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Chronic Ethanol Exposure Leads to Divergent Control of Dopaminergic Synapses in Distinct Target Regions

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Abstract

Neuroadaptations following chronic exposure to alcohol are hypothesized to play important roles in alcohol-induced alterations in behavior, in particular increased alcohol drinking and anxiety like behavior. Dopaminergic signaling plays a key role in reward-related behavior, with evidence suggesting it undergoes modification following exposure to drugs of abuse. A large literature indicates an involvement of dopaminergic signaling in response to alcohol. Using a chronic inhalation model of ethanol exposure in mice, we have begun to investigate the effects of alcohol intake on dopaminergic signaling by examining protein levels of tyrosine hydroxylase (TH) and the dopamine transporter (DAT), as well as monoamine metabolites in three different target fields of three different dopaminergic nuclei. We have focused on the dorsal lateral bed nucleus of the stria terminalis (dBNST) because of the reported involvement of dBNST dopamine in ethanol intake, and the nucleus accumbens (NAc) and dorsal striatum because of their dense dopaminergic innervation. After either a chronic intermittent exposure (CIE) or continuous exposure (CCE) regimen, mice were killed, and tissue punches collected from the dBNST, NAc and striatum for Western analysis. Strikingly, we found divergent regulation of TH and DAT protein levels across these three regions that was dependent upon the means of exposure. These data thus suggest that distinct populations of catecholamine neurons may be differentially regulated by ethanol, and that ethanol and withdrawal interact to produce differential adaptations in these systems.

Introduction

Alcoholism is a complex disease characterized by chronic relapse to alcohol intake despite known, and often severe, negative consequences. It is believed that adaptations within specific neuronal circuitries underlie this aberrant behavior, and a large focus of the research community is on the delineation of these specific circuitries (Koob and Kreek, 2007). A number of neurotransmitter systems are also thought to be involved, including classical neurotransmitters GABA and glutamate and a number of neuromodulators, including CRF and NPY (Heilig and

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Koob, 2007). In addition, the dopaminergic system has also been strongly implicated in alcohol abuse, particularly in mediating its acute reinforcing properties. Acute alcohol exposure can enhance the firing rate of dopaminergic cells in both the ventral tegmental area (VTA) (Brodie et al., 1999) and the substantia nigra (Mereu et al., 1984). This is thought to correspond to an increase in dopamine release in a number of brain regions, including the nucleus accumbens (Melendez et al., 2002; Gonzales et al., 2004), striatum (Melendez et al., 2003), the central nucleus of the amygdala (Yoshimoto et al., 2000) and the bed nucleus of the stria terminalis (BNST) (Carboni et al., 2000).

While positive reinforcement is thought to be a driving force in acute intake, ethanol seeking after chronic exposure has been proposed to involve more complex processes, included alleviation of negative reinforcement provided by withdrawal from alcohol intake (Koob and Kreek, 2007). Further, repeated cycles of exposure and withdrawal have been proposed to “kindle” circuitries key to the likelihood of subsequent alcohol intake, such as those subserving anxiety, HPA axis regulation and reward (Breese et al., 2005). Currently less is known of the participation of dopamine in chronic actions of ethanol, or conversely, what effects chronic ethanol exposure has on dopaminergic systems. Chronic ethanol exposure has been reported to alter dopaminergic function via modulation of the activity of VTA dopaminergic cells (Brodie, 2002; Shen, 2003; Hopf et al., 2007). In addition to somatic effects on dopaminergic neurons, a number of studies have suggested that chronic ethanol administration can alter dopaminergic function at the level of the dopamine synapse (Casu et al., 2002; Budygin et al., 2003; Budygin et al., 2007). In particular, several studies have provided evidence for enhanced dopamine uptake in the nucleus accumbens (Carroll et al., 2006; Budygin et al., 2007).

To date, the bulk of the studies on ethanol effects on dopaminergic transmission have either focused on VTA dopaminergic input to the nucleus accumbens, or nigral input to the dorsal striatum, with an implicit assumption that ethanol produces common regulation of all dopaminergic neurons. In addition to these two primary sources of dopamine, recent findings have drawn interest to the role of a third population of dopamine neurons, the A10dc group in the periaqueductal gray in anxiety and reward related behaviors (Flores et al., 2006). Thus we set out to systematically explore the effects of chronic ethanol exposure and withdrawal on these three different groups by examining markers of dopamine transmission in their respective target fields; the dorsal striatum, nucleus accumbens, and dorsal-lateral bed nucleus of the stria terminalis (dBNST).

We utilized a chronic inhalation model to provide controlled, sustained ethanol delivery to mice, as described by Becker and colleagues (Becker, 1994). Mice were either subjected to a chronic intermittent exposure (CIE) or to a continuous exposure (CCE) regimen, after which they were killed, and tissue punches collected from the dBNST, NAc and dorsal striatum for Western and HPLC-based neurochemical analysis. Strikingly, we found divergent regulation of DAT protein levels across these three regions that was dependent upon the means of exposure. These data thus suggest that distinct populations of dopamine neurons may be differentially regulated by ethanol, and that ethanol and withdrawal interact to produce differential adaptations in these systems.

Methods

Chronic Intermittent Ethanol Procedure

Procedures were performed as outlined in the INIA-Stress standard operating procedure (www.iniastress.org). Briefly, male C57Bl/6J mice (6-7 weeks old, Jackson Laboratories) were given an injection daily of either pyrazole (control group, CON, 1 mmol/kg) or pyrazole + ethanol (ethanol group, EtOH, 1mmol/kg + 0.8 g/kg, respectively). Thirty minutes following the injection, in their home cages, mice were placed in a chamber filled with volatilized ethanol

(EtOH, 20.3 ± 0.2 mg/L) or volatilized water (CON). Airflow through the chambers was maintained at 5.5 L/min and volatilization at 1.5 L/min. Mice were allowed food and water *ad libitum*. After 16 hours of exposure, mice were removed from the chambers and placed in fresh cages. Four 16 hour exposures occurred with 4 eight hour withdrawals following each exposure. Mice were killed immediately after the final exposure for blood ethanol content and corticosterone measurement, and 4-6 hours or 4 days after the last exposure for corticosterone measurement and brain tissue collection (see Figure 1A).

Chronic Continuous Ethanol Procedure

Treatment and chamber conditions of chronic continuous ethanol (CCE) mice were similar to that of CIE mice, however mice were only removed from the chambers twice per day for very brief periods (cage change and injection). After 64 hours of continuous exposure, mice were removed from the chambers. Mice were killed immediately post the final exposure for blood ethanol content and corticosterone measurement, and 4-6 hours or 4 days after the last exposure for corticosterone measurement and brain tissue collection (see Figure 1B).

Blood Ethanol Measurement

Immediately after being removed from the chamber, mice underwent cervical dislocation and decapitation to collect trunk blood. After centrifugation at 4000 rpm for 10 min at 4°C, plasma was collected. Ten μ L of plasma was used in the blood ethanol kit (Diagnostic Chemicals Limited Kit #229-29, Oxford, CT). The rest of the plasma was stored at -80°C until further analysis.

Corticosterone Assay

Plasma corticosterone was measured with an ImmuChem Double Antibody 125I RIA Kit (MP Biomedicals) by the hormone assay and analytical services core, Vanderbilt MMPC, grant # U24DK59637

Tissue Collection

Mice were decapitated under anesthesia (Isoflurane). The brains were quickly removed and placed in ice-cold sucrose-artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃ saturated with 95% O₂/5% CO₂. Slices 300 μ m in thickness were prepared using a vibratome (Leica). Rostral slices containing nucleus accumbens (Bregma 1.10 mm to 1.0 mm) (Franklin and Paxinos, 1997) were identified using the lateral ventricle and anterior commissure. Caudal slices containing anterior portions of BNST (Bregma 0.26 mm to 0.02 mm) (Franklin and Paxinos, 1997) were identified using the internal capsule, anterior commissure, fornix and stria terminalis as landmarks as previously described (Egli and Winder, 2003; Egli et al., 2005; Weitlauf et al., 2004). Slices were frozen on a block cooled with dry ice. Once the slice was frozen, 0.5mm punches were taken from the NAc, dBNST, and dorsal striatum (dStr) as previously described (Grueter et al., 2006; Olsen and Winder, 2007). Punches were stored at -80°C until further analysis (Representative punches shown in Figure 2).

Monoamine measurement

Tissue monoamine measurements were collected with two dedicated Waters high performance liquid chromatographic systems equipped with autosampler and a 464 pulsed electrochemical after extraction of the tissue by The Center for Molecular Neuroscience/Vanderbilt Kennedy Center Neurochemistry Core Lab.

Immunohistochemistry

Male C57Bl/6J mice (6-7 weeks old, Jackson Laboratories) transcardially perfused with phosphate buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Following a 24 hour post-fixation, brains were transferred to 20% sucrose and stored at 4°C for up to one week. Using a cryostat, 25µm coronal brain slices containing BNST were made. Slices were free-floated for immunohistochemical staining. Sections were quenched in 0.6% hydrogen peroxide, blocked in 5% normal goat serum with 0.2% Triton-X-100 (block), exposed to primary antibody in block overnight at 4°C, and exposed to secondary antibody in block overnight at 4°C. Following antibody exposures, the ABC elite kit was used to amplify signal (Vector, Burlingame, CA), and visualization occurred using a diaminobenzoic acid peroxidase substrate kit (Vector, Burlingame, CA). Sections were then mounted on slides, sealed with PolyAquamount, and left overnight to dry. To ensure that differences in staining intensity were due to antigen expression, multiple slices were used concurrently using the same conditions. In addition, control conditions of slices being exposed to all conditions with the exception of the primary antibody were utilized. The primary antibodies used were mouse α -tyrosine hydroxylase (1:8000, Immunostar Corporation, Hudson, WI) and rat α -dopamine transporter (1:500, Chemicon International, Temecula, CA). The biotinylated secondary antibodies used were goat α -rat and goat α -mouse (1:1000, Vector, Burlingame, CA).

Tissue Homogenization and Western Blotting

Punches were homogenized in 2% SDS, 2mM sodium orthovanadate, 2mM NaF, 1mM benzanamide, 10mg/ml aprotinin, 10mg/ml leupeptin. Whole homogenate was analyzed. Protein levels were determined with a BCA protein assay kit, diluted to equal concentrations, mixed with sample buffer (62.5 mM Tris-Cl, pH 6.8, glycerol, 5% SDS, 0.5% bromophenol blue, and 5% β -mercaptoethanol) and 20 µg was run on a 10% polyacrylamide resolving gel. Protein was transferred to two Immobilon PVDF membranes (Pall Corporation, Pensacola, FL) in series. The first blot was probed with specific primary antibodies, whereas the second blot was stained for total protein using colloidal gold (Bio-Rad, Hercules, CA) to grossly verify equal lane loading. In all cases, the first blot was stripped and reprobed with additional primary antibodies. Antibodies used include mouse α -TH (1:2000, Immunostar) and rat α -DAT (1:4000, Chemicon). In order to combine blocks of experiments from different blots, samples on each blot were normalized to their experimental timepoint control. For example, 4-6 hour intermittent control and ethanol samples were normalized to 4-6 hour intermittent control while 4 day continuous control and ethanol samples were normalized to 4 day continuous control.

Results

Male C57bl/6J mice were chronically exposed to ethanol vapor as described above (Figure 1A). Both of the ethanol exposure paradigms, CIE and CCE, resulted in blood ethanol concentrations (BECs) of 182 ± 9 mg/dL immediately following removal from the chamber following the initial exposure ($n = 6$). The corresponding blood alcohol level (0.21%) in a human would render the user legally intoxicated. The concentration of ethanol in the chamber was monitored throughout experiments in such that the environmental exposure was consistent.

Given the role that corticosterone has been hypothesized to play in ethanol withdrawal, we examined corticosterone levels at two time points (4-6 hours and 4 days following removal from ethanol chamber) from each treatment condition. We found a significant increase in corticosterone levels 4-6 hours (Figure 1C)(CIE control: 137 ± 14 ng/mL, $n = 20$, CIE ethanol: 206 ± 14 ng/mL, $n = 21$, CCE control: 143 ± 30 ng/mL, $n = 5$; CCE ethanol: 330 ± 29 ng/mL, $n = 4$), but not 4 days (CIE control: 86 ± 18 ng/mL, $n = 13$, CIE ethanol: 84 ± 17 ng/mL, $n = 14$, CCE control: 80 ± 30 ng/mL, $n = 5$; CCE ethanol: 85 ± 38 ng/mL, $n = 3$), following removal from the chambers in both treatment groups. Using a two-way ANOVA there was a significant

interaction of model and treatment - $F_{1,49}=4.26136$, $p=0.0447$. Further, there was a significant main effect of model (intermittent versus continuous) - $F_{1,49}=5.07$, $p=0.029$. There is a significant main effect of treatment (control versus ethanol) - $F_{1,49}=19.92011$, $p<0.0001$. After Fisher's LSD Post-hoc analysis, it was determined that CCE at 4-6 hours was significantly different from all other groups ($p<0.01$), CIE was significantly different from chronic intermittent control and CCE at 4-6 hours ($p<0.01$) and there was no significant difference between intermittent and continuous control ($p>0.05$). The control animals have slightly elevated corticosterone levels 4-6 hours upon removal from the chamber as compared to 4 days following removal. This may be reflective of a level of stress caused by the chronic ethanol exposure paradigm and underlies the importance of comparing the ethanol treated animals to the sham control animals. However, in the biochemical experiments below, there were no significant differences between the control values at the different timepoints. For this reason we compared the ethanol groups to the corresponding time matched control measurements.

As shown in Figure 2, punches were taken from the nucleus accumbens, dorsal striatum and dBNST. HPLC analysis of dopamine and dopamine metabolite tissue content levels revealed, as expected, greater levels of basal dopamine and dopamine metabolite levels in the NAc and dSTR than in the dBNST (Figure 2E). Nonetheless, these monoamines were readily detectable in the dBNST as well. Further, we demonstrate, using immunohistochemistry, that both TH and DAT are expressed in the dBNST (Figure 3).

Effect of ethanol treatment on DAT and TH protein expression in the nucleus accumbens

CIE treatment resulted in a significant upregulation of DAT protein expression in the nucleus accumbens 4-6 hours (179 ± 38 % of control values at 4-6 hours, $n = 13$, $p < 0.05$) and 4 days (169 ± 28 % of control values at 4 days, $n = 11$, $p = 0.05$), following removal from the ethanol chambers (Figure 4A). Curiously, we found that DAT expression in the nucleus accumbens was significantly decreased 4-6 hours (43 ± 13 % of control values at 4-6 hours, $n = 6$, $p < 0.05$), but not 4 days (76 ± 22 % of control values at 4 days, $n = 5$), following CCE treatment. These data suggest that DAT levels are bidirectionally modulated by ethanol exposure and withdrawal.

We next examined TH expression levels in the nucleus accumbens and found that neither CIE nor CCE altered expression at 4-6 hours (CIE: 118 ± 14 % of control values at 4-6 hours, $n = 15$; CCE: 94 ± 8 % of control values at 4-6 hours, $n = 6$) or 4 days (CIE: 100 ± 8 % of control values at 4-6 hours, $n = 9$; CCE: 100 ± 10 % of control values at 4-6 days, $n = 5$) following removal from the chambers (Figure 4B).

Effect of ethanol treatment on DAT and TH protein expression in the dorsal striatum

Neither CIE nor CCE treatment resulted in an alteration of DAT protein expression in the dorsal striatum 4-6 hours (CIE: 87 ± 16 % of control values at 4-6 hours, $n = 7$; CCE: 112 ± 16 % of control values at 4-6 hours, $n = 8$) or 4 days (CIE: 93 ± 19 % of control values at 4 days, $n = 8$; CCE: 131 ± 17 % of control values at 4 days, $n = 6$) following removal from the ethanol chambers (Figure 5A). Additionally, we found no changes in TH expression levels in the dorsal striatum following either CIE or CCE treatment at 4-6 hours (CIE: 94 ± 13 % of control values at 4-6 hours, $n = 7$; CCE: 97 ± 4 % of control values at 4-6 hours, $n = 8$) or 4 days (CIE: 92 ± 12 % of control values at 4 days, $n = 8$; CCE: 91 ± 13 % of control values at 4 days, $n = 6$) following removal from the chambers (Figure 5B).

Effect of ethanol treatment on DAT and TH protein expression in the dorsal bed nucleus of the stria terminalis

Neither CIE nor CCE treatment resulted in statistically significant alterations of DAT protein expression in the dBNST 4-6 hours (CIE: 62 ± 18 % of control values at 4-6 hours, $n = 15$;

CCE: 98 ± 23 % of control values at 4-6 hours, $n = 7$) or 4 days (CIE: 53 ± 12 % of control values at 4 days, $n = 13$; CCE: 141 ± 41 % of control values at 4 days, $n = 6$) following removal from the ethanol chambers (Figure 6A). Additionally, we found no changes in TH expression levels in the dBNST following CIE nor CCE treatment at 4-6 hours (CIE: 71 ± 13 % of control values at 4-6 hours, $n = 15$; CCE: 100 ± 9 % of control values at 4-6 hours, $n = 7$) or 4 days (CIE: 83 ± 13 % of control values at 4 days, $n = 14$; CCE: 100 ± 9 % of control values at 4-6 hours, $n = 6$) following removal from the chambers (Figure 6B).

Effect of Chronic Intermittent Ethanol treatment on tissue content of dopamine and dopamine metabolites

We next examined the tissue content of dopamine and the dopamine metabolites, DOPAC and HVA, in nucleus accumbens punches obtained from mice that have undergone the CIE treatment. We found no significant changes in dopamine (control 4- 6 hours: 49 ± 6 ng/mg, $n = 7$; ethanol 4 – 6 hours: 56 ± 12 ng/mg, $n = 6$; control 4 days: 53 ± 9 ng/mg, $n = 9$; ethanol 4 days: 51 ± 5 ng/mg, $n = 12$), DOPAC (control 4- 6 hours: 2.7 ± 0.3 ng/mg, $n = 7$; ethanol 4 – 6 hours: 2.9 ± 0.5 ng/mg, $n = 6$; control 4 days: 3.9 ± 0.5 ng/mg, $n = 9$; ethanol 4 days: 4.5 ± 1.1 ng/mg, $n = 12$) or HVA (control 4- 6 hours: 4.4 ± 1.2 ng/mg, $n = 7$; ethanol 4 – 6 hours: 3.4 ± 0.6 ng/mg, $n = 6$; control 4 days: 4.3 ± 0.8 ng/mg, $n = 9$; ethanol 4 days: 3.9 ± 0.8 ng/mg, $n = 12$) at any of the time points investigated (results normalized to control values are shown in Figure 7A).

We then examined the tissue content of dopamine and the dopamine metabolites, DOPAC and HVA, in dorsal striatum punches obtained from mice that have undergone the CIE treatment. We found no significant changes in dopamine 4-6 hours following removal from the chambers (control 4- 6 hours: 63 ± 6 ng/mg, $n = 9$; ethanol 4 – 6 hours: 58 ± 8 ng/mg, $n = 9$), however we did note a significant increase in dopamine content 4 days following removal from the chambers (control 4 days: 60 ± 4 ng/mg, $n = 13$; ethanol 4 days: 73 ± 5 ng/mg, $n = 12$, student's t-test, $p < 0.05$). There were no significant differences in DOPAC (control 4- 6 hours: 3.8 ± 0.4 ng/mg, $n = 9$; ethanol 4 – 6 hours: 4.7 ± 0.7 ng/mg, $n = 9$; control 4 days: 4.0 ± 0.4 ng/mg, $n = 13$; ethanol 4 days: 5.4 ± 0.8 ng/mg, $n = 12$) or HVA (control 4- 6 hours: 5.6 ± 0.9 ng/mg, $n = 9$; ethanol 4 – 6 hours: 9.6 ± 2.2 ng/mg, $n = 9$; control 4 days: 8.7 ± 1.2 ng/mg, $n = 13$; ethanol 4 days: 9.0 ± 1.1 ng/mg, $n = 12$) at either time point (results normalized to control values are shown in Figure 7B).

We next examined the tissue content of dopamine and the dopamine metabolites, DOPAC and HVA, in dBNST punches obtained from mice that have undergone the CIE treatment. We found no significant changes in dopamine (control 4- 6 hours: 13 ± 2 ng/mg, $n = 8$; ethanol 4 – 6 hours: 17 ± 3 ng/mg, $n = 9$; control 4 days: 10 ± 1 ng/mg, $n = 11$; ethanol 4 days: 13 ± 2 ng/mg, $n = 13$), DOPAC (control 4- 6 hours: 1.0 ± 0.2 ng/mg, $n = 8$; ethanol 4 – 6 hours: 1.5 ± 0.2 ng/mg, $n = 9$; control 4 days: 1.1 ± 0.2 ng/mg, $n = 11$; ethanol 4 days: 1.5 ± 0.2 ng/mg, $n = 13$) or HVA (control 4- 6 hours: 2.9 ± 0.5 ng/mg, $n = 8$; ethanol 4 – 6 hours: 3.5 ± 0.6 ng/mg, $n = 9$; control 4 days: 2.8 ± 0.4 ng/mg, $n = 11$; ethanol 4 days: 3.3 ± 0.7 ng/mg, $n = 13$) at any of the time points investigated (results normalized to control values are shown in Figure 7C).

Discussion

The results of this study demonstrate that chronic ethanol vapor exposure alters the expression of DAT in both a location-specific and exposure-specific fashion in male C57Bl/6j mice. Specifically, chronic intermittent ethanol vapor treatment increased DAT expression in the nucleus accumbens but not the dorsal striatum or the dBNST. Additionally, chronic continuous ethanol vapor treatment decreased DAT expression in the nucleus accumbens with no significant effects in the dorsal striatum or the dBNST. These findings support the idea that

chronic ethanol exposure and subsequent withdrawal can alter dopaminergic transmission in reward-related circuitry.

Ethanol exposure paradigm

Several groups have hypothesized that the development of alcoholism is dependent not only on alcohol exposure, but rather repeated cycles of exposure and withdrawal. In order to determine if there were alterations in proteins involved in dopaminergic transmission in reward-related circuitry that were sensitive to the pattern of exposure we evaluated the effect of a continuous versus an intermittent exposure. Both of these paradigms resulted in decreased body weight compared to control animals (data not shown) and increased corticosterone levels. It is unclear as to the event, ethanol exposure versus withdrawal, that precipitated the increase in corticosterone levels, as acute ethanol exposure causes an increase in corticosterone levels (Gorin-Meyer et al., 2007).

Nucleus accumbens

A number of studies have shown alterations in dopamine dynamics in the nucleus accumbens following chronic ethanol exposure consistent with our findings. Specifically, several recent studies (Carroll et al., 2006; Budygin et al., 2007) demonstrated that chronic ethanol exposure or consumption increased dopamine uptake in the nucleus accumbens in rats. Similar findings have also been demonstrated in non-human primates (Budygin et al., 2003), suggesting that neuroadaptations in dopaminergic systems generalize across species and thus are of particular interest. Our results suggest that these changes in dopamine clearance are due, at least in part, to increases in levels of DAT expression.

Further, our data suggest this effect is restricted to dopamine transmission in the nucleus accumbens relative to the striatum and dBNST. The reason for this change still remains to be determined, however a recent study (Hopf et al., 2007) demonstrated that VTA neurons exhibit increased NMDA-induced bursting during ethanol withdrawal, suggesting that the alteration in DAT expression could be a homeostatic change in response to increasing amounts of dopamine released. Interestingly, we found that DAT levels in the nucleus accumbens were decreased following chronic continuous ethanol exposure. This suggests that the increases in DAT levels are due to an interaction between ethanol exposure and withdrawal, rather than just the alcohol exposure. This is consistent with the findings that intermittent ethanol exposure causes a greater increase in drinking behavior when compared to continuous exposure (Lopez and Becker, 2005). Moreover, an alteration occurring in withdrawal dependent fashion could play a key role in the kindling-like process that is believed to be critical for the development of alcoholism. We did not note any changes in TH expression or levels of dopamine or dopamine metabolites in the nucleus accumbens at any time point. Given the lack of temporal and spatial resolution of the neurochemical analysis we performed it is difficult to draw conclusions, but it is worth noting that this is consistent with results obtained from other groups showing no change in dopamine release properties (Budygin et al., 2007).

Dorsal Striatum

In contrast to our findings in the nucleus accumbens, there were no alterations in DAT or TH expression in the dorsal striatum following either CIE or CCE exposure paradigms. This finding appears to contrast to a recent study that demonstrated an increase in dopamine clearance in the caudate putamen (Budygin et al., 2007). One potential explanation is that the increase in striatal dopamine clearance reported by Budygin et al. could be mediated through a post-translational mechanism involving alterations in DAT function and/or subcellular localization. For example, ethanol has been reported to acutely regulate DAT function (Mayfield et al., 2001; Maiya et al., 2002).

Since the substantia nigra is the primary source of dopamine in the striatum, these data suggest that distinct populations of dopamine synapses differentially adapt to ethanol exposure and withdrawal. This could be due to differences in target feedback, in presynaptic responsiveness, in dopamine somatic responses, or a combination of these. While we did not observe alterations in DAT levels in the striatum of C57Bl/6J mice, it is important to note that this lack of a change in DAT expression levels could be due to subtle genetic or species related differences in dopaminergic neuroadaptation, as a recent study demonstrated that prolonged alcohol consumption altered DAT binding in the caudate-putamen in Wistar Kyoto but not Wistar rats (Jiao et al., 2006). Curiously, we noted a small but significant increase of dopamine levels in the dorsal striatum 4 days following removal from the ethanol chambers. This may reflect an increase in dopaminergic tone in the dorsal striatum.

Dorsolateral BNST

There were no statistically significant changes noted in DAT or TH expression in the dBNST following either CIE or CCE exposure paradigms. This finding would suggest that the dopaminergic afferents to the dBNST are differentially regulated than those of the closely related structure, the nucleus accumbens. This is consistent with a study that demonstrated no alterations in TH expression in the extended amygdala, specifically the central nucleus of the amygdala, following chronic ethanol exposure (Zhou et al., 2006). While the dBNST and the nucleus accumbens share a common source of dopamine, the VTA, several studies have shown that the dBNST receives roughly half of its dopamine from the periaqueductal gray (PAG) (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006). Our results demonstrating that DAT in the dBNST is unchanged during ethanol withdrawal raise the interesting possibility that there is divergent modulation of DA afferents to the BNST; with the VTA originating fibers having upregulated DAT expression with the PAG originating fibers having reduced DAT expression. Indeed, a sensitization of the PAG has been suggested to play a role in the negative states associated with ethanol withdrawal (Cabral et al., 2006). Alternatively, distinct target or terminal effects as outlined above could play a role.

Conclusion

Here we demonstrate alterations in DAT expression that are both regionally and treatment specific. These changes may reflect alterations in dopaminergic function induced by the combination of alcohol exposure and subsequent withdrawal. Moreover, these findings suggest that region specific alterations in dopaminergic function may underlie some of the behavioral abnormalities associated with chronic ethanol exposure.

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A.

Chronic Intermittent Exposure (CIE)

B.

Chronic Continuous Exposure (CCE, 64 hours)

C.

Figure 1. Diagram of Ethanol Exposure

- A. Time-course of the two chronic ethanol exposure paradigms employed in this study. The Chronic Intermittent Exposure (CIE) paradigm was comprised of four 16 hour ethanol exposure sessions, separated by 8 hour periods of withdrawal from ethanol.
- B. The Chronic Continuous Exposure (CCE) paradigm was a single 64 hour ethanol exposure followed by a single withdrawal episode. Samples were collected at two different time points for each treatment condition, 4-6 hours following removal from the ethanol chambers, and 4 days following removal from chambers.
- C. Corticosterone levels (in ng/ml) at 4-6 hours and 4 days following removal from CIE and CCE treatment paradigm. * indicates a significant difference between chronic intermittent ethanol treatment values compared to the chronic intermittent control values. # indicates a significant difference between chronic continuous ethanol treatment values compared to chronic continuous control values and chronic intermittent ethanol treatment values.

Figure 2. Diagram of tissue punches and representative slices

- A. Diagram adapted from mouse brain atlas demonstrating the size and location of the tissue punch employed in the nucleus accumbens as shown by the grey circle.
- B. Representative tissue punch of the nucleus accumbens from a coronal brain slice.
- C. Diagram adapted from mouse brain atlas demonstrating the size and location of the tissue punch employed in the dorsal striatum and dorsal bed nucleus of the stria terminalis as shown by the grey circles.
- D. Representative tissue punch of the dorsal striatum and dorsal bed nucleus of the stria terminalis from a coronal brain slice.
- E. Dopamine, DOPAC and HVA content of tissues punches analyzed via HPLC.

Figure 3. DAT and TH are expressed in the dorsal bed nucleus of the stria terminalis

- A.** (1,2) DAT is present in the dBNST albeit in lower levels than in the striatum. This labeling is absent when the slices were not incubated with the primary antibody (3).
- B.** (1,2) TH is present in both the dBNST and the ventral BNST. This labeling is absent when the slices were not incubated with the primary antibody (3).

Figure 4. Chronic ethanol exposure alters DAT expression in the Nucleus Accumbens

- A.** Representative immunoblots for DAT from nucleus accumbens of either CIE, CCE or control animals 4-6 hours and 4 days following removal from ethanol chambers. Quantitative analysis of DAT expression in the nucleus accumbens following CIE and CCE treatment 4-6 hours and 4 days following removal from ethanol chambers. DAT expression levels in the nucleus accumbens were significantly ($p < 0.05$) increased at the early (4-6 hours) and late (4 days) withdrawal time-points following CIE. DAT expression levels in the nucleus accumbens were significantly ($p < 0.05$), decreased at the early (4-6 hours) but not the late (4 days) withdrawal time-points following CCE.

- B.** Representative immunoblots for TH from nucleus accumbens of either CIE, CCE or control animals 4-6 hours and 4 days following removal from ethanol chambers. Quantitative analysis of TH expression in the nucleus accumbens following CIE and CCE treatment 4-6 hours and 4 days following removal from ethanol chambers. TH expression levels in the nucleus accumbens were not altered at either early or late withdrawal time-points following CIE or CCE

A.

Intermittent

Continuous

B.

4-6h withdrawal

4d withdrawal

Intermittent

Continuous

Figure 5. Chronic ethanol exposure does not alter expression of DAT or TH in the dorsal striatum

- A.** Representative immunoblots for DAT from dorsal striatum of either CIE, CCE or control animals 4-6 hours and 4 days following removal from ethanol chambers. Quantitative analysis of DAT expression in the dorsal striatum following CIE and CCE treatment 4-6 hours and 4 days following removal from ethanol chambers. DAT expression levels in the dorsal striatum were not altered at either early or late withdrawal time-points following CIE or CCE.
- B.** Representative immunoblots for TH from dorsal striatum of either CIE, CCE or control animals 4-6 hours and 4 days following removal from ethanol chambers. Quantitative analysis of TH expression in the dorsal striatum following CIE and CCE treatment 4-6 hours and 4 days following removal from ethanol chambers. TH expression levels in the dorsal striatum were not altered at either early or late withdrawal time-points following CIE or CCE.

Figure 6. Chronic ethanol exposure does not alter expression of DAT or TH in the dorsal bed nucleus of the stria terminalis

- A.** Representative immunoblots for DAT from BNST of either CIE, CCE or control animals 4-6 hours and 4 days following removal from ethanol chambers. Quantitative analysis of DAT expression in the BNST following CIE and CCE treatment 4-6 hours and 4 days following removal from ethanol chambers. There was no alteration in DAT expression in the BNST at either early or late withdrawal time-points following CIE or CCE.
- B.** Representative immunoblots for TH from BNST of either CIE, CCE or control animals 4-6 hours and 4 days following removal from ethanol chambers. Quantitative analysis of TH expression in the BNST following CIE and CCE treatment 4-6 hours and 4 days following removal from ethanol chambers. TH expression levels in the

BNST were not significantly altered at the early (4-6 hours) but not the late (4 days) withdrawal time-points following CIE or CCE.

Figure 7. Effects of chronic ethanol exposure on tissue content of dopamine and dopamine metabolites

- A.** Tissue punches from the nucleus accumbens were analyzed for dopamine and the dopamine metabolites, DOPAC and HVA, following CIE treatment. The experimental results (filled bars), shown normalized to the respective controls (clear bars), indicate no significant difference in dopamine, DOPAC or HVA at 4-6 hours post withdrawal or 4 days post withdrawal.
- B.** Tissue punches from the dorsal striatum were analyzed for dopamine and the dopamine metabolites, DOPAC and HVA, following CIE treatment. The results, shown normalized to the respective controls, indicate no significant difference in dopamine, DOPAC or HVA at 4-6 hours post withdrawal. However, there is a significant increase in dopamine ($p < 0.05$) but not DOPAC or HVA content 4 days post withdrawal.
- C.** Tissue punches from the dBNST were analyzed for dopamine and the dopamine metabolites, DOPAC and HVA, following CIE treatment. The results, shown normalized to the respective controls, indicate no significant difference in dopamine, DOPAC or HVA at 4-6 hours post withdrawal or 4 days post withdrawal.