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Long-term alcohol consumption alters dorsal striatal dopamine release and regulation by D2 dopamine receptors in rhesus macaques

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The dorsal striatum (DS) is implicated in behavioral and neural processes including action control and reinforcement. Alcohol alters these processes in rodents, and it is believed that the development of alcohol use disorder involves changes in DS dopamine signaling. In nonhuman primates, the DS can be divided into caudate and putamen subregions. As part of a collaborative effort examining the effects of long-term alcohol self-administration in rhesus macaques, we examined DS dopamine signaling using fast-scan cyclic voltammetry. We found that chronic alcohol self-administration resulted in several dopamine system adaptations. Most notably, dopamine release was altered in a sex- and region-dependent manner. Following long-term alcohol consumption, male macaques, regardless of abstinence status, had reduced dopamine release in putamen, while only male macaques in abstinence had reduced dopamine release in caudate. In contrast, female macaques had enhanced dopamine release in the caudate, but not putamen. Dopamine uptake was also enhanced in females, but not males (regardless of abstinence state). We also found that dopamine D2/3 autoreceptor function was reduced in male, but not female, alcohol drinkers relative to control groups. Finally, we found that blockade of nicotinic acetylcholine receptors inhibited evoked dopamine release in nonhuman primates. Altogether, our findings demonstrate that long-term alcohol consumption can sex-dependently alter dopamine release, as well as its feedback control mechanisms in both DS subregions.

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INTRODUCTION

Alcohol is one the most widely used and abused drugs in the world and the number of annual alcohol-attributed deaths exceeds 3 million [1]. In the United States of America, alcohol use disorder (AUD) accounts for annual economic losses of ~$250 billion [2] and ~88,000 deaths [3].

Drugs of abuse, including alcohol, increase dopamine release in the striatum [4]. However, as recreational use progresses to compulsive use and abuse, it is believed that alcohol effects on the brain produce different outcomes than in alcohol naive states. A common feature of treatment-seeking individuals with AUD is the proclivity for multiple abstinence and relapse cycles over a long period of time, often months to years. Rodent experimental models of AUD are consequently limited by the lifespan of the experimental subject. Furthermore, there are substantial differences in alcohol metabolism between rodents and humans that likely affect alcohol consumption patterns. To address these shortcomings, we used a nonhuman primate (NHP) model of long-term alcohol consumption as part of the Integrative Neuroscience Initiative on Alcoholism (INIA) consortium. The use of NHPs provides a level of translational validity that cannot be achieved in rodent studies [5]. This is achieved by the volitional consumption of alcohol (to intoxication) for a long time period (>1 year). This time scale allows for long-term examination of drinking behavior and, eventually, ex vivo analysis of the long-term consequences of AUD on neurobiological processes, including dopamine release.

AUD involves dysfunction of several brain regions, including ventral and dorsal striatal areas rich in dopamine neurotransmission. The DS is divided into the caudate nucleus and putamen in primates and dorsomedial and dorsolateral striatum in rodents. The dorsomedial striatum, as part of the associative circuitry, has relatively more involvement in mediating flexible behavioral adaptations to environmental contingencies, while the dorsolateral striatum, part of the sensorimotor circuitry, has relatively more involvement in mediating habitual or inflexible, stimulus-response-driven behaviors [6]. In AUD, the function of these brain regions may be altered such that alcohol seeking and excessive consumption become habitual outcomes in specific contexts. Indeed, we have previously reported subregion-specific alterations in glutamatergic and GABAergic signaling following long-term alcohol use or treatment in rodents and NHPs that may contribute to the development or maintenance of AUD. For example, following chronic alcohol consumption, GABAergic inhibitory signaling onto dorsolateral striatum/putamen medium spiny

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neurons is diminished, resulting in a disinhibition of this habit-associated brain region [7–9].

The role of dopamine in AUD is complex and has been reviewed in detail elsewhere [10–13]. Briefly, acute alcohol increases dopamine release across the striatum [14] primarily due to increased firing of midbrain dopaminergic neurons, an effect that may underlie the initial reinforcing properties of alcohol. In individuals that drink alcohol frequently, however, tolerance develops, and more alcohol is consumed. Concomitantly, adaptations in glutamatergic, GABAergic, and dopamine transmission occur [15] and greater or continued amounts of alcohol can result in allostatic changes to preserve normal brain function. This allostasis is characterized by aberrant glutamate, GABA, and opioid signaling, as well as, a dysfunction in nigrostriatal and mesolimbic dopamine transmission [16, 17]. The mechanisms underlying this dysregulation of dopamine transmission are not well understood, particularly in a primate brain. Therefore, in the current study, we used fast-scan cyclic voltammetry (FSCV) to study dopamine release dynamics in striatal slices from long-term alcohol drinking and control rhesus macaques. This method allows for examination of dopamine release and its regulation on a subsecond time scale that has seldom been used in NHPs [18–24]. Furthermore, FSCV allows for the study of dopamine uptake using Michaelis–Menten based kinetic modeling of uptake parameters, allowing researchers to assess dopamine transporter function. Finally, we can pharmacologically probe the contribution of different regulatory systems, including the D2 dopamine autoreceptor and nicotinic acetylcholine receptor (nAChR), to dopamine release.

MATERIALS AND METHODS

For full details see Supplementary Materials and Methods.

Subjects

Male and female rhesus macaques (*Macaca mulatta*; 5.5–8.5 years old at study onset) obtained from the Oregon National Primate Research Center were used in the current studies. All procedures were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee.

Alcohol self-administration

Rhesus macaques from three cohorts were induced and maintained on an alcohol self-administration protocol as previously described [7, 25, 26] and outlined in Fig. 1A. Monkeys were single-housed for 22 h each day in a room controlled for temperature (20–22 °C), humidity (65%), and an 11-h light cycle (lights on 06:00–17:00). Monkeys were housed with their drinking panels and each cohort occupied the same room with visual, auditory, and olfactory access to other members of the cohort as previously described [26, 27]. Animals were induced to drink using schedule-induced polydipsia. Briefly, subjects were trained to self-administer food pellets by pulling a dowel. After 1 month of food pellet training, pulling the dowel resulted in access to water delivery from one of two drinking spouts. After another month, one waterspout was replaced with a 4% ethanol solution (w/v) and subjects could self-administer up to 0.5 g/kg per day for 1 month. Then, for each of the following 2 months, the amount of ethanol that could be self-administered increased to 1.0 and then 1.5 g/kg per day. This induction period was followed by a 1-year open access period, where female and male macaques (cohorts 1 and 2, respectively) could self-administer 4% ethanol ad libitum for 22 h per day [26]. Blood samples were taken for ethanol concentrations (BEC) at 7 h into the 22 h session every 5–7 days to assess levels achieved by the pattern of ethanol intake at that time. Immediately following the open access periods, Cohorts 1 and 2 macaques were taken to necropsy as previously described [28]. The third cohort consisted of male macaques with a 14-month open access period followed by three, 1-month forced abstinent periods interspersed with two, 3-month open access periods. After the final abstinent period, the macaques were taken to necropsy. Control macaques were either housing controls or panel trained like alcohol self-administration subjects but did not have access to an alcohol solution. Detailed alcohol self-administration data for cohorts 1–3 in this manuscript are available online through the Monkey Alcohol Tissue Research Resource (MATRR) at www.MATRR.com. Rhesus cohorts 6b and 7b are cohorts 1 and 2 in this study, respectively, and Rhesus cohorts 10 and 14 were grouped into Cohort 3 [29].

Ex vivo slice preparation and FSCV

The detailed necropsy procedures used to harvest tissues [28] and obtain ex vivo slices [8] have been previously described. Briefly, subjects were anesthetized with sodium pentobarbital (30–50 mg/kg, i.v.), then transcardially perfused with cooled, oxygenated perfusion solution (see Supplementary Materials for composition) before craniotomy, brain extraction, and blocking using a macaque coronal plane brain matrix (Electron microscopy Sciences, Hatfield, PA). A block containing the caudate and putamen was microdissected from the left hemisphere and sectioned with a VT1200S (Leica, Buffalo Grove, IL) in a sucrose cutting solution aerated with 95% O_2/5% CO_2 (see Supplementary Materials for composition). A ceramic blade (Camden Instruments Limited, Lafayette, IN) was used for sectioning 250 µm slices that were equilibrated at 33 °C for 1 h in equilibration ACSF before being moved to room temperature for an additional hour before beginning experiments.

Carbon fiber electrode (CFE) preparation is described in Supplementary Materials and Methods. After the equilibration period, brain slices were transferred to the recording chamber and perfused at a rate of ~1.5 mL/min with voltammetry ACSF (composition in Supplementary Materials and Methods). Once the brain slice was in place, a bipolar stainless-steel stimulating electrode (Plastics One, Roanoke, VA) was placed in the region of interest and the CFE was placed ~300 µm from the stimulating electrode (Fig. 1B). FSCV was carried out as previously described (details in Supplementary Materials and Methods and [30]). Dopamine release was evoked every 5 min by applying a 2 ms monophasic electrical current pulse with a DS3 Constant Current Stimulator (Digitimer, Hertfordshire, UK). A stimulus intensity that evoked a submaximal response was applied until four consecutive responses with <10% variation in evoked transient peak were obtained. Slices where the dopamine responses changed by more than 10% from the initial stimulation in either direction (i.e., slices that displayed run down or run up) were excluded from the study. After stable responses were established, a stimulation intensity–current response curve was measured from each slice and a stimulation intensity yielding a response 40–60% of the maximum was chosen. A baseline period of five consecutive, stable responses was then collected before beginning any experiments.

For the determination of dopamine transient uptake kinetics, the modeling module in DEMON was used as previously described [30]. The 600 µA stimulation intensity was used for all Vmax determinations. Briefly, the dopamine affinity for the transporter (Km; set to 0.16 µM) was held constant and the dopamine peak height was determined empirically for each file and used for determination of Vmax (dopamine uptake rate), which was altered to best fit the empirically obtained dopamine transients. To examine D2/3 dopamine autoreceptor function, the D2/3 dopamine receptor agonist, quinpirole (30 nM), was bath applied for 30 min and was followed by application of the D2-like dopamine receptor antagonist sulpiride (2 µM) for 15 min. To examine differences between tonic and phasic release, we applied stimuli at varying frequencies before and after the application of the β2 subunit-containing nAChR antagonist, dihydro-β-erythroidine hydrobromide (DHβE; 1 µM).

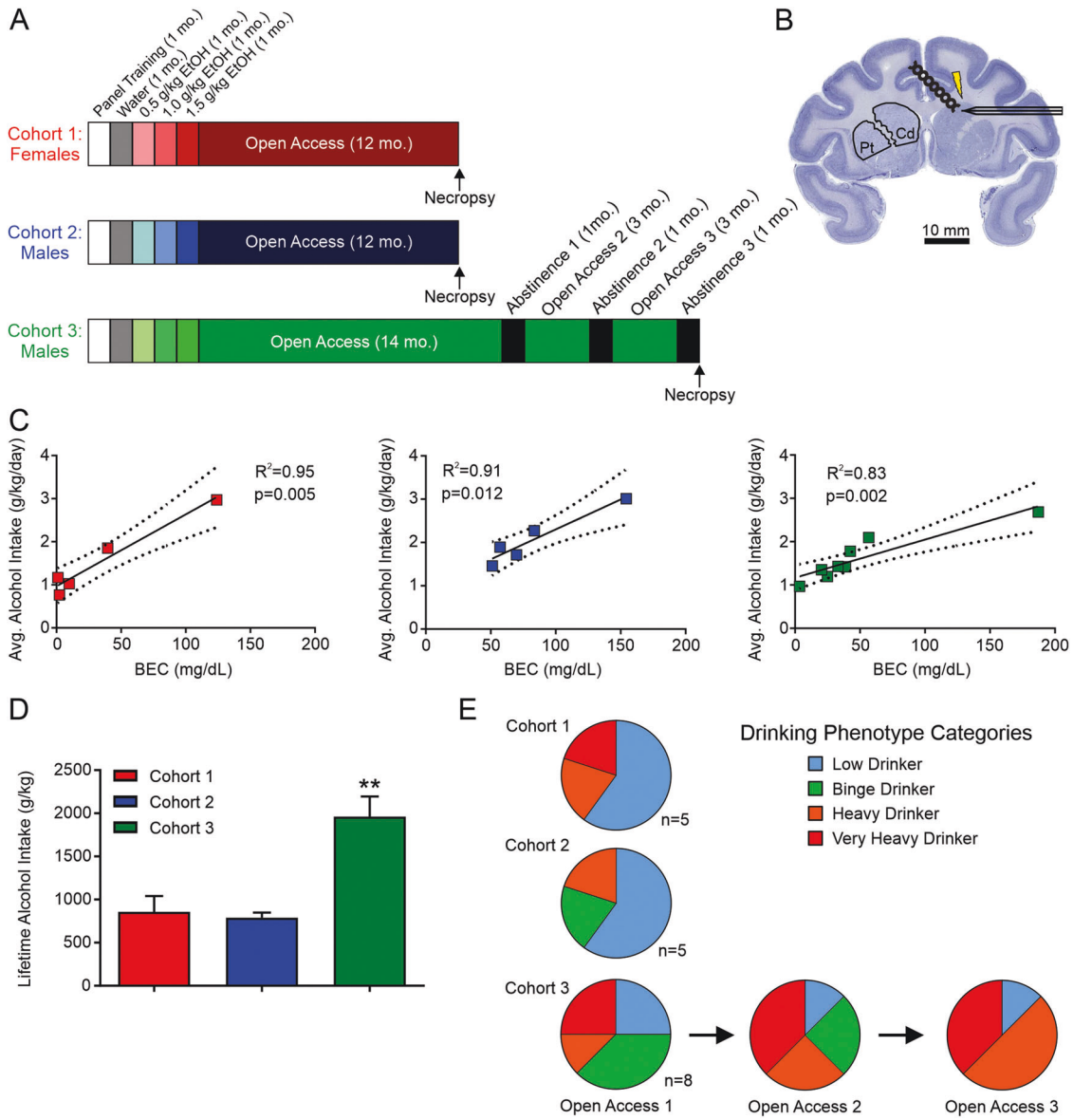

Fig. 1 Overview of experimental design and alcohol consumption measures for all cohorts. A Experimental design and timelines used in this study. Cohort 1 (red) consisted of females with 1 year of ad libitum alcohol open access necropsied during the active drinking time. Cohort 2 (blue) consisted of males treated identically to the females in Cohort 1. Cohort 3 (green) consisted of males that underwent an extended ad libitum alcohol open access with intermittent forced abstinent periods and were necropsied following 1 month of abstinence. **B** Example of the brain section obtained with the caudate (Cd) and putamen (Pt) outlined and the typical FSCV recording configuration. Image obtained from www.brainmaps.org. **C** The average alcohol intake at blood sampling (7 h after session onset) positively correlated with blood ethanol concentrations for each cohort. **D** The average lifetime alcohol intake was similar between the active drinking female and male cohorts (cohorts 1 and 2, respectively), but both were lower than the multiple abstinence males (Cohort 3). **E** Alcohol-consuming monkeys were categorized according to their alcohol consumption and intoxication patterns during the open access period. These categories are stable throughout the first open access period but currently we demonstrate that multiple abstinence periods push animals toward more severe drinking phenotypes. $^{**}p < 0.01$. Error bars represent the SEM.

DHβE was applied to slices to isolate dopamine axons from the influence of nAChRs. After 15 min in DHβE, we began train stimulations. For each slice, only one recording site was used. Multiple slices per subject were sometimes used with no more than two slices per subject/brain region included in any experiment. CFEs were calibrated post hoc against a solution of 1 μM dopamine dissolved in voltammetry ACSF.

Gene expression analyses

To determine if long-term alcohol consumption and repeated abstinence adversely affected cholinergic interneurons, we examined mRNA levels for the vesicular acetylcholine transporter (vAChT) and the acetylcholine synthesis enzyme, choline acetyltransferase (ChAT), as surrogate markers for cholinergic interneurons in some of the Cohort 3 subjects. We also examined mRNA levels for various nAChR subunits (α4, α5, α7, and β2). Detailed methods for these assays are available in Supplementary Materials and Methods.

Drugs and reagents

Dopamine-HCl and (±)-sulpiride were obtained from Sigma-Aldrich (St. Louis, MO). Quinpirole and DHβE were obtained from Tocris Bioscience (Minneapolis, MN). All drugs were dissolved in voltammetry ACSF.

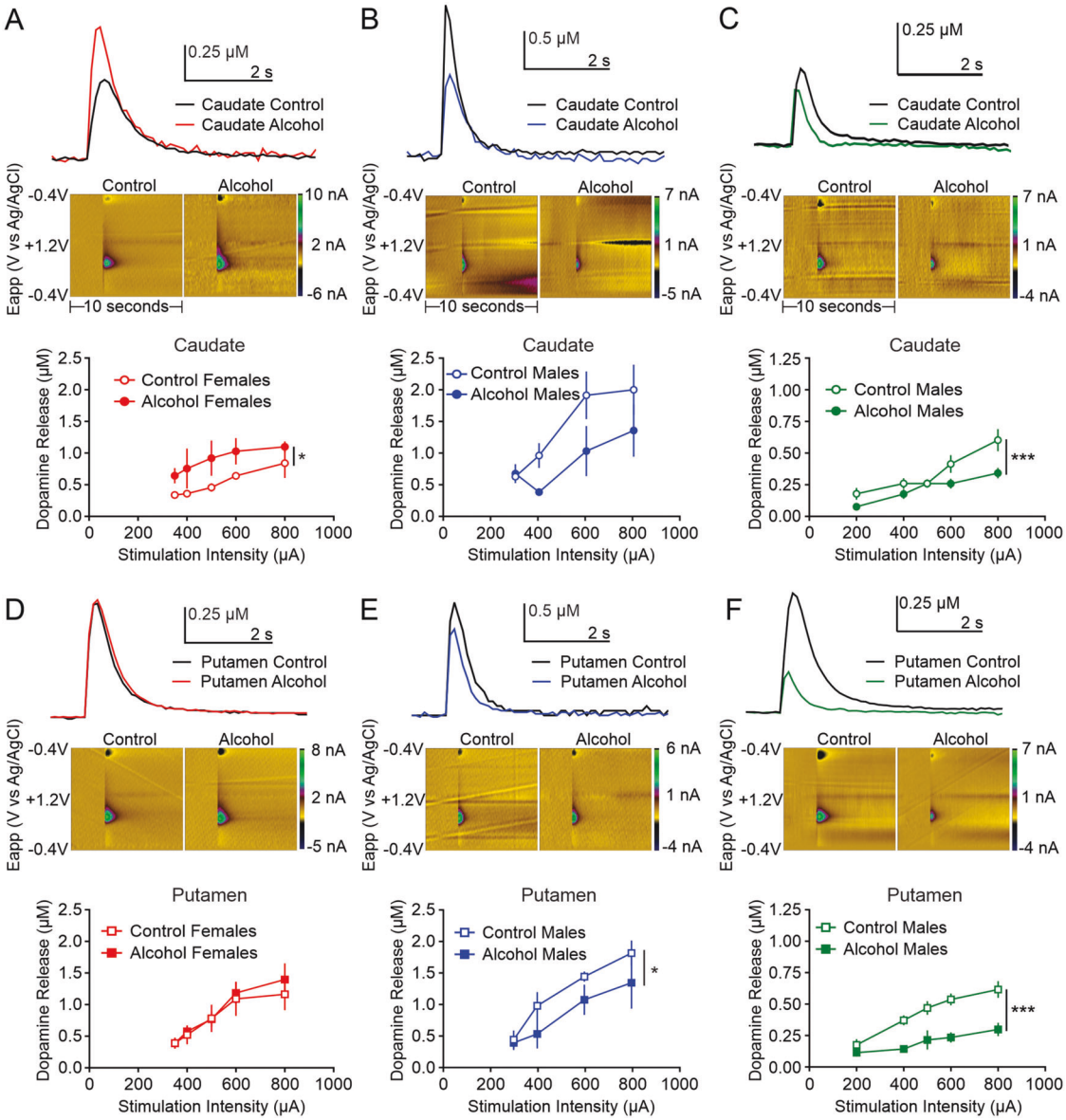

Fig. 2 Dopamine release is altered in subjects with long-term alcohol consumption in both active drinking status and after multiple abstinences. Representative color plots depicting dopamine oxidation and reduction potentials during FSCV recordings (+0.6 V and −0.2 V, respectively) across time are shown along with representative dopamine transients for each cohort, treatment group, and region (**A–F**). Evoked dopamine release was increased in the caudate (**A**), but not putamen (**D**), of female macaques following long-term alcohol consumption. In male macaques, 1 year of long-term alcohol consumption did not significantly alter evoked dopamine release in the caudate; however, dopamine release was decreased in the putamen of alcohol subjects relative to controls (**B**, **E**). In male macaques with long-term alcohol consumption and multiple abstinence periods, dopamine release was significantly reduced relative to control subjects in the caudate and putamen (**C**, **F**). *$p < 0.05$, ***$p < 0.001$. Error bars represent the SEM.

Statistics

GraphPad Prism 7 (GraphPad Software) was used for all statistics. A one-factor ANOVA with Tukey's post hoc test was used to compare the average lifetime alcohol intake between cohorts. Two-factor ANOVAs (stimulation intensity and treatment group) were used for the input–output curve experiments examining dopamine release. For the dopamine uptake rate (Vmax) data, two-factor ANOVAs (treatment and brain region) were used. For experiments in Fig. 4, the final quinpirole treatment time points (i.e., after 30 min in quinpirole) were analyzed with a two-factor ANOVA (treatment group and region). For experiments in Fig. 5A, a two-factor ANOVA (region and treatment group) was used. For Fig. 5D experiments, unpaired *t*-tests were used.

RESULTS

Alcohol consumption, blood ethanol concentrations, and drinking patterns

The average alcohol consumption during the open access periods (normalized to body weight) for subjects from each cohort positively correlated with BECs, demonstrating that monkeys consumed the alcohol they responded for and that our intake measures are accurate (Fig. 1C). The average lifetime alcohol intake differed between cohorts ($F(2,15) = 11.42$, $p = 0.001$) with post hoc testing revealing no significant difference between cohorts 1 and 2 (no abstinence females and males, respectively; $p = 0.97$) (Fig. 1D). Consequently, any differences between cohorts 1 and 2 cannot be attributed to differences in average daily alcohol intake. Subjects

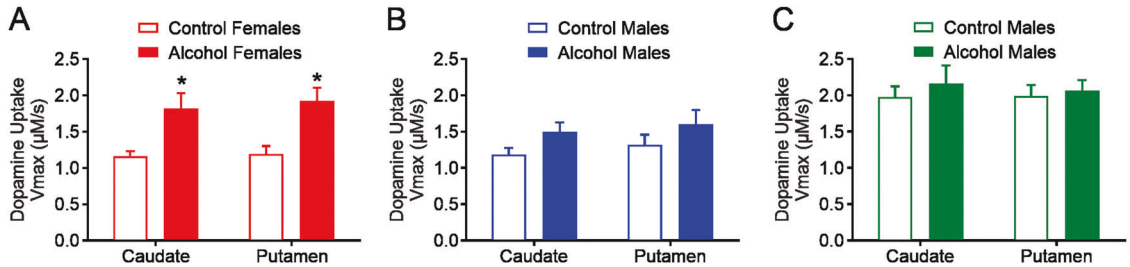

Fig. 3 The dopamine uptake rate (Vmax) was assessed for each cohort. In female macaques, Vmax was significantly increased in caudate and putamen following long-term alcohol consumption (**A**). The Vmax for alcohol-consuming male macaques, however, was similar to control subjects in both male cohorts (**B**, **C**). *$p < 0.05$. Error bars represent the SEM.

from Cohort 3 (multiple abstinence males) had a higher average lifetime alcohol intake relative to cohorts 1 and 2, likely due to the greater number of drinking days ($p = 0.004$ and 0.003, respectively). The drinking patterns of the subjects were subjected to clustering analyses demarcating categorical drinking levels as low, binge, heavy, or very heavy drinkers based on the average daily alcohol consumption, percentage of days over predetermined thresholds, and for the binge drinking category evidence of attaining 80 mg/dL BEC [25, 26, 31]. These drinking categories show remarkable stability across ~12 months of 22 h/day "open access" to ethanol and water in the absence of imposed withdrawal periods [25]. However, a month of forced abstinence can alter the drinking category assignment and transiently increase ethanol intakes across all drinking categories [32]. The current examination of these initial drinking categories in Cohort 3, also documents an exacerbating effect of forced abstinence on ethanol intake such that the number of subjects in the heavy and very heavy drinking categories increased across open access periods (Fig. 1E).

Dopamine release was altered in a sex-dependent manner in chronic alcohol self-administering macaques

Chronic alcohol consumption resulted in increased dopamine release in the caudate of female rhesus macaques ($n = 4$ control and 6 alcohol slices, main effect of treatment $F(1,27) = 7.91$, $p = 0.012$; no effects of stimulation intensity or interaction $F(4,27) = 1.68$, $p = 0.18$ and $F(4,27) = 0.07$, $p = 0.99$, respectively; Fig. 2A). In putamen, there was a main effect of stimulation intensity ($n = 5$ control and 8 alcohol slices, $F(4,47) = 7.59$, $p < 0.001$) but no significant effects of treatment or interaction ($F(1,47) = 0.36$, $p = 0.55$ and $F(4,47) = 0.14$, $p = 0.99$, respectively; Fig. 2D). In long-term drinking male macaques with no abstinence we found no significant effects of treatment ($n = 4$ control and 3 alcohol slices, $F(1,13) = 2.34$, $p = 0.149$), stimulation intensity ($F(3,13) = 2.584$, $p = 0.098$), or an interaction ($F(3,13) = 0.31$, $p = 0.819$) in caudate (Fig. 2B). In putamen (Fig. 2E), however, we found significant effects of treatment ($n = 6$ control and 3 alcohol slices, $F(1,19) = 4.52$, $p = 0.046$) and stimulation intensity ($F(3,19) = 10.49$, $p < 0.001$) but no interaction ($F(3,19) = 0.34$, $p = 0.798$). In the long-term drinking male macaques in abstinence, dopamine release was significantly decreased in both caudate and putamen. In caudate (Fig. 2C), there were significant main effects of treatment and stimulation intensity ($n = 6$ control and 8 alcohol slices, $F(1,45) = 12.88$, $p < 0.001$ and $F(4,45) = 15.12$, $p < 0.001$, respectively) but no interaction effect ($F(4,45) = 1.538$, $p = 0.208$). In putamen (Fig. 2F), there were significant main effects of treatment ($n = 9$ control and 12 alcohol slices; $F(1,68) = 51.46$, $p < 0.001$) and stimulation intensity ($F(4,68) = 12.29$, $p < 0.001$) but no interaction effect ($F(4,68) = 2.086$, $p = 0.092$).

Chronic alcohol self-administration increased dopamine uptake in a sex-dependent manner

Chronic alcohol consumption increased dopamine uptake rates in the caudate and putamen of the female cohort ($n = 3$ control and 5 alcohol slices; main effect of treatment; $F(1,12) = 13.29$, $p = 0.0034$; Fig. 3A). There was no effect of region ($F(1,12) = 0.143$, $p = 0.712$) or interaction effect ($F(1,12) = 0.038$, $p = 0.849$). In the no abstinence male cohort, no significant main effect of treatment group ($n = 5$ control caudate, 5 alcohol caudate, 11 control putamen, and 8 alcohol putamen; $F(1,25) = 2.925$, $p = 0.099$), region ($F(1,25) = 0.476$, $p = 0.496$), or interaction ($F(1,25) = 0.006$, $p = 0.938$) on uptake was observed (Fig. 3B). Dopamine uptake rates in the repeated abstinence male macaques were not affected by treatment or region ($n = 12$ control caudate, 15 alcohol caudate, 16 control putamen, and 13 alcohol putamen slices; $F(1,52) = 0.489$, $p = 0.487$ and $F(1,52) = 0.046$, $p = 0.832$, respectively, for treatment and region factors). There was also no significant interaction effect ($F(1,52) = 0.085$, $p = 0.771$; Fig. 3C).

Dopamine D2/3 autoreceptor sensitivity was decreased in chronic alcohol self-administering male macaques

To examine the function of the Gi/o-coupled D2 autoreceptor, the D2/3 dopamine receptor agonist quinpirole (30 nM) was applied to slices, resulting in a reduction of evoked dopamine release in all groups (Fig. 4). Chronic ethanol exposure did not alter the quinpirole-induced decrease in evoked dopamine release in female macaques (Fig. 4A, D). There were no significant effects of treatment, region, or interaction ($n = 6$ control caudate, 8 alcohol caudate, 3 control putamen, and 6 alcohol putamen slices; $F(1,19) = 0.003$, $p = 0.955$, $F(1,19) = 0.002$, $p = 0.968$, and $F(1,19) = 0.238$, $p = 0.631$, respectively). However, in the no abstinence male cohort, the quinpirole effect was decreased following alcohol treatment in both dorsal striatal subregions (Cohort 2, $n = 2$ control caudate, 3 alcohol caudate, 2 control putamen, and 4 alcohol putamen slices; main effect of treatment $F(1,7) = 26.96$, $p = 0.0013$; no main effect of region or interaction $F(1,7) = 5.36$, $p = 0.054$ and $F(1,7) = 1.05$, $p = 0.34$, respectively; Fig. 4B, E). Similarly, in the multiple abstinence group of male macaques, alcohol treatment also decreased the quinpirole effect (Cohort 3, $n = 5$ control caudate, 7 alcohol caudate, 5 control putamen, and 5 alcohol putamen slices; main effect of treatment $F(1,18) = 8.11$, $p = 0.011$; no main effect of region or interaction $F(1,18) = 0.14$, $p = 0.71$ and $F(1,18) = 0.01$, $p = 0.93$, respectively; Fig. 4C, F). We then followed quinpirole application with the D2/3 receptor antagonist, sulpiride (2 μM), and observed partial reversal of the quinpirole-induced dopamine release depression in all groups.

Local nAChRs modulate dopamine release in rhesus macaques

Application of DHβE reduced single-pulse evoked dopamine release. There were no main effects of region or treatment group on DHβE (1 μM) inhibition of dopamine release ($n = 5$ control caudate, 12 alcohol caudate, 5 control putamen, and 9 alcohol putamen slices; $F(1,27) = 0.219$, $p = 0.643$ and $F(1,27) = 0.779$, $p = 0.389$); however, there was a trend toward an interaction effect in the putamen of alcohol-consuming subjects ($F(1,27) = 4.027$, $p = 0.055$; Fig. 5A). Train stimulation at phasic frequencies

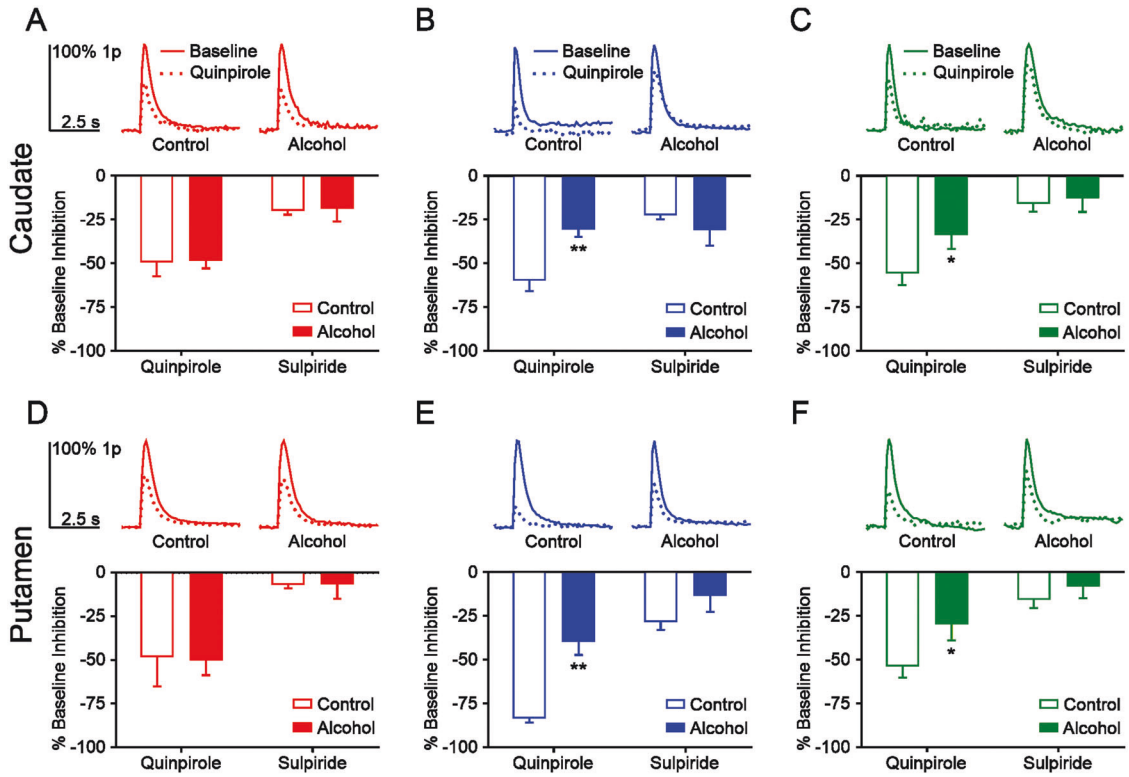

Fig. 4 Presynaptic D2/3 dopamine receptor sensitivity is decreased following long-term alcohol consumption in a sex-dependent manner. The D2/3 dopamine receptor agonist, quinpirole (30 nM), decreased stimulated dopamine release and this effect was partially reversed by the D2/3 dopamine receptor antagonist, sulpiride (2 μM), during washout. Data shown were collected after 30 min in quinpirole and after 15 min in sulpiride solutions. In female macaques, there was no alcohol effect on D2 dopamine receptor sensitivity in the caudate (**A**) or putamen (**D**). In both groups of male macaques, however, D2/3 dopamine receptor-mediated inhibition of dopamine release was decreased following long-term alcohol consumption in the caudate (**B**, **E**) and putamen (**C**, **F**) relative to control subjects. *$p < 0.05$, **$p < 0.01$. Error bars represent the SEM.

(>20 Hz) increased dopamine release across striatal subregion and treatment groups (Fig. 5B, C).

Striatal expression of cholinergic interneuron markers and nAChRs is not altered following long-term ethanol consumption and abstinence

Steady-state levels of mRNA for the vAChT, ChAT, and the nAChR subunits α4, α5, α7, and β2 in putamen and caudate nucleus were analyzed using qRT-PCR. There were no significant differences in mRNA expression between alcohol-consuming and control subjects for either vAChT or ChAT, suggesting that cholinergic interneurons are not lost with alcohol treatment. Examination of gene expression changes in nAChR subunits did not yield any significant group differences for any of the examined genes. However, there were trends toward decreases in α5 caudate ($t(14) = 2.134$, $p = 0.051$; Fig. 5D) and α4 putamen ($t(14) = 1.839$, $p = 0.087$; Fig. 5E) in alcohol drinking subjects from Cohort 3.

DISCUSSION

Summary of findings

We found that long-term alcohol consumption altered dorsal striatal dopamine release and uptake in a sex- and subregion-dependent manner. We further found that regulation of dopamine release by D2/3 dopamine autoreceptors was altered by long-term alcohol consumption in male, but not female, rhesus macaques regardless of abstinence status. These results are largely in agreement with the literature, though some disparities exist. For example, long-term alcohol self-administration resulted in decreased dopamine uptake rates in the dorsolateral caudate of male cynomolgus macaques [22, 24]. This group also found no difference in the quinpirole-mediated inhibition of dopamine release between alcohol and control male cynomolgus macaques [24]. It is likely that species, striatal subregion, and intake duration (6 months in the previous study versus 1 year in the present study) differences may account for many of the dissimilarities between studies. It should also be noted that our study is the first to examine long-term alcohol effects on dopamine release in the putamen of NHPs and to demonstrate that acetylcholine driven dopamine release is conserved across rodent and NHP species.

Dopamine and AUD

AUD is a chronic relapsing brain disease. One factor contributing to the development of AUD may be the change in synaptic signaling in the caudate and putamen that could contribute to a bias toward sensory-motor circuit control of behavior and inflexible alcohol consumption [33, 34]. As an important regulator of behavioral output, dysregulation of dopamine neurotransmission is implicated in theories of AUD development [13, 16, 35]. Acutely, in vivo alcohol administration dose-dependently increases cortical, mesolimbic, and nigrostriatal dopamine in rodents [36]; an effect attributed to enhanced dopamine neuron firing [37]. However, in rodent and macaque brain slices, an acute alcohol challenge following chronic alcohol exposure (inhalation or drinking) decreases dopamine release in the nucleus accumbens (NAc) in vivo and ex vivo preparations [24, 38]. Beyond the NAc, chronic alcohol exposure has varied effects on dopamine release that are brain region and species dependent. Throughout the striatum, dopamine release is generally decreased following chronic alcohol use or treatment. This has been shown in rodents

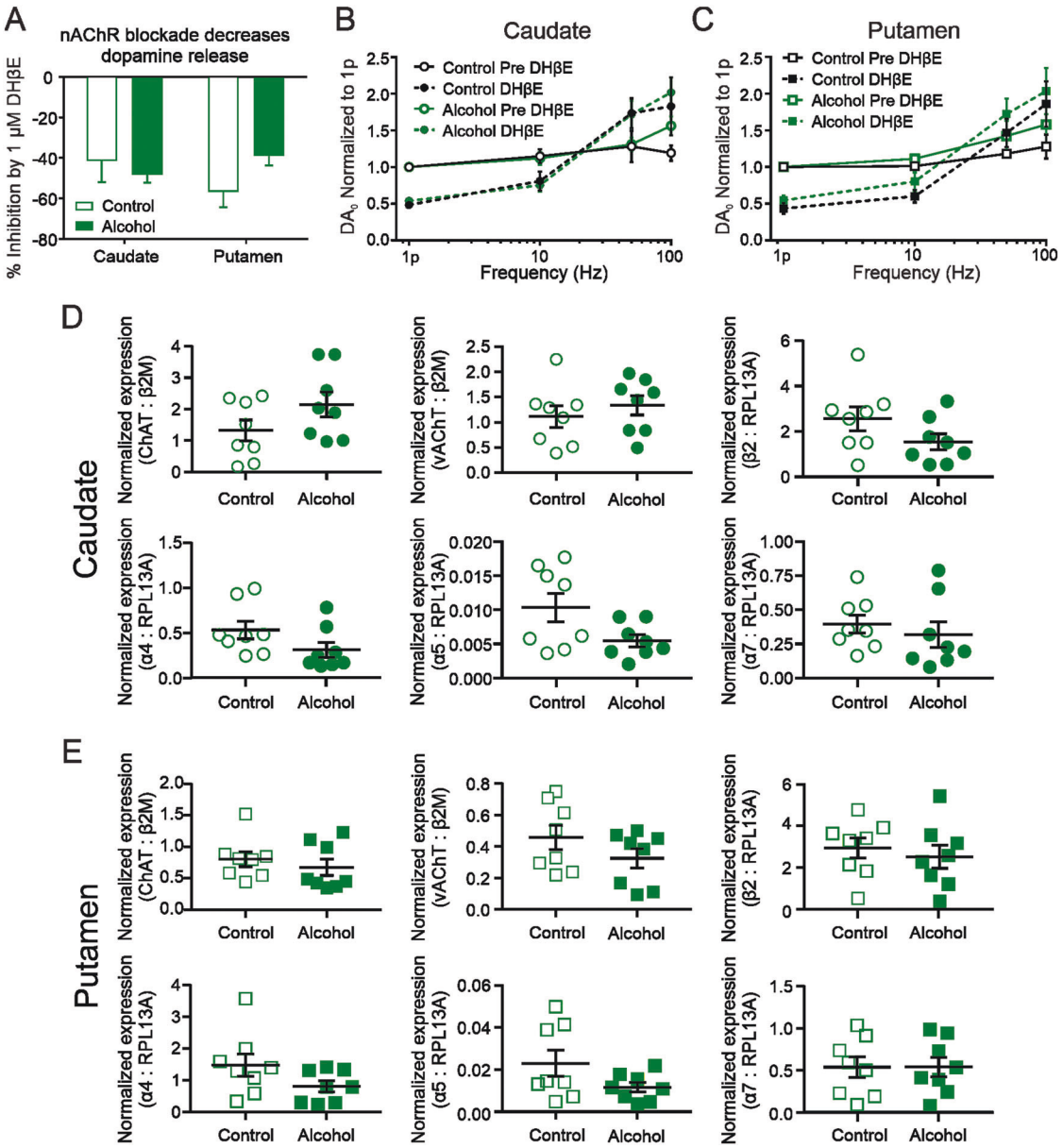

Fig. 5 nAChR antagonism decreases dopamine release. The β2 subunit-containing nAChR antagonist DHβE (1 μM) depressed dopamine release in caudate and putamen of control and ethanol subjects (**A**). Dopamine release was compared across varying train stimulations (6 pulses at the indicated frequencies) before and after nAChR blockade with DHβE (1 μM) in caudate and putamen (**B**, **C**; values normalized to single-pulse values before DHβE application). Gene expression of cholinergic interneuron markers and several nAChR subunits was not changed following chronic alcohol consumption and abstinence (**D**, **E**). Error bars represent the SEM.

[38–40], NHPs [22], and humans [41]. In contrast to the dorsal striatum, dopamine release in the NAc is increased following chronic alcohol use in male cynomolgous macaques [22, 24]. The current study indicates that long-term alcohol consumption decreased dopamine release in the putamen of male rhesus macaques (regardless of abstinence status) and in the caudate of the multiple abstinence monkeys. Interestingly, we found an increase in dopamine release in the caudate and no change in the putamen of female macaque drinkers. The effects of these alcohol-induced changes in dopamine release must be considered with other factors contributing to dopamine signaling (e.g., dopamine uptake/transporter activity).

Indeed, our analysis of dopamine transient dynamics revealed faster dopamine uptake in caudate and putamen of alcohol-consuming female, but not male, macaques. Thus, any apparent dopamine uptake differences in the male macaque groups presented here are a function of faster clearance times due to decreased dopamine release and not faster dopamine clearance rates per se. Interestingly, across multiple studies, chronic alcohol use resulted in enhanced dopamine uptake rates, though this effect has been found to vary between species and striatal subregions (for review, see [10]). Nonetheless, our observed adaptations in dopamine uptake may contribute to the apparent changes in dopamine release following long-term alcohol consumption. Faster dopamine uptake in the female subjects would have the net effect of decreasing the duration of neuromodulation produced by this transmitter. However, the increased uptake rate could be countered by the observed enhanced release, at least in female caudate. Nonetheless, altered dopamine kinetics or release could affect dopamine-dependent synaptic plasticity [42] that might subsequently affect new learning and behavioral flexibility. Indeed, in the multiple

abstinence cohort, in which alcohol treated subjects had significantly less dopamine release, a separate study found that alcohol-consuming subjects had poorer cognitive flexibility relative to controls [43, 44].

Presynaptic regulation of dopamine release by dopamine and acetylcholine

The D2 dopamine receptor is expressed widely in the striatum, including medium spiny neurons [45], cholinergic interneurons [46], and dopamine terminals [47]. Activation of this Gi/o-coupled GPCR inhibits dopamine release via autoreceptors on dopamine terminals [48]. Interestingly, imaging studies in humans have associated lower D2-like dopamine receptor binding in AUD subjects relative to controls [49], though most of these findings likely reflect postsynaptic D2 dopamine receptors and not the presynaptic autoreceptors [50]. Thus, we sought to determine if chronic alcohol use in macaques was associated with altered D2/3 dopamine autoreceptor regulation of dopamine release. We found that D2/3 dopamine autoreceptor function was decreased with long-term alcohol consumption in the male, but not female, cohorts. This effect persisted with multiple abstinence periods and thus may represent a long-lasting consequence of AUD. Given that these D2 autoreceptors are mainly activated during periods of intense afferent activation (e.g., during activity bursts, [51, 52]), this change may lead to filtering of dopaminergic signals with decreased D2R activation during tonic dopamine neuron firing and increased activation during phasic activity. Thus, one consequence of reduced autoreceptor function (and subsequent filtering) would be to enhance neural responses to salient environmental events (e.g., alcohol availability) that might stimulate burst-firing of dopaminergic neurons following chronic drinking. Interestingly, this is the only significant change observed in the actively drinking male cohort, and this effect is not observed in the female chronic drinkers. Thus, it is possible that this difference in the D2/3 autoreceptor function might represent an allostatic adaptation to compensate for the decreased dopamine release observed in the male, but not female, subjects. It will be interesting to determine if responses to alcohol-associated environmental events differ in female and male drinkers with and without abstinence.

Given our findings showing differences in dopamine release, it might be assumed that these effects are attributable to changes in presynaptic dopamine terminals. It should be noted, however, that our study utilized electrical stimulation to induce dopamine release. This stimulation method is nonspecific and activates all axons and neurons near the stimulus electrode, including cholinergic interneurons. Thus, it is possible that electrically stimulated dopamine release could be due to several effectors beyond depolarization of the dopamine terminal. Indeed, a major role for nAChRs on dopamine terminals in regulating dopamine release has been demonstrated in rodents [53–57]. This disynaptic mechanism involves acetylcholine released from cholinergic interneurons activating nAChRs on dopamine axons to induce dopamine release. Thus, any changes to cholinergic signaling in striatum might also influence changes in dopamine release. Indeed, a recent study examining optogenetically evoked dopamine release in mice found no change in dopamine release in the NAc core and medial shell following chronic alcohol treatment, suggesting that the chronic alcohol effect may be due to mechanisms upstream of the dopamine terminal [58]. However, we found no significant differences in the cholinergic contribution to dopamine release between multiple abstinence and control males in Cohort 3 but we did find a trend toward reduced cholinergic driven dopamine release in the putamen of alcohol-consuming subjects. Similarly, in a limited set of putamen slices from the female cohort, we observed a potential reduction in cholinergic driven dopamine release in alcohol monkeys relative to controls (Fig. S1). We thus examined the activity of dopamine axons by blocking nAChRs. Once isolated from cholinergic influence, dopamine terminals from the multiple abstinence male subjects in control and alcohol treatment groups responded similarly to varying frequency stimulation. Our findings with blockade of β2-containing nAChRs resemble previous findings in rodent striatum both with respect to antagonist inhibition and decreased inhibition at higher/phasic stimulation frequencies. Thus, the cholinergic contribution to dopamine release is conserved in primate striatum. We further explored the effect of long-term ethanol consumption on striatal cholinergic systems by examining gene expression of several nAChR subunits (α4, α5, α7, and β2) and markers for cholinergic interneurons (ChAT and vAChT). We found no significant differences in ChAT or vAChT expression between control and alcohol treated subjects, suggesting that long-term alcohol consumption does not adversely affect cholinergic interneurons. Similarly, we did not see any significant changes in mRNA levels of the nAChR subunits. This may be due to the ubiquitous expression of nAChRs in the striatum which would limit our ability to detect changes in specific cell types. Nonetheless, further work and more subjects per group (particularly in female subjects) are required to determine if the alcohol-induced changes in dopamine release are attributable to changes in the function of the presynaptic dopamine terminal or other factors that indirectly modulate dopamine release.

Behavioral and neurobiological consequences of altered dopamine signaling

The consequences of the alterations in dopamine signaling we observed may be numerous. Neurobiologically, striatal dopamine alters intracellular signaling that affects synaptic plasticity [42]. Activation of D1 dopamine receptors increases the excitability of the direct pathway medium spiny projection neurons (MSNs) [59], while D2 receptor activation inhibits GABAergic synaptic transmission within striatum through presynaptic actions on indirect pathway MSNs. In addition, D2 receptors can alter striatal dopamine and acetylcholine levels and inhibit cortical glutamatergic transmission directly or indirectly [60–62]. Decreased dopamine release (in the repeated abstinence cohort) or increased uptake (in the alcohol females) can limit these dopamine actions, hindering normal synaptic plasticity of cortical glutamatergic signaling (directly or indirectly), which may inhibit cognitive or behavioral flexibility as previously reported for some of the subjects in this study [43, 44]. Furthermore, the balance of altered dopamine changes and subsequent effects on cellular excitability and fast synaptic transmission in the caudate and putamen will likely dictate the relative behavioral control by the associative and sensorimotor circuits. In this context, the decreases in release in the putamen of the repeated abstinence male monkeys may limit behavioral plasticity to a greater extent in this region relative to the caudate. This could be one factor contributing to the development of invariant alcohol consumption following long-term drinking with repeated abstinence observed in a previous study of cynomolgous macaques [8]. In this context, the different dopaminergic changes in actively drinking versus repeated abstinence males are intriguing. The decreased D2 effect in the actively drinking males would tend to increase dopamine levels at least under some conditions, while the changes in all three measures in the repeated abstinence males would have a net overall effect of decreasing dopamine levels, which could be a key factor contributing to the progression to more severe drinking phenotypes at each postabstinence drinking stage in this cohort.

The reduction in D2 autoreceptor function may lead to reduced dopamine inhibitory feedback, which might lead to enhanced salience of stimuli that trigger phasic dopamine neuron firing in alcohol-consuming males (e.g., alcohol-related cues/contexts) thus strengthening conditioned associations or maladaptive learning. Indeed, alcohol-associated stimuli increased striatal activity in detoxified alcoholic males more than in healthy males [63]. These individuals also displayed less D2R availability in ventral striatum

and had greater alcohol craving severity. This might suggest that the female subjects here, which lacked changes in D2 autoreceptor function, may not be affected by alcohol-related stimuli in the same way as males. Indeed, in socially drinking individuals, sex differences in neural reactivity to alcohol cues have been described [64, 65]. For example, in males, but not females, alcohol craving was strongly associated with striatal activity [64].

Future directions and considerations

Given that treatment-seeking individuals with AUD invariably go through repeated periods of abstinence and relapse, it is important for animal models of AUD to incorporate this element into the experimental design as these abstinence periods may contribute to the neurobiology of AUD. Indeed, in rodent models, alcohol abstinence or withdrawal periods are often followed by enhanced rebound alcohol drinking, the alcohol deprivation effect [66]. This alcohol deprivation effect has also been observed in cynomolgus macaques [8]. Accordingly, the macaques in Cohort 3 underwent three, 1-month long abstinent periods during the experiment. When compared alongside the male macaques from Cohort 2, which did not undergo multiple abstinence periods, we can begin to assess the effect of the abstinence periods on our measured outcomes, as well as, the persistence of these outcomes. For example, the subjects from Cohort 3 demonstrated an escalation in the severity of drinking category following each "relapse" period (Fig. 1E). This effect has been examined in greater detail elsewhere and was found to be driven primarily by the first month of drinking, post abstinence [32]. Nonetheless, it is interesting to note that the previously reported drinking data from Cohort 3 rhesus macaques showed an alcohol deprivation effect-like phenomenon in which subjects robustly increased their ethanol consumption for 1 month following each abstinence period [32]. Furthermore, the trend toward decreased dopamine release in the males with no abstinence might have become significant had those subjects been put through abstinence periods like the male subjects in Cohort 3 of this study.

Future experiments will need to assess the relationship between the changes in dopaminergic transmission and other striatal excitability and synaptic alterations following chronic alcohol exposure and intake. While this may be difficult to do in NHPs, where experimental manipulations are limited, parallel experiments in rodent models may be able to provide useful information. For example, we know that GABAergic transmission in striatum is altered in a similar fashion after chronic alcohol exposure in mice and monkeys, and similar effects on dopamine release are observed in some strains of mice and monkeys. Thus, the connection between the *trans*-species conserved changes can be explored in the more tractable rodent models.

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AUTHOR CONTRIBUTIONS

Designed research: AGS, YM, VCCC, AFS, KAG, and DML. Performed research: AGS, YM, GL, GSS, AFS, and KAG. Analyzed data: AGS, GSS, AFS, and YM. Wrote the paper: AGS, YM, and DML.

ADDITIONAL INFORMATION

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