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Chronic alcohol drinking slows brain development in adolescent and young adult non-human primates

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Title: Chronic alcohol drinking slows brain development in adolescent and young adult non-human primates.

Abbreviated title: Adolescent alcohol slows brain development in NHPs

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Abstract

The transition from adolescence to adulthood is associated with brain remodeling in the final stages of developmental growth. It is also a period when a large proportion of this age group engages to binge (occasional consumption of 4-5 drinks leading to intoxication) and heavy (binge drinking on ≥5 days in a month) alcohol drinking. Here we report on magnetic resonance imaging of developmental changes in the brain occurring during late adolescence and early adulthood (3.5-7.5 years) in a rhesus macaque model of alcohol self-administration. Monkeys were imaged prior to alcohol exposure, and following ~6 and ~12 months of daily (22 hr/day) access to ethanol and water. The results revealed that the brain volume increases by 1 ml per 1.87 years throughout the late adolescence and early adulthood in controls. Heavy alcohol reduced the rate of brain growth by 0.25 ml/year per 1 g/kg of daily ethanol. Cortical volume increased throughout this period with no significant effect of alcohol drinking on the cortical growth rate. In subcortical regions, age-dependent increases in the volumes of globus pallidus, thalamus, brainstem and cerebellum were observed. Heavy drinking attenuated the growth rate of the thalamus. Thus, developmental brain volume changes in the span of late adolescence to young adulthood in macaques is altered by excessive alcohol, an insult that may be linked to the continuation of heavy drinking throughout later adult life.

Significance Statement

Alcohol abuse during late adolescence and early adulthood is a risk factor for development of alcohol dependence. This longitudinal study used a macaque model of alcohol self-administration and *in vivo* MR-imaging to quantify the impact of chronic alcohol on developmental changes occurring within the brain during this period. Chronic alcohol self-intoxication reduced the growth rate of brain, cerebral white matter and subcortical thalamus. Thus, daily alcohol drinking during the critical transition to adulthood significantly impacts critical areas of sensory motor integration, concomitant with a decrease in cortical white matter, documenting in the primate brain neural circuitry implicating in the propagation of alcohol use disorder.

Introduction

Adolescence is associated with experiencing alcohol binging to extreme intoxication (Patrick and Terry-McElrath 2017) coincident with brain maturation processes. I*n vivo* magnetic resonance imaging (MRI) studies in humans and other species have confirmed that the brain volume continues to increase throughout childhood and adolescence (Pfefferbaum et al. 1994, Mengler et al. 2014, Scott et al. 2016). The volumetric changes are attributed to synaptic pruning, leading to a reduction of gray matter in the cerebral cortex and an increase in white matter volume, concurrent in some brain regions, with protracted myelination of late-developing fiber systems (Giedd et al. 1999, Lebel et al. 2008, Sullivan et al. 2011, Yeatman et al. 2014, Levman et al. 2017, Narvacan et al. 2017). In addition, specific age-related growth-trajectories have been demonstrated for the medial frontal cortex, the thalamus, amygdala, hippocampus and cerebellum (Barnea-Goraly et al. 2014, Squeglia et al. 2015). Heavy alcohol use in human subjects ranging in age from 9 to 23 attenuates white matter growth, increases age-related decline in cortical volume, and reduces cortical thickness (Luciana et al. 2013, Squeglia et al. 2015, Pfefferbaum et al. 2016). These studies correspond to imaging experiments in rodents demonstrating that vapor alcohol exposure during adolescence affects cerebral cortical thickness (Vetreno et al. 2017). Chronic alcohol self-administration also attenuated brain growth in a selected line of alcohol-preferring rats (Pfefferbaum et al. 2006). To our knowledge, there are only a couple of reports that found effects of alcohol on volumetric characteristics of subcortical nuclei. One study found reduced brainstem and caudate volumes in heavy alcohol drinking adolescents (Squeglia et al. 2014) and another found a reduction in the volume of the cerebellum, hypothalamus and hippocampus in alcohol-exposed rodents (Vetreno et al. 2017).

78 The majority of *in vivo* MRI brain-developmental experiments in humans are cross-sectional
79 due to the resources and time required for a longitudinal design. An exception is the multisite
80 National Consortium on Alcohol and Neurodevelopment (N-CANDA) designed to quantify
81 longitudinal effects of low and heavy alcohol use in children throughout adolescence on
82 development of cortical regions and the white matter. Importantly, this study includes scanning
83 subjects prior to any alcohol use (about age 9) (Pfefferbaum et al. 2018). The N-CANDA studies
84 included alcohol use assessments, with the caveat that quantity and frequency of alcohol intakes are
85 estimates in human subject studies where high rates of inaccurate self-reported alcohol use might
86 significantly impact the results (Clark et al. 2016, Bertol et al. 2017). The nonhuman primate (NHP)
87 model of alcohol self-administration can control a variety of variables contributing to brain imaging
88 outcomes, most notably the precise measurements of alcohol intake, diet, daily schedules, and health
89 care (Grant and Bennett 2003). Therefore, translational MRI research utilizing this model for
90 tracking developmental brain changes might be especially valuable (see Zahr and Pfefferbaum
91 2017). However, there are relatively few MRI studies that report on overall anatomical change in the
92 maturing NHP brain, especially during late adolescence and early adulthood (Knickmeyer et al.
93 2010, Scott et al. 2016, Uematsu et al. 2017), much less the effect of alcohol on brain growth
94 parameters. Thus, although reduced cortical gray matter volume was demonstrated in heavy drinking
95 rhesus macaques, the study was underpowered to specifically address changes in brain growth when
96 chronic ethanol drinking begins in late adolescence (Kroenke et al. 2014).

97 This gap in our knowledge was addressed in the present study that measured volumetric
98 trajectories occurring in the rhesus macaque brain during the period of late adolescence to early
99 adulthood and how these changes are affected by chronic alcohol self-administration. We tested the
100 hypothesis that chronic alcohol self-administration dose-dependently decreases whole brain, cortical

and white matter volumes in the macaque. We also predicted that subcortical structures undergoing growth during this age range would also be vulnerable to alcohol-dependent effects on volume in the heavy drinkers.

Methods

Animals

Male (n=58) and female (n=13) rhesus monkeys (*Macaca mulatta,* N=71, cohorts 4, 5, 6a, 6b, 7a, 7b, 10 and 14, see www.matrr.com for details) were obtained from the Oregon National Primate Research Center breeding colony. All monkeys except cohort 4 were enrolled in the study at 3.5 to 5.5 years of age and 3 to 6 kg in body weight. All animals were housed indoors in rooms with controlled temperature (20-22°C), humidity (65%), and an 11-h light cycle with lights on at 07:00 a.m. Each subject was housed in a metal cage (0.8 × 0.8 × 0.9 m) and fed a diet of nutritionally complete 1 g banana-flavored pellets (TestDiet, USA) and fresh fruit. Food and fluid availability was dependent on experimental phase as described below. All monkeys were weighed weekly and ethanol intakes based on body weight (g/kg) were calculated from the contemporary weights. All procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee, 2011) and approved by the Oregon National Primate Research Center Animal Care and Use Committee.

Alcohol self-administration

Equipment. The housing cages were equipped with operant panels as previously described (Vivian et al. 2001, Shnitko et al. 2017). Each panel incorporated a centrally located dowel and 2 drinking spouts, with a food receptacle located below one of the spouts. The receptacle was connected to a 1 g pellet dispenser (Med Associates Inc., USA). Below the receptacle there was a recessed well that could detect the insertion of a finger via the breaking of an infrared beam. Each drinking spout was connected via tubing to a plastic bottle placed on a digital scale (Ohaus Adventurer, USA) located outside the cage. The bottle contained either filtered tap water or 4% ethanol (w/v diluted in water), refilled with fresh solutions daily. All programming used a National Instruments interface and

LabView software (LabView 2011, SP1, National Instruments, TX, USA). Thus, the operant panels were used for the ethanol and water self-administration and food delivery. To initiate food or fluid delivery, the dowel had to be pulled (closing an electrical circuit) and held (see Grant et al. 2008 for details).

Procedure to induce alcohol self-administration. Alcohol self-administration began after a baseline MRI assessment was completed. As previously described, a schedule-induced polydipsia procedure was used to induce monkeys to drink 4% (w/v) ethanol (Vivian et al. 2001, Grant et al. 2008). The induction sessions were given daily over a period of approximately 4 months (~120 sessions, 7 days a week). During each session 1-gram banana flavored pellets were delivered at a fixed-time interval of 300 sec until a required volume of either water or 4% (w/v) ethanol was consumed. Initially, monkeys were induced to drink water for 30 sessions (1 session a day), and then water was replaced with 4% ethanol (for monkeys assigned to ethanol experimental group). Monkeys were required to consume the ethanol solution at the volume sufficient to obtain 0.5 g/kg a day (30 days), 1.0 g/kg a day (30 days) and 1.5 g/kg a day (30 days) of ethanol. Control animals were required to drink water at the volumes corresponding to the ethanol doses. When an animal consumed the required volume, the ethanol spout became inoperative but water was available through the other spout, and the remaining daily ration of food became available two hours later (Grant et al. 2008).

Open access to alcohol self-administration. When induction to ethanol drinking was completed, all animals were allowed daily "open access" to water and ethanol (4% w/v, or water for control subjects) for 22 hours a day, 7 days a week (Grant et al. 2008). During the open access period, the daily food ration (banana flavored pellets) was divided equally into three meals and the first meal was available at the beginning of each daily session and subsequent meals were available at 2-h intervals. The pellets were available under a fixed ratio 1 schedule. Initially, animals were allowed ~

6 months of concurrent access to water and 4% (w/v) ethanol, for a total of 201±19 sessions (N=61, including only adolescent cohorts, i.e., cohort 4 excluded). Subsequently, additional 171±16 self-administration sessions were allowed (~ 12 months of open access). The study timeline for each cohort is shown in Figure 1A.

Blood ethanol concentration (BEC). Prior to MRI and ethanol self-administration sessions all animals were trained to comply with veinipuncture procedure without the use of anesthesia. Initially, subjects were trained to present their leg through an opening in the front wall of the housing cage. They then were trained to comply with blood sampling to be reinforced with raisins or trail mix. Blood samples were collected from the saphenous vein 30, 60, 90 min after the start of the induction sessions and 7 h after the start of the 22 h drinking sessions in 4-5 day intervals. The blood samples were collected throughout the 3 months of ethanol induction and period of open access to ethanol. Ethanol content was assessed by gas chromatography (5890 Series II, Hewlett-Packard, Avondale, PA).

MRI data acquisition and processing.

Prior to an imaging session, each animal was anaesthetized with ketamine (15 g/kg, IM) in the housing environment. The animal was transferred to the ONPRC MRI Core facility, intubated and maintained anesthetized with 1-1.5% isoflurane throughout imaging procedures (≤ 2 hours). The images were acquired using a 3T Siemens Magnetom MRI system (Erlangen, Germany). For all subjects in this study, the MR imaging protocol included an acquisition of T_1- weighted magnetization-prepared rapid gradient-echo (MP-RAGE) with one of the four imaging protocols described in Table 1. The imaging data were acquired at baseline (prior to alcohol self-administration, MRI_1), post-6 (MRI_2) and 12 months of self-administration (MRI_3). Within each cohort, image acquisition settings were kept constant, except for cohorts 6a, 7a, and 7b, in which the

MRI system was upgraded from a Trio to a Tim Trio between the MRI_1 and MRI_2 time points and different MRI protocols were used for data acquisition in the cohorts. Between cohorts, experiments took advantage of upgrades in RF-coil or scanner capabilities as they became available.

MRI data processing. Figure 1B shows a schematic representation of the processing for volumetric analysis within a NHP brain. At the beginning, all T1-weighted images collected at a given time point were averaged within a subject after motion correction and intensity bias correction. The motion correction was implemented by the rigid-body registration with "antsRegistrationSyN.sh" which is an ANTS standard function (Avants et al. 2008). The intensity bias field of each T1-weighted image was corrected using a B-spline approximation routine and a hierarchical optimization scheme implemented by "N4BiasFieldCorrection" in ANTS (Tustison et al. 2010). Next, a registration-based skull-stripping procedure was applied to the averaged MP-RAGE images successively from MRI_1 to MRI_3. At the beginning, the INIA19 template, which includes a T1-weighted head image, brain mask, and NeuroMap labels, was used a reference (Rohlfing et al. 2012). All corrected and averaged T1-weighted images in MRI_1 were nonlinearly registered to the INIA19 head image using "antsRegistrationSyN.sh". With the resulting transformation parameters, the INIA19 brain mask was then reversely aligned to each subject image to generate the brain mask using a nearest neighbor interpolation method. For MRI_2 and MRI_3, skull-stripping was performed with the same method but updated references (MRI_2 with MRI_1 as reference, and MRI_3 with MRI_2 as reference). Based on the registration results, the label map of the INIA19 template was transformed to the space of each MRI_1 image. With the same method, the label maps of brain images in MRI_2 and MRI_3 were generated based on the resulting transformation parameters. Subsequent to the analysis of brain volume changes, a secondary analysis was performed to determine whether individual brain regions are differentially susceptible to age or ethanol exposure. Accordingly, we

197 explored effect of ethanol on age-related changes on a finer scale by parcellating the brain volume
198 into 10 well-resolved regions of interest (ROIs): WM, cortex, putamen, caudate nuclei, globus
199 pallidus, thalamus, amygdala, hippocampus, brainstem and cerebellum, illustrated in Figure 1C. The
200 ROI boundaries were defined prior to the statistical analysis of the determined volumes.

201 *Statistical data analysis*

202 Imaging data for all subjects collected longitudinally across 3 time points were analyzed using a
203 linear mixed model (LMM). A series of LMMs were used in this study to estimate effects of age and
204 chronic ethanol drinking on volumetric characteristics of the primate brain where $Y_{i,j}$ is brain volume
205 (V_B), white matter volume (V_{WM}) or volume of a ROI (V_{ROI}).

206 **LMM 1**: $Y_{i,j} \sim \beta_0 + \beta_1 age_{i,j} + b_j subject + \varepsilon_{i,j}$

207 **LMM 2**: $Y_{i,j} \sim \beta_0 + \beta_1 age_{i,j} + \beta_2 intake_{i,j} + \beta_3 age_{i,j} \times intake_{i,j} + b_j subject + \varepsilon_{i,j}$

208 **LMM 3.1.**: $Y_{i,j} \sim \beta_0 + \beta_1 age_{i,j} + \beta_2 group_{i,j} + \beta_3 age_{i,j} \times group_{i,j} + b_j subject + \varepsilon_{i,j}$

209 **LMM 3.2.** $Y_{i,j} \sim \beta_0 + \beta_1 age_{i,j} + \beta_2 group_{i,j} + \beta_3 sex_{i,j} + \beta_4 age_{i,j} \times group_{i,j} + \beta_5 age_{i,j} \times sex_{i,j} + \beta_6 group_{i,j} \times sex_{i,j}$
210 $+ \beta_7 age_{i,j} \times group_{i,j} \times sex_{i,j} + b_j subject + \varepsilon_{i,j}$

211 First, we estimated age-related change in the brain volume (V_B) of control (CTR) animals using
212 LMM 1 which incorporates a fixed effect of age (β_1) and random intercept (b_j). Next, we explored
213 whether the age effect on $Y_{i,j}$ depends on ethanol intake using LMM 2 with fixed effects of age and
214 average ethanol intake (β_2), as well as an age-by-intake interaction (β_3), and random intercept.
215 Subjects were categorized as low, binge, heavy and very heavy drinkers based on each individuals
216 ethanol intake parameters during the open access as described in previous studies (Baker et al.
217 2014). Briefly, an animal was considered as a low drinker if its average ethanol intake per day was <
218 2 g/kg; an animal was considered a binger if similar ethanol intake per day (<2 g/kg) resulted in at
219 least a single BEC value higher than 80 mg%. The demarcation for heavy and very heavy drinkers

was an average ethanol intake >3 g/kg during 30% of total given 22-h sessions and >4 g/kg during 20% of total given 22-h sessions, respectively. The low and binge drinkers were collapsed into a group of non-heavy drinkers (NHD, n=19), and and heavy and very heavy drinkers were grouped as heavy drinkers (HD, n=26). This parallels previous work in humans (Squeglia et al. 2015) and in nonhuman primates (Cervera-Juanes et al. 2017, Shnitko et al. 2018). The third linear mixed model, LMM 3.1., used the categorical variable (group) to determine how heavy and non-heavy alcohol drinking affects age-related changes in volume of the brain, white and gray matter. If a significant effect of group or an age-by-group interaction was found, then the age-dependent changes were compared to the CTR group as a reference. Finally, we used LMM 3.2. to explore if sex has a significant effect on age-related changes in volume of the brain and interacts with effects of heavy and non-heavy alcohol drinking. All statistical analysis was performed in IBM SPSS Statistics, version 24 (IBM, Corp, NY, USA). All significant effects of main factors and interactions were confirmed with a *F*-test within each model indicated above. For the analysis of age-dependent change in the total brain volume, a significance threshold of $p<0.05$ was considered significant. For the analysis of age-dependent change in the volume of the 10 ROIs, a significance threshold of $p<0.005$ (adjusting for 10 ROIs) was considered significant. The significant fixed effects and interactions found in linear mixed model analyses were followed by post-hoc t-tests in which p values were adjusted for the number of comparisons, and differences of $p<0.05$ were considered significant.

Results

Brain growth in late adolescent/young adult rhesus monkeys.

Subjects were initially studied (MRI_1) at ages ranging from 3.9 to 5.9 years and reached MRI_3 at ages ranging from 5.6 to 7.5 years (Figure 1A). First, we wanted to establish whether brain continues to grow

in the NHPs over the 3.9 to 7.5 years age range (Figure 2A). The average brain volume across all CTR animals was 104.6±9.4 ml at the MRI_1, 105.9±11.4 ml at MRI_2 and 107.1±8.5 ml at MRI_3. Analysis of brain volume (V_B) using LMM 1 revealed a significant effect of age on V_B (β=1.87, se=0.17, p<0.00001, 95%CI[1.5-2.1]); V_B of CTR animals increases by 1 ml per 1.87 years.

Voluntary ethanol intake during open access.

After the induction period, 55 of the 71 animals were allowed to voluntarily self-administer ethanol during a 12-month period (open access in Figure 1A). The average number of open access drinking days was 179±13 prior to MRI_2, and 164±11 between MRI_2 and MRI_3. The average ethanol intake of NHD animals was 1.8±0.5 g/kg and 2.1±0.4 g/kg prior MRI_2 and between MRI_2 and MRI_3, respectively (Table 2). Low ethanol intake was reflected in the low levels of BEC collected 7 hours after the initiation of drinking sessions. The group average BECs of NHD animals was 30±7.6 mg% (individual averages ranged from 3 mg% to 73 mg%) and 37±7.2 mg% (individual averages ranged from 3 mg% to 100 mg%) pre and post MRI_2, respectively. The average ethanol intake of HD animals was 3.0±0.3 g/kg and 3.4±0.4 g/kg during the same periods. Higher ethanol intake in the HD group resulted in relatively high group average BECs of 75±19 mg% (individual averages ranged from 21 mg% to 159 mg%) averaged across drinking sessions prior to MRI_2 and 105±24.2 mg% (individual averages ranged from 6 mg% to 196 mg%) averaged across sessions between MRI_2 and MRI_3.

Heavy drinking attenuates growth in the late adolescent/young adult rhesus brain in an age-dependent manner.

In order to explore whether chronic ethanol self-administration affects brain growth in non-human primates, we used LMM2 with age at MRI sessions 1-3, average ethanol intake, and the potential interaction between them as predictors of V_B. This analysis identified that age (β=1.9, se=0.2, p<0.00001,

95%CI[1.5-2.3] and ethanol intake (β=1.2, se=0.5, p<0.05, 95%CI[0.24-2.2]) significantly predicted V_B and these effects were interdependent as demonstrated by a statistically significant age-by-intake interaction (β=-0.25, se=0.08, p<0.01, 95%CI[-0.4-(-0.09)]). Thus, brain growth occurred in all NHP subjects studied over this range however, it was attenuated by a factor of 0.25 mL/year per daily g/kg ethanol. Figure 2E shows a correlation between individual brain growth (β_i) and average ethanol intake of the subject across 12 months of open access (Spearmen correlation r_s=-0.41, p<0.01).

An additional analysis was performed in which ethanol-drinking monkeys were categorized as non-heavy and heavy drinkers. Drinking group (CTR, NHD and HD) was used as a fixed factor in LMM 3.1, to compare age-related brain growth between subjects with different drinking statuses. Figure 2B,C shows brain growth trajectories obtained based on data collected longitudinally during the 3 MRI sessions in NHD and HD monkeys. The LMM 3.1. analysis identified that age significantly predicts V_B ($F_{1,123}$=141.9, p<0.0001); however, the age-related V_B was dependent on drinking group (age x group: $F_{2,123}$=5.5, p<0.01). Multiple comparison between groups (Fig. 2D) shows that the age-dependent increase of V_B in HD was significantly attenuated when compared to CTR (β'=-0.9, se=0.3, adjusted p=0.004, 95%CI [-1.5-(-0.3)]), while a similar rate of increase of V_B was observed in CTRs and NHDs (β'=-0.3, se=0.64, p=0.32, 95%CI [-0.9-0.3]). Importantly, the age-dependent changes in the V_B significantly differ between two groups of NHD animals (late adolescent and adult) as depicted in Fig. 2D. A linear mixed model analysis revealed a significant effect of the groups ($F_{1,71}$=8.6, p<0.01) on the age-related changes in V_B. The brain continues to grow in younger NHD animals throughout the period from 4 to 7.5 years of age. In adult subjects, the growth stopped and V_B declined by 0.27 ml per year. This differed from the adolescent NHD by 1.8ml per year (β'=1.8, se=0.4, p=0.0001, 95%CI [-0.9-2.6]). Importantly, as indicated in Table 1, multiple hardware configurations were utilized as a result of MRI system upgrades over the course of this study. An analysis of the potential effect of

MRI protocol on the results reported here was conducted using a mixed model analysis. This revealed no significant effect of data acquisition parameters (effect of MRI protocol, $F_{3,73}$= 1.4, p=0.24, effect of age, $F_{1,123}$=68.7, p<0.0001, effect of group, $F_{2,100}$=1.4, p=0.25, age by group interaction, $F_{1,123}$=5.5, p<0.01).

Lastly, we utilized the small subset of female subjects in this study (n=13) to examine the possibility that the effects of alcohol exposure on brain growth was sex-dependent. The analysis using LMM 3.2 revealed a significant effect of sex on V_B ($F_{(1,122)}$=91.4, p<0.0001), but no significant interactions between sex and age ($F_{(1,122)}$=0.4, p=0.5), sex and group ($F_{(2,120)}$=1, p=0.35) or a sex x age x group interaction ($F_{(2,122)}$=1.7, p=0.2). These findings correspond to previous reports demonstrating sex-dependent differences in the brain volume of humans and nonhuman primates (Leonard et al. 2008).

The white matter and the thalamus are the most susceptible to chronic drinking during late adolescence

Previous studies have demonstrated varying age-dependent changes in the volume of different brain structures (Sowell et al. 2004, Sullivan et al. 2011, Dennison et al. 2013, Barnea-Goraly et al. 2014, Yeatman et al. 2014, Bernard et al. 2015), and have also shown that heavy alcohol drinking specifically attenuates white matter growth, and decreases the volume of other brain structures such as the cortex, brainstem, caudate, hippocampus and cerebellum (Luciana et al. 2013, Kroenke et al. 2014, Vetreno et al. 2017). In order to determine whether ethanol differentially affects development of different brain regions, the brain was parcelated into 10 ROIs, and ethanol effects were examined within each of them separately, using LMM 3.1. The results of tests of fixed-effects are presented in Table 3. According to our criteria, there were no significant age-related changes in the volume of caudate nucleus, hippocampus, putamen, cortex and amygdala as indicated by lack of significant effects of age and group by age interaction (p value less than 0.005). However, age-dependent

changes in volumes were identified for the cerebellum, brainstem, globus pallidus, thalamus and white matter. The cerebellum, brainstem, globus pallidus exhibit a statistically significant effect of age, but no age-by-group interaction. As shown in Table 4, the rate of growth in these structures was estimated to be 0.14 ml/year, 0.012 ml/year and 0.16 ml/year in the cerebellum, globus pallidus and brainstem, respectively. Thus, the brainstem volume undergoes the highest change (5% per year from the baseline level) during the age period from 3.9 to 7.9 years compared to two other structures: the globus pallidus (3.2% per year) and the cerebellum (1.6% per year).

As shown in the Table 3, the white matter and thalamus also exhibit significant age-related changes in their volumes; however, the volumetric changes were ethanol intake-dependent. Table 5 demonstrates the estimated effects of age on volume for these ROIs within CTR, NHD, and HD groups. The analysis with LMM 3.1. revealed a striking effect of heavy drinking on the age-related growth in the white matter and the thalamus. The whole brain white matter continued to increase during period from 3.9 to 7.9 years in all subjects (Figure 3B).

In the CTR group, the estimated rate of white matter volume (V_{WM}) growth was 0.6 ml/year and it was slightly lower in NHD by 0.07ml/year compared to CTR. However, the rate of growth was robustly attenuated in HD compared to CTR by 0.25 ml/year. In order to confirm that there were no group differences in the V_{WM} at baseline (MRI_1) prior to ethanol self-administration we performed analysis of variance. The analysis revealed no significant differences between three groups of monkeys ($F_{(2,58)}=1.1$, $p=0.35$), where average V_{WM} at MRI_1 was 19.9±2 ml in control subjects, 20.4±1.7 ml in NHD and 20.9±2.2 ml in HD. Figure 3C shows the change in the volume of WM that occurred between baseline (MRI_1) and the end of alcohol or water (control) self-administration (a ~12 month period). In the CTR monkeys, V_{WM} increased by 4.7±0.4%. Similar to CTR, 4.0±0.5% increase in the V_{WM} was observed in NHDs. In HDs, the increase in the V_{WM} was significantly

smaller (2.6±0.3%) compare to CTR. One-way ANOVA revealed a significant effect of group ($F_{2,58}$=7.8, p<0.001, results of post hoc analysis are indicated in Figure 3C).

Thalamic growth also occurred in the NHP brain over this period (Figure 4B). In the CTR group, the estimated rate of growth was 0.06 ml/year. It was significantly decreased in both types of alcohol drinkers NHD (by 0.02 ml/year) and HD (0.03 ml/year). We confirmed that there were no group differences in the thalamic volume (V_T) at baseline (MRI_1) prior to ethanol self-administration using analysis of variance. The analysis revealed no significant differences between three groups of monkeys ($F_{(2,58)}$=0.3, p=0.73), where average V_T at MRI_1 was 1.7±0.2 ml in control subjects, 1.7±0.15 ml in NHD and 1.7±0.13 ml in HD. The V_T increased by 5±0.7% between baseline (MRI_1) and the end of self-administration in CTR animals and by 2.8±0.6% in NHDs. In HDs, the 1.8±0.6% increase in the V_T was significantly smaller, as revealed as a significant effect of group in one-way ANOVA ($F_{2,58}$=5.5, p<0.01, results of post hoc analysis are indicated in Figure 4C).

Discussion

Rhesus macaques have been widely used as a translational model for investigating the neural substrates of human behavior and, particularly, alcohol use and abuse (Grant and Bennett 2003, Wright and Taffe 2014, Chandler et al. 2017, Shnitko et al. 2018, Thomas and Czoty 2018). The present longitudinal study was focused on measurement of brain changes that occur during late adolescence and early adulthood in rhesus monkeys (3.5-7.5 years of age) because this stage of development confers maximum risk for heavy drinking in human and macaques (Helms et al. 2014, NSDUH 2017). We quantified changes in the volume of brain structures in macaques over this period of life and characterized the effects of chronic alcohol use on these changes. First, we demonstrated that the macaque's brain continues to grow well into young adulthood (at least until 7.5 years of age), as previous studies have not measured brain volume increases beyond adolescence

in the macaque (until 5 years of age) (Malkova et al. 2006, Bakken et al. 2016, Scott et al. 2016). Second, the reduced rate of brain growth due to heavy ethanol drinking could be quantified with this analysis, and was found to be 0.25ml/year per g/kg of daily ethanol intake. Finally, these results extend the chronic effects of heavy alcohol intake from brain volume reductions in adult rhesus macaques to significant impact on brain growth at an age range associated with a high risk of establishing a pattern of unhealthy alcohol consumption (Kroenke et al. 2014).

Even though the *rate* of brain volumetric growth decreases during development (Bakken et al. 2016), the present data clearly show that brain continues to grow throughout late adolescence and early adulthood (Figure 2A). A diverse set of neurodevelopmental processes contribute to this volume change. For example, the cortical volumes of gray and white matter undergo nonlinear changes across human lifespan, with gray matter volume decreasing during late adolescence and early adulthood, while white matter volume continues to increase beyond adolescence and early adulthood (Sowell et al. 2003, Sowell et al. 2004). A regressive neuronal process contributing to the gray matter volumetric changes is synaptic pruning that begins in childhood and continues in adulthood (Sowell et al. 2001, Chung et al. 2017). In rhesus macaques, initiation of synaptic pruning occurs between 1 and 3 years of age and accelerates during puberty (Eckenhoff and Rakic 1991, Zecevic and Rakic 1991). At the same time, myelination of many white matter fascicles is quite protracted over adolescent development in human and NHPs (Sowell et al. 2001, Miller et al. 2012), which contributes to the increase in white matter volume throughout adolescence. As stated above, the present data are the first to document normal developmental changes in the brain volume beyond 5 years of age in rhesus macaques.

These neurodevelopmental underpinnings are thought to form the biological bases of robust behavioral and cognitive changes that occur concurrently and are accompanied by susceptibility to

maladaptive behaviors in adolescents. Importantly, the age at onset of alcohol drinking is considered as one of the significant predictors of heavy alcohol drinking in adulthood (Poikolainen et al. 2001, Englund et al. 2008, Morean et al. 2012, but see, Maimaris and McCambridge 2014). Furthermore, recent NCANDA experiments found robust effects of chronic alcohol abuse on brain volumes in human adolescents (Squeglia et al. 2015, Pfefferbaum et al. 2016, Pfefferbaum et al. 2018). In similarity to human subjects, in macaque individuals that begin drinking to intoxication as late adolescents/young adults have a greater risk for future heavy drinking during mature adulthood (Helms et al. 2014, Baker et al. 2017). Further, the present study extended this finding to an estimate of the dose-dependence of the ethanol effect on brain growth in rhesus macaques (Figure 2).

There are numerous factors that could contribute to the observed impact of chronic alcohol drinking on brain growth. For example, cortical volume, which occupies the greatest volume fraction of the total brain in rhesus macaques (Rakic 1995, Toro et al. 2008), could account for the observed impact of alcohol on the brain growth. In human cross-sectional data, heavy alcohol drinking was associated with smaller volumes of the lateral frontal and temporal cortices (Squeglia et al. 2015). However, here we established that cortical volume did not increase from 3.9 to 7.5 years in rhesus monkeys (Table 3) and the global measure of cortical volume was not sensitive to the effects of chronic alcohol drinking. Another significant contributor to the total brain volume is the white matter volume. For example, morphometric studies using MRI have demonstrated that the volume of white matter increases faster with the total brain growth then gray matter volume (Rilling and Insel 1999). An impact of chronic ethanol on the development of white matter subsequently contributes to the attenuated brain growth. In this study, daily alcohol drinking for over 12 months reduced white matter growth in the NHP brain (Figure 3) and these results correspond to attenuated growth of white matter in heavy drinking human subjects (Squeglia et al. 2015). Overall, the unique analyses

of growth rate, rather than a single measure of volume, appears to be a key factor in documenting the adverse effects of alcohol on relatively late brain growth studied here.

The volume of subcortical structures also showed age-dependent growth in 4 out of 8 subcortical gray matter regions of interest included in the study (specifically, globus pallidus, thalamus, brainstem and cerebellum). These findings partially correspond to a human cross-sectional pediatric study where the volumes of thalamus, brainstem and cerebellum increase in a period from 4 to 18 years (Brain Development Cooperative 2012). The data presented here help establish that subcortical nuclei during adolescence are susceptible to voluntary intakes of alcohol. The effect of chronic alcohol on the volumes of subcortical gray matter regions in the human adult brain largely resulted in reduced volumes. Specifically, daily drinking of both heavy and non-heavy amounts of alcohol dramatically reduces the rate of growth in the thalamus, a finding which parallels the effect observed in the adult human brain with reductions in caudate, putamen, thalamus, cerebellum and hippocampus (Sullivan et al. 2000, Chanraud et al. 2007, Yang et al. 2016). In adolescents, smaller hippocampal, thalamic and putamen volumes are reported in male alcohol users (Nagel et al. 2005), however, larger thalamic and putamen volumes have been reported in adolescent female drinkers (Fein et al. 2013). In present study, chronic heavy alcohol drinking attenuated age-related growth of the thalamus in the macaques (Table 5). Overall the changes in the growth rate of the thalamus and white matter due to chronic alcohol drinking in the macaques have some key similarities to the human data, but the macaque also offers quantitative information related to ethanol dose. Further, the observation that a subset of structures shows an increased rate of volumetric growth suggests that additional studies are needed to understand the allopathic balance between responses of subcortical structures to chronic alcohol in the late adolescent brain.

In summary, the NHP model of alcohol self-administration, in combination with longitudinal

427 measures by MRI utilized in this study highlights the rate of volume changes within the developing
428 primate brain to isolate dose-dependent effects of chronic voluntarily alcohol drinking in rhesus
429 macaques. These effects are concentrated within cerebral white matter and the thalamus involved in
430 critical control over sensory and limbic integration with behavioral choice and output. Thus, future
431 research is needed to determine if these volumetric changes might lead to altered functioning within
432 neural circuitry underlying excessive alcohol drinking.

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630

631 **Figure legend**

632 **Figure 1.** Schematic representations of experimental timeline (A) and MRI data processing (B, C).
633 A. The timelines of the experiment are shown for each of 8 cohorts of subjects admitted to and
634 completed the study at different periods from 2008 to 2017. As indicated in the legend, the blown
635 squares represent an MRI sessions in the timeline of experiment and the numbers within them
636 indicate a MRI protocol used at the time. B. The MR image processing is described in details in the
637 text (see methods section). C. Representation of the monkey's brain map used for segmentation of
638 ROIs in individual subjects (top: coronal, middle: axial, bottom: 3D image showing cortical surface,
639 the cerebellum and brainstem). The ROIs were labeled in INIA19 template brain.

640 **Figure 2.** Age-dependent brain growth in the non-human primates. A-C. Plots of individual brain
641 volume changes across age in control (CTR, n=16), non-heavy (NHD, n=19) and heavy (HD, n=26)
642 drinking NHPs. Brain volume (V_B) of each subject was measured 3 times as depicted by empty
643 circles. An individual regression line is drawn across the three V_B values for each subject. D.
644 Average V_B-by-age linear regression estimated for controls, non-heavy and heavy drinkers with 95%
645 confidence interval depicted by the shaded area around the line. E. Correlation between age-
646 dependent brain growth (β_i) and individual daily ethanol intake averaged across 12 months of
647 drinking. The β_i is based on slope of the individual regression line as shown in A-C. The ethanol
648 intake is estimated based on daily consumption of 4% (w/v) ethanol solution averaged across ~372
649 drinking sessions with exception of induction period (r_s=0-0.41, $p<0.01$).

650 **Figure 3.** Heavy ethanol intake reduces rate of the white matter growth in NHP brain. A. 3D
651 representation of the cortical white matter in the brain. B. The estimated rate of white matter growth
652 in the control, nonheavy and heavy drinking NHPs. The shadows above and below the regression
653 lines depict 95% confidence interval. C. The effect of chronic ethanol on average change of white

matter volume occurred in three groups of subjects from baseline until the end of ethanol/water self-administration. The dots represent change in the volume measured in individual monkeys. Asterisks show results of Bonferroni post hoc test where p values adjusted for the multiple comparisons were $<0.01^{**}$ and $<0.05^{*}$.

Figure 4. Ethanol drinking attenuates thalamic growth in adolescent/early adult NHP. A. 3D representation of the thalamus in the brain. B. The estimated rate of the thalamic growth in the control, nonheavy and heavy drinking NHPs. The shadows above and below the regression lines depict 95% confidence interval. C. The effect of chronic ethanol on average change of thalamic volume occurred in three groups of subjects from baseline until the end of ethanol/water self-administration. The dots represent change in the volume measured in individual monkeys. Asterisks show results of Bonferroni post hoc test where p values adjusted for the multiple comparisons were $<0.01^{**}$.

Tables

Table 1. As indicated in the table, the ONPRC MRI system was upgraded throughout the study and different radiofrequency coils were used to enhance signal to noise ratio. The parameters of MR image acquisition were adjusted accordingly.

Table 2. [a]One-way ANOVA revealed no significant difference in age at the beginning of the study (MRI 1) between three groups of subjects ($F_{(2,60)}=1.3$, $p=0.28$).

[b]RM ANOVA revealed significant effect of MRI (2 vs 3, $F_{(1,43)}=11.3$, $p<0.01$) and group (NHD vs HD, $F_{(1,43)}=67.9$, $p<0.0001$) on ethanol intake with no significant interaction ($F_{(1,43)}=0.06$, $p=0.8$).

Table 3. The table includes the results of test for fixed effects used in the LMM3.1: age, group and age by group interaction. Note that volume of a single ROI was used as a dependent variable in LMM3.1. [a] The DFn and DFd are 1 and 123 (age), 2 and 96 (group), and 2 and 123 (interaction),

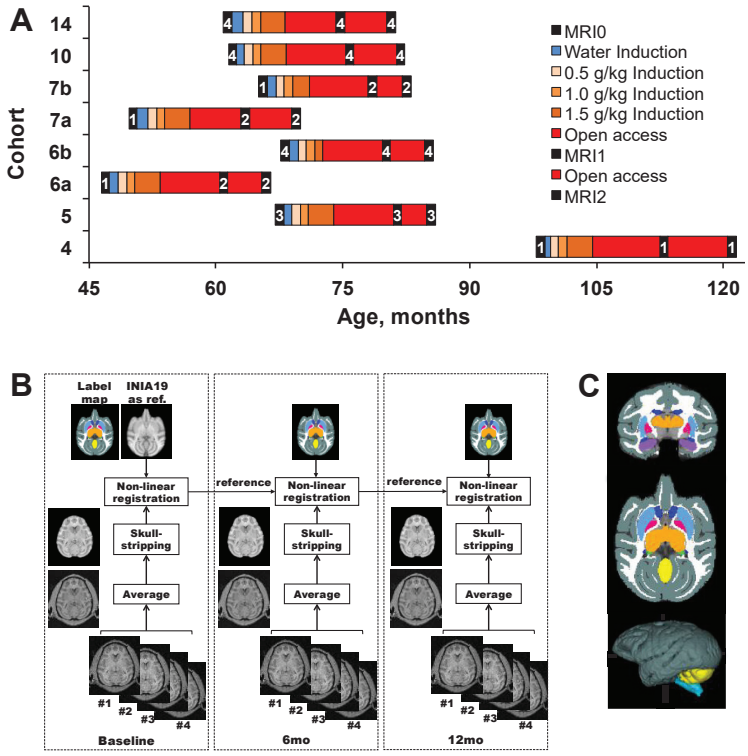
677 respectively. Note that only $p<0.005$ were considered significant (adjustment for 10 ROIs included
678 in the analysis).

679 **Table 4.** The table includes the parameters of estimates of fixed effects obtained in the LMM1 with
680 random intercept and fixed effect of age on V of listed ROIs. The rate of volume change is expressed
681 as ml/year.

682 **Table 5.** The table includes the results obtained with the LMM 3.1 with random intercept and fixed
683 effect of age, group and their interaction on V of the ROIs. β'= βNHD or HD-βCTR. The rate of
684 volume change is expressed as ml/year. Average estimated age-dependent growth trajectories
685 obtained in the groups of NHD and HD were compared to CTR group with t-test and p values were
686 adjusted accordingly.

687 **Figures**

688 **Figure 1.**



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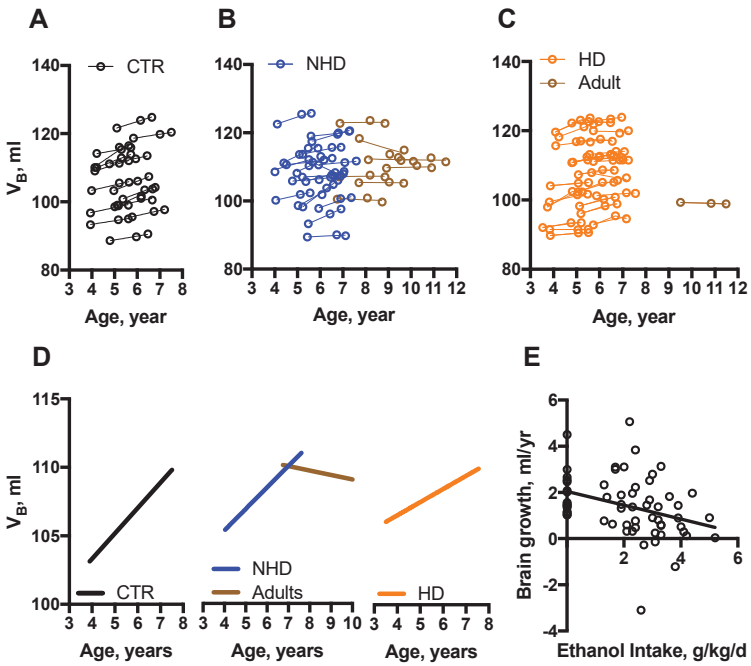
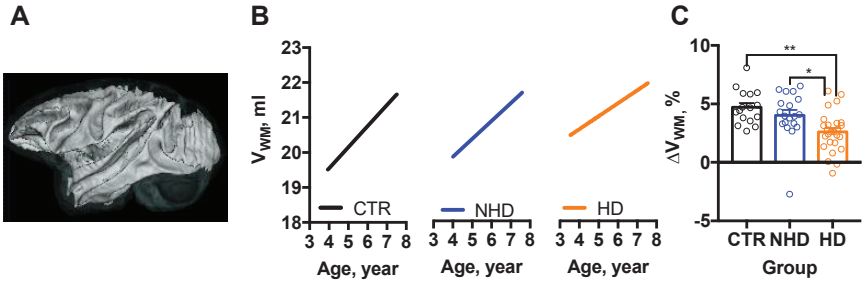


693 **Figure 2.**

694

695 **Figure 3.**



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698 **Figure 4.**

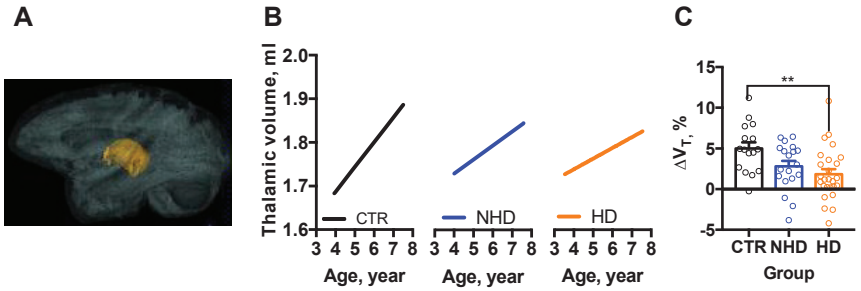

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Table 1. Protocols of MR image acquisition.

Protocol	MRI system	RF coil	TR(ms)	TE(ms)	TI(ms)	Voxel size(mm)	Flip Angle°
1	Trio	Quadrature Knee	2500	4.38	1100	0.5x0.5x0.5	12
2	TimTrio	Quadrature Knee (Tim)	2500	3.86	1100	0.5x0.5x0.5	12
3	Trio	8 Channel Knee Array	2500	4.38	1100	0.5x0.5x0.5	12
4	TrioTim	15 Channel Knee Array	2500	3.86	1100	0.5x0.5x0.5	12

Table 2. Data sample characteristics.

MRI	Age (SD), year			Ethanol Intake (SE), g/kg/d		
	CTR	NHD	HD	CTR	NHD	HD
1	4.7(0.6)	5.1(0.6)	4.8(0.8)	0.0	0.0	0.0
2	5.8(0.6)	6.2(0.6)	5.9(0.7)	0.0	1.8(0.5)	3.3(0.8)
3	6.3(0.6)	6.6(0.6)	6.4(0.7)	0.0	2.1(0.4)	3.6(0.8)

Table 3. Tests of Fixed Effects in LMM 3.1.

ROI [a]	Group[a]		Age [a]		Interaction	
	F	p	F	p	F	p
WM	**4.4**	**<0.05**	**405.2**	**<0.00001**	**9.6**	**<0.00001**
Thalamus	**3.7**	**<0.05**	**108.8**	**<0.00001**	**8.2**	**<0.00001**
Globus pallidus	1.6	0.2	148.4	<0.00001	3.7	<0.05
Brainstem	1.1	0.3	700.7	<0.00001	3.9	<0.05
Cerebellum	1.6	0.2	120.5	<0.00001	1	0.4
Amygdala	1.6	0.2	12.7	<0.01	3.2	<0.05
Cortex	1.9	0.2	11.4	<0.01	2.2	0.1
Putamen	0.8	0.4	10.8	<0.01	1.9	0.2
Caudate	2.3	0.1	0.7	0.4	0.7	0.5
Hippocampus	1.8	0.2	0.2	0.6	2.3	0.1

Table 4. Estimates of Fixed Effects in LMM 1.

ROI	β	SE	p	95%CI
Cerebellum	0.14	0.01	<0.00001	[0.12-0.17]
Globus pallidus	0.012	0.001	<0.00001	[0.009-0.13
Brainstem	0.16	0.006	<0.00001	[0.15-0.018]

Table 5. Estimates of fixed effects in LMM 3.

	CTR				NHD				HD			
ROI	β	SE	p	95%CI	β'	SE	adjusted p	95%CI	β'	SE	adjusted p	95%CI
WM	0.6	0.050	<0.00001	[0.5-0.7]	-0.07	0.06	0.44	[-0.2-0.04]	-0.25	0.06	<0.00001	[-0.4-(-0.13)]
Thalamus	0.06	0.007	<0.00001	[0.04-0.07]	-0.02	0.01	<0.01	[-0.4-(-0.01]	-0.03	0.01	<0.00001	[-0.05-(-0.02)]