurobiological underpinnings of addictive behavior. The development of bacterial artificial chromosome-eGFP (enhanced green fluorescent protein) transgenic mice that express eGFP driven by endogenous D1 dopamine receptor (D1R) promoters has now allowed investigation of the cell type-specific synaptic modifications in the NAc in response to drugs of abuse. In this study, we used whole-cell *ex vivo* slice electrophysiology in *Drd1*-eGFP mice to investigate cell type-specific alterations in NAc synaptic plasticity following ethanol exposure. Electrophysiological recordings were made from eGFP-expressing medium spiny neurons (D1+ MSNs) and non-eGFP expressing (putative D2 receptor-expressing) (D1– MSNs) from the shell subregion of the NAc. We observed low frequency-induced long-term depression (1Hz-LTD) of α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)-mediated excitatory postsynaptic currents (EPSCs) solely in D1+ MSNs. However, 24 hours following 4 consecutive days of *in vivo* chronic intermittent ethanol (CIE) vapor exposure, 1Hz-LTD was conversely observed only in D1– MSNs, and now absent in D1+ MSNs. Complete recovery of the baseline plasticity phenotype in both cell types required a full 2 weeks of withdrawal from CIE vapor exposure. Thus, we observed a cell type-specificity of synaptic plasticity in the NAc shell, as well as, a gradual recovery of the pre-ethanol exposure plasticity state following extended withdrawal. These changes highlight the adaptability of NAc shell MSNs to the effects of ethanol exposure and may represent critical neuroadaptations underlying the development of ethanol dependence.

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Keywords

synaptic plasticity; drug dependence; mesocorticolimbic; long-term depression; metaplasticity; neuroadaptation

The nucleus accumbens (NAc) is an important brain region of convergence for the widespread projections involved in reward processing and guidance of goal-directed behaviors (Ikemoto, 2007). In the NAc, γ -aminobutyric acid (GABA)-ergic medium spiny neurons (MSNs) receive dopaminergic innervations from the ventral tegmental area and glutamatergic inputs from the prefrontal cortex, hippocampus, and amygdala. Limbic region afferents to the NAc interface with motor control circuitry to regulate goal-directed behavior (Mogenson et al., 1980; Nicola et al., 2000; Wise, 2004). Persistent adaptive changes within the NAc in response to drugs of abuse are posited to underlie drug dependence (Koob and Le Moal, 2001; Kauer and Malenka, 2007). These adaptations involve neuronal signaling and synaptic mechanisms similar to those implicated in neural models of learning and memory, in particular long-term synaptic plasticity (Nestler, 2001; Hyman et al., 2006). Specifically, long-term depression (LTD) of α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)-mediated excitatory postsynaptic currents (EPSCs) normally present in drug-naïve animals is absent in MSNs from animals sensitized to the behavioral effects of psychostimulants (Thomas et al., 2001; Brebner et al., 2005). In addition, LTD in the NAc core remains occluded in rats that had self-administered cocaine 21 days earlier (Martin et al., 2006). Thus, it is increasingly apparent that drug-induced modifications of synaptic plasticity may share a common mechanism that underlies drug dependence.

NAc MSNs are commonly divided into two major categories based on their expression of releasable peptides, dopamine receptor subtype expression, and their axonal projection targets (Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1990). Dopamine D1 receptor (D1R)-expressing and dopamine D2 receptor (D2R)-expressing MSNs characterize the striatonigral (direct) and striatopallidal (indirect) pathways, respectively (Alexander et al., 1986; Groenewegen et al., 1999). To facilitate investigation into the functional differences between D1R- and D2R-expressing MSNs, bacterial artificial chromosome transgenic mice in which expression of enhanced green fluorescent protein (eGFP) is controlled by D1R or D2R promoters have been created (Gong et al., 2003). Studies capitalizing on this advancement provide insights into the specific synaptic characteristics of both MSN subtypes in the dorsal striatum and NAc core (Cepeda et al., 2008; Grueter et al., 2010). One such report described how activation of D1+ MSNs in the NAc enhances cocaine sensitization and conditioned place preference (CPP), whereas activating D2+ MSNs diminishes these behaviors (Lobo et al., 2010). Additionally, aberrant expression of *N*-methyl-D-aspartate receptor (NMDAR) LTD in the NAc may facilitate the transition of rats to an “addicted” behavioral state (Kasanetz et al., 2010).

While there is extensive literature detailing how alterations in NAc signaling are important in behavioral responses to psychostimulants, reports investigating NAc synaptic plasticity and ethanol are few. In fact, our lab published the first report demonstrating a disruption in NAc shell NMDAR-dependent LTD following *in vivo* ethanol exposure. We observed that

chronic intermittent ethanol (CIE) exposure reverses the polarity of synaptic plasticity from depression to potentiation (Jeanes et al., 2011). In addition, modulation of ethanol-related behaviors can occur in a dopamine receptor-specific fashion within the NAc. One study demonstrated that siRNA-mediated downregulation of D1 receptors in the NAc decreases ethanol intake and preference, behavioral sensitization, and acquisition of ethanol-induced CPP (Bahi and Dreyer, 2012); while another study showed that blocking D1 receptors in the NAc dose-dependently attenuates reinstated ethanol seeking in rats (Chaudhri et al., 2009). Taken together, these observations formed the impetus for our investigation of the cell type-specificity of NAc shell LTD and its potential conversion to synaptic potentiation subsequent to CIE vapor exposure.

In the current study, we used *Drd1*-eGFP transgenic mice expressing eGFP in direct pathway D1 receptor-expressing MSNs (D1+ MSNs) to determine synaptic properties in both D1+ and non-eGFP expressing (putative D2 receptor-expressing) (D1- MSNs). We investigated the cell type-specific expression of low-frequency-induced LTD (1 Hz-LTD) in the NAc shell of ethanol-naïve and CIE-exposed mice at several withdrawal time points. Collectively, we aimed to determine whether significant alterations in synaptic plasticity occurred in cell type-specific manner either before or after CIE exposure. Additionally, we sought to detail how quickly disruptions in NAc 1Hz-LTD following CIE exposure recovered to pre-CIE levels after extended withdrawal. These findings could provide crucial insight into the cell type-specific synaptic adaptations within the NAc shell thought to contribute to the development of ethanol dependence.

Experimental Procedures

Subjects

Dopamine D1a receptor (*drd1a*) promoter-dependent eGFP BAC transgenic mice, generated by the GENSAT (Gene Expression Nervous System Atlas) project, were purchased from the Mutant Mouse Regional Resource Center and outcrossed onto the Swiss Webster background to create hemizygous progeny. Mice were housed under a 12-h light/dark cycle (lights on at 0700 hours) and cared for by the University of Texas at Austin Animal Resource Center. Food and water were available *ad libitum*, and all of the following experimental procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

Brain Slice Preparation

Parasagittal slices (210–250 μ m thick) containing the NAc were prepared from the brains of 4–8 week-old male mice from either the C57BL/6J mouse line (The Jackson Laboratory, Bar Harbor, ME) or our in-house mouse line derived from hemizygous *Drd1*-eGFP transgenic mice backcrossed onto the Swiss Webster background (Harlan Laboratories, Indianapolis, IN). Mice were lightly anesthetized with isofluorane, and the brains were rapidly removed and placed in ice-cold (4°C) oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 87 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 25 dextrose, 75 sucrose, bubbled with 95% O₂/5% CO₂. Sagittal slices were cut and then transferred to an incubation ACSF for a minimum of 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, 32°C. Unless otherwise noted, all drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Patch Clamp Electrophysiology

We conducted all recordings at 31–33°C 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 and improve 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 and current clamp recordings were obtained from NAc shell eGFP-expressing MSNs (referred to as D1+ MSNs) and non-eGFP expressing MSNs (referred to as D1– MSNs) visually identified using a mercury lamp with a GFP filter on an Olympus BX-50WI microscope (Leeds Instruments, Irving, TX) mounted on a vibration isolation table. MSNs represent ~90–95% of the neurons in the NAc and have distinctly smaller cell bodies (about 10 μm in diameter) than cholinergic or GABA interneurons. MSNs were also identified by their highly negative resting membrane potential (less than –75 mV), presence of inward rectification to hyperpolarizing current injection, and prolonged ramp to firing first action potential. MSNs from the most rostral and ventral areas of the NAc were chosen to make sure all recordings arose from the NAc shell. Only one neuron per slice was used for recording and once 1 Hz-LTD induction stimuli were initiated in a slice, that slice was discarded whether the recording lasted the full length or not. 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 MΩ and contained (in mM): 135 KMeSO₄, 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.

For the experiments using the active (GluR2_{3Y}) and inactive (GluR2_{3A}) peptides to disrupt AMPAR internalization, these reagents were obtained by generous gift of Yu Tian Wang at the University of British Columbia. The peptides were delivered by bath perfusion at 10 μM and delivered as the Tat-protein congener. Thus, GluR2_{3Y} constituted of 9 amino acids (YKEGYNVYG), and was attached to an HIV-1 Tat peptide (YGRKKRRQRRR) to enable the peptide to permeate cells. The scrambled peptide, Tat-GluR2, comprised the same 9 amino acids in random sequence (VYKYGGYNE) and served as a control. These peptides have been documented to modulate GluR2 internalization and behavioral sensitization to psychostimulants previously (Brebner et al., 2005; Choi et al., 2013). The concentration selected (10 μM) was determined by the Wang lab in preliminary studies to maximally prevent AMPA internalization.

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 μm 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 excitatory postsynaptic currents (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 “pairing stimulation”). EPSCs were then monitored for 30–45 minutes post-pairing (at 0.1 Hz).

Peak EPSC amplitude values were determined using Clampfit 10.2 software (Axon Instruments). For each plasticity 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. A paired student's t test (p value < 0.05) was used to compare the normalized EPSC amplitudes from 20 to 30 minutes (minutes 40–50 on figures) after the pairing protocol to the 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 (either depression or potentiation).

For each experiment, the 40–50 minute time period was used to compare the magnitude of plasticity after different drug exposures. Each cell was given a single average normalized EPSC value from min 40–50, which were then compiled for each experimental group and 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

Mice were exposed to chronic intermittent ethanol vapor in a manner that has been demonstrated to induce ethanol dependence (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 95–120 mL/min. The resulting ethanol vapor then combined with a separate air stream to give a total flow rate of 2.5–3 L/min which was delivered to mice in airtight mouse chamber units (Allentown Inc., Allentown, NJ). These chambers resembled normal acrylic cages but contain an additional airtight 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 μ L tail blood sample using an Analox AM1 alcohol analyzer (Analox, Lunenburg, 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 four consecutive days. Air control mice received only the pyrazole injection but were otherwise handled exactly as the ethanol group. On the fifth day, animals were returned to home cages for 24-h, 72-h, 1 week, or 2 weeks (depending on experiment). On the day corresponding to each specific withdrawal time point, electrophysiological experiments were performed as described above.

Results

Synaptic Plasticity of NAc Shell MSNs in Slices from C57BL/6J Mice

Low frequency stimulation (500 pulses @ 1 Hz) paired with postsynaptic depolarization to -50 mV (denoted “pairing”) induces an NMDA receptor-dependent LTD of AMPA-mediated responses in the NAc (Thomas et al., 2000; Brebner et al., 2005; Jeanes et al., 2011). The impetus of the current study stemmed from our suspicion that the observed control LTD magnitudes from our prior study (Jeanes et al., 2011) were derived from two distinct populations of MSNs which displayed distinctly different time courses of LTD expression and degree (separated by 2 standard deviations on the basis of the final degree of synaptic depression observed; Figure 1A). Furthermore, we confirmed a bimodal distribution of the average “post-pairing” EPSC amplitudes (minutes 40–50 of electrophysiological recording) upon post-hoc analysis of those data from C57BL/6J mice (Figure 1B). To further characterize 1Hz-LTD of EPSCs in MSNs from the NAc shell of C57BL/6J mice, we obtained a peptide derived from the C-terminus of the AMPA receptor GluR2 subunit (denoted GluR2_{3Y}) that inhibits clathrin-mediated AMPA receptor endocytosis and NMDAR-dependent LTD in the NAc (Brebner et al., 2005; Choi et al., 2013). Following pre-treatment of slices (45–120 minutes) with the active form of this “interference” peptide (10 μ M), we did not observe 1-Hz LTD in NAc shell MSNs ($108.68 \pm 7.75\%$ of baseline; $n=5$; student's t test: $p > 0.05$ vs baseline). Yet, in the presence of an inactivated form of the peptide (denoted GluR2_{3A}, 10 μ M), we did observe 1Hz-LTD ($60.80 \pm 9.29\%$ of baseline; $n=5$; student's t test: $p < 0.001$ vs baseline; one-way ANOVA: $F_{(1,8)} = 19.59$, $p = 0.002$ vs GluR2_{3Y}) (Figure 2).

NMDA Receptor-Mediated 1 Hz-LTD is Preferentially Expressed by D1+ MSNs in the NAc Shell of *Drd1*-eGFP transgenic mice

While recording from either D1+ or D1– MSNs in NAc shell of *Drd1*-eGFP transgenic mice, we used an identical stimulation protocol to induce synaptic plasticity (1Hz-LTD) as that described in the previous section. In all of the following experiments, the 40–50 minute time period of each recording (denoted “2” on figures) was used to determine either presence of synaptic plasticity within a group (compared to baseline, minutes 0–10, denoted “1” on figures) or differences in plasticity expression between different experimental groups. We observed a robust LTD of EPSCs in D1+ MSNs ($64.02 \pm 5.63\%$ of baseline; $n=8$; student's t test: $p < 0.001$ vs baseline). Neither depression nor potentiation was observed in the D1– MSNs ($90.99 \pm 4.23\%$ of baseline; $n=7$; student's t test: $p = 0.08$ vs baseline) (Figure 3A). LFS 1 Hz-LTD in D1+ MSNs differed significantly from D1– MSNs (one-way ANOVA: $F_{(1,13)} = 13.99$, $p = 0.002$). In the presence of the NMDA receptor antagonist, DL-APV (100 μ M), 1 Hz-LTD was completely occluded ($95.35 \pm 1.56\%$ of baseline; $n=6$; one-way ANOVA: $F_{(1,12)} = 22.03$, $p = 0.001$ vs D1+ control) (Figure 3B). Similarly, perfusion of ethanol (40 mM) into the recording chamber blocked 1 Hz-LTD in D1+ MSNs ($100.29 \pm 7.27\%$ of baseline; $n=7$; $F_{(1,13)} = 17.37$, $p = 0.001$ vs D1+ control) (Figure 3C).

Activation of dopamine D1 receptors on D1+ MSNs via bath application of the selective D1-like receptor agonist, $\pm$ SKF38393 (50 μ M), did not significantly alter the magnitude of LTD expression in D1+ MSNs ($71.86 \pm 5.96\%$ of baseline; $n=5$; student's t test: $p < 0.05$ vs baseline; one-way ANOVA: $F_{(1,11)} = 0.89$, $p = 0.37$ vs D1+ control) (Figure 3D). Baseline AMPA-mediated EPSC amplitudes of D1+ MSNs were also unaffected by the presence $\pm$ SKF38393 (50 μ M) (Control, 177 pA vs. SKF, 173 pA; data not shown). However, in the presence of the selective D1-like receptor antagonist, SCH23390 (10 μ M), we observed a slight reduction in 1-Hz LTD in D1+ MSNs ($80.26 \pm 1.71\%$ of baseline; $n=5$; student's t test: $p < 0.001$ vs baseline; one-way ANOVA: $F_{(1,13)} = 5.88$, $p = 0.03$ vs D1+ control) (Figure 3E). Yet, the degree of LTD of EPSCs was not different when the D1 agonist and antagonist were compared (one-way ANOVA: $F_{(1,8)} = 2.26$, $p = 0.17$ SKF vs SCH). A summary of the average normalized post-pairing EPSC amplitudes between minutes 40–50 in all groups is shown (Figure 3F).

LTD Expression Switches from D1+ to D1– MSNs Following *In Vivo* Chronic Intermittent Ethanol Exposure and Recovers Over Extended Withdrawal

We next tested whether disruptions in synaptic plasticity expression following CIE vapor exposure occurred preferentially in either D1+ or D1– MSNs. In an Air vapor control group, we observed 1 Hz-LTD in D1+ MSNs ($60.82 \pm 6.75\%$ of baseline; $n=5$; student's t test: $p < 0.01$ vs baseline; one-way ANOVA: $F_{(1,11)} = 0.14$, $p = 0.72$ vs D1+ control) but not in D1– MSNs ($93.01 \pm 4.57\%$ of baseline; $n=5$; student's t test: $p < 0.01$ vs baseline; one-way ANOVA: $F_{(1,10)} = 0.11$, $p = 0.75$ vs D1– control; one-way ANOVA: $F_{(1,8)} = 19.51$, $p = 0.002$ vs D1+ Air Vapor) (Figure 4A) consistent with our findings of 1Hz-LTD in D1+, but not D1– MSNs from ethanol-naïve mice.

In contrast to Air vapor controls, we observed a reversal of cell type-specific 1 Hz-LTD expression in slices prepared from mice 24 hours following 4 consecutive days of *in vivo* CIE vapor exposure. LFS 1 Hz- LTD was not observed in D1+ MSNs ($96.31 \pm 4.61\%$ of baseline; $n=7$; student's *t* test: $p > 0.05$ vs baseline; one-way ANOVA: $F_{(1,9)} = 26.73$, $p < 0.001$ vs D1+ Air Vapor); whereas, 1 Hz-LTD was elicited in D1- MSNs ($56.18 \pm 6.97\%$ of baseline; $n=7$; student's *t* test: $p < 0.01$ vs baseline; one-way ANOVA: $F_{(1,10)} = 19.02$, $p = 0.001$ vs D1- Air Vapor; one-way ANOVA: $F_{(1,12)} = 26.95$, $p < 0.001$ vs D1+ 24hr WD) (Figure 4B).

Increased withdrawal (72 hours) from CIE vapor exposure resulted in modest synaptic depression of EPSCs in D1+ MSNs ($83.95 \pm 4.58\%$ of baseline; $n=5$; student's *t* test: $p < 0.05$ vs baseline) that had yet to return to Air vapor control levels (one-way ANOVA: $F_{(1,9)} = 10.53$, $p = 0.01$ vs D1+ Air Vapor). In D1- MSNs, 1 Hz-LTD of EPSCs remained at 72 hours withdrawal ($75.54 \pm 5.16\%$ of baseline; $n=5$; student's *t* test: $p < 0.01$ vs baseline; one-way ANOVA: $F_{(1,8)} = 8.04$, $p = 0.02$ vs D1- Air Vapor) (Figure 5A). There was no difference in 1 Hz-LTD expression between D1+ and D1- MSNs (one-way ANOVA: $F_{(1,8)} = 1.86$, $p = 0.21$ D1+ 72hr WD vs D1- 72hr WD).

Not until one week of withdrawal did the magnitude of 1 Hz-LTD return to Air control levels in D1+ MSNs ($74.97 \pm 5.23\%$ of baseline; $n=6$; student's *t* test: $p < 0.01$ vs baseline; one-way ANOVA: $F_{(1,10)} = 3.77$, $p = 0.08$ vs D1+ Air Vapor). Yet, 1-Hz LTD was still observed in D1- MSNs that did not differ from D1+ MSNs ($80.36 \pm 11.94\%$ of baseline; $n=6$; student's *t* test: $p < 0.05$ vs baseline; one-way ANOVA: $F_{(1,9)} = 1.01$, $p = 0.34$ vs D1- Air Vapor; one-way ANOVA: $F_{(1,10)} = 0.21$, $p = 0.66$ vs D1+ 1 week WD) (Figure 5B).

Two weeks of withdrawal from CIE vapor exposure were necessary to reveal a robust 1 Hz-LTD in D1+ MSNs ($53.32 \pm 5.63\%$ of baseline; $n=5$; student's *t* test: $p < 0.001$ vs baseline) indistinguishable from Air vapor controls (one-way ANOVA: $F_{(1,9)} = 0.97$, $p = 0.35$ vs D1+ Air Vapor). Additionally, we observed a lack of 1 Hz-LTD expression in D1- MSNs similar to the air vapor control group ($99.33 \pm 14.51\%$ of baseline; $n=4$; student's *t* test: $p > 0.05$ vs baseline; one-way ANOVA: $F_{(1,7)} = 0.28$, $p = 0.61$ vs D1- Air Vapor) (Figure 5C). At two weeks withdrawal, 1 Hz-LTD expression differed significantly between D1+ and D1- MSNs (one-way ANOVA: $F_{(1,7)} = 13.71$, $p = 0.008$, D1+ 2 week WD vs D1- 2 week WD). A summary of the average normalized post-pairing EPSC amplitudes between minutes 40–50 in all CIE treatment groups, as well as, air control and ethanol-naïve groups is shown (Figure 5D).

DISCUSSION

Mechanism of 1Hz-LTD in the NAc shell

Our previous work demonstrates the expression of 1Hz-LTD in the NAc shell of C57BL/6 mice and its modulation subsequent to *in vivo* CIE vapor exposure (Jeanes et al., 2011). In the current study, we provided evidence suggesting that this form of long-term depression involves clathrin-mediated endocytosis of AMPA receptors from postsynaptic membranes. Our observation that the GluR2_{3Y} peptide interferes with 1-Hz LTD expression in the NAc shell aligns with previous studies showing a similar inhibition of NMDAR-dependent low-

frequency LTD by the same peptide (Brebner et al., 2005; Choi et al., 2013). Thus, we believe the underlying mechanism of 1Hz-LTD in the NAc remained consistent even as we transitioned to utilizing a different mouse strain throughout our investigation into the cell type-specific nature of this form of long-term synaptic plasticity.

One technical observation deserves discussion concerning the differences in the time course of LTD expression between the data included in Figure 1 versus the data in the remaining figures. In Figure 1, the large LTD group shows a transient post-LFS delay in the expression of LTD which is in general not nearly as apparent in the following figures. In our view, this time period immediately following the LFS is dominated by two different forms of plasticity – a short-term suppression of EPSC amplitude most likely due to transmitter depletion occurring during the prolonged 500 stimuli 1 Hz (8.33 min) LFS train and the retraction of AMPA receptors from the synapse as long-term depression becomes fully expressed. Indeed, it is also apparent that D1– MSNs or D1+ MSNs recorded in the presence of NMDA antagonists (Figure 3 and 4) show a short term post-LFS depression which rapidly abates as the EPSC amplitude returns to the pre-train baseline. Thus, some component of the post-train depression is unrelated to NMDA LTD. Nevertheless, even in MSNs expressing NMDA LTD, we have observed such differences in LTD expression which are likely due to slight differences in slice preparation, electrode placement and other technical differences throughout the years these experiments have been performed in this lab.

Cell type-specificity of LTD differs between NAc core and shell

Our observations of D1+ MSN-specific LTD in the NAc shell differs from both the dorsal striatum and NAc core where LTD is specific to D2+ MSNs (Kreitzer and Malenka, 2007; Grueter et al., 2010). This discrepancy may be partially explained by the different plasticity induction protocols used (high frequency (100 Hz) in striatum and 10 Hz in NAc core), or by the difference between using transgenic mice expressing eGFP under either the D1 or D2 receptor promoter (the report in NAc core analyzed D2+ and D2– MSNs). However, these results could implicate an important functional difference between the synapses within subregions of the NAc. In fact, because of their morphological and circuitry similarities, the NAc shell, bed nucleus of the stria terminalis, and central amygdala, are commonly referred to as a functional unit, called the extended amygdala complex (Hopkins and Holstege, 1978; Heimer and Alheid, 1991), which has been implicated in emotional processing of stimuli and drug addiction (Koob, 2013). Additionally, increased dopamine release subsequent to burst firing of VTA neurons has been shown to occur solely in the NAc shell but not the NAc core or dorsal striatum (Zhang et al., 2009). Given that the NAc core is continuous with the dorsal striatum, and core MSNs differ from the shell in their cellular morphology, neurochemistry, projection patterns, and functions (Heimer et al., 1991; Zahm and Brog, 1992; Meredith, 1999); it is not surprising that our findings regarding LTD expression differ as well. In fact, we consider this emerging idea central to our focus on NAc shell plasticity and ethanol exposure, as the shell is more prominently associated with the early component of reward processing while the core has a more prominent role in conditioned responding (Carlezon et al., 1995; Rodd-Henricks et al., 2002; Sellings and Clarke, 2003; Ikemoto, 2007). Administration of most drugs of abuse, including ethanol, lead to an increase in extracellular dopamine concentrations in the NAc (Imperato and Di Chiara, 1986; Weiss et

al., 1993). Thus, cell type-specific synaptic differences between the NAc core and shell may underlie the different aspects of behavioral responses to drugs of abuse (Grueter et al., 2013).

Basal dopamine tone and LTD in D1+ MSNs

Similar to our previous report (Jeanes et al., 2011), D1+ MSN-specific 1 Hz-LTD was NMDAR-dependent and completely occluded by ethanol (40mM). Although LTD was unaffected by prior D1-like receptor activation, it was slightly reduced in the presence of a D1-like receptor antagonist, suggesting that a basal dopaminergic tone may modulate the expression of 1 Hz-LTD. However, since the degree of change is small and LTD was still readily apparent, the main conclusion is that neither strong activation nor blockade of D1 receptors has a dramatic effect on LFS LTD expression in the slice preparation. Since D1 receptor antagonists have been shown to reduce operant responding for ethanol (Rassnick et al., 1993; Samson et al., 1993), it remains that in the intact animal where DA tone is fully present, modulation of LTD in D1+ MSNs may constitute a synaptic mechanism through which the NAc circuitry is altered to facilitate ethanol consumption. Nonetheless, the role of basal D1 receptor activation in vivo since we have previously documented that D1-receptor activation can significantly diminish NMDA receptor-sensitivity to ethanol and thereby even modulate the ability of ethanol to inhibit NMDA 1Hz-LTD (Maldve et al., 2002; Zhang et al., 2005; Jeanes et al., 2011).

CIE treatment reverses cell type-specificity of LTD

We observed a remarkable switch from only D1+ MSNs expressing 1 Hz-LTD under control conditions to only D1- MSNs expressing LTD following a 4-day CIE vapor exposure. The CIE vapor exposure model stably increases voluntary ethanol consumption in mice following withdrawal (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009b). In our own hands, we observed an increase in voluntary ethanol consumption following a single 4-day CIE exposure (Jeanes et al., 2011), which is identical to the ethanol exposure method used in the current study. However, an important distinction between the present study and those previous is the mouse strain used, as C57BL/6J mice are known to voluntarily consume ethanol while Swiss Webster (SJL) mice drink very little voluntarily (Belknap et al., 1993). We believe this important phenotypic difference could explain our observation of an occlusion of LTD in D1+ MSNs rather than a conversion to synaptic potentiation, as we have previously shown (Jeanes et al., 2011).

The implications of this discrepancy are quite exciting, as perhaps those genetic determinants that confer an elevated drinking behavior to C57BL/6J mice may also contribute to the reversal of synaptic plasticity polarity observed following CIE exposure. However, two studies report that, in Swiss Webster mice, either intra-NAc or intraperitoneal administration of the specific dopamine D1 antagonist (SCH-23390) blocked the expression of behavioral sensitization to ethanol (Camarini et al., 2011; Abrahao et al., 2013). Additionally, the loss of NMDAR-dependent plasticity in the NAc is associated with acquisition of ethanol-induced locomotor sensitization in Swiss Webster mice (Abrahao et al., 2013). Thus, although the Swiss Webster mice used in our study traditionally lack a

robust voluntary ethanol consumption behavior, certain important ethanol-related behaviors remain intact.

Specific neural adaptations may encode long-term ethanol exposure

Our experiments demonstrated a gradual recovery of baseline cell type-specific plasticity expression over several time periods until complete recovery was observed at two weeks of withdrawal from CIE vapor exposure. Here we speculate how ethanol exposure (CIE) may modulate drinking via these plasticity changes. Our data suggest that a vital period of neuroadaptation exists following ethanol exposure in which the NAc shell integrates afferent stimuli in a manner opposite to that before drug exposure. First, we propose that D1+ MSNs have a substantially lower threshold for NMDA-dependent plasticity in comparison to D1– MSNs. Additionally, we suggest that CIE treatment enhances NMDA receptor function for both cell types – this resulted in the CIE-induced net conversion of synaptic depression to potentiation we previously observed in C57 mice (Jeanes et al., 2011). Conversely, D1– MSNs appear to be normally subthreshold for LTD induction and only following CIE exposure will they express LTD (Figure 6). Yet, the simple occlusion of LTD without conversion of LTD to LTP in the Swiss Webster D1+ MSNs we observed here is notable and in contrast to our observation of robust LTD to LTP conversion in wild-type C57 mice – even when we could not identify their D1 receptor identity (Jeanes et al., 2011). In our prior report, CIE-induced drinking surpassed intoxicating levels (~3 g/kg in 2 hr) whereas Swiss Webster mice will not ingest ethanol in our hands. Thus, there appears to be a distinct linkage between the degree of CIE-induced plasticity and enhancement of ethanol intake between mouse strains. We have now obtained the C57 Drd1a-tdTomato mice and are presently engaged in dissecting D1+/- plasticity and ethanol intake phenotypes following multiple bouts of CIE with prolonged two-bottle choice measures.

Our results indicate that 1Hz-LTD in the NAc shell requires an NMDAR-dependent clathrin-mediated endocytosis of AMPARs. Calcium influx through postsynaptic NMDARs can result in three possible intracellular calcium levels that affect synaptic plasticity differently: high (LTP), intermediate (no plasticity), and low (LTD) (Lisman, 1989; Cho et al., 2001). Given that animals chronically treated with ethanol show a selective increase in NMDAR expression and function (see (Nagy, 2008) for review); it is reasonable to suggest that our CIE exposure induces similar alterations in NMDARs, albeit perhaps abbreviated in nature. Increasing the duration or frequency of CIE exposure could significantly increase NMDAR function, thus providing the catalyst for the expression of synaptic potentiation through the presence of a higher intracellular calcium concentration achieved during 1Hz conditioning stimulation. In fact, multiple bouts of CIE and withdrawal are typically necessary for subsequent escalation of voluntary ethanol consumption in mice (Griffin et al., 2009a). Our observations include a steady increase in LTD expression in D1+ MSNs and a similarly gradual decline in LTD expression in D1– MSNs as withdrawal from CIE exposure progressed. We posit that further CIE exposure may cement these synaptic alterations in the NAc shell and contribute to the complex neuronal modifications that underlie increased voluntary ethanol consumption following CIE.

Cell type-specificity of drug-related behaviors

Drugs of abuse, mainly psychostimulants, have been proposed to elicit LTD in the NAc, which is associated with the expression of behavioral sensitization (Thomas et al., 2001; Brebner et al., 2005; Martin et al., 2006; Kourrich et al., 2007). Additionally, in response to cocaine administration, D2+ MSN-specific alterations of a different form of LTD were shown in the NAc core (Grueter et al., 2010), while D1+ and D2+ MSNs displayed bidirectional changes in basal excitatory transmission in the NAc shell (Kim et al., 2011). Optogenetic activation of direct pathway D1+ MSNs or indirect pathway D2+ MSNs facilitates and inhibits cocaine-induced CPP, respectively (Lobo et al., 2010). In addition, chronic cocaine-induced inability to induce NMDAR-LTD in the NAc is thought to contribute to the transition to addiction (Kasanetz et al., 2010); whereas, reversal of cocaine-evoked synaptic potentiation in D1+ MSNs can abolish cocaine-induced locomotor sensitization (Pascoli et al., 2012). Another recent report demonstrated that overexpression of the transcription factor, FosB, in the NAc shell and core modulates synaptic strength and behavioral responses to cocaine in a cell type-specific manner (Grueter et al., 2013). These findings suggest that cell type-specific alterations in the expression of synaptic plasticity in the NAc shell are likely crucial cellular mechanisms that contribute to the development of drug dependence.

In relation to ethanol-seeking behaviors, there is very little information concerning the pattern of excitability of shell MSNs. Janak's seminal early report in rats trained for operant ethanol self-administration focused solely on shell MSNs (Janak et al., 1999), whereas a later report by Robinson and Carelli included individual analysis of both core and shell MSNs (Robinson and Carelli, 2008). Janak et al. observed very complex neuronal responses of shell MSNs and noted the most definitive changes were (1) increased excitability immediately prior to execution of the operant task and (2) inhibition of firing in response to ethanol (though this change was most likely not a pharmacological effect). She interpreted the shell as participating in many aspects of creation "of the motor sequences chained together to accomplish ethanol-reinforced behavior". Robinson and Carelli reported remarkably similar fundamental observations as Janak on the pattern of firing rate changes associated with the operant task (including both increases and decreases in firing rate). Robinson pooled all changes associated with the lever pressing and found an apparent linkage between ethanol-selectivity of MSNs and ethanol preference that was apparent in both core and shell. Given that context, our present findings indicate that CIE vapor exposure could potentially change the setpoint of baseline excitability for such information processing at specific stages of ethanol-seeking behaviors. In fact, there is direct evidence that ethanol can directly alter NAc excitability, Henriksen and colleagues reported that large experimenter-administered doses of ethanol (up to 44 mM and slightly greater than the levels produced by CIE vapor herein) always inhibited glutamate-iontophoretic stimulation of core MSNs (Criado et al., 1995) (no published reports exist in the shell); thus we might assume prolonged similar inhibitory responses to CIE vapor herein. Finally, Janak and colleagues have also provided an additional basis for D1-dopamine receptor selectivity in behavioral responding for ethanol since D1 receptor blockade in the shell reduced active lever pressing (Chaudhri et al., 2009). Taken together, while we have much more to learn about the role of shell MSNs in ethanol-related behaviors, the present work adds to these

pioneering studies and may help to explain some of their complexity, in that we must consider the D1 and D2-type of MSN in information processing as well.

Conclusions

An occlusion of LTD (as observed in D1+ MSNs) following CIE exposure could be interpreted as simply the resistance to change in synaptic function or perhaps LTD was already saturated from ethanol exposure resulting in the inability to induce further depression. The appearance of LTD (as observed in D1– MSNs) may represent an increase in activity at glutamatergic synapses (either through presynaptic neurotransmitter release or postsynaptic AMPA or NMDA receptor function) so that a synaptic depression is now possible. It is not yet entirely clear which interpretation best fits these findings, as future studies should continue to elucidate the precise nature of these cell type-specific synaptic alterations.

Ethanol-induced changes in NAc synaptic plasticity could establish an overt disruption in coordinated information flow in the reward-related neuronal network of the extended amygdala. Our observations are consistent with a ‘metaplastic’ modification of NAc synapses affecting their susceptibility to express either LTP or LTD in response to subsequent stimuli (Abraham and Bear, 1996). Perhaps abnormal reward-related learning is encoded through synaptic metaplasticity in the NAc shell, which would shape the potential of the same synapses to modulate their efficacy in response to future stimuli. The plastic changes in the neuronal signaling within the NAc could explain the aberrant associations that an animal makes during drug exposure. Given that the NAc shell is thought to be one of the first brain regions to integrate novel reward-related stimuli, these data could be a remarkable representation of this idea on a synaptic level. Following ethanol exposure, an abnormal balance of synaptic plasticity between D1+ and D1– MSNs could contribute to dysfunction in downstream neuronal circuits. The present observation that D1+ MSN-specific LTD expression gradually returns over two weeks of withdrawal prompts an intriguing possibility that permanent changes in D1+ and D2+ MSN synaptic plasticity expression could somehow underlie the maintenance of the aberrant voluntary ethanol consumption behavior that is a hallmark of dependence. Unfortunately, since Swiss Webster mice do not voluntarily ingest pharmacological concentrations of ethanol, a combined analysis of plasticity changes in drinking animals was not possible in the present study. We have since adopted the C57 line of Drd1a-tdTomato mice and studies on that experiment with this line are presently ongoing.

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Highlights

- D1 MSNs of the NAc shell display robust baseline NMDAR-LTD whereas presumed D2 MSNs do not
- NMDAR-LTD is dependent upon GluR2 internalization
- Chronic intermittent ethanol exposure occludes D1 MSN LTD and induces D2 MSN LTD
- Return to normal plasticity after intermittent EtOH vapor requires nearly 2 weeks
- Glutamatergic plasticity may contribute to early stages of ethanol reward learning

Fig. 1.

In panel (A) post-hoc analysis of previous work from our lab revealed two potential sub-populations of NAc shell MSNs in C57BL/6J mice. The recordings were divided based on the post-pairing average (min 40–50) EPSC amplitude being greater or less than 2 standard deviations (SD's) from baseline (min 0–10). The magnitude of synaptic depression in the “Large LTD” (greater than 2 SD's from baseline) neurons ($55.1 \pm 4.9\%$ of baseline, $n=11$, student's t test: $p < 0.001$ vs baseline) was significantly greater than the “Small LTD” (less than 2 SD's from baseline) neurons ($88.7 \pm 3.2\%$ of baseline, $n=10$, student's t test: $p < 0.01$ vs baseline; one-way ANOVA: $F_{(1,19)} = 45.77$, $p < 0.001$ vs “Large LTD” group). Sample EPSC traces of averaged baseline “1” (min 0–10) and post-pairing “2” (min 40–50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each group. * $p < 0.01$, ** $p < 0.001$ vs. baseline (min 0–10); ### $p < 0.001$ vs. Small LTD. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). (B) Histogram showing distribution of average EPSC amplitudes between min 40–50 for control recordings of 1Hz-LTD in MSNs of the NAc shell from C57BL/6J mice. Amplitude bins ranged from 31 to 103% of baseline with a bin size of 6. The greatest decrease in post-pairing average EPSC amplitude observed

was 38% of baseline, while the smallest was 97% of baseline. A polynomial trendline highlights the two populations of LTD responses observed in MSNs.

Fig. 2.

In panel (A) active interference peptide (GluR2_{3Y}) inhibits 1Hz-LTD expression in NAc shell MSNs from C57BL/6J mice. Inactive peptide (GluR2_{3A}) does not prevent 1-Hz LTD expression. Sample EPSC traces of averaged baseline “1” (min 0–10) and post-pairing “2” (min 40–50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each experimental group. Scale bars represent 10 ms (horizontal) and 50 pA (vertical) in all traces. (B) Cumulative bar graph representing the percentage change $\pm$ SEM for average EPSC amplitude between baseline (min 0–10) and post-pairing (min 40–50) for each experimental group. ** $p < 0.005$ vs. baseline (min 0–10); ## $p < 0.005$ vs. GluR2_{3Y} peptide.

Fig. 3. In panel (A) 1Hz-LTD of evoked AMPA receptor-mediated EPSCs was observed in D1+ but not D1- MSNs of the NAc shell from *Drd1*-eGFP transgenic mice. (B) Pairing stimulation, in the presence of DL-APV (100 μ M), in D1+ MSNs did not elicit 1Hz-LTD. (C) Ethanol (40 mM) blocked 1Hz-LTD in D1+ MSNs. (D) In the presence of the D1-like agonist, $\pm$ SKF38393 (50 μ M), 1Hz-LTD in D1+ MSNs was not affected. (E) 1Hz-LTD in D1+ MSNs was reduced in the presence of the D1-like antagonist, +SCH23390 (10 μ M). Sample EPSC traces of averaged baseline “1” (min 0–10) and post-pairing “2” (min 40–50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each experimental group. Scale bars represent 10 ms (horizontal) and 50 pA (vertical) in all traces. (F) Bar graph representing the percentage change $\pm$ SEM for average EPSC amplitude between baseline (min 0–10) and post-pairing (min 40–50) for each experimental group. * $p < 0.05$, ** $p < 0.005$ vs. baseline (min 0–10); # $p < 0.05$, ## $p < 0.005$ vs. D1+ control.

Fig. 4.

In panel (A) 24 hrs following 4 consecutive days of air vapor exposure (16 hr on, 8 hr off, with pyrazole only injections) 1Hz-LTD was observed D1+, but not in D1– MSNs similar to ethanol-naïve controls. (B) 24 hrs following 4 consecutive days of ethanol vapor exposure (16 hr on, 8 hr off, with 1.5 g/kg 20% ethanol and pyrazole injections) 1 Hz-LTD was not present D1+ MSNs but was observed in D1– MSNs. Sample EPSC traces of averaged baseline “1” (min 0–10) and post-pairing “2” (min 40–50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 10 ms (horizontal) and 50 pA (vertical) in all traces.

Fig. 5.

In panel (A) 72 hrs following CIE vapor exposure, pairing stimulation induces modest LTD in D1+ and a greater depression in D1– MSNs. (B) One week following CIE exposure, 1Hz-LTD is again observed in D1+ MSNs, but a modest LTD remains in D1– MSNs. (C) Two weeks following CIE exposure LTD was completely recovered in D1+ MSNs, as well as, the absence of LTD was observed in D1– MSN. Sample EPSC traces of averaged baseline “1” (min 0–10) and post-pairing “2” (min 40–50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 10 ms (horizontal) and 50 pA (vertical) in all traces. (F) Cumulative bar graph representing the percentage change $\pm$ SEM for average EPSC amplitude between baseline (min 0–10) and post-pairing (min 40–50) for each experimental group. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ vs. Air control of same cell type; ## $p < 0.005$, ### $p < 0.001$ D1+ vs. D1– MSNs.

Fig. 6. Proposed model of cell-specific alterations in NAc MSN synaptic plasticity induced by chronic intermittent ethanol exposure in C57BL/6 and Swiss Webster (SW) mice. According to the Artola-Brocher-Singer rule, induction of LTP or LTD depends on the ability of the postsynaptic membrane to reach different sequential thresholds (Artola and Singer, 1993). Using three codes of synaptic communication (LTD, LTP, or no plasticity), the dynamic shaping of the intrinsic synaptic connections in the NAc may control the final output signals to function in gating salient information, while ignoring unwanted impulses, through activation or depression of target neurons (Berretta et al., 2008). Our previous data and those presented herein suggest a potential model of how ethanol modulates the thresholds of NAc MSN synaptic plasticity expression in C57BL/6 and Swiss Webster