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Research Article

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Intermittent Access to Ethanol Induces Escalated Alcohol Consumption in Primates

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Abstract

Background: Escalation of voluntary alcohol drinking is characteristic of alcohol addiction and can be induced in rodents using intermittent access to alcohol. This model has been used to evaluate candidate therapeutics, but key systems involved in the transition into alcohol addiction, such as CRF, differ in their organization between rodents and primates. We examined the ability of an intermittent access schedule to induce escalation of voluntary alcohol drinking in non-human primates and used this model to assess the role of corticotropin releasing hormone (CRF) signaling in this process.

Methods: Four young adult male rhesus macaques were given access to an 8.4% alcohol solution every other weekday (EOD; M, W, F), while four other young adult males were given the same solution every weekday (ED; M-F). Subjects were then administered a CRF1 antagonist, antalarmin.

Results: EOD increased alcohol intake by up to 50% over baseline, with a more pronounced increase immediately following reintroduction of alcohol. For the morning/daytime sessions, EOD subjects increased their consumption by 83% over baseline. Differences between ED and EOD schedules emerged quickly, and EOD-induced escalation resulted in pharmacologically active BAC's. EOD-induced alcohol consumption was insensitive to CRFR1 blockade by antalarmin, but subjects with high CSF levels of CRF were more responsive.

Conclusions: Similar to what has been observed in rodents, intermittent access results in an escalation of voluntary alcohol drinking in non-human primates. In contrast to findings in rats, recruitment of the CRF system does not seem to be involved in the escalated alcohol drinking observed under these conditions, though individual differences in CRF system activity may play a role.

Keywords

Alcohol; Dependence; Rhesus macaque; Intermittent access; CRF

Introduction

Alcohol use disorders account for a major share of global disease burden [1], and their treatment continues to pose a challenge. Animal studies hold the hope of identifying mechanisms that can be targeted by novel treatments and offer tools to evaluate candidate therapeutics [2,3]. A key challenge for these studies has been to establish models in which levels and patterns of brain alcohol exposure are sufficient to trigger neuroadaptive processes that promote alcohol addiction in humans. Development of paradigms that achieve this objective has ultimately been successful in rats [4] and mice [5]. These models have identified central stress and aversion systems as candidate mediators of ethanol-induced neuroadaptations. Among these, an involvement of corticotropin-releasing hormone (CRF) and its type 1 receptors has accumulated particularly consistent support [6,7].

It is, however, unclear to what extent rodent findings are able to predict clinical efficacy in human behavioral health disorders. Multiple failures in this arena have recently led to a marked decreased activity in CNS drug development [8]. The limited success rates are not well understood, but species differences may be an important contributing factor. In that context, the central stress systems show considerable differences between catarrhine primates (old world monkeys, apes and humans) and other species [9]. Studies in non-human primates may, therefore, be critically important in the assessment of whether candidate therapeutics for alcohol use disorders identified in rodent models will show clinical efficacy in humans [10,11].

A key feature shared by several rodent models of escalating alcohol drinking is the use of intermittent access or exposure. This results in a cyclical pattern of brain alcohol exposure that mimics that which is observed in humans and thought to promote the transition into alcohol addiction [12]. Using these types of paradigms, it has been demonstrated that repeated cycles of intoxication and withdrawal induce neuroadaptive changes that result in persistently escalated alcohol consumption. Paradigms using intermittent forced exposure to ethanol vapor have, for instance, been shown to result in blood alcohol concentrations (BAC's) in the range of 150-200 mg/dL [5,13-18]. These levels of exposure are thought to be sufficient to promote a shift to negatively reinforcement drinking, which underlies the transition to an addicted state; The neuroadaptations induced in these models have collectively been labeled "the post-dependent state" [4].

Because key mediators of the transition to negatively reinforce drinking in rodents, such as CRF and its receptors, differ in their expression and distribution between rodents and catarrhine primates [9], an intermittent access model of escalated alcohol consumption in primates would be an important tool to assess the clinical potential of candidate therapeutics. A major barrier to developing this type of model is that methods commonly used in rodent intermittent access studies, such as forced exposure to alcohol vapor, are not practically feasible in catarrhine primates, due to their relative size and requirements for housing conditions.

However, while the rodent escalation models cited above have generally relied on passive exposure to alcohol, some of the earliest studies to recognize the phenomenon of escalated consumption resulting from intermittent access investigated different schedules

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of access to alcohol for oral self-administration. In those studies, rats given voluntary access to alcohol on intermittent schedules (i.e., alcohol was available for 24 or 48 hours and then removed for 24 or 48 hours) were found to consume as much as 4 times more alcohol than rats drinking on continuous schedules [19,20]. This type of model successfully predicted the clinical efficacy of the nicotinic partial agonist varenicline as a therapeutic for alcohol use disorders [21,22].

Here, we examined the feasibility of inducing escalated alcohol consumption in rhesus macaques using an intermittent alcohol access schedule. We then determined whether, similar to what has been observed in rodents [23], intermittent exposure-induced drinking reflects a recruitment of the CRF system in this species. We used an every-other day access schedule for nine weeks to evaluate its ability to induce escalated alcohol consumption. We also examined whether cerebrospinal-fluid (CSF) levels of CRF changed as a result of intermittent exposure, and whether alcohol consumption induced using this paradigm was sensitive to CRF1 receptor blockade.

Materials and Methods

Subjects

The subjects were 8 young adult male rhesus macaques (4 mother-reared and 4 peer-reared, see [24]), ranging in age from 4.9 to 5.3 years of age (mean age=5.2 years). All subjects were first exposed to alcohol (8.4% ethanol-water-aspartame solution) in a 1 hour, limited access paradigm for 4 weeks while housed in their male-only social group, using methods described previously [25]. There were four weeks between being allowed access to alcohol in the social group and being placed into single cages. Animals were housed in the same room as the other members of their social group being tested, and they were acclimated to this condition for two weeks prior to initiation of the study. Research was carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Alcohol schedule and assessment of self-administration

For a schematic of the study design, (Figure S1). Subjects were housed individually in single cages and were given access to the 8.4% ethanol-water-aspartame solution for a total of 22.5 hours a day. Data acquisition occurred in two blocks of time: 9:00 AM until 2:30 PM (DAY session), and 2:45 PM until 7:45 AM the next morning (NIGHT session). The light cycle for the test room was 7:00am to 7:00pm, and food was provided at 7 am and 2 pm. Each cage was equipped with two lick-initiated lixit bottles to dispense the ethanol solution, and the amount of solution consumed by each subject was recorded by computer. Regular drinking water was also available to the subjects at all times.

For the first three weeks (baseline phase), subjects were provided with daily access (Monday through Friday) to the ethanol solution to establish baseline consumption levels within a 22.5-hour exposure paradigm. Subjects were then randomly assigned to one of two groups: Every Other Week Day Access (EOD, n=4, 2 MR, 2 PR) and Every Week Day Access (ED, n=4, 2 MR, 2 PR) for alcohol testing, for days Monday through Friday. Alcohol was available to subjects in the EOD group on Monday, Wednesday, and Friday while the ED group had access Monday through Friday. The test phase lasted for nine weeks. Assessment of alcohol intake was performed only on Wednesdays and Fridays, in order to ensure that alcohol deprivation over the weekend days and external factors that could disrupt drinking behavior were not confounded. Alcohol intake measured on Tuesdays and Thursdays was excluded because only the ED animals had access to alcohol on those days, and alcohol consumption levels measured on Mondays were not considered in order to eliminate the weekend deprivation effect [26], which could obscure EOD access effects (Figure S2).

CSF sampling and CRF radioimmunoassay

CSF samples (3ml) were obtained from the cisterna magna under ketamine anesthesia (10 mg/kg, IM) using a 22 gauge needle. CSF collection was performed 10 days prior to the initiation of the study and at the end of the ninth week of EOD testing. Samples were collected within fifteen minutes of entering the room and were immediately aliquoted into polypropylene tubes and frozen in liquid nitrogen. Samples were stored at -70°C until assayed. CSF was assayed for CRF by radioimmunoassay. Each sample was run, in duplicate, with a RIA kit from Phoenix Pharmaceuticals, Burlingame, CA. (RK-019-06). Reported assay sensitivity is 26.51 pg/ml.

Antalarmin administration

The CRF1 antagonist antalarmin was synthesized as described [27]. During weeks 10 and 11, animals received antalarmin in a counterbalanced within-subject design (20 mg/kg IM; [28,29]). Antalarmin was put into suspension in sterile saline by stirring followed by sterile filtration and was administered at 8:30 AM on either Wednesday of the first week and Friday of the second week, or on Friday of the first week and Wednesday of the second. Animals received saline injections on treatment days on which they did not receive antalarmin.

Assessment of blood alcohol concentrations

On Friday of the third baseline week and that of the third, sixth, and ninth weeks of EOD testing, subjects were anesthetized at 12:00 PM, and blood samples were collected from the femoral vein to assess blood ethanol concentrations BAC, (Figure S1). Samples were also obtained during saline and antalarmin treatment days (weeks 10 and 11). On these Fridays, subjects experienced a slightly shortened DAY session (9:00 to 12:00 PM) and were not provided access to the ethanol solution again until 9:00 the following Monday. All BAC's were quantified by an enzymatic method using a commercial kit (Sigma 332, U.S.A., Ultraviolet, Endpoint method). Due to technical difficulties with the assay, the BAC values from samples collected during week 9 (late testing) were unavailable.

Statistical analysis

To examine effects of intermittent access on levels of alcohol consumption, repeated measures ANOVA was performed with testing condition (EOD vs. ED) as the between-subjects variable and time as the within-subjects factor. Analysis was performed for baseline, early (weeks 2 and 3), middle (weeks 5 and 6) and late (weeks 8 and 9) testing weeks. We analyzed Daily Total consumption (over the entire 22.5 hours) as well as consumption during the two separate time blocks (DAY session and NIGHT session) using data averaged over the Wednesday and Friday testing sessions.

To determine whether EOD access affected CSF levels of CRF, we performed repeated measures ANOVA, with condition (EOD vs. ED) as the between subjects variable and alcohol exposure (pre-test vs. week 9 of EOD testing) as the within subjects variable. To determine whether antalarmin influenced alcohol self-administration and whether EOD access affected antalarmin response, we performed a separate repeated measures ANOVA, with condition (EOD vs. ED) as the between subjects variable and treatment (Saline vs. Antalarmin) as the within subjects variable. Because individual differences in CRF

system function could influence antalarmin response, CSF levels of CRF assessed during week 9 were used as a co-variate in the analysis.

To control for a possible contribution of effects from early peer-rearing on alcohol consumption and CSF levels of CRF, analyses were repeated with rearing condition included as a co-variable, but no effects were found and the inclusion of this variable did not reduce the residual variance. It was removed from the analyses (PR vs. MR effect, F (1,6)=0.196, p=0.67). All analyses were performed using JMP statistical software. Where appropriate, *post-hoc* analyses were performed using the Tukey-Kramer method (p<0.05).

Results

Effects of intermittent access on voluntary alcohol consumption

There was a trend for a main effect of the test group for Daily Total alcohol consumption (F[1,18]=4.38, p=0.08) and a main effect for test phase (F[3,18]=11.6, p=0.0002). There was also an interaction between the test group and test phase (F[3,18]=4.5, p=0.02). *Post-hoc* analyses revealed that EOD subjects showed progressive increases in their levels of alcohol intake (Tukey-Kramer, p<0.05), while levels of consumption among ED subjects remained unchanged (Figure 1A).

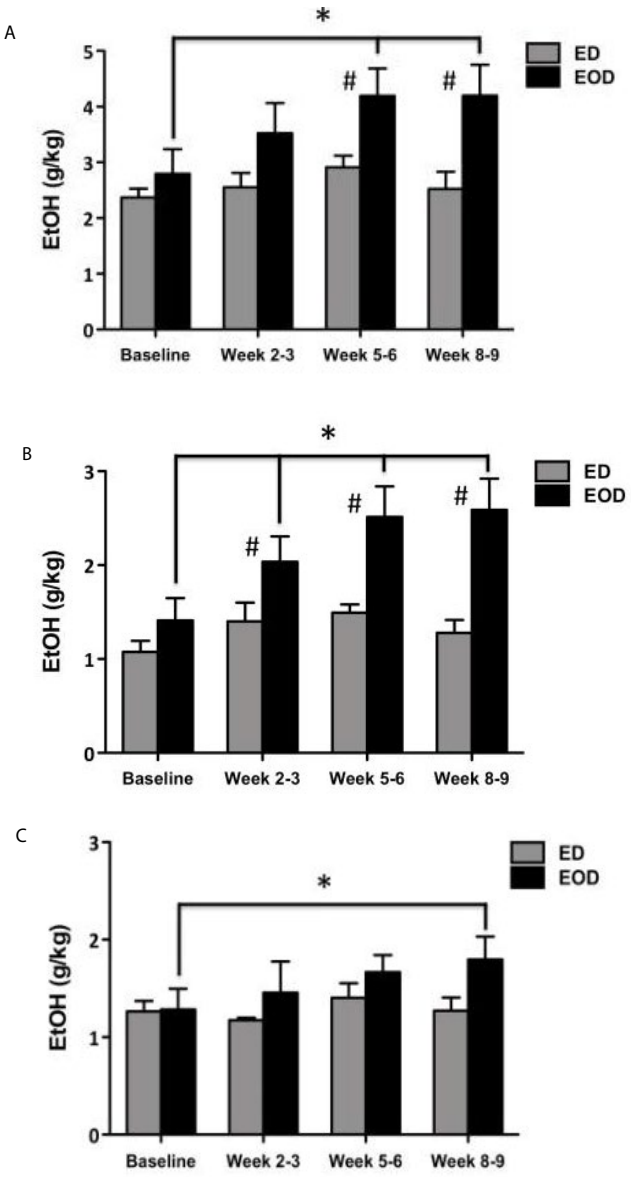

Figure 1: Group means of daily total alcohol consumption (g/kg/day) for baseline, early, middle and late testing phases. Shown are data collected in both every-day (ED) and every-other day (EOD) administration paradigms for **A.** total daily consumption and that collected during the

B. DAY (9:00 am to 2:30 pm) or

C. NIGHT sessions (2:45 pm to 7:45am the next day). There were interactions between test group and test phase for alcohol consumption (A and B). EOD subjects showed progressive increases in their levels of alcohol intake, while levels of consumption among ED subjects remained unchanged. (*P<0.05 within group, #P<0.05 between group).

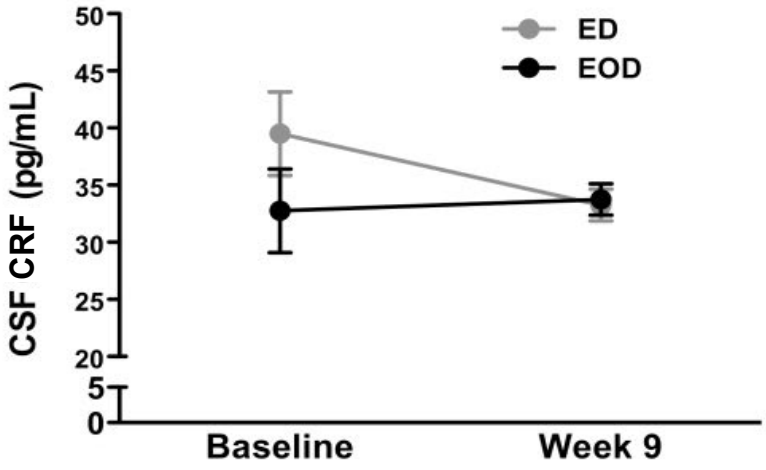

Figure 2: Effects of Intermittent Access on CRF System functioning. Shown are CSF levels of CRF assessed during baseline testing and following intermittent, every-other day access. There were no effects of alcohol exposure (pre vs. Week 9) or of intermittent access (ED, every day vs. EOD, every-other-day) on CSF levels of CRF, nor were there any interactions.

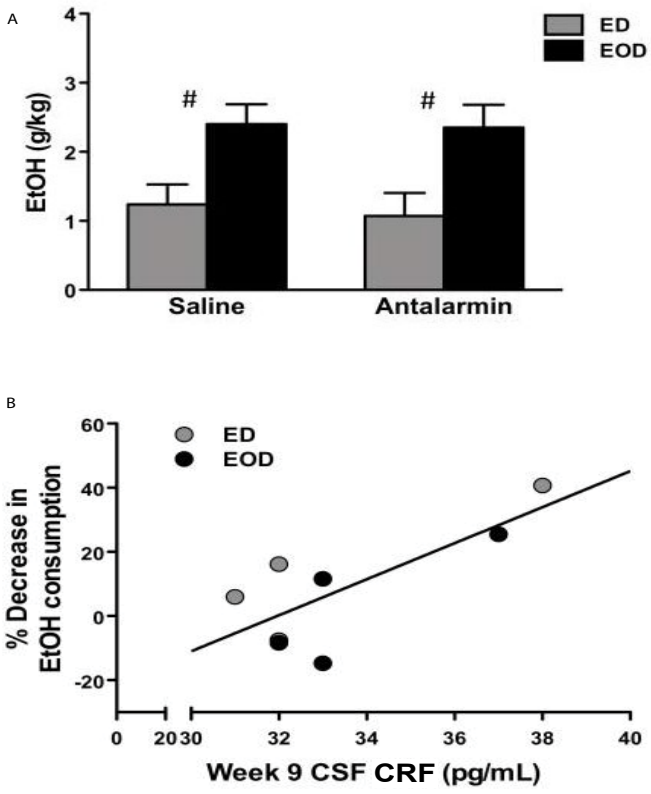

Figure 3: Effects of Intermittent Access on CRF System Functioning and Antalarmin Response. Shown are

A. levels of alcohol consumption assessed on days of saline and antalarmin administration and

B. percent decrease in alcohol consumption as a function of CSF levels of CRF. Data collected for animals assessed under an intermittent paradigm (EOD, every-other-day) and those with daily access (ED, every day vs.) are represented. Although there were no differential effects of antalarmin treatment as a function of intermittent access (EOD consumption was higher overall), individual differences in CSF levels of CRF predicted antalarmin response. (# P<0.05 between group).

No difference was observed between test groups during baseline weeks of testing, but EOD subjects consumed more alcohol than ED subjects by weeks 2 and 3 of testing ((Figure 1A), Tukey-Kramer, $p<0.05$.), and all subjects achieved blood alcohol concentrations in excess of 100 mg % (mean BAC=154.8, (Figure S1)).

When the effects of intermittent access on levels of consumption during the DAY and NIGHT sessions were evaluated separately, similar results were found during for the DAY session. There was a main effect for test group ($F[1,18]=8.0$, $p=0.03$), a main effect for test phase ($F[3,18]=16.6$, $p<0.0001$), and an interaction between the test group and test phase ($F[3,18]=6.41$, $p=0.004$). *Post-hoc analyses* of data collected during the DAY session showed that EOD subjects consumed more alcohol in the early and late test phases when compared to baseline levels ((Figure 1B), Tukey-Kramer, $p<0.05$). For data collected during the NIGHT session, there was no effect of the test group ($F[1,18]=1.8$, $p=0.28$), but there was a main effect for test phase ($F[3,18]=3.354$, $p=0.04$) and a trend for an interaction between test phase and the test group ($F[3,18]=1.8$, $p=0.18$). EOD subjects exhibited increased levels of alcohol consumption relative to baseline, but not until the late phase of testing (Figure 1C).

Effects of intermittent access on CRF levels in CSF

Overall, there were effects neither of alcohol exposure (pre vs. Week 9) nor of intermittent access (EOD vs. ED) on CSF levels of CRF (effect of alcohol, $F(1,6)=0.887$, $p=0.38$; effect of EOD access, $F(1,6)=1.692$, $p=0.24$) nor were there any interactions (alcohol x EOD, $F(1,6)=1.69$, $p=0.24$) (Figure 2).

Effects of the CRF1 antagonist antalarmin on alcohol consumption

There was no overall effect of antalarmin treatment on alcohol consumption ((Figure 3A), effect of antalarmin, $F(1,6)=0.78$, $p=0.41$; EOD x antalarmin interaction, $F(1,6)=0.08$, $p=0.78$). However, when CSF levels of CRF were included as a co-variate in the analyses, there was a significant antalarmin x CRF interaction ($F(1,5)=8.41$, $p<0.03$), and a significant effect of antalarmin emerged ($F(1,5)=7.84$, $p<0.04$). This observation was followed up using regression analyses, which demonstrated that antalarmin more effectively decreased alcohol consumption among subjects with higher CSF levels of CRF ((Figure 3B), simple regression for % Decrease in Consumption with Antalarmin, $R^2=0.782$, $p<0.03$). Even after controlling for CSF levels of CRF, there was no EOD x treatment interaction ($F(1,5)=0.53$, $p=0.50$).

Discussion

We report a robust escalation of voluntary alcohol consumption in rhesus macaques allowed intermittent access to alcohol. The magnitude of this escalation was similar to that observed in rats under a similar schedule [19,20] and resulted in pharmacologically relevant BAC's. In contrast to what has been observed in rats [23], intermittent access – induced escalation in this model was not sensitive to CRF1 antagonism. Interestingly, however, CSF levels of CRF predicted individual response to antalarmin independent of access schedule.

Candidate medications for behavioral disorders have had high clinical failure rates, pointing to an urgent need for improved translational tools [30]. Studies performed in catarrhine primates have produced results that translate to the human condition [31-33] and may, therefore, offer a valuable intermediate step between rodent and human studies. To be useful for the development of alcoholism medications, primate models need to result in an escalation of voluntary alcohol consumption to levels that yield pharmacologically active BAC's and brain exposure patterns similar to those experiences by humans in the course of developing alcohol addiction. Established approaches to inducing escalated alcohol consumption in rodents, such as selective breeding or forced inhalation, are not practical in studies using non-human primates. Our data demonstrate that intermittent access offers a simple, yet effective, method for achieving pharmacologically relevant BAC's in non-human primates (Figure S1). These intermittent access subjects increased their alcohol consumption in a step-wise manner during the test phase, increasing their total daily consumption by 50% over baseline values. Differences between intake on intermittent versus continuous access occurred within the first two - three weeks of testing and resulting BAC's for intermittent access subjects were in a range shown to promote long-term neuroadaptation in rodents, 100-200 mg% [34].

Our model is not unique in inducing levels of alcohol intake in non-human primates that have utility for alcohol studies. It has previously been reported that a fixed-time schedule of food delivery can be used to induce significant consumption of ethanol in cynomolgus monkeys [35,36]. Over the course of 9 months, this procedure ultimately resulted in BAC's that in some individuals reached in excess of 200 mg%. However, high individual variation was also observed, with low (5 mg%) BAC's in some animals. The long drinking duration required and high individual variation observed in this model may render it particularly useful as a tool to study individual susceptibility factors, and mechanisms through which these mediate risk. In contrast, intermittent access – induced escalation appears to be faster and less variable, making it attractive for evaluation of novel candidate medications.

In addition to total consumption, we also examined whether intermittent access influenced the pattern of alcohol intake. During the early phases of the study, intermittent access subjects showed increased alcohol intake upon renewed alcohol access (i.e., during the DAY sessions). However, there was no effect of intermittent access to the NIGHT sessions, neither during the early nor the middle phases of induction. In fact, we found that animals did not increase their NIGHT consumption levels until the late phase of induction (weeks 8-9). It should be noted that, during the later weeks of testing (weeks 6-11), high morning BAC, in excess of 150 mg% were still maintained, despite animals not having afternoon access on the prior day. This clearly shows that sufficient levels of consumption were maintained in the morning.

Studies performed in cynomolgous macaques have demonstrated that chronicity of ethanol exposure affects the sensitivity of CRF-containing neurons in the paraventricular nucleus of the hypothalamus [37], though the extra-hypothalamic circuits shown to drive escalated alcohol intake in rodents were not studied in these subjects. This being said, while we were able to induce escalated alcohol consumption using rhesus macaques in the present study, we did not obtain evidence for a significant contribution of the CRF system to alcohol drinking under these conditions. Following intermittent access resulting in BAC in excess of 150 mg%, there was neither an increase in the CSF levels of CRF, nor was alcohol drinking generally sensitive to CRF1 antagonism. The latter is in contrast to findings in rats [23] and mice [38]. As stated in the introduction, catarrhine primates differ in their levels and distributions of CRF receptors relative to other species. It is therefore possible that the CRF system, while critical

for escalation that results from intermittent access in rodents, is not equally important for this process in catarrhine primates. Should this type of species differences, in fact exist, then the consistent ability of CRF1 antagonists to block escalated alcohol consumption observed in rodents may have limited prospects of being translated into clinical efficacy in humans. In fact, recent experimental medicine findings are in line with this possibility [39,40].

Our study has obvious limitations. Studies in rodents have, for instance, suggested that up-regulation of the CRF system following intermittent brain alcohol exposure involves increased expression of CRF and the CRF1 receptor within the amygdala complex [41]. In the present study, we measured CRF levels in the CSF as an indirect measure of central expression, and we were not able to obtain a measure of CRF1 receptor expression. However, an up-regulated expression of CRF peptide, of CRF1 receptors, or both would be predicted to result in an increased sensitivity (i.e. left-shift of the dose-response curve) to CRF1 antagonism. This is, indeed, what has consistently been observed in rat studies [42-44]. In contrast, this was not observed in the present study, despite the use of the CRF1 antagonist antalarmin at a dose that has previously been shown to produce behavioral anti-stress effects in rhesus macaques, 20 mg/kg [28]. In fact, even a lower dose, 10 mg/kg, has been reported to partially block heroin choice precipitated in rhesus macaques by heroin-withdrawal [29]. Together, these data make it appear unlikely that a recruitment of the CRF system contributed to the escalation of alcohol drinking observed following intermittent access. It remains a possibility, however, that neuroadaptations involving the CRF system might occur with a longer duration of intermittent access to ethanol. Our findings may also have to be interpreted with caution because they were obtained in a small number of subjects, so that the potential influence from factors such as age or functional genetic variation could not be considered.

An incidental, but potentially important, finding was that central CRF levels predicted the response to CRF1 blockade, independent of access schedule. Because of the limited number of subjects studied, this observation also needs to be considered with caution. It may, however, provide support for the notion that CRFR1 promotes alcohol consumption in subpopulations of individuals rendered responsive by factors that influence the functional state of their CRF system. Follow-up analyses demonstrated that CRF levels assessed prior to the onset of intermittent access did not predict antalarmin response ($p=0.4$, data not shown), suggesting that the system may have been differentially modulated by alcohol among test subjects, resulting in differential sensitivity to the drug. Differential sensitivity of the CRF system to up-regulation as an individual predictor of response has been discussed previously, and may be related to interactions between environmental and genetic factors [45,46].

Because of their close phylogenetic relationship and neurobiological similarities to humans, rhesus macaques may be especially valuable as an intermediate step between rodent assays and clinical medications development. We present here a robust and practical model to induce pharmacologically relevant levels of alcohol intake in this species. Our initial analysis using this model does not provide support for a general role of CRF-system recruitment as a factor behind escalated alcohol intake resulting from intermittent access. The findings do, however, suggest the possibility that the differential sensitivity of the CRF system to repeated brain alcohol exposures may be an individual factor that predicts response to CRF1 antagonist treatment in alcohol dependent subjects. If confirmed, this finding would have important implications for the design of clinical studies to evaluate efficacy of CRF1 antagonists for alcohol addiction.

Conflict of Interest

The authors declare that, except for income received from primary employers, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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