

Effect of repeated abstinence on chronic ethanol self-administration in the rhesus monkey

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Received: 13 July 2017 / Accepted: 19 September 2017 / Published online: 19 October 2017
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Abstract

Rationale Abstinence-based approaches to treating alcohol use disorder (AUD) are highly prevalent, but abstinence from chronic drinking may exacerbate subsequent levels of alcohol intake in relapse.

Objective Use a non-human primate model that encompasses a range of chronic voluntary ethanol drinking to isolate biological responses to repeated cycles of imposed abstinence as a function of baseline voluntary alcohol drinking levels.

Methods Over a 26-month protocol, young adult male rhesus macaques were first induced to drink alcohol and then given continuous access to 4% (w/v) ethanol ($n = 8$) or water ($n = 4$) for approximately 14 months, followed by three 28- to 35-day abstinence phases, with 3 months of ethanol access in between. Ethanol intake and blood ethanol concentration (BEC) were the primary dependent variables. Observational signs of physical dependence and circulating ACTH and cortisol were monitored.

Results Prior to abstinence, stable, categorical, individual differences in voluntary ethanol intake under chronic access conditions were found. Following abstinence, categorical “non-heavy” drinking subjects increased drinking transiently (increased between 0.7 and 1.4 g/kg/day in first month after

abstinence) but returned to baseline after 3 months. Categorical “heavy” drinkers, however, maintained drinking 1.0–2.6 g/kg above baseline for over 3 months following abstinence. Signs of physical dependence were rare, although huddling and social withdrawal increased in ethanol and control subjects. The most prominent effect on hormonal measures was heightened cortisol during abstinence that increased to a greater extent in ethanol subjects.

Conclusion Involuntary abstinence increases drinking in the absence of overt physical withdrawal symptoms, and heavy drinkers are more robustly affected compared to non-heavy drinkers.

Keywords Forced abstinence · Ethanol · Monkey · HPA axis · Self-administration · Relapse · Macaque · Cortisol · Extinction

Introduction

Alcohol use disorder (AUD) is a chronic, progressive disease that is often characterized by cycles of heavy drinking, remission, and relapse (American Psychological Association 2013). A primary approach to treatment of AUD across all modalities is a period of imposed abstinence, from rehabilitation clinics to outpatient support groups (e.g., alcoholics anonymous). Abstinence is also the benchmark for success in many new clinical trials aimed to develop therapeutics for AUD (Food and Drug Administration 2006). Despite the prevalence of forced abstinence in therapeutic approaches, data are accumulating that involuntary treatment (i.e., medical, family or court ordered abstinence) results in lower remittance of problem drinking compared to voluntary treatment

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00213-017-4748-9>) contains supplementary material, which is available to authorized users.

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(Matzger et al. 2005; Witkiewitz and Marlatt 2006; Delucchi and Kaskutas 2010).

The probability of relapse following treatment is determined by a complex interaction between internal and external variables that are difficult to disentangle or predict (Marlatt and Gordon 1985; Larimer et al. 1999). Following alcohol-dependent patients over many years shows that the individual's choice to enter treatment voluntarily is critical to longer term success (Polich et al. 1981; Delucchi and Kaskutas 2010). However, for many AUD patients, achieving abstinence does not necessarily mean successful remission. For example, after short-term abstinence (1–5 months), patients were just as likely to relapse as those that never abstained (Polich et al. 1981). Further, in some cases, a month of complete abstinence is immediately followed by a month of no abstinent days (Witkiewitz 2005). Thus, the efficacy of abstinence-based treatment is questionable, and utilizing animal models can isolate the response to abstinence separate from behavioral therapies and variance in environmental factors (family life, job security, etc.).

Specifically, translational studies in robust animal models have been used to better understand the mechanisms underlying excessive alcohol intake, the role of abstinence, and drinking trajectories post-remittance. Many rodent animal models utilize repeated periods of chronic ethanol exposure interspersed with periods of complete abstinence to increase alcohol self-administration (Becker and Lopez 2004; Griffin et al. 2009; Anderson et al. 2016). However, the most significant increases in drinking in rodents are reported after involuntary (i.e., passive vapor chamber) ethanol exposure, a period of alcohol withdrawal (1–3 days), followed by very limited ethanol access (< 2 h). These pre-clinical models initially emphasized an exacerbation of physiological withdrawal symptoms over repeated withdrawal periods, such as handling-induced convulsions (Becker and Hale 1993; Becker 1998). However, the emphasis in pre-clinical studies on physiological withdrawal symptoms are not well matched to the abstinence syndromes noted in human subjects (Holleran and Winder 2017), which emphasize psychological features such as negative affect, lower coping, and risk of relapse (Witkiewitz 2005, Witkiewitz and Masyn 2008). An alternate approach has been using deprivation periods to produce an alcohol deprivation effect (ADE), in which drinking is transiently increased following drug removal (Sinclair and Senter 1968; Spanagel and Holter 1999; Sanchis-Segura and Spanagel 2006). However, the magnitude of the escalation in drinking is modest, though it appears under conditions of brief or extended abstinence (3 to 60 days, Spanagel and Holter 1999). Additionally, many animal models measure alcohol relapse under extinction conditions in which alcohol is not actually consumed, which is not well matched in the clinical literature (Hauser et al. 2016). Finally, the common use of inbred rodent strains inherently limits the individual variability in ethanol drinking before

abstinence, as well as the behavioral response to alcohol withdrawal.

To address some of these issues, we utilized a monkey model of alcohol self-administration that captures individual differences in voluntarily drinking to excessive levels associated with phenotypic alcoholic intakes (Grant et al. 2008; Baker et al. 2014) prior to imposed abstinence and observed the effects on subsequent alcohol self-administration. This model has helped unravel the biological contribution to individual variability in voluntary daily ethanol drinking (Cervera-Juanes et al. 2016, 2017; Nimitvilai et al. 2017; Pleil et al. 2016; Siciliano et al. 2016a, b), including endocrine measures of stress response as predictors (Helms et al. 2014) and consequences (Jimenez and Grant 2017; Jimenez et al. 2017a, b) of ethanol self-administration. Thus, the current study sought to characterize the response to repeated cycles of abstinence on voluntary drinking, associated blood ethanol concentrations and HPA activity, due to the translational potential to human studies of abstinence (Sinha et al. 2009).

Materials and methods

Animals

Twelve experimentally naïve young adult male rhesus macaques (*Macaca mullatta*; 4.0–5.5 years at assignment) were selected for this study from a pedigreed population at the Oregon National Primate Research Center (Beaverton, OR) and did not have common parents or grandparents. This study was an extension of a previous experiment examining the effect of repeated abstinence in male cynomolgus monkeys (Cuzon Carlson et al. 2011). Since a cohort design was used, this study was populated with only young adult males to address species differences prior to addressing other important organismal variables such as sex or age. Co-housed controls were also added to the original design that had only experimental subjects. All monkeys were housed individually within the same room, allowing visual, auditory, and olfactory contact with conspecifics. The housing room was maintained at a constant temperature (20–22 °C) and humidity (65%) with an 11-h light/dark cycle (lights on at 7:00 AM). Animals were housed in either a single (0.8 × 0.8 × 0.8 m) or double (1.6 × 0.8 × 0.8 m) wide cage within a quadrant rack, depending on body weight. All monkeys were trained to present their legs through an opening in the front of the cage for awake blood collection and to operate a panel located on a side wall of the housing cage. Each panel was equipped with two drinking spouts, stimulus lights, food pellet receptacle, and a device that measures discrete light beam breaks scheduled for food delivery (further details in Grant et al. 2008; Baker et al. 2014). Fluid reservoirs resting on digital scales (Ohaus Corporation, Parsippany, NJ) and pellet dispensers were

located outside of the housing cage. Fluid was recorded with an accuracy of 0.1 ml and 1 s.

After training, animals were assigned to either an ethanol group ($n = 8$) or yoked control group ($n = 4$). The experimental procedures were identical for ethanol and control groups, except that the control group had water available through both spouts. In addition, at the start of the experiment, yoked controls were assigned to a specific ethanol subject. Every morning, each control was given a specific volume of maltodextrin solution (10% *w/v* in water) calculated weekly to match the average daily calories consumed in the form of ethanol (calories/kg/day) of the assigned ethanol subject over the past week. Maltodextrin volumes were administered in a separate bottle hung on the outside of the cage at the session start. All solutions were made fresh daily.

Ethanol self-administration

All monkeys were induced to drink either ethanol or water using a schedule-induced polydipsia (SIP) procedure, which has been described in detail (Grant et al. 2008; Baker et al. 2014, 2017). The key feature of this procedure is that 1-g banana-flavored food pellets are delivered under a fixed-time (FT) schedule (typically FT 300 s) until a specified dose of ethanol is consumed. Induction began with water (volume equivalent to 1.5 g/kg from 4% (*w/v*) ethanol) and then escalated to 0.5, 1.0, and 1.5 g/kg ethanol, with each condition given in at least 30 session increments. The induction of water was 56 sessions to allow the adjunctive drinking patterns to stabilize in all individuals prior to ethanol induction. The 1.5 g/kg induction was 67 sessions to allow for an extensive examination of adrenal hormonal response (Jimenez et al. 2017b). Control monkeys were induced to drink an equivalent volume of water in each phase of the schedule induction procedure. At the end of induction (183 consecutive sessions, Fig. 2a), the FT pellet delivery was discontinued, both ethanol and water spouts were operative for 22 h/day, and the total daily food allotment available as three meals at 0, 2, and 4 h into the self-administration session. All monkeys were weighed weekly without sedation to calculate gram per kilogram ethanol intakes.

Abstinence

After 425 sessions of daily access to ethanol (~ 14 months), the first abstinence phase began (Fig. 2a). All independent variables remained constant except the ethanol bottle was replaced with a second water bottle. Yoked controls no longer received daily maltodextrin during the abstinence phase. Abstinence 1 lasted for a total of 35 days (sessions 426–460), which included an additional week for magnetic resonance imaging (MRI) as part of a larger study. Days 1–28 were used in data analysis, and MRIs were conducted over days 29–35. On session 461, the

ethanol bottle was replaced for ~ 3 months and yoked maltodextrin was reinstated. Abstinence 2 was 28 days in total, and abstinence 3 was 35 days (28 days of abstinence, with MRIs on days 29–35). These abstinence periods were otherwise identical to abstinence 1, with a final ethanol access phase between them (Fig. 2a). The final ethanol access phase was also extended due to MRI imaging on days 63–74 post-abstinence 2 (Fig. 2a).

Behavioral observations

Live behavioral observations were conducted by trained staff as previously reported (Cuzon Carlson et al. 2011). Pre-abstinence observations occurred typically 3–5 days before abstinence, following two baseline sessions to habituate subjects to the observers. All observers were involved in animal husbandry and familiar to the subjects. Abstinence observations (abstinence 1–3) took place 1–3 days after ethanol was removed at 8 AM and 4 PM (time points: 24, 32, 48, 56, and 72 h post-ethanol). Time of day was matched in the pre-abstinence observations for three morning observations and two evening observations in each experimental phase. Morning and afternoon observation sessions were conducted to capture spontaneous behavior before and after daily meals (daily 22-h sessions ran from 10 AM–8 AM).

Prior to observations, training video recordings were used to establish consistent criteria based on the ethogram (Table 1; adapted from Cuzon Carlson et al. 2011 and Ruedi-Bettschen et al. 2013). Each monkey was observed for a total of 20 min, the first 10 min by one observer, and the second 10 min by a second observer. Six observers were present for approximately 45 min (4 monkeys/observer, 12 monkeys). The order of the observations was rotated and counterbalanced. All behaviors were recorded on a binary scale (0 = did not occur, 1 = did occur) to weight each behavior equally and minimize the between-rater variability. Behaviors within three categories (physical signs of dependence, huddling/social withdrawal, and normal behaviors; Table 1) were summed for each monkey by observation session (i.e., 24 h abstinence) and then by pre-abstinence or abstinence period. This approach increased statistical power with the small number of subjects and extensive ethogram.

Blood sampling

All blood samples for hormone analysis (3 ml) were collected from the femoral vein during routine weekly morning blood draws (7–9 AM) from the home cage without sedation. Samples were kept on ice until centrifuged (15 min, 5 °C, 3000 rpm) and then stored at – 80 °C until assayed on an automatic Roche platform by the Endocrine Core at Oregon National Primate Research Center (Beaverton, OR).

Blood samples for blood ethanol concentration (BEC) analysis (20 μ l) were collected 7 h into the drinking session

Table 1 Behavioral ethogram

Behavior	Description
Signs of physical alcohol dependence	
Piloerection	Raised body hair
Intentional tremor	Spontaneous shake of hand when reaching or extending arm
Tremor	Spontaneous shake of any body part
Vomiting	Any vomiting or gagging
Huddling/social withdrawal	
Huddling	Hugging self with head between knees
Social withdrawal	Sitting at back of cage, unresponsive to any cage noise or objects being moved by other monkeys in the room
Normal (or species-typical) behaviors	
Cage exploration	Tactile or oral exploration of their environment
Dowel pull	Pulling the dowel at the center of the panel
Left spout	Drinking from the left spout
Right spout	Drinking from the right spout
Masturbation	Tactile contact with genitals
Self-grooming	Picking, scraping, or spreading of animal's own hair
Vocalizing	Any vocalization originating in the throat, most commonly cooing and grunting

from the medial saphenous vein and diluted in 500 μ l sterile water, placed in airtight containers and stored at -4°C until assayed using headspace gas chromatography (Agilent Technologies, Santa Clara, CA). Samples were analyzed using linear regression against a standard curve that included 25, 50, 100, 200, and 400 mg/dl.

Drinking categories

Drinking categories for each ethanol monkey were established using drinking and BEC data from the first 22-h open access period (425 sessions), as previously described (Grant et al. 2008; Baker et al. 2014). Very heavy drinkers (VHD) have a daily ethanol intake average ≥ 3 g/kg, with $\geq 10\%$ of drinking days exceeding 4 g/kg. Heavy drinkers (HD) are monkeys that reach 3 g/kg for $\geq 20\%$ of open access days. Binge drinkers (BD) have ethanol intakes of ≥ 2 g/kg for $\geq 55\%$ of drinking days and have a BEC ≥ 80 mg/dl at least once per year. Low drinkers (LD) are all monkeys that do not meet the criteria for any of the previous categories (Baker et al. 2014).

Data analysis

All analyses were conducted using GraphPad Prism 6. Drinking data were collapsed into non-heavy (low and binge drinkers) and heavy (heavy and very heavy drinkers) groups for all analyses (Baker et al. 2017). Drinking and BEC data were confirmed for normality and then entered into a one-way repeated measures ANOVA with experimental phase as the independent variable. To emphasize the transitory nature of drinking post-abstinence, we analyzed 28 sessions immediately prior to abstinence (pre-abstinence), the first 28 sessions

after abstinence ended (days 1–28), and the final 28 sessions of the open access period (post abstinence 1: days 56–84; post-abstinence 2: days 76–104). Post hoc comparisons were made using Dunnett's test. For intakes under extinction conditions (Fig. 3), the first 28 sessions of abstinence were used in paired *t* tests within each abstinence phase, excluding days 29–35 in abstinence 1 and 3 when MRIs were occurring.

Behavioral observations were confirmed for normality and then analyzed as separate two-way repeated measures ANOVAs for each abstinence period (abstinence 1, 2, and 3) with group (heavy drinkers, non-heavy drinkers, controls) and experimental phase (pre-abstinence and abstinence) as factors within each category. Post hoc comparisons were made with the control group as appropriate (Sidak's).

For ACTH and cortisol analyses, blood samples were taken 3 days before abstinence (pre-abstinence), 24 h into abstinence, 21–22 days into abstinence, and within 1 week of ethanol being reintroduced (post-abstinence) (Fig. 4). There are no post-abstinence samples for abstinence 3 since the experiment had ended. All samples were log transformed for normality and then analyzed as a two-way repeated measures ANOVA with group (heavy, non-heavy, and control) and experimental phase as factors within each abstinence (abstinence 1–3). Post hoc comparisons were made with pre-abstinence or the control group (Sidak's).

Results

Baseline ethanol drinking

Prior to the first abstinence, individual daily ethanol intakes (g/kg/day) were measured during the first open access period (425

consecutive sessions), and 66–70 blood samples/monkey were taken over this period for BEC analysis. These data were used to assign each monkey to a drinking category (see the “Materials and methods” section). There were 2 VHD, 1 HD, 3 BD, and 2 LD (Fig. 1). To accommodate statistical analysis, the four categories were collapsed into two: heavy (VHD and HD) and non-heavy (BD and LD), consistent with previous publications (Beattie et al. 2015; Baker et al. 2014, 2017; Cervera-Juanes et al. 2016a, b, 2017). The summary drinking and BEC data are shown in Fig. 1a, b respectively. Figure 1c depicts the percent of total drinking days at each categorical drinking level.

Effect of abstinence on ethanol drinking and blood ethanol concentrations

To determine the effect of abstinence on subsequent ethanol drinking and BEC, 28-day epochs before and after each abstinence phase were analyzed in heavy and non-heavy drinkers (timeline in Fig. 2a). Pre-abstinence drinking (28 days) was not different from the 12-month averages, demonstrating the stability of each monkey’s drinking category prior to abstinence ($p > 0.05$, Fig. 2b, c). Overall, both abstinence 1 and abstinence 2 initially increased drinking in both non-heavy

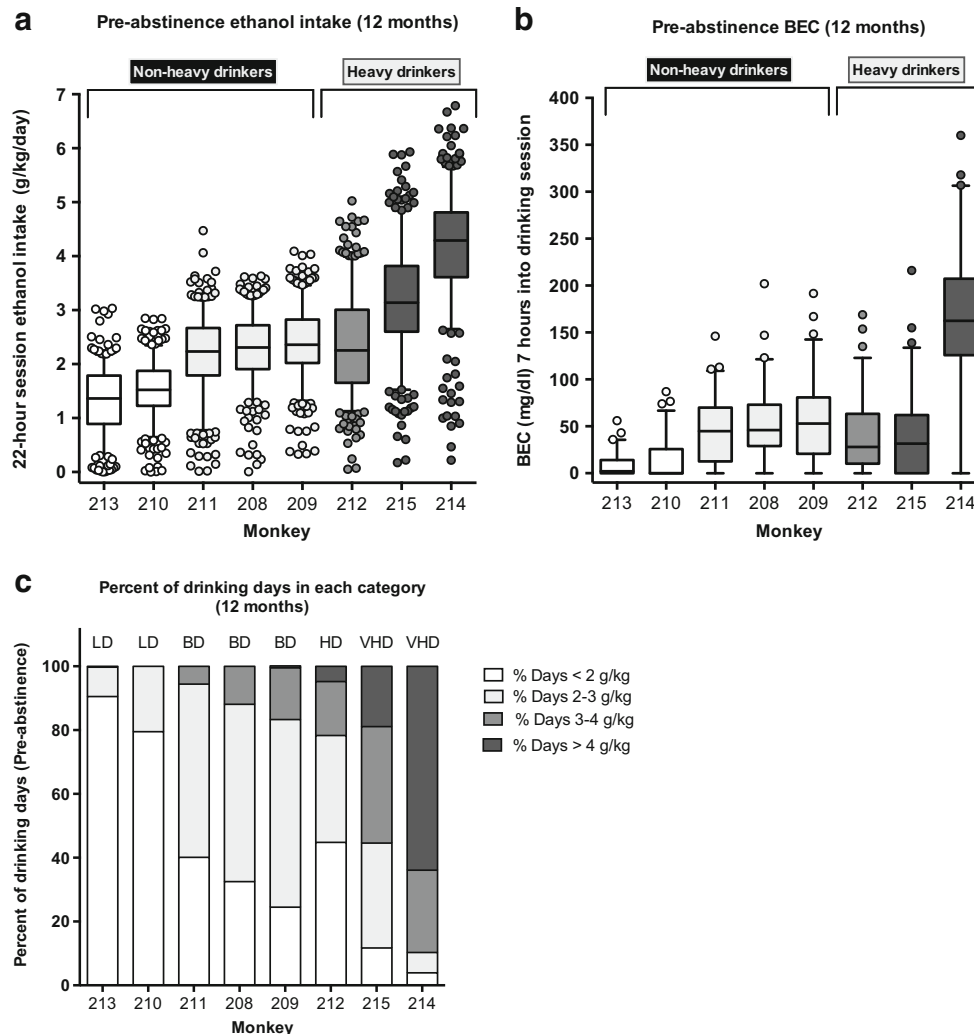


Fig. 1 Effect of abstinence on ethanol intake and blood ethanol concentration (BEC) in heavy and non-heavy drinkers. **a** Experimental timeline. The number of days of each phase of the experiment is indicated below each section. Additionally, the 28-day epochs used in current analysis are highlighted. Hash symbol (#) indicates that these periods were extended to allow for MRI brain imaging. MRIs took place on days 29–35 in abstinence 1 and 3, and on days 63–74 in the final ethanol open access period, and these days were excluded from the analysis. **b**, **c** Ethanol intake in heavy (**b**; $n = 3$) and non-heavy drinkers (**c**; $n = 5$) before and after each abstinence period. The post-abstinence period is separated into two phases: the first 28-days following abstinence (post-

abs days 1–28) and the last 28-days of the open access period (post-abs days 56–84 (abs 1) and 76–104 (abs 2)). Each data point represents a 28-day mean for each monkey and the bars represent a group mean \pm SEM. **d**, **e** BEC in **d** heavy and **e** non-heavy drinkers before and after abstinence (pre-abs, early post-abs, and late post-abs). Each data point represents a mean of 4–6 samples per monkey collected 7 h into the drinking session. **f** Water intake before and after abstinence for heavy and non-heavy drinkers. **g** Total water intake (ml/kg) for control subjects ($n = 4$) from both spouts before and after each abstinence. Asterisks represent significant changes from pre-abstinence (pre-abs), * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$

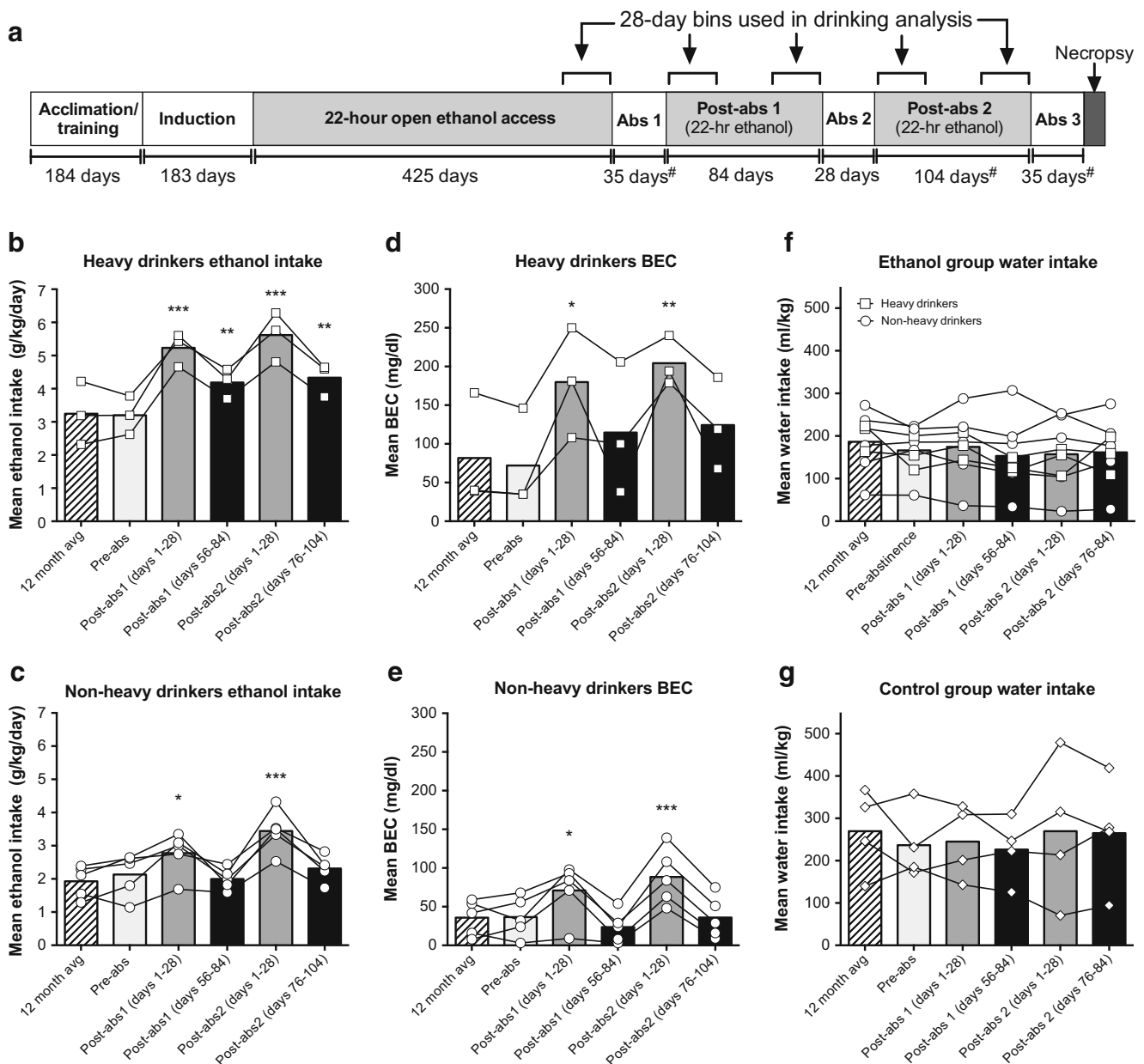


Fig. 2 Boxplots of ethanol intake and BEC during the 12 months before abstinence. **a** Boxplot of 425 sessions prior to abstinence for each monkey colored by drinking category (each plot includes between 410 and 415 drinking sessions excluding days where sedation occurred or there was a computer/technical error in data collection). **b** Boxplot of 66–70 blood samples assayed for BEC over 425 sessions before abstinence. Whiskers in both graphs represent 5–95% of sessions/samples, with the outliers as

individual data points. The monkeys that were collapsed into Non-heavy and Heavy categories are indicated above the graphs. Low drinkers (LD) are in white, Binge drinkers (BD) are in light gray, Heavy drinkers (HD) are in dark gray, and Very heavy drinkers (VHD) are in black. **c** Percentage of drinking sessions at each categorical drinking level for each monkey, as indicated by color (legend at right)

and heavy drinkers (Fig. 2b, c; Main effect of abstinence, non-heavy: $F(4,16) = 14.19$, $p < 0.0001$; heavy: $F(4,8) = 47.87$, $p < 0.0001$). In non-heavy drinkers, this effect is only observed in early post-abstinence (Fig. 2c; post-abs 1 (days 1–28): $p < 0.05$; post-abs 2 (days 1–28): $p < 0.001$). Drinking returned to pre-abstinence levels at the end of the post-abstinence ethanol access periods (days 56–84 (abs 1) and 76–104 (abs 2): $p > 0.05$). Heavy drinkers, however, had

elevated drinking that remained elevated over the entire post-abstinence ethanol access (Fig. 2b; post-abs 1 (days 1–28): $p < 0.001$; post-abs 1 (days 56–84): $p < 0.01$; post-abs 2 (days 1–28): $p < 0.001$; post-abs 2 (days 76–104): $p < 0.01$). Water intake was not different between heavy and non-heavy drinkers and was unchanged following abstinence (Fig. 2f). Additionally, there was no effect of abstinence on water intake in the control group (Fig. 2g).

In general, the effect of abstinence on BEC reflected the ethanol intake results. Both abstinence periods increased BEC in non-heavy and heavy drinkers (Fig. 2d, e; main effect of abstinence, non-heavy: $F(4,16) = 14.54$, $p < 0.0001$; heavy: $F(4,8) = 7.985$, $p < 0.01$). In non-heavy drinkers, this effect is observed in the first 28 days following abstinence (Fig. 2e; post-abs 1 (days 1–28): $p < 0.05$; post-abs 2 (days 1–28): $p < 0.001$), while BECs in the last month of ethanol access returned to pre-abstinence levels. A similar effect is found with the heavy drinkers, where the average BEC is only statistically increased in the early post-abstinence period (Fig. 2d; post-abs 1 (days 1–28): $p < 0.05$; post-abs 2 (days 1–28): $p < 0.01$).

Extinction conditions: water drinking on ethanol spout during abstinence

Extinction conditions were imposed on the ethanol animals during the abstinence periods such that ethanol was removed from its vehicle (i.e., water); therefore, both spouts supplied only water. Intake (ml/kg) on the former ethanol

spout during the 28-day abstinence was compared to ethanol intake (ml/kg) 28 days prior to water substitution (Fig. 3). As a group, non-heavy drinkers did not significantly decrease intake on the ethanol spout during any of the abstinence periods (Fig. 3a; $p > 0.05$), and individually, only two monkeys showed any evidence of extinction (Fig. 3a). Heavy drinkers as a group showed significant decrease on the ethanol spout only in the third abstinence phase ($t(2) = 6.39$, $p < 0.05$), but one subject significantly decreased drinking on the ethanol spout during all three abstinences (Fig. 3b). Representative graphs of different drinking patterns during abstinence are shown in Fig. 3c, d. There were no changes in drinking on the previous water spout in either ethanol drinking group (Fig. S1a-b).

Control subjects did not demonstrate any extinction on either water spout during abstinence (Fig. S1c-d). However, during the first abstinence, control monkeys increased their water intake on the “ethanol” spout (Fig. S1c). These monkeys no longer received maltodextrin on the outside of their cage, and this increased water intake may correspond to the loss of additional fluid each morning.

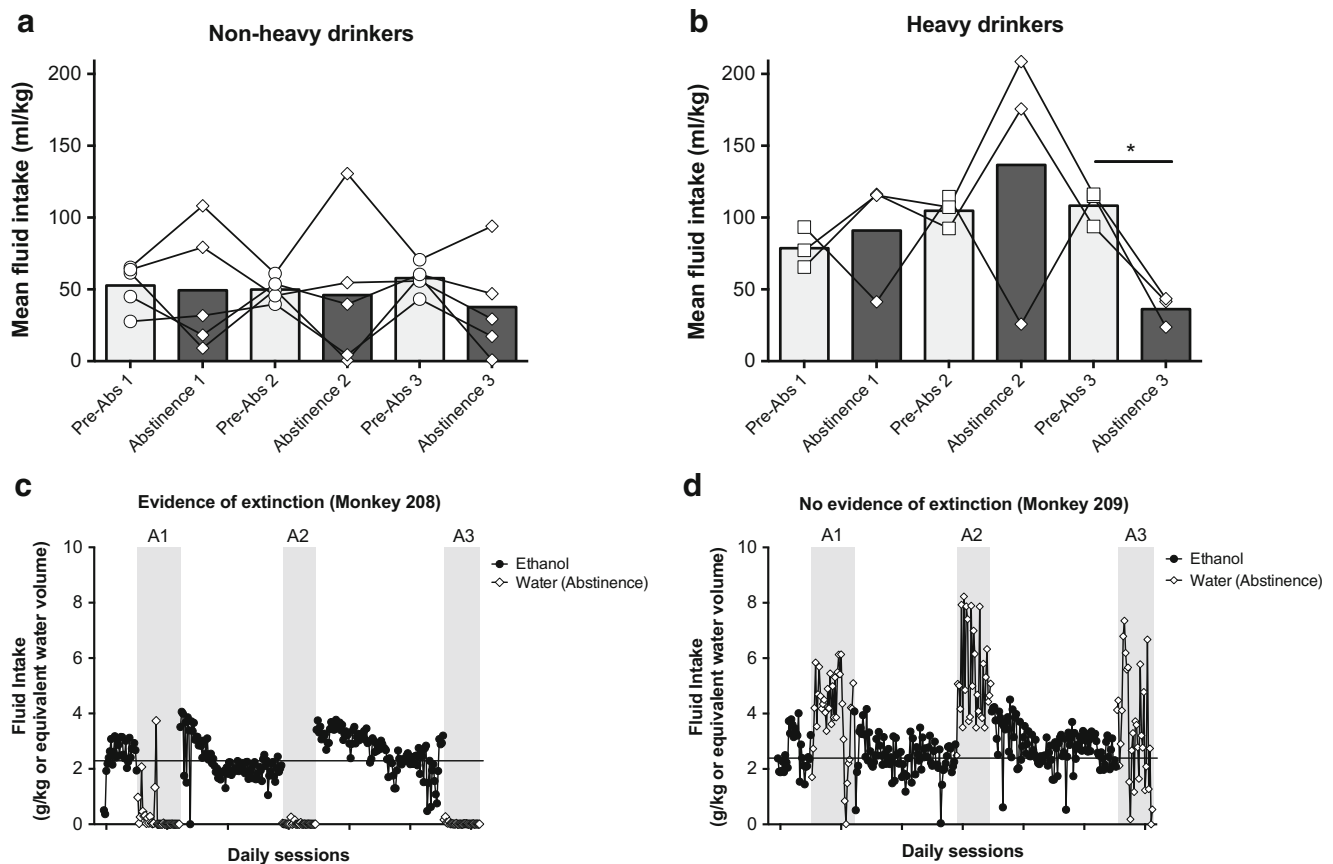


Fig. 3 Water intake on ethanol spout during abstinence. **a, b** Total fluid intake (ml/kg) before (pre-abstinence) and during abstinence for each of the three abstinence periods for **a** non-heavy and **b** heavy drinkers. Each data point represents a 28-day mean for each monkey and the bars represent a group mean \pm SEM. Asterisks represent significant changes from

pre-abstinence (pre-abs), $*p < 0.05$. **c, d** Representative drinking patterns are shown for two monkeys: one that had evidence of extinction during abstinence (**c**, Monkey 208) and another that had no evidence of extinction (**d**, Monkey 209). Solid horizontal lines in both panels represent each monkey’s 12-month average ethanol intake prior to abstinence

Behavioral observations

Behavioral observations took place immediately prior to abstinence (pre-abstinence) and during abstinence. There was a near absence of any physical signs of alcohol dependence (Table 1; tremor, intentional tremor, piloerection, or vomiting) during any observation period (Table 2). The main observation was an increase in huddling/social withdrawal during both abstinence 1 ($F(1,9) = 24.56, p < 0.001$) and abstinence 3 ($F(1,9) = 10.64, p < 0.01$), but not abstinence 2 ($p > 0.3$) (Table 2). There was no effect of group on huddling/social withdrawal in any of the abstinence periods ($p > 0.3$), indicating that this effect is not specific to ethanol subjects (Table 2).

Normal, or species-typical, behaviors appeared stable across pre-abstinence and abstinence periods (Table 2), except for a decrease in normal behaviors during abstinence 3 ($F(1,9) = 25.69, p < 0.001$). A significant group interaction ($F(2,9) = 4.73, p < 0.05$), indicated that only the ethanol subjects decreased normal behaviors during abstinence 3 (heavy drinkers $p < 0.05$; non-heavy drinkers $p < 0.01$).

Hormonal response to repeated abstinence

In general, ACTH and cortisol were differentially responsive to ethanol consumption and abstinence (Fig. 4). Specifically, ACTH was altered during abstinence 1 in all subjects ($F(3,27) = 9.82, p < 0.001$) with a significant increase at 24 h into abstinence (abstinence 1 (24 h): $p < 0.01$) that persisted through day 21 ($p < 0.01$; Fig. 4a). However, when

ethanol was reintroduced, ACTH returned to pre-abstinence levels (post-abstinence 1: $p > 0.05$). This effect is driven primarily by the ethanol group, though only a trend level interaction was found so no post hoc comparisons were made ($F(6,27) = 2.27, p = 0.066$). During abstinence 2, there was an interaction between group and phase ($F(6,27) = 3.818, p < 0.01$), but post hoc tests between ethanol and control groups were not significant ($p > 0.05$).

Cortisol, however, was highly reactive to abstinence throughout all three abstinence phases (Fig. 4b; (abstinence 1: $F(3,27) = 16.42, p < 0.0001$; abstinence 2: $F(3,27) = 28.16, p < 0.0001$; abstinence 3: $F(2,18) = 97.09, p < 0.0001$). During each abstinence phase, a similar pattern was found: cortisol increased at 24 h of abstinence, continued to rise in week 3, and then fell post-abstinence. However, over repeated abstinence cycles, morning cortisol patterns begin to diverge between ethanol and control groups, particularly in heavy drinkers. Specifically, during abstinence 1, the three groups are tightly matched ($p = 0.89$). In abstinence 2, there is an interaction between experimental group and abstinence ($F(6,27) = 3.32, p < 0.05$). During the second abstinence, control monkeys and non-heavy drinkers remain matched, but heavy drinkers have higher cortisol ($p < 0.001$). In abstinence 3, cortisol rose in all groups ($F(2,18) = 97.09, p < 0.0001$) and was significantly higher than pre-abstinence cortisol at both 24 h ($p < 0.001$), and 21–22 days ($p < 0.001$) into abstinence. Additionally, there was a group difference ($F(2,9) = 8.45, p < 0.01$), such that heavy drinkers ($p < 0.05$) and non-heavy drinkers ($p < 0.01$) had higher cortisol during abstinence compared to controls.

Table 2 Behavioral observations during alcohol abstinence

Number of observed behaviors collapsed across experimental phase and group (Mean ± SD)						
Signs of physical alcohol dependence (max score = 40)	Pre-abs 1	Abs 1	Pre-abs 2	Abs 2	Pre-abs 3	Abs 3
Control ($n = 4$)	0.0 ± 0.0	0.8 ± 1.0	0.8 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5
Non-heavy drinkers ($n = 5$)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4	0.2 ± 0.4	0.4 ± 0.9	0.6 ± 0.9
Heavy drinkers ($n = 3$)	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.6	0.0 ± 0.0	0.7 ± 0.6	0.7 ± 0.6
All subjects ($n = 12$)	0.0 ± 0.0	0.3 ± 0.4	0.4 ± 0.3	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.2
Huddling/social withdrawal (max score = 20)	Pre-abs 1	Abs 1	Pre-abs 2	Abs 2	Pre-abs 3	Abs 3
Control ($n = 4$)	1.0 ± 1.2	3.5 ± 2.5	3.3 ± 2.1	3.8 ± 4.2	3.8 ± 1.3	5.2 ± 2.6
Non-heavy drinkers ($n = 5$)	0.6 ± 0.9	4.4 ± 1.1	4.8 ± 2.8	4.6 ± 2.9	2.0 ± 2.6	5.8 ± 3.0
Heavy drinkers ($n = 3$)	0.0 ± 0.0	3.7 ± 3.6	2.3 ± 2.5	4.0 ± 1.7	1.0 ± 0.0	4.3 ± 0.6
All subjects ($n = 12$)	0.5 ± 0.5	3.9 ± 0.5***	3.5 ± 1.2	4.1 ± 0.4	2.3 ± 1.4	5.1 ± 0.7***
Normal (or species-typical) behaviors (max score = 80)	Pre-abs 1	Abs 1	Pre-abs 2	Abs 2	Pre-abs 3	Abs 3
Control ($n = 4$)	41.3 ± 13.4	36.8 ± 5.9	33.3 ± 2.6	33.3 ± 9.9	34.3 ± 10.0	32.5 ± 9.7
Non-heavy drinkers ($n = 5$)	40.0 ± 6.9	31.4 ± 8.2	35.0 ± 5.0	31.8 ± 6.8	39.6 ± 10.2	27.4 ± 6.3##
Heavy drinkers ($n = 3$)	34.7 ± 7.0	39.7 ± 5.5	31.3 ± 13.1	34.7 ± 11.0	38.7 ± 11.0	29.7 ± 10.7#
All subjects ($n = 12$)	38.6 ± 3.5	35.9 ± 4.2	33.2 ± 1.8	33.2 ± 1.4	37.5 ± 2.9	29.9 ± 2.6***

*** $p < 0.001$, indicating a significant difference between abstinence and the preceding pre-abstinence measure. # $p < 0.05$; ## $p < 0.01$, indicating a significant difference compared to controls during abstinence

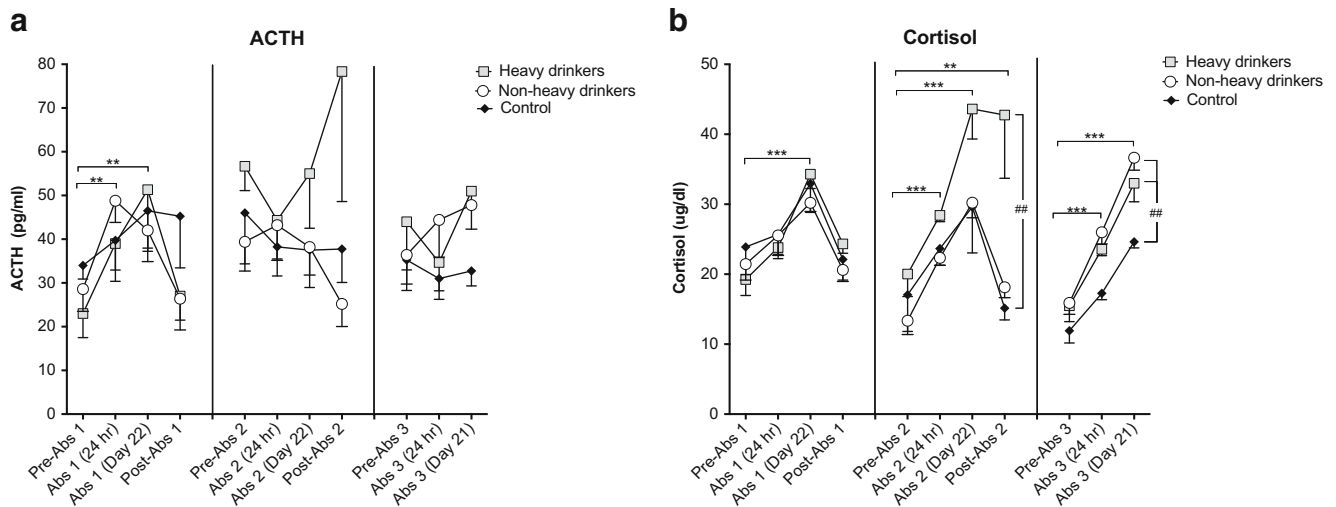


Fig. 4 Pituitary and adrenal hormone response to repeated abstinence during morning blood draws. **a** ACTH and **b** cortisol before abstinence (pre-abs), 24 h into abstinence, 21–22 days into abstinence, and within 1 week of resumed ethanol access (post-abs). Each of these time points

was matched for all abstinence phases (abstinence 1–3). All data are presented as mean \pm SEM. Asterisks indicate a significant change from pre-abstinence. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Hashes represent a significant group difference compared to the control group: # $p < 0.01$

Discussion

The examination of repeated cycles of abstinence on drinking and stress hormones in this study represents an extension of previous work from our laboratory establishing a non-human primate model of chronic ethanol self-administration (Grant et al. 2008). Over replicate cohorts, the individual differences in daily ethanol intakes in our monkey population represent statistically defined, stable categories of ethanol drinking phenotypes under continuous access conditions of 22 h/day (Grant et al. 2008; Baker et al. 2014, 2017). These studies have reliably and repeatedly produced cohorts of monkeys with a predictable range of drinking (Baker et al. 2014, 2017), including the present cohort with a consistent distribution of heavy and non-heavy drinkers. Average ethanol intakes in this cohort range from 1.3 to 4.2 g/kg/day, which are approximately equivalent to 5–17 drinks/day in humans. This range extends from binge drinking to clinically problematic drinking, which averages approximately 15 drinks per day (Kwako et al. 2014). The between-subject variance prior to abstinence represents a critical strength of the current study, allowing us to examine the effects of prolonged, repeated cycles of abstinence on both non-heavy and heavy drinkers within the same experiment.

The most prominent effect of abstinence was the robust increase in ethanol intake and BEC in the 28 days following abstinence (Fig. 2b, c). The increase in ethanol intake resulted in six out of eight monkeys having mean BECs consistently above 80 mg/dl, compared to only one monkey at this mean level before abstinence (Fig. 2d–e). The final post-abstinence drinking phase resulted in a range of mean BECs from 48 to

240 mg/dl. Human data show BECs from 50 to over 150 mg/dl are associated with impairments in memory, attention, and motor coordination, increasing the risk of injury to self and others up to and including severe impairments in judgment, amnesia (blackouts), and loss of consciousness (National Institute on Alcohol Abuse and Alcoholism 2015). Thus, the increased mean BECs seen here and documented over the first 28-day period following abstinence are highly relevant. A similar self-administration protocol in cynomolgus monkeys also found that repeated abstinence resulted in higher BECs in relapse (Cuzon Carlson et al. 2011). Under a different protocol and laboratory, eight male rhesus monkeys were provided prolonged (i.e., over 1 year) concurrent access to 16 or 32% (v/v) ethanol and water (Kornet et al. 1990). In this study, short alcohol deprivation periods (1, 2, and 7 days) increased post-abstinence ethanol intake proportional to the number of days abstinent. Overall, macaque monkeys robustly display individual differences in voluntary ethanol intakes, and the present study also demonstrates increased alcohol drinking following abstinence. Importantly, however, the effect of repeated abstinence on drinking has not yet been characterized in female macaques, indicating a critical future direction for these findings.

In contrast to the few reports in non-human primates, rodent models have a long history of investigating repeated alcohol deprivations (i.e., alcohol deprivation effect, ADE) following chronic daily drinking (Sinclair and Senter 1968; Spanagel and Holter 1999; Spanagel 2000). In general, ADE is classically associated with a transient increase in ethanol intake that slowly recovers over up to 4 weeks of resumed ethanol access (Spanagel and Holter 1999), which is notably similar to the effect observed

here. However, ADE using rodent models has not been applied to between-subjects variability in drinking. The current data with macaques suggest that an alcohol deprivation effect is prominent following abstinence across all drinking levels, including low-to-moderate drinkers. While the initial increase in drinking eventually resolves in most subjects, the heaviest drinkers demonstrated sustained (over 3 months) increases in ethanol intake and resultant BECs that rose after each subsequent abstinence (Fig. 2). The heavy drinkers all had average BECs exceeding 100 mg/dl in the 1 month after abstinence 1, and exceeding 175 mg/dl following abstinence 2. This sustained effect of abstinence on heavy drinkers indicates a marked divergence from the traditional ADE effect and was not found in non-heavy drinkers. The differential sensitivity to increased drinking following repeated involuntary abstinence between heavy and non-heavy drinkers provides additional validation of the drinking categories developed using our macaque model (Baker et al. 2014; Cervera-Juanes et al. 2016a, b).

The sustained escalation of ethanol intake post-abstinence in heavy drinkers resembles rodent alcohol dependence models of involuntary ethanol exposure, such as CIE (chronic intermittent ethanol). In this design, alcohol dependence is experimentally induced through passive ethanol exposure via inhalation chambers to maintain blood ethanol concentrations above 250 mg% for 24–72 h (Becker 1998; Becker and Lopez 2004). This procedure can produce elevations in self-administration 72 h after removal from the inhalation chambers (Becker and Lopez 2004; Griffin et al. 2009). Importantly, the current protocol of self-administration in macaques demonstrates a similar robust escalation in drinking, induced exclusively through voluntary ethanol intake. In addition, there are minimal overt signs of physical dependence in this model, which deviates from the rodent dependence studies. These data provide a translational link between rodent models of alcohol dependence that emphasize physical dependence and studies of AUD in humans that emphasize signs of negative affect during abstinence (Holleran and Winder 2017). A primary emphasis of the macaque model is the self-selection into heavy and non-heavy categorical drinking emerging from a heterogeneous population that reflects human populations of moderate to heavy drinkers.

Interestingly, there was minimal evidence of extinction across the first two abstinence phases, based on similar (or greater) average fluid intakes through the ethanol spout before and during abstinence. In abstinence 3, water intake on the ethanol spout decreased selectively in heavy drinkers, suggesting that extinction was learned in this group by the third abstinence cycle (Fig. 3b). The initial resistance to extinction is consistent with the development of inflexible (or habitual) behavior over the course of prolonged ethanol intake. There is

evidence that chronic ethanol drinking produces a shift from flexible, easily adjusted behavior to habitual highly patterned behavior that is less modifiable in response to changes in reinforcement contingencies (Gerdeman et al. 2003; Everitt and Robbins 2005; Corbit et al. 2012; O'Tousa and Grahame 2014). In addition, research in baboons has shown that alcohol-associated cues can maintain responding at levels above cues associated with other reinforcers (i.e., Tang solution) and this behavior is highly resistant to change (Holtyn et al. 2014). Thus, the prolonged daily ethanol paired with the complex cues in the home cage associated with ethanol availability likely contributed to the resistance to extinction reported here. Resistance to extinction in abstinence may be mediated by synaptic changes within the striatum to favor habitual, inflexible behavior (Yin et al. 2007; Cuzon Carlson et al. 2011). Specifically, chronic ethanol and abstinence in monkeys is associated with increased synaptic firing within the putamen (rodent dorsolateral striatum) but not the caudate (rodent dorsomedial striatum), representing a shift toward habitual striatal circuitry (Cuzon Carlson et al. 2011). Thus, resistance to extinction may reflect the state of the primate brain once ethanol is made available (e.g., relapse conditions) that leads to a rapid return to excessive ethanol consumption.

Signs of overt physical dependence were minimal in this cohort, similar to a previous report on the absence of withdrawal signs in rhesus monkeys given over 1 year ethanol access (Kornet et al. 1990, 1991). The most notable change in behavior was an increase huddling and social withdrawal, which were also seen in control subjects. These data suggest that the conditions of co-housing ethanol and control subjects were not optimal for detecting changes in behavior due specifically to ethanol abstinence. Recent work in mice has demonstrated transfer of alcohol withdrawal states between experimental and control groups within the same housing environment (Smith et al. 2016). Additionally, control subjects had their daily yoked-ration of highly palatable maltodextrin removed during abstinence, and this removal of a valued commodity may have contributed to the control monkeys also showing increased huddling/social withdrawal in the abstinence periods (Lemolo et al. 2012).

Lastly, the effects of abstinence on circulating ACTH and cortisol were variable and wide-ranging in both drinkers and controls. Overall, ACTH was more variable compared to cortisol, and ACTH levels were not consistently related to the experimental phases. In general, these data support a common finding of dissociation between ACTH and cortisol over chronic ethanol drinking in the macaque model (Helms et al. 2012; Helms et al. 2014). The results are also consistent with clinical alcohol-dependent populations that have elevated cortisol during acute abstinence from ethanol (Iranmanesh et al. 1989; Adinoff et al. 1991, 2003), as well previous work in cynomolgus monkeys (Cuzon Carlson et al. 2011). In alcohol-dependent patients, cortisol often falls from acute to protracted

abstinence (Adinoff et al. 2003). However, other studies demonstrated that abstinent AUD patients maintain higher basal cortisol relative to controls a month after their last drink, even if some recovery within subjects is observed (Sinha et al. 2009), consistent with the elevation in cortisol levels across the course of 28 days of abstinence seen here in the monkey. Interestingly, the cortisol response of the control monkeys tightly match the ethanol subjects during the first abstinence, perhaps due to heightened stress though the entire housing room that extends beyond ethanol subjects alone (Smith et al. 2016). However, by the third experience of abstinence, ethanol monkeys continue to show heightened cortisol response during abstinence in contrast to controls. This group difference is primarily due to ethanol monkeys showing a nearly doubled range of morning cortisol levels between ethanol access periods and abstinence. These findings indicate that repeated abstinence periods with interspersed daily alcohol drinking may be necessary to show ethanol-specific activation of the HPA axis and circulating cortisol as opposed to stress due to the loss of any important commodity. This finding as implications for the clinical literature because nearly all AUD patients have undergone several relapse cycles throughout the course of their disease progression superimposed upon multiple commodity losses (e.g., employment, significant relationships, health) as a consequence of their alcohol drinking.

In conclusion, we have presented data from heterogeneous sample of male rhesus monkeys demonstrating that repeated 28–35 day abstinences following daily access to ethanol for over 1 year produced consistently elevated drinking in all monkeys, independent of intake level before abstinence. Importantly, abstinence did not result in a decrease in drinking in any subject, a result that is consistent with longitudinal analysis of human AUD patients (over 11 years) indicating that involuntary abstinence was a consistent predictor of increased drinking (Delucchi and Kaskutas 2010). Further, significant changes in drinking were present despite the absence of spontaneous signs of physical alcohol dependence. Lastly, morning cortisol did not differentiate drinkers from control subject until the third abstinence experience, highlighting the importance of repeated abstinences in animal models of AUD. In conclusion, these data provide evidence that abstinence, as a target for treatment, can exacerbate reinstated drinking particularly in the heaviest drinking subjects. Instead, treatment approaches that emphasize harm reduction rather than complete abstinence may improve treatment outcomes and decrease risk of relapse behavior.

Acknowledgements The authors would like to acknowledge Andrew Woodall, Molly McGinnis, and Devin Owen who provided essential technical assistance in carrying out these experiments. These studies were funded by grants from the US National Institutes of Health F31 AA024660, AA019431, AA013641, and U01 AA013510.

Authors' contribution K.A.G designed the experiment. D.C.A. conducted the experiment and analyzed the data. S.W.G conducted preliminary data processing and analysis. D.C.A and K.A.G. wrote the paper. All authors critically reviewed the paper and approved the final version for publication.

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