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Repeated Binge-Like Ethanol Drinking Alters Ethanol Drinking Patterns and Depresses Striatal GABAergic Transmission

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Repeated cycles of binge alcohol drinking and abstinence are key components in the development of dependence. However, the precise behavioral mechanisms underlying binge-like drinking and its consequences on striatal synaptic physiology remain unclear. In the present study, ethanol and water drinking patterns were recorded with high temporal resolution over 6 weeks of binge-like ethanol drinking using the 'drinking in the dark' (DID) protocol. The bottle exchange occurring at the beginning of each session prompted a transient increase in the drinking rate that might facilitate the acquisition of ethanol binge-like drinking. Ethanol drinking mice also displayed a 'front-loading' behavior, in which the highest rate of drinking was recorded during the first 15 min. This rate increased over weeks and paralleled the mild escalation of blood ethanol concentrations. GABAergic and glutamatergic transmission in the dorsal striatum were examined following DID. Spontaneous glutamatergic transmission and the density of dendritic spines were unchanged after ethanol drinking. However, the frequency of $GABA_A$ receptor-mediated inhibitory postsynaptic currents was depressed in medium spiny neurons of ethanol drinking mice. A history of ethanol drinking also increased ethanol preference and altered the acute ethanol effects on GABAergic transmission differentially in dorsolateral and dorsomedial striatum. Together, the study shows that the bottle exchange during DID promotes fast, voluntary ethanol drinking and that this intermittent pattern of ethanol drinking causes a depression of GABAergic transmission in the dorsal striatum.

INTRODUCTION

Alcohol is the most widely abused drug in the United States, as 60% of Americans over the age of 12 reported drinking and 25% reported binge drinking (defined as $\geqslant 5$ drinks/occasion yielding blood ethanol concentrations (BECs $\geqslant 80$ mg/dl) (NIAAA, 2010). The prevalence of ethanol consumption and the deleterious effect on the drinker and society (Rehm, 2011) highlight the need for a better understanding of ethanol's cellular targets and neurophysiological alterations. Until recent, research has been limited by the low levels of voluntary ethanol consumption typical of most mouse strains (Crabbe *et al*, 2011).

A recently developed mouse model of intermittent ethanol access elicits binge-like drinking and pharmacologically relevant BECs (Rhodes *et al*, 2005). Termed 'Drinking in the Dark (DID),' this model takes advantage

of the innate nocturnal nature of mice that display the highest levels of consumption and physical activity during the dark phase of the circadian cycle. In a 2- or 4-h drinking session, mice typically reach BECs higher than 80 mg/dl and show signs of behavioral intoxication such as motor impairment (Rhodes *et al*, 2007). Its use is growing and more and more studies are applying this paradigm to investigate neuronal circuits and signaling molecules affected by ethanol drinking (Mulligan *et al*, 2011; Cozzoli *et al*, 2012; Sprow and Thiele, 2012). Despite its success in inducing voluntary binge-like ethanol drinking, the behavioral mechanisms underlying the acquisition of this behavior are not understood. It is thought that DID works by limiting access to ethanol but it is unclear how the limited access induces binge drinking.

Previous studies examined the microstructure of drinking during DID using either lickometers to record individual licks or monitoring the volume change over the entire session (Rhodes *et al*, 2007; Griffin *et al*, 2009; Barkley-Levenson and Crabbe, 2012). However, the temporal resolution of these analyses has been low (30 min bins, but see Griffin *et al*, 2009) and limited the detection of transient changes in the drinking pattern. Furthermore, the drinking behavior was evaluated during 1–4 sessions

(usually over 1 week); as a result, there is no information on the evolution or stability of drinking patterns over weeks of DID. However, Griffin et al, 2009 showed that repeated passive vapor exposure to ethanol increases voluntary drinking during limited access and transiently changes the drinking pattern. The goal of this study is to investigate the ethanol drinking patterns of mice during DID with higher temporal resolution to determine whether the patterns are stable or changing and to better understand the mechanisms underlying binge-like drinking.

Chronic ethanol exposure affects brain pathways and neuronal circuits related to reward, stress (reviewed in Cui et al, 2013). Specifically, the dorsolateral (DLS) and dorsomedial striatum (DMS) are implicated in addiction (Koob and Volkow, 2010; Volkow et al, 2012). The dorsal striatum region is involved in ethanol drinking and it is the site of ethanol neuroadaptations (Wang et al, 2010; Cuzon Carlson et al, 2011; Cui et al, 2013; Depoy et al, 2013; Fanelli et al, 2013). Recently, we reported that non-human primates with >2 years of intermittent ethanol drinking exhibited a higher density of dendritic spines and altered putamen glutamatergic and GABAergic neurotransmission, a region homologous to the mouse DLS (Cuzon Carlson et al, 2011). Here, this study test the hypothesis that chronic intermittent binge-like ethanol drinking in mice alters basal ganglia connectivity and changes inhibitory and/or excitatory synaptic transmission in the dorsal striatum. Furthermore, the experiments described here investigate the possible regional specificity of the ethanol actions, given the distinctive connectivity of the DMS and DLS that receive and send projection to different brain regions (Chen et al, 2011).

MATERIALS AND METHODS

Mice

All experiments were performed in accordance with guidelines from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) Animal Care and Use Committee. Male C57BL/6J mice ($n = 137$), obtained from Jackson Laboratory (Bar Harbor, ME), were used in all experiments and were housed on a reversed 12-h light/dark cycle (0630–1830 dark) with *ad libitum* food. Water was provided *ad libitum* except during the DID.

DID Model

Mice (5–8 weeks old) were randomly assigned to the water or ethanol group, individually housed, and acclimated to the reverse light/dark cycle (0630–1830 dark) for 5–7 days before the start of DID. The first DID cohort was performed at UNC, and the rest was carried out at NIAAA using similar protocols. Three hours into the dark cycle (0930), water bottles were replaced with either water or ethanol (20% v/v in tap water) for 4 consecutive days. On the first 3 days, access to these bottles was limited to a 2 -hour (2 h) session. On the fourth day, access was extended to a 4 -hour (4 h) session after which mice underwent 3 days of abstinence. This 7-day cycle was repeated for 6 weeks (Figure 1a). Note that this is a single-bottle procedure and thus mice receiving 20% ethanol solution have no access to water during the

DID session (2–4 h). Tubes were fitted with metal sippers (straight, open tip), were filled with either water or ethanol (20% v/v in tap water), and were weighed before and after each drinking session. Plastic tubes (60ml conical) were used at the beginning of the study and were soon retired in favor of glass bottles (25 × 100 mm, Pyrex) that reduce the leakage of liquid during the sessions. Volume was calculated using $V_c = ((W_i - W_f) - W_l)/\delta$, where V_c is volume consumed, W_i is the bottle weight at the beginning of the session, W_f is the bottle weight at the end of the session, W_l is the average weight of the leaked ethanol or water solution and δ is the density for water (1 g/ml) or 20% ethanol solution (0.97336 g/ml) at 25–30 °C. The ethanol intake in g/kg was calculated according to $I = (V_c \times \delta_e)/W_m$; where I is ethanol intake in g/kg, V_c is the volume consumed of 20% ethanol, δ_e is the density of ethanol (0.789 g/ml), and W_m is the weight of the mouse in kg. For every session, the volume leaked was measured using a pair of dummy bottles placed on empty cages. The average leak volumes for the ethanol and water solutions were subtracted from each ethanol and water volume measurement, respectively. Occasionally, a bottle leaked a large volume that deviated far from the average volume measurement. Video monitoring during the sessions showed that mice climbing on the sipper tube sometimes caused these large leaks. Thus, single session data with z-scores value greater than two were excluded from the data set.

Blood samples were collected from tail nicks immediately following one session each week: after a 2-h session (first, second, and third sessions of the week) or after the 4-h session, depending on the cohort. BEC values were measured using a blood sample analyzer (Model GM7 Micro-Stat, Analox Instruments, Lununburg, MA). Body weight was measured weekly.

Lickometer

A custom-built lickometer was set up for individual cages (Figure 2a). A grated stainless steel platform was connected to the ground of an analog/digital converter (DIGIDATA 1322A, Molecular Devices, Sunnyvale, CA) and placed in the cage directly under the sipper tube to ground the mouse during drinking. The input signal from the analog/digital converter was clipped to the metal sipper tube. When a mouse licked the sipper while standing on the grounded platform, the circuit was closed and the lick was recorded as a current pulse using the pClamp v9 software (Molecular Devices). Data were collected at 10 Hz an hour before, during, and an hour after every DID session. Single current transients were defined as lick events and lick events occurring within 2 s or less were grouped into a 'bout'.

Locomotor Activity

Horizontal locomotor activity was recorded in home cages using infrared beam cross detectors (10 beams, 1 inch apart; model Opto M3, Columbus Instruments) during the two-bottle choice experiment and for the last week of DID. Total and ambulatory (consecutive) beam breaks were binned every 10 s for the duration of the DID session. Data are expressed as the number of infrared beam breaks per unit time.
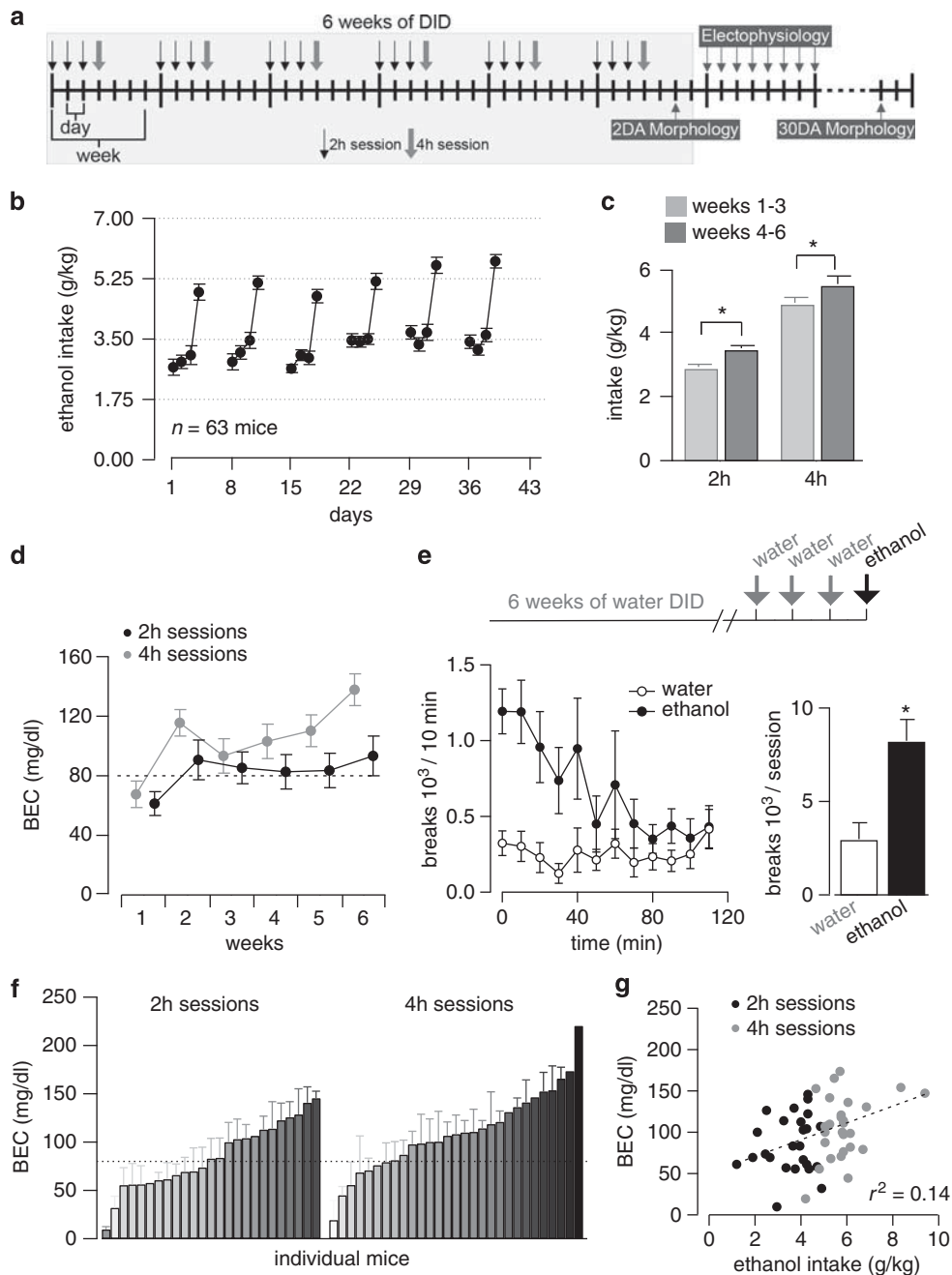
Figure 1 Ethanol intake and BECs increase over weeks of repeated exposure. (a) Schematic diagram of the 6-week long 'Drinking in the Dark' protocol with arrows representing 2 h (black) and 4 h (gray) sessions and marking the time for the morphological and electrophysiological studies. (b) Daily ethanol consumption during 2 h and 4 h sessions. (c) Average daily ethanol intake was significantly greater during the last 3 weeks (dark gray) than for the first 3 weeks (light gray) during both the 2-h and 4-h sessions. *Denotes $P < 0.01$. (d) Average BEC recorded after 2 h (black) and 4 h (gray) sessions over the weeks. Dotted line marks 80 mg/dl, the legal limit for intoxication in humans ($n = 25–29$ mice). (e) *Top*, timeline of the locomotor activity experiments. Mice received water under the DID schedule and were habituated to the beam break recording equipment for 6 weeks before the first ethanol exposure. *Left*, locomotor activity (mean ± SEM beam breaks per 10 min) during DID 2 h sessions with access to water bottles (open circles) and a single 2 h session with access to ethanol (black circles) ($n = 6$ mice). *Right*, total beam breaks per 2 h session for session with access to water (open) and for sessions with 20% ethanol (solid). (f) Individual variability in average BEC achieved during 2 h and 4 h sessions ($n = 25–29$ mice). Dotted line marks the legal limit for intoxication in humans, 80 mg/dl. (g) Average BEC per mouse plotted as a function of the average ethanol intake for 2 h (black) and 4 h (gray) sessions ($n = 25–29$ mice). Dotted line represents the linear fit ($r^2 = 0.14$). Markers/bars and lines represent mean ± SEM.

Electrophysiology

Water and ethanol drinking mice that underwent DID for 6 weeks were killed and studied at least 48 h after the last DID session to assure that the ethanol consumed and its

metabolites have been cleared. Electrophysiological experiments were then carried out from one mouse a day throughout week 7 from the start of DID (4–8 days after the last DID session). Mice were anesthetized with isoflurane and decapitated, and brains were removed.
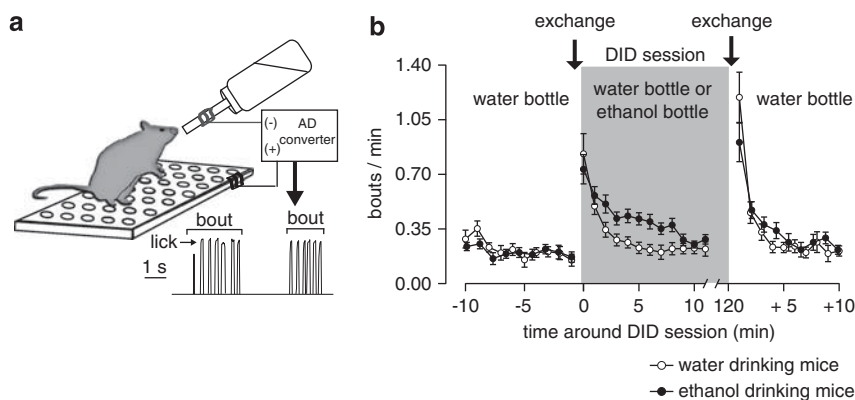
Figure 2 Bottle exchange induces increased drinking. (a) Schematic diagram of the lickometer set-up consisting of an analog-digital converter connected to the bottle sipper and the cage floor. Single licks to the sipper are detected as current transients and they are grouped into bouts. (b) Drinking rate expressed in bouts per minute (mean ± SEM) recorded around the time of the bottle exchange (arrow) that signals the start and the end of the drinking in the dark (DID) session for control mice (open) that received water during the DID session (shaded area) and for the ethanol mice (solid) that received 20% ethanol. Note that all mice received water before and after the DID session.

Coronal 250 μm thick slices were obtained using a vibrating blade microtome (Leica VT 1200S, Leica Microsystems, Buffalo Grove, IL) in ice-cold cutting solution containing in mM: 194 sucrose, 30 NaCl, 4.5 KCl, 26 NaHCO$_3$, 1.2 NaH$_2$-PO$_4$, and 10 glucose. Slices were placed in aerated aCSF containing in mM: 124 NaCl, 4.5 KCl, 1 MgCl$_2$, 26 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 10 glucose, and 2 CaCl$_2$ and allowed to recover at 33 °C for 1 h. Slices were then placed at room temperature until experimental use. Slices were transferred to a recording chamber fixed to the stage of an upright microscope (Axioskop2, Zeiss, Thornwood, NY) and continuously perfused with aCSF containing the GABA$_A$ receptor blocker picrotoxin (100 μM; Sigma, St Louis, MO) to isolate spontaneous excitatory postsynaptic currents (sEPSCs) or a combination of DL-2-amino-5-phosphono-pentanoic acid (DL-APV; 50 μM; Tocris, Ellisville, MO), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX; 5 μM, Tocris), and tetrodotoxin (TTX; 1 μM, Tocris) to isolate miniature inhibitory post-synaptic currents (mIPSCs). The temperature of the perfusate was maintained at 28–30 °C and was not allowed to fluctuate > 1 °C during a given experiment (Automatic Temperature Controller, Warner Instruments, Camden, CT). Medium spiny neurons (MSNs) were identified using differential interference contrast optics. Patch pipettes were pulled from borosilicate glass capillaries (1.5 mm outer diameter, 0.86 mm inner diameter, Sutter Instruments, Novato, CA) and filled with a CsCl-based internal solution containing in mM: 150 CsCl, 10 HEPES, 2 MgCl$_2$, 0.3 Na-GTP, 3 Mg-ATP, and 0.2 BAPTA-4K. The patch pipettes had a resistance of 2–4 MΩ. Recordings were made using an AxoPatch 200B amplifier (Molecular Devices). Whole-cell membrane currents were filtered at 2 kHz, digitized using Clampex v9.0 at 5 kHz, and analyzed with MiniAnalysis (Synaptosoft v6.0.7, Decatur, GA). Ethanol (50 mM) and other drugs were applied to slices via bath superfusion. In experiments examining ethanol acute ethanol exposure, mISPCs were recorded for 5 min, followed by application of ethanol for 15 min, and subsequent washout in normal aCSF with ionotropic glutamate receptor blockers.

DiOlistic Labeling

Water and ethanol drinking mice that underwent DID for 6 weeks were killed 48 h after the last DID session to assure that the ethanol consumed and its metabolites have been cleared. Labeling was performed as described previously (Seabold *et al*, 2010). Briefly, mice were perfused with phosphate-buffered saline (PBS) followed by ice-chilled fixative solution (4% paraformaldehyde, 4% sucrose in PBS). Brains were removed and fixed for an additional 30 min before being washed thoroughly with PBS at 4 °C. Brains were incubated for 10 min in 15% (w/v) sucrose solution and overnight in 30% (w/v) sucrose before storage in PBS at 4 °C until processing. Brains were sliced along the coronal plane to obtain 200 μm thick sections (Vibrotome 1000 plus, Leica). Tungsten beads (1.7 μm in diameter, Bio-Rad, Hercules, CA) coated with DiI (1-1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; Invitrogen, Grand Island, NY) were shot through a membrane filter with a 3-μm pore size (EMD Millipore, Billerica, MA) using a biolistic Helios gene gun (Bio-Rad) at 150–180 psi helium gas pressure used to sparsely label cells. Slices were then rinsed twice with PBS and mounted on slides using ProLong Antifade Gold (Invitrogen).

Morphological Analysis

Image acquisition and analysis were performed in a systematic manner with researchers blind to treatment. The distal portion of two to three dendrites (third and fourth order) per cell was collected in at least four cells per mouse. Regions with dense DiI staining in which individual neurons could not be demarcated were avoided. Image stacks (512 × 512, z-spacing = 0.7 μm; x-y scaling = 0.14 μm/pixel) of dendrite segments were acquired using a confocal microscope (Zeiss LSM 510 META) with a 63X water objective (NA = 1.2) and 2X zoom corresponding to a 71.4 × 71.4 μm image field. DiI was excited using a DPS 561 nm laser line and fluorescence emission was collected with long-pass 575 nm filter. Spine morphology was analyzed using ImageViewer (Dobi *et al*, 2011), a custom

software for spine analysis written in Matlab (MathWorks). Spines were defined as lateral protrusions from the dendritic shaft and were identified manually in the 3D image stacks. Spines extending in the z axis were not analyzed because morphology could not be measured accurately. While total spine density was thus under-estimated, this error remained constant across experimental conditions. Spine head width and spine length were also measured in the 3D stacks by drawing a transverse line across the thickest part of the spine head (parallel to the dendrite) and a longitudinal line along the whole spine, respectively. Head width and length were then automatically determined from the fluorescence distribution along the transversal and longitudinal lines.

Two-Bottle Choice Experiments

One cohort of mice ($n = 9$ water, 9 ethanol) underwent DID for 6 weeks followed by 1 week of abstinence, during which all mice received only water under the same schedule as DID. The following week, all mice received 20% ethanol for 2 h before they were given simultaneous access to two bottles (water and 20% ethanol) for the next 3 days. Both bottles were exchanged with fresh solution daily and weights were recorded every 24 h. The position of water and ethanol bottles was counterbalanced across mice in each cohort to avoid side preference and remained constant for each mouse throughout the experiment. Preference was calculated as the volume of 20% ethanol solution consumed over the total volume (water and ethanol) consumed in the last 24 h of testing.

Statistical Analysis

Behavioral data were analyzed using Microsoft Excel and Igor Pro 6.2 (WaveMetrics, Tigard, OR). Statistical analysis was performed using Prism 5 (GraphPad, LaJolla, CA), PASW Statistics 18, and SigmaStat 3.0 (SPSS, Chicago, IL). Independent two-population t-tests or paired t-tests were performed when comparing two groups. When applicable, data were fitted with a linear equation and the coefficient r^2 and p are calculated. A one-factor ANOVA was performed on the morphological data. Comparisons between two or more repeated factors (session, week, phase, etc.) by treatment were made using two-factor ANOVAs or Generalized Estimating Equations (GEEs) with either a linear or ordinal logistic regression model. Pair-wise comparisons of significant interactions and main effects were performed on estimated marginal means using Bonferroni's correction.

RESULTS

Mice were randomly assigned to either the water or ethanol experimental group. Mice in each group were given access to either water or ethanol (20%), respectively, on a weekly schedule that started with 2 hours (2 h) access a day for 3 days, followed by 1 day 4 hour (4 h) access session, and then 3 days of abstinence, for a total of 6 weeks (Figure 1a). Water and ethanol mice had similar body weights at the beginning and the end of the 6-week DID treatment (water: 20.5 ± 0.4 g and 24.7 ± 0.3 g; ethanol: 21.0 ± 0.4 g and 24.7 ± 0.3 g at week 1 and week 6, respectively;

$n = 49$–65), in agreement with previous results showing ethanol consumption during DID does not affect weight (Lyons et al, 2008).

Mice Increase Voluntary Oral Ethanol Consumption

Voluntary ethanol consumption averaged 3.22 ± 0.08 g/kg during the 2-h sessions and almost doubled to 5.21 ± 0.16 g/kg ($n = 63$) during the 4-h sessions (Figure 1b). An increase in ethanol consumption was observed after the first 3 weeks of exposure (Figure 1b). During 2 h sessions, ethanol intake was 2.93 ± 0.08 g/kg for weeks 1–3 and increased to 3.51 ± 0.11 g/kg for weeks 4–6; during the 4-h sessions, intake was 4.94 ± 0.14 g/kg for weeks 1–3 and increased to 5.52 ± 0.21 g/kg for weeks 4–6 ($n = 63$, two-way ANOVA: $F_{(1,250)} = 16.63$, $P < 0.0001$; weeks: $P < 0.01$; session duration: $P < 0.01$; Figure 1c). BECs were measured weekly at the end of either the 2-h or 4-h sessions and showed a matching increase over the weeks of voluntary drinking (Figure 1d). In addition, with the exception of the first week, average BEC values after both 2 h and 4 h sessions were equal to or higher than 80 mg/dl, consistent with the criteria for binge drinking in humans (NIAAA, 2010) and previously shown to impair performance on the accelerated rotarod and balance beam in C57BL6 mice (Rhodes et al, 2007). Thus, this model of intermittent limited access to ethanol leads to a modest escalation of ethanol intake and pharmacologically relevant BEC.

In addition to impairing motor performance, some doses of ethanol have been shown to induce locomotor activation, as seen with other drugs of abuse (Linsenbardt and Boehm, 2012). We evaluated the effect of ethanol drinking on locomotor activity in a group of control water mice that had been acclimated to the bottle exchanges for several weeks and had never been exposed to ethanol (Figure 1e). Mice were given access to ethanol for the first time during a 2-h session in which they achieved an average BEC of 45.4 ± 2.27 mg/dl ($n = 6$). The locomotor activity during this single ethanol session was higher than in a previous 2 h session in which mice received water (ethanol = $8.2 \pm 1.1 \times 10^3$ breaks; water = $3.0 \pm 0.85 \times 10^3$ breaks, $n = 6$; paired t-test: $t_5 = 4.4$, $P = 0.007$; Figure 1e). Ethanol-induced hyper-locomotion was restricted to the first 40 min of the session, the time when mice consumed most of the ethanol (see below). These results indicate that voluntary ethanol consumption under the conditions established using the DID protocol induces locomotor activation in mice, in agreement with a recent report (Linsenbardt and Boehm, 2012).

We observed substantial variability in the average BECs of individual mice of this inbred strain following 2 h or 4 h sessions (9.6–145 mg/dl; $n = 25$ mice for 2 h session and 19–220 mg/dl, $n = 29$ mice for 4 h session, Figure 1f). Half and two-thirds of the mice averaged BEC values higher than 80 mg/dl during the 2-h and 4-h sessions, respectively. A linear correlation was found between the average intake and BEC over 6 weeks of DID but the coefficient of determination was low, indicative of a weak correlation ($r^2 = 0.14$, $P = 0.005$, $n = 54$ mice; Figure 1g). Thus, the cumulative ethanol intake throughout the session could explain only a small fraction of the variability observed in the BEC.

Bottle Exchange Causes a Transient Increase in Drinking Rate

To understand the factors that regulate BEC in the DID paradigm and the mechanisms responsible for the small, but consistent, escalation of drinking, we sought information on the drinking pattern of mice within each session. Custom-built lickometers were set up in each cage to record individual licks of the bottle sipper (Figure 2a). Lick events occurring within 2 s were clustered into bouts (Figure 2a, inset). The drinking rate (the number of bouts per minute) was calculated for water and ethanol drinking mice.

Analysis of the drinking patterns in 24 control mice and 30 ethanol drinking mice (5 cohorts) revealed that the exchange of bottles, an integral part of the DID protocol that relies on limited access, leads to a transient increase in the number of bouts per minute (Figure 2b). This increase in drinking rate was observed in both ethanol and water drinking control mice every time the bottles were replaced at the beginning and the end of each session, regardless of the solution within the bottles. The exchange of one water bottle for another water bottle produced a sharp increase (4-fold) in the drinking rate from 0.22 ± 0.03 bouts/min before the bottle exchange to 0.8 ± 0.1 bouts/min after the bottle exchange. The rate of drinking returned to baseline 4 min after the bottle exchange. A similar effect of the bottle exchange on drinking behavior was observed during the first 4 min following the bottle exchange in which a water bottle was replaced by one containing 20% ethanol (average rate 0.49 ± 0.12 and 0.56 ± 0.07 bouts/min for water and ethanol, respectively, $n = 24$–30 mice; Figure 2b). These findings indicate that the bottle exchange affects the drinking behavior of mice and might act as a stimulus that could also represent a cue for the beginning and the end of each ethanol drinking session.

Highest Rate of Ethanol Drinking at the Start of Each Session

The high temporal resolution of the lickometer recordings also revealed that in mice with repeated access to ethanol, the drinking rate remained elevated for 10 min following the initial bottle exchange (4–14 min into the session), while in water drinking mice the drinking rate dropped to baseline quicker, within 4 min during the 2-h and 4-h sessions (Figure 3a). Also, it became apparent that while water drinking mice showed a steady rate of drinking throughout the session, mice drinking ethanol exhibited a bi-phasic drinking behavior. To quantify this phenomenon, the minutes 5–12 of each session were referred to as the early phase (the first 4 min right after the bottle exchange was not considered because they reflected the bottle exchange effect) and minutes 112–119 were referred to as the late phase. During the early phase, the drinking rate was higher in ethanol drinking than in water drinking mice (0.33 ± 0.02 bouts/min for ethanol; 0.23 ± 0.02 bouts/min for water, $n = 30$–24) while during the late phase the rate was lower in ethanol than in water drinking mice (0.08 ± 0.01 bouts/min for ethanol; 0.18 ± 0.02 bouts/min for water, $n = 30$–24; two-way repeated measures ANOVA, $F_{(1,104)} = 62.17$, $P = 0.0001$; treatment: $P < 0.001$; session phase: $P < 0.001$; Figure 3b). As a consequence of this 'front-loading' behavior observed in ethanol drinking mice, nearly half (44%) of the ethanol was consumed during the first quarter (30 min) of each session; in contrast, mice drank water at a constant rate and consumed only a quarter of the total water volume (27%) during same time frame (Figure 3c). This front-loading behavior has previously been seen for ethanol drinking and also self-administration of other drugs of abuse such as cocaine and it is thought to indicate the acquisition of the self-administration behavior (Rhodes et al, 2007; Griffin et al, 2009; Barkley-Levenson and Crabbe, 2012).

This front-loading behavior was extinguished when ethanol was removed after 6 weeks of DID. For this experiment, ethanol drinking mice who underwent 6 weeks of DID were given access to water under the same DID schedule during week 7 (extinction session) and the drinking patterns were determined. During DID, ethanol drinking mice displayed front-loading behavior and had more bouts than water mice during the first 10 min of the DID sessions (1.9 ± 0.4 bouts for water; 3 ± 0.3 bouts for ethanol; Figure 3d). However, when access to water was offered under the same DID scheduled during the week 7 of extinction, mice showed a constant pattern of water drinking and consumed a similar number of bouts than water mice during the first 10 min of the extinction sessions (2.2 ± 0.8 bouts for water; 1.9 ± 0.4 bouts for ethanol; Figure 3d). These results suggest that the front-loading behavior is specific for the ethanol and not a consequence of the DID schedule.

Changes in the drinking patterns of ethanol were also observed within each week of DID (Figure 3e). During the first DID session of each week, which was preceded by 3 days of abstinence, mice made the highest number of ethanol bouts during the early phase (2.5 ± 0.3 bouts during early phase, $n = 30$ mice). Ethanol bouts dropped significantly by the fourth consecutive drinking session of the week (1.6 ± 0.2 bouts during the early phase, $n = 30$, three-way repeated measures ANOVA, factors: treatment, session, and phase (nested within session); three-way interaction $F_{(7,125)} = 8.68$, $P < 0.001$; Bonferroni's test $P = 0.02$ for ethanol early phase of session 1 vs 4; Figure 3e). In contrast, ethanol drinking during the late phase or water drinking during the early and late phases was similar throughout the sessions (Figure 3e).

We searched for specific changes in the drinking behavior that could develop over the repeated sessions of DID and that could account for the escalation of intake and BEC. The mean duration of the ethanol bouts remained constant over the weeks (2.4 ± 0.08 s), as did most other factors evaluated such as total drinking time, the number of licks per bout, and the latency to drink. Interestingly, the degree of the front-loading behavior appeared to increase over the consecutive weeks of DID, specifically with ethanol drinking during the early phase of the 4-h session of the week, in which the number of ethanol bouts was the lowest during week 1 (0.92 ± 0.25 bouts), more than doubled by week 3 (2.0 ± 0.41 bouts), and remained elevated from weeks 3 to 6 of DID (Figure 3f). This increase in ethanol front-loading drinking behavior was not apparent in the 2-h sessions (Figure 3g).

A strong correlation was found between the number of ethanol bouts recorded during the early phase of each session and the BEC values achieved by individual mice
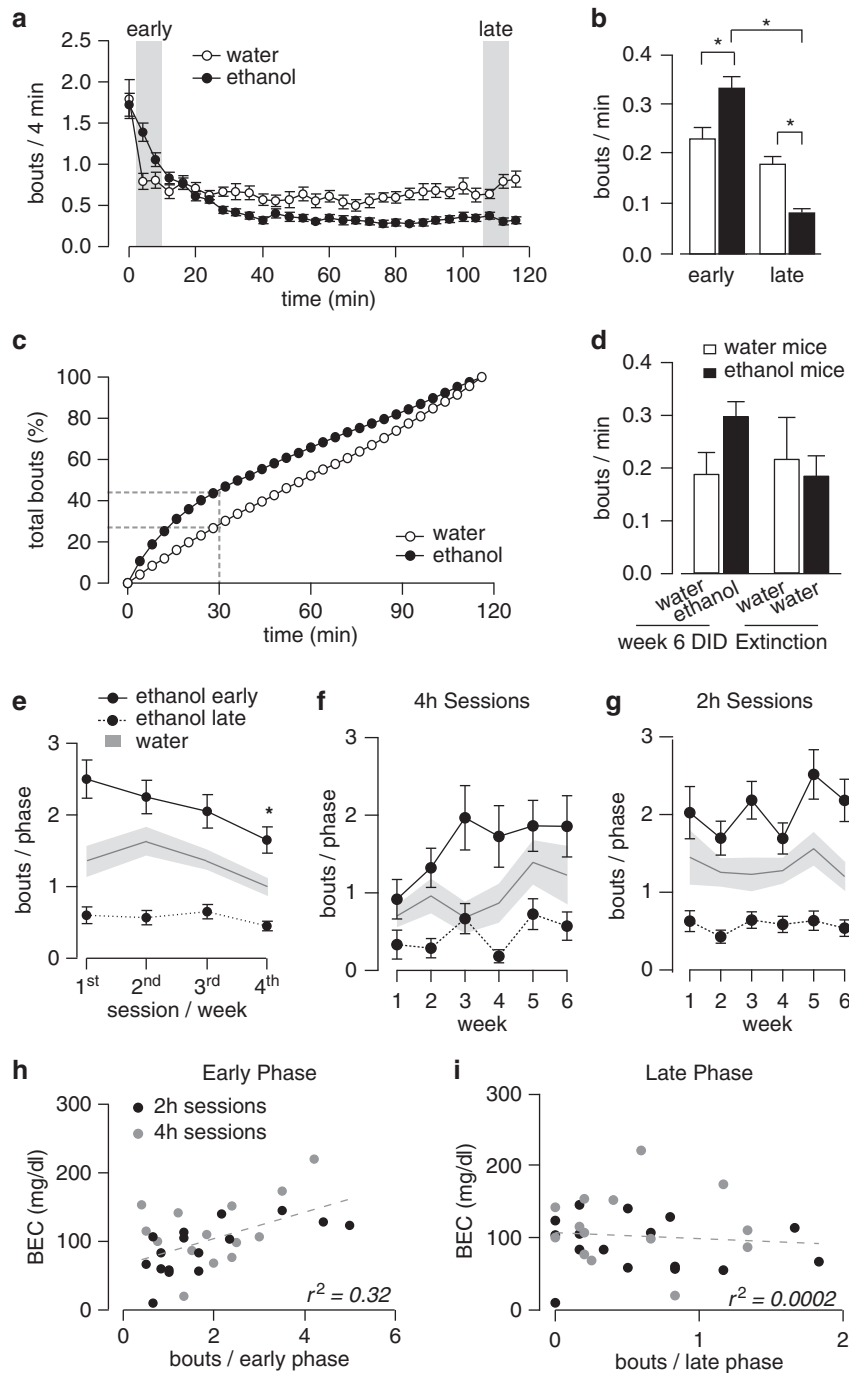
Figure 3 Large ethanol bouts during the early phase of each session. (a) Drinking rate (mean bouts per 4 min) recorded during the 2-h drinking in the dark (DID) session in water (open) and ethanol (solid) mice. Shaded areas mark the early phase (5–12 min) and the late phase (112–119 min) of the session. (b) Number of bouts during the early (E) and late (L) phase for water (open) and ethanol (solid) mice. *Denotes $P < 0.0001$. (c) Cumulative consumption over the 2-h sessions expressed as the percent of total bouts over time for control water mice (open) and ethanol mice (solid). (d) Number of bouts recorded during the first 10 min of the session for water (open, $n = 7$ mice) and ethanol (solid, $n = 8$ mice) mice during week 6 of DID (left) and during the following week of abstinence in which only water was available to all mice with the same DID schedule. Bars represent mean ± SEM for the three combined 2 h sessions of the week. (e) Average number of ethanol bouts during the early (solid line) and late (dashed line) phase of the first, second, third, and fourth sessions of each week (the first session is the average of data from all 6 weeks; likewise for sessions two, three, and four). Gray-shaded area shows mean ± SEM of the number of water bouts during combined early and late phases for the first to the fourth session of each week. *Denotes $P = 0.022$. (f, g) Number of ethanol bouts (mean ± SEM) consumed during the early (solid line) and late (dashed line) phases of the (f) 4-h session (fourth session of the week) and the (g) 2-h sessions (first, second, and third sessions of the week) over weeks 1–6. Gray-shaded area shows mean ± SEM of the number of bouts for control water mice during the early and late phases combined. (h, i) BEC plotted as a function of number of ethanol bouts consumed during (h) the early phase and (i) the late phase of the 2-h session (black) and the 4-h session (gray). Dashed line is the linear fit to the data with $r^2 = 0.32$ for the early phase; $r^2 = 0.0002$ for the late phase.
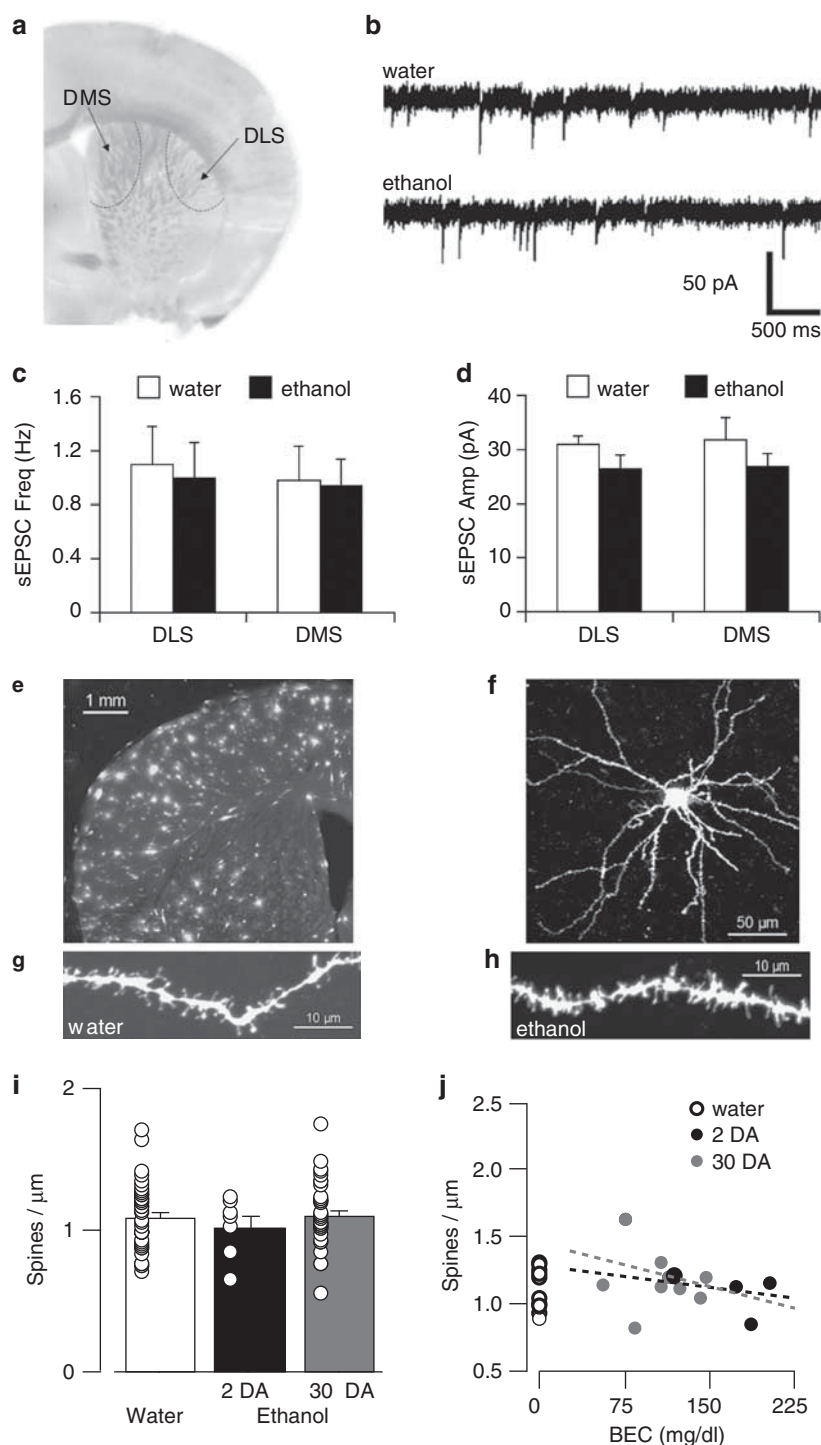
Figure 4 No change in spontaneous glutamatergic transmission or spine density in dorsolateral striatum (DLS) medium spiny neurons after drinking in the dark (DID). (a) Coronal brain section with demarcated DLS and dorsomedial striatum (DMS) regions where the recordings were made. (b) Representative traces of sEPSC recorded from MSNs located in the DLS of water drinking (top) and ethanol drinking (bottom) mice 4–8 days after the last drinking session. (c) Frequency of sEPSC (mean ± SEM) recorded in medium spiny neurons in the DLS and DMS of water control mice (white, $n = 21$ neurons/9 mice) and ethanol mice (black, $n = 22$ neurons/9 mice). (d) Amplitude of sEPSC (mean ± SEM) recorded from neurons in the DLS and DMS of water control mice (white) and ethanol mice (black). (e) Low-magnification fluorescence image of coronal brain section showing sparse DiI labeling of neurons in the dorsal striatum achieved using DiOlistic staining. (f) Confocal image of a representative medium spiny neuron. (g, h) Confocal image of representative dendrite and dendritic spines in (g) water drinking and (h) ethanol drinking mice. (i) Spine density (mean ± SEM) of MSN in DLS of water (white) and ethanol mice after 2 days of abstinence (2 DA, black) and 30 days of abstinence (30 DA, gray) ($n = 42$, 11, and 37 neurons, respectively). (j) Spine density in DLS for water (white) and ethanol (black for 2 DA and gray for 30 DA) as a function of the average BEC ($n = 11$, 4, and 8 mice, respectively). Dashed line represents linear regression for 2 DA data (black, $r^2 = 0.2$) and for 30 DA data (gray, $r^2 = 0.02$).

($r^2 = 0.32$, $P = 0.006$, $n = 22$; Figure 3h). This was a stronger correlation than the previously observed correlation between intake and the BEC ($r^2 = 0.14$, $P = 0.005$, Figure 1g). Please note that the lickometer data were recorded in a subset of all mice that underwent DID and thus the difference in sample size between the plot of BEC vs Intake (Figure 1g) and the plot of BECs vs bouts/phase plot (Figure 3g and h). In contrast, bouts during the late phase showed no correlation with BEC ($r^2 = 0.0002$, $P = 0.95$, $n = 22$; Figure 3i). Thus, the rate of ethanol drinking during the first 15 min of each session has a strong influence on the BECs measured 2 or 4 h later. The improved temporal resolution of the lickometer recordings compared with cumulative volume measurements allows for a thorough dissection of drinking behavior and patterns. The analysis suggests that changes in drinking rate may be responsible for the escalation of ethanol intake and BEC observed with repeated cycles of DID.

Striatal Glutamatergic Transmission and Spine Density of MSNs Are Unchanged

The dorsal striatum is an important brain region involved in controlling motor output and it is susceptible to the effects of ethanol. We have previously shown that both glutamatergic and GABAergic synaptic transmission were altered in the striatum of non-human primates with extended access to ethanol for 3 years (Cuzon Carlson et al, 2011). Thus, we examined GABAergic and glutamatergic synaptic transmission in MSNs of the dorsal striatum in ethanol drinking mice and their water control counterparts after 6 weeks of DID.

Spontaneous glutamatergic excitatory postsynaptic currents (sEPSCs) were recorded in the presence of the GABA_A receptor antagonist, picrotoxin, in MSNs of the DLS and DMS in acute brain slices from control water drinking mice and ethanol drinking mice 4–8 days after the last DID session (Figure 4a and b; $n_{water} = 21$ neurons, 9 mice; $n_{EtOH} = 22$ neurons, 9 mice). The frequency of sEPSCs was similar in ethanol drinking mice (DLS: 0.99 ± 0.27 Hz; DMS: 0.94 ± 0.20 Hz) and water drinking mice (DLS: 1.1 ± 0.3 Hz; t-test; $P = 0.77$; DMS: 1 ± 0.3 Hz; t-test; $P = 0.91$; Figure 4c). The amplitude of sEPSCs was also unchanged (DLS: 30.8 ± 1.7 pA in water mice; 26.3 ± 2.7 pA in ethanol mice; $P = 0.15$; DMS: 31.7 ± 4.3 pA in water mice; 26.7 ± 2.6 pA in ethanol mice; $P = 0.32$; Figure 4d). Analysis of the area, rise time, and decay time constant of the sEPSCs revealed a similar kinetics in water and ethanol drinking mice (DLS: area $= 132.7 \pm 10.8$ and 137 ± 16; rise time $= 2.2 \pm 0.2$ ms and 2.5 ± 0.2 ms; decay time $= 4.3 \pm 0.3$ ms and 5.4 ± 0.4 ms for water and ethanol, respectively; $P = 0.82$, 0.40, and 0.06, respectively; DMS: area $= 154.1 \pm 22$ and 127.3 ± 16.3; rise time $= 2.4 \pm 0.3$ ms and 2.3 ± 0.2 ms; decay time $= 5.1 \pm 0.8$ ms and 4.8 ± 0.5 ms for water and ethanol, respectively; $P = 0.34$, 0.80, and 0.76, respectively).

The density of dendritic spines in MSNs was evaluated using fluorescent labeling and confocal imaging 2 days and 30 days after the last DID session (2 DA and 30 DA). A short and a long abstinence period was investigated because of the extensive literature on the effects of other drugs of abuse such as cocaine on spine density in the striatum observed after 4 weeks of the last exposure (Robinson and Kolb, 1999;

Lee et al, 2006; Dobi et al, 2011; Waselus et al, 2013). Sparse fluorescent labeling of MSNs was achieved using the DiOlistic technique in fixed brain slices of ethanol drinking and controls water drinking mice at the two time points (Figure 4e). Brightly labeled MSNs in the DLS were identified based on their morphology and confocal images of two distal dendritic segments (second to fourth order dendrites) were acquired and analyzed per neuron (Figure 4f–h; $n_{water} = 42$ neurons, 11 mice; $n_{EtOH} = 48$ neurons, 12 mice). Data from water control mice at 2 DA and 30 DA were similar so they were combined. No difference in the spine density of DLS neurons was detected between water drinking mice and ethanol drinking mice at 2 DA or 30 DA (1.09 ± 0.04 spines/μm for water, 1.03 ± 0.08 spines/μm for ethanol at 2 DA, 1.12 ± 0.04 spines/μm for ethanol at 30 DA; $n = 42$, 11, and 37 neurons, respectively; one-way ANOVA, $F_{(1,83)} = 0.46$, $P = 0.63$). Furthermore, there was no correlation between average BEC of each mouse and spine density in neurons in DLS ($P = 0.54$ for 2 DA and 0.69 for 30 DA; Figure 4j, $n = 11$, 4, and 8 mice, respectively). Thus, 6 weeks of intermittent access to ethanol produced binge-like ethanol drinking patterns but did not significantly alter spontaneous excitatory transmission or the density of spines in MSNs of DLS.

Striatal GABAergic Transmission is Depressed Following DID

GABA_A receptor-mediated mIPSCs were recorded in MSNs from the DLS and DMS in the presence of blockers for AMPA and NMDA glutamate receptors as well as tetrodotoxin to block sodium channels (Figure 5a and b). Recordings were made following 6 weeks of repeated ethanol drinking, 4–8 days after the last DID session. The frequency of mIPSCs was significantly reduced in DLS and DMS of ethanol drinking mice compared with water drinking controls (Figure 5c; water DLS $= 2.86 \pm 0.31$ Hz, $n = 17$ cells/8 mice; ethanol DLS $= 1.89 \pm 0.19$ Hz, $n = 21$ cells/6 mice, t-test, $P = 0.009$; water DMS $= 1.52 \pm 0.25$ Hz, $n = 17$ cells/8 mice; ethanol DMS $= 0.89 \pm 0.13$ Hz, t-test, $P = 0.04$, $n = 12$ cells/5 mice). A significant difference was also found in mIPSCs frequency between DLS neurons and DMS neurons of water drinking mice, indicative of the distinctive properties of these striatal regions (t-test, $t_{22} = 3.36$, $P = 0.003$). No other significant differences were observed between striatal regions nor treatments with regard to mIPSC amplitude (Figure 5d; water DLS $= 34.81 \pm 2.57$ pA, ethanol DLS $= 31.86 \pm 2.90$ pA, t-test, $P = 0.49$; water DMS $= 33.05 \pm 2.40$ pA, ethanol DMS $= 29.25 \pm 2.59$ pA, t-test, $P = 0.30$), area, rise time, and decay time recorded in the DLS or DMS (Table 1). Thus, GABAergic transmission is depressed in both regions of the dorsal striatum after DID. To determine potential dependence with BEC levels achieved during the DID session, the mean mIPSC frequency of striatal neurons in DLS and DMS was plotted as a function of the mean BEC achieved by each mouse (Figure 5e and f). There was no correlation between mIPSC frequency and BECs in the DMS (DMS: $r^2 = 0.33$, $P = 0.31$, $n = 5$ mice). There was a trend for a negative correlation in the DLS that was driven by a larger reduction in mIPSC frequency in mice with BEC of around
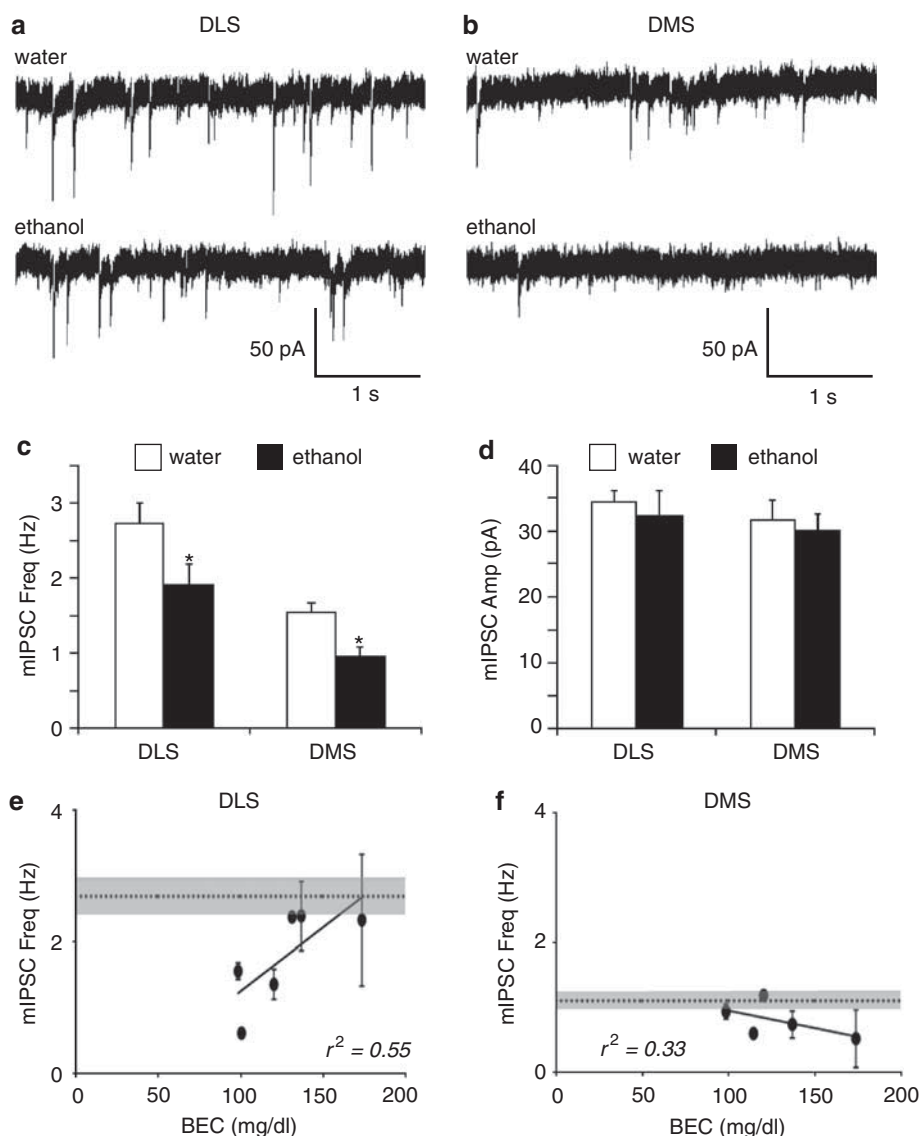
Figure 5 GABA transmission in dorsolateral striatum (DLS) and dorsomedial striatum (DMS) is depressed after binge-like ethanol consumption. (a, b) Representative traces of miniature inhibitory post-synaptic currents (mIPSCs) recorded from medium spiny neurons (MSNs) located in the DLS (a) and DMS (b) of water and ethanol drinking mice 4–8 days after the last drinking session. (c) Frequency of mIPSCs recorded in medium spiny neurons in the DLS and DMS of water control mice (white) and ethanol mice (black). (d) Amplitude of mIPSCs recorded from neurons in the DLS and DMS of water control mice (white) and ethanol mice (black). (e, f) Mean mIPSCs frequency in neurons of the DLS (e) and DMS (f) as a function of the mean BEC achieved during the DID session for ethanol drinking mice. Dotted line and shade mark the mean and SEM, respectively, for the mIPSCs frequency of neurons in water control mice. All data are expressed as mean ± SEM. *Denotes $P < 0.05$.

100 mg/dl; however, this trend was not significant (DLS: $r^2 = 0.55$, $P = 0.09$, $n = 6$ mice).

Acute Effect of Ethanol on Striatal Synaptic Transmission

As chronic ethanol drinking altered inhibitory GABAergic transmission in the striatum, we speculated that acute ethanol would also affect inhibitory synapses in DLS and DMS but there is little information on this topic other than observation that acute ethanol inhibits stimulus-evoked IPSCs in DLS (Blomeley et al, 2011). The acute effects of ethanol were studied first in naïve mice. Ethanol (50 mM) application in the DLS inhibited mIPSC frequency ($70.50 \pm 11.51\%$ of baseline; paired t-test, $P = 0.03$; $n = 10$;

Figure 6a, c, and d). This is the opposite of the acute actions of ethanol in several other brain regions (reviewed in Weiner and Valenzuela, 2006 and Kumar et al, 2009) and to what it was measured in the DMS. Indeed, in neurons of the DMS, acute ethanol application increased mIPSC frequency ($134.74 \pm 7.33\%$ increase from baseline; paired t-test, $P = 0.002$; Figure 6e, g, and h). The amplitude of mIPSCs was unaffected in either DLS or DMS (DLS: $110 \pm 12.87\%$ of baseline; paired t-test, $P = 0.46$; DMS: $100.03 \pm 2.52\%$ of baseline; paired t-test, $P = 0.85$; Figure 6c and g). The effect of acute ethanol exposure is reversible upon washout (DLS wash: $97.98 \pm 1.12\%$ of baseline; paired t-test, $P = 0.12$; DMS wash: $99.38 \pm 0.74\%$ of baseline; paired t-test, $P = 0.85$). Thus, acute ethanol exerts opposite effects on distinct regions of the dorsal striatum.

Table I Binge-Like Ethanol Consumption Does Not Alter the Kinetics of mIPSCs Recorded in MSNs Located in the DLS or DMS

mIPSC characteristic	DLS			DMS		
	Water	Ethanol	*P*-value	Water	Ethanol	*P*-value
Area	193.54 ± 18.02	194.08 ± 22.80	0.99	197.18 ± 12.15	166.70 ± 17.85	0.16
Rise time	2.85 ± 0.15	2.87 ± 0.16	0.95	2.92 ± 0.14	2.94 ± 0.14	0.95
Decay time	5.73 ± 0.44	6.06 ± 0.30	0.52	6.51 ± 0.39	5.90 ± 0.47	0.33

Abbreviations: DLS, dorsolateral; DMS, dorsomedical steriatum.
Data are expressed as mean ± SEM. Statistical comparison between water and ethanol drinking mice in the DLS and DMS was assessed using *t*-test.

In DID mice with a history of ethanol drinking, acute ethanol (50 mM) no longer inhibited mIPSC frequency in the DLS ($106 \pm 8.89\%$ of baseline; paired *t*-test, $P = 0.52$; Figure 6b–d). In DMS, however, the acute ethanol effect was reversed in ethanol drinking mice such that ethanol application decreased rather than increased mIPSC frequency ($81.52 \pm 12.37\%$ of baseline, paired *t*-test, $P = 0.05$; Figure 6f–h). The amplitude of mIPSCs was not significantly changed by acute application of ethanol in either the DLS or the DMS of ethanol drinking mice (DLS: $97.45 \pm 9.52\%$ of baseline; paired *t*-test, $P = 0.80$; DMS: $84.32 \pm 10.80\%$ of baseline; paired *t*-test, $P = 0.22$; Figure 6c and g). Thus, ethanol exposure has a different effect on mice with a previous ethanol history than those exposed for the first time.

Intermittent Ethanol Drinking Alters Ethanol Preference

To assess changes in voluntary ethanol consumption that could occur as a consequence of ethanol exposure under the DID schedule, ethanol consumption was measured in a two-bottle choice paradigm in water and ethanol mice. Mice underwent 6 weeks of DID with access to water or ethanol followed by a week of abstinence and subsequent testing in a two-bottle choice paradigm for 3 days ($n_{water} = 9$ mice, $n_{EtOH} = 9$ mice; Figure 7a). During the week of abstinence, ethanol and water mice consumed similar volumes of water during the 2-h and 4-h sessions (water 2 h = 0.39 ± 0.07 ml, ethanol 2 h = 0.40 ± 0.08 ml; water 4 h = 0.71 ± 0.14 ml, ethanol 4 h = 0.80 ± 0.12 ml; Figure 7b). However, during the two-bottle choice test, mice with a history of ethanol drinking displayed higher preference for the ethanol solution than ethanol naïve water mice (preference = 0.25 ± 0.04 for water mice and 0.44 ± 0.06 for ethanol mice, $n = 9$, 9 mice; $t_{16} = 2.4$, $P = 0.03$; Figure 7c). Note that the values of preference (ethanol volume/(water + ethanol volume)) were always under 0.5, indicating that the mice always consumed a larger volume of water than 20% ethanol. Mice with a history of DID consumed more ethanol than ethanol naïve mice (ethanol = 37 ± 3 g/kg/day; water = 22 ± 1 g/kg/day; $t_{16} = 4.5$; $P = 0.0004$; Figure 7d).

DISCUSSION

This study, using data from lickometer recordings, dissects the drinking behavior of mice with access to ethanol during repeated DID sessions. During these drinking sessions, male C57BL/6J mice consumed 3–3.5 mg/kg of ethanol in 2 h and 5–5.5 mg/kg of ethanol in 4 h sessions, in agreement with previous reports (Rhodes *et al*, 2005). The lickometer analysis showed that control mice drink water at a constant rate throughout most of the session. However, an unexpected result was revealed by a closer examination of the drinking pattern of control mice with access to water only. Following the exchange of one water bottle for another water bottle, mice displayed a transient increase in the number of sipper licks lasting 2–4 min. Mice drinking ethanol showed the same increase in drinking during the first 2–4 min following the bottle exchange but it was followed by a longer lasting increase in ethanol drinking. It is tempting to speculate that the bottle exchange might represent a signal or stimulus that contributes to the success of DID, possibly by acting as a predictive cue of ethanol availability that facilitates the acquisition of voluntarily ethanol drinking behavior.

The analysis also showed that mice drink ethanol at a higher rate than water during the 15 min that follow the bottle exchange in each session and, as a consequence, mice consume half of the total ethanol volume within the first quarter (30 min) of each session. This ethanol front-loading behavior is not evident during the first week of DID, but rather develops over time as indicated by the more than doubling of the drinking rate during the early phase between weeks 1 and 6. One possibility is that this behavioral adaptation in the drinking pattern is shaped by previous ethanol experience and represents a form of learning. Another possibility is that it represents a rapid form of tolerance to either the pharmacological effects of ethanol or the taste of ethanol. A recently published study found that ethanol preference and intake was greatly enhanced for a 20% ethanol solution but only slightly for a 10% ethanol solution, pointing away from tolerance after 6 weeks of DID (Cox *et al*, 2013).

Our data are in agreement with a previous study employing lickometers to measure drinking during DID in that (1) ethanol bouts are shorter than water bouts and (2) mice take fewer ethanol than water bouts during the second half of the session (Rhodes *et al*, 2007). However, in disagreement with our findings, Rhodes *et al* (2007) reported stable rates of ethanol consumption throughout the 4-h session. We speculate that the low temporal resolution analysis in the Rhodes *et al* (2007) study (30 min bins) might account for the inability to detect the transient spike in the drinking rate early during the session. Also, the previous recordings were performed during the first DID sessions in which the front-loading behavior is less apparent.
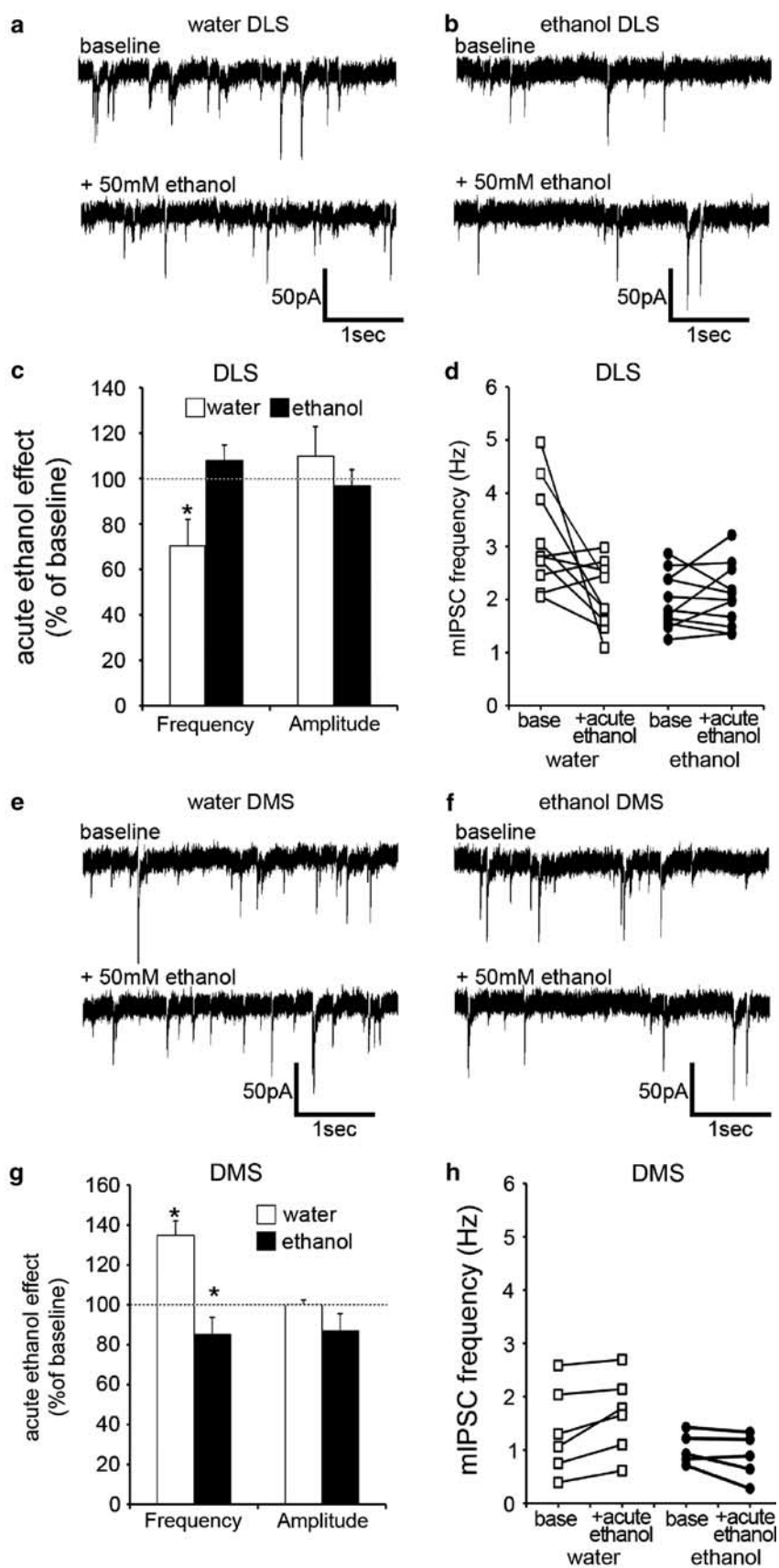
The rate of drinking during the early phase of the session was the best predictor of BEC, which increased over the weeks of exposure. A recent report showed that a mouse line selected for high DID (HDID-1) consumed slightly more ethanol during the second half of the 4-h session (Barkley-Levenson and Crabbe, 2012). Because BECs are measured at the end of the session, it could be expected that higher drinking in the second half of the session would have a larger impact on this measure. However, the results obtained here challenge the idea that this is true for all mouse strains. In our experiments performed using male C57BL/6J mice, BECs correlated best with the rate of drinking during the early phase of the 4-h session indicating that the amount of drinking during the first 15 min is critical in determining the BECs 2–4 h later. It is important to note that this does not mean that the maximal BEC is reached during the first 15 min. Mice drink ethanol at a low but constant rate during most of the session (3/4 of 2 h sessions and 15/16 of a 4-h session). Thus, ethanol consumed at low rate during the remaining of the session also influences the amount of ethanol consumed and BEC achieved. Furthermore, beyond any possible strain difference, the current study provides direct evidence that mice can change the rate of ethanol consumption during the acquisition of voluntary ethanol drinking behavior.

A previous history of ethanol drinking under DID also increased ethanol consumption and preference. After 6 weeks of DID, mice showed increased ethanol intake in a two-bottle paradigm. A recently published study also showed increased ethanol preference and consumption after 3 and 6 weeks of DID (Cox *et al*, 2013). The results are evidence consistent with plasticity stemming from repeated binge-like drinking and add to the current literature by showing that a history of voluntary binge-like ethanol drinking causes changes in the drinking pattern and enhances ethanol consumption in C57BL/6J mice.

The current study also revealed a mild escalation of ethanol intake over the 6 weeks of DID. However, two recent studies found no statistical difference in ethanol intake and BEC between weeks 1 and 6 of DID (Lowery-Gionta *et al*, 2012; Sparrow *et al*, 2012). It is possible that the larger sample size of this current study enhanced the statistical power. Furthermore, the use of glass bottles instead of plastic bottles for the DID procedure (see Materials and Methods) dramatically reduced the leak volume and improved the accuracy of the intake measurements in the current study.

Individual variability with regard to both ethanol intake and BEC achieved was observed among mice of this inbred strain. For example, a third of the C57BL/6J mice tested here failed to achieve BEC of 80 mg/dl during the 4-h DID session. This variability among individuals of an inbred strain is in agreement with a previous report (Rhodes *et al*, 2007) and could be used to determine the contributions of environmental factors that could lead to more efficient acquisition of voluntary drinking behavior. We also showed that mice achieving BEC of 50 mg/dl on the first ethanol DID session exhibited a significant locomotor activation. Thus, these results suggest that most, if not all, subjects in the study were affected by the ethanol exposure.

It is important to understand changes in synaptic transmission that might contribute to changes in ethanol drinking pattern brought about by the DID paradigm. Thus, we examined GABAergic and glutamatergic synaptic transmission in the striatum, a brain region known to be affected by ethanol exposure and self-administration (Wang *et al*, 2010; Cuzon Carlson *et al*, 2011; Cui *et al*, 2013; Depoy *et al*, 2013; Fanelli *et al*, 2013). Electrophysiological analyses showed that inhibitory GABAergic synapses are depressed following the DID treatment in both striatal subregions, DLS and DMS. Thus, there is an overall disinhibition that could contribute to the increased output from the dorsal striatum. This increased output would, in turn, alter the basal ganglia control of cortical activity, ultimately contributing to enhanced seeking and intake of ethanol. This hypothesis is in agreement with a growing body of evidence that points to an important role of the dorsal striatum in controlling ethanol intake (Jeanblanc *et al*, 2009; Chen *et al*, 2010; Corbit *et al*, 2012; Nielsen *et al*, 2012; Jeanblanc *et al*, 2013).

We found no significant correlation between mIPSC frequency and BECs in either striatal subregions. However, in the DMS, there was a slight trend for a positive correlation as mice that achieved higher BEC displayed a larger reduction in mIPSC frequency. In the DLS, there was an opposite trend as mice that achieved BEC of around 100 mg/dl showed a larger reduction in mIPSC frequency than those with higher BEC. While this trend was also not significant, it could represent a preliminary evidence of compensatory changes that are triggered only at higher BECs.

It was surprising to find that the basal frequency of inhibitory GABAergic mIPSCs in the DLS is twice that observed in the DMS, suggesting that neurons in the DLS are under a stronger inhibitory tone. Acute application of ethanol to striatal slices decreased the frequency of GABAergic mIPSCs in the DLS but increased the frequency in the DMS of ethanol naïve animals. These opposing actions equalize mIPSC frequency in DLS and DMS. By enhancing the inhibitory tone in DMS, acute ethanol might

Figure 6 The acute effects of ethanol on GABA transmission are impaired after binge-like ethanol consumption. (a, b) Representative traces of miniature inhibitory post-synaptic currents (mIPSCs) recordings from medium spiny neurons (MSNs) in the dorsolateral striatum (DLS) of water (a) and ethanol drinking mice (b) before (top traces) and after acute application of 50 mm ethanol (bottom traces). (c) The effect of 50 mm ethanol application on mIPSC frequency normalized to baseline frequency in the DLS of water (white) and ethanol drinking (black) mice. (d) Frequency of mIPSCs recorded from individual MSNs in the DLS during baseline (base) and acute exposure to 50 mm ethanol (+ acute ethanol) in water drinking (white) and ethanol drinking (black) mice. (e, f) Representative traces of mIPSC recordings from MSNs in the DMS of water (e) and ethanol drinking mice (f) before (top) and after (bottom) the acute application of 50 mm ethanol. (g) The effect of 50 mm ethanol application on mIPSC frequency normalized to baseline frequency in the DMS of water (white) and ethanol drinking (black) mice. (h) Frequency of mIPSCs recorded from individual MSNs in the DMS during baseline (base) and acute exposure to 50 mm ethanol (+ acute ethanol) in water drinking (white) and ethanol drinking (black) mice. Data are expressed as mean ± SEM. *Denotes a statistical difference from baseline mIPSC frequency, $P < 0.05$.
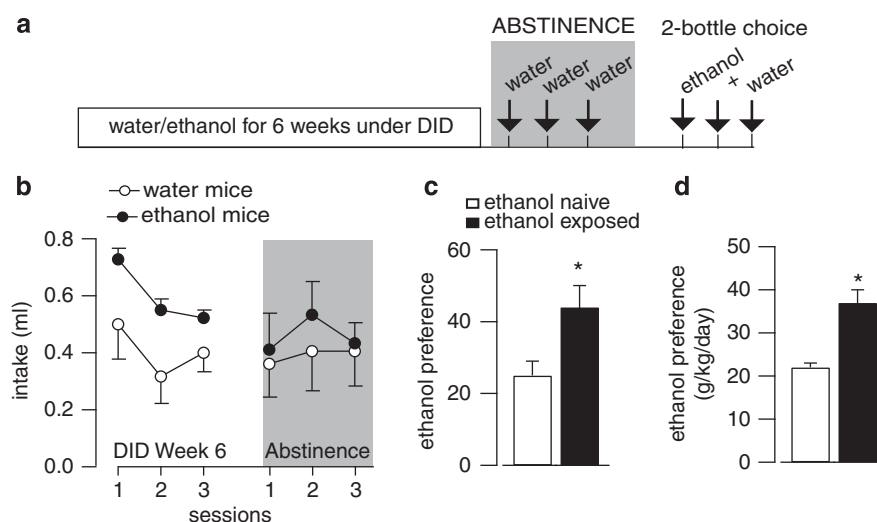
Figure 7 Higher ethanol consumption in mice with a history of binge-like ethanol consumption. (a) Timeline of the two-bottle choice test performed after 6 weeks of drinking in the dark (DID) and 1 week of abstinence. (b) Intake volume during the last week (6) of DID for water (open) and ethanol (solid) mice and during the week of abstinence in which only water was available. Markers and lines represent the mean ± SEM for every 2 h session. (c) Ethanol preference determined on the third day of two-bottle choice experiment for ethanol naïve mice (open, $n = 9$ mice) and for ethanol pre-exposed mice (solid, $n = 9$ mice). *Denotes $P = 0.02$. (d) Ethanol consumed by ethanol naïve mice (open, $n = 9$ mice) and by ethanol pre-exposed mice (solid, $n = 9$ mice) during the two-bottle choice experiment. *Denotes $P = 0.0004$.

be responsible for shutting down DMS neurons, while simultaneously disinhibiting the DLS.

The potentiating effect of acute ethanol in DMS is reminiscent of the enhancement of GABA release produce by acute ethanol exposure in other brain regions (Siggins et al, 2005; Kelm et al, 2011). However, the acute ethanol decrease in mIPSC frequency observed in DLS is a more unique finding. Further work will be needed to determine the mechanisms underlying this decrease (eg, altered neuromodulator levels or activity) and the cellular and molecular bases of the differences in GABAergic transmission and acute ethanol effect in DLS and DMS. In animals with a history of binge-like ethanol drinking, the effects of acute ethanol on GABAergic synaptic currents were absent in the DLS, and switched from potentiation to depression in the DMS. In the DLS, the lack of acute response may reflect occlusion, given that the baseline mIPSC frequency is already depressed in ethanol drinking mice. The change in the acute ethanol action in the DMS cannot be explained by occlusion, as the baseline mIPSC frequency decreased following the DID procedure. One possible explanation is that DID exposure fosters tolerance to the acute ethanol potentiation leaving behind a small inhibitory effect.

The properties of spontaneous glutamatergic transmission were similar in the DLS and DMS of water and ethanol mice. Furthermore, there were no significant changes in the density of dendritic spines, the postsynaptic locus of glutamatergic synapses, in neurons of the dorsal striatum after 6 weeks of intermittent ethanol drinking. Other studies have previously shown ethanol-induced changes in glutamatergic transmission, glutamate receptors, and/or spine density in dorsal striatal regions. For example, short-term, repeated ethanol exposure altered AMPA receptor synaptic localization and NMDA receptor activity (specifically NR2B-containing receptors) in the dorsomedial striatum (Wang et al, 2010, 2012). Our group has previously reported that non-human primates that consumed ethanol for several

years exhibited an enhanced glutamatergic transmission and spine density in the putamen, a region analogous to the DLS in rodent (Cuzon Carlson et al, 2011).

Several factors may contribute to the different results, including animal species, age of drinking onset, extended vs short access, and time after the last binge. With the currently available data, it is not possible to identify the responsible factor with certainty. From all the parameters, the extent of the ethanol drinking (in terms of exposure duration and total amount consumed) emerges as a likely relevant factor that can be tested in future studies. Nevertheless, despite the differences, one relevant conclusion that transpires from all these studies is that ethanol drinking leads to enhanced striatal output.

Altogether, this dissection of the ethanol drinking behavior during DID identified the bottle exchange as a likely mechanism by which intermittent access facilitates the acquisition of ethanol drinking behavior by inducing mice to drink at a higher rate. Over the weeks of repeated ethanol access, mice increased the rate of drinking at the beginning of the DID sessions and faster ethanol drinking was associated with higher BEC and enhanced ethanol preference. The behavioral changes were accompanied by a depression of inhibitory GABAergic transmission in the dorsal striatum, which would like contribute to enhanced striatal output.

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