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Long-Evans Rats Acquire Operant Self-Administration of 20% Ethanol Without Sucrose Fading

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A major obstacle in the development of new medications for the treatment of alcohol use disorders (AUDs) has been the lack of preclinical, oral ethanol consumption paradigms that elicit high consumption. We have previously shown that rats exposed to 20% ethanol intermittently in a two-bottle choice paradigm will consume two times more ethanol than those given continuous access without the use of water deprivation or sucrose fading (5–6 g/kg every 24 h *vs* 2–3 g/kg every 24 h, respectively). In this study, we have adapted the model to an operant self-administration paradigm. Long-Evans rats were given access to 20% ethanol in overnight sessions on one of two schedules: (1) intermittent (Monday, Wednesday, and Friday) or (2) daily (Monday through Friday). With the progression of the overnight sessions, both groups showed a steady escalation in drinking (3–6 g/kg every 14 h) without the use of a sucrose-fading procedure. Following the acquisition phase, the 20% ethanol groups consumed significantly more ethanol than did animals trained to consume 10% ethanol with a sucrose fade (1.5 *vs* 0.7 g/kg every 30 min) and reached significantly higher blood ethanol concentrations. In addition, training history (20% ethanol *vs* 10% ethanol with sucrose fade) had a significant effect on the subsequent self-administration of higher concentrations of ethanol. Administration of the pharmacological stressor yohimbine following extinction caused a significant reinstatement of ethanol-seeking behavior. Both 20% ethanol models show promise and are amenable to the study of maintenance, motivation, and reinstatement. Furthermore, training animals to lever press for ethanol without the use of sucrose fading removes a potential confound from self-administration studies.

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INTRODUCTION

The current standard models of ethanol-seeking behaviors use rodents in a variety of paradigms that relate to various aspects of consumption and relapse. The operant self-administration paradigm is a commonly used model in which animals are trained to lever press for ethanol reinforcement (Samson *et al*, 1988). This model has been invaluable in the alcohol research field, as it has enabled researchers to explore the motivational aspects of ethanol seeking in rodents, with the use of fixed and progressive ratio schedules and reinstatement paradigms. The operant model has had an important role in the preclinical validation and characterization of the two medications approved by the US Food and Drug Administration for the treatment of alcohol use disorders (AUDs) since 1994: naltrexone (ReVia) (Bienkowski *et al*, 1999; Burattini *et al*, 2006; Dayas *et al*, 2007; Holter and Spanagel, 1999; Katner *et al*, 1999; Le *et al*, 1999; Liu and Weiss, 2002) and acamprosate (Campral) (Bachteler *et al*, 2005; Czachowski *et al*, 2001; Heyser *et al*, 1998; Holter *et al*, 1997; Rassnick *et al*, 1992). However, this model suffers from several limitations, including the need for sucrose fading and water deprivation to initiate drinking behavior, and low baseline ethanol consumption in outbred rat strains following removal of these initiation procedures.

Since its introduction in the mid-1980s, sucrose fading has largely been adopted as the primary means of getting rats to acquire operant ethanol self-administration (Samson, 1986). Using this method, animals are trained to lever press in operant chambers by shaping with sweetened solutions (sucrose or saccharin). Ethanol is added later to these sweetened solutions and the sucrose/saccharin is gradually faded out until the animal is pressing for an unsweetened, dilute ethanol solution. These methods lead to high ethanol consumption while the sucrose is present but drinking drops precipitously once the sweetener is removed (Carrillo *et al*, 2008; Koob and Weiss, 1990; Samson, 1986; Samson *et al*, 1999). In addition, there is emerging evidence that indicates that sucrose may be addictive in rodents (Avena *et al*, 2008; Colantuoni *et al*, 2002). Others have found that sucrose may cause similar brain activation and be more rewarding to rodents than drugs that are

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commonly abused by humans, such as opioids (Spangler *et al*, 2004) and cocaine (Lenoir *et al*, 2007). The addition of these sweetened solutions may introduce a confound to studies exploring ethanol-reinforced behaviors.

We have recently adapted an intermittent access two-bottle choice model that was first described in the 1970s (Amit *et al*, 1970; Wise, 1973), and have shown that rats will consume 20% ethanol without the use of sucrose fading or water deprivation (Nielsen *et al*, 2008; Simms *et al*, 2008; Steensland *et al*, 2007). Using this method, we found that outbred rats would increase their drinking by two- to threefold over those given continuous access to ethanol (Simms *et al*, 2008). In this study, we attempt to adapt this model of intermittent ethanol access to an operant setting where we hope to elucidate the motivational aspects of ethanol consumption and reinstatement.

MATERIALS AND METHODS

Animals and Housing

Adult, male, ethanol-naive, Long-Evans rats (Harlan, Indianapolis, IN), weighing 150–175 g on arrival (Harlan), were individually housed in ventilated Plexiglas cages (Thoren Caging Systems, Hazelton, PA) in a climate-controlled room on a 12-h light–dark cycle (lights on at 0700 hours). Rats were given at least 1 week to acclimate to individual housing conditions and handling procedures. Food and water were available *ad libitum* in the home cage throughout the entire paradigm. Operant sessions occurred between 0800 and 1200 hours, with the exception of initial self-administration training as outlined below. All procedures were pre-approved by the Ernest Gallo Clinic and Research Center Institutional Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs

Yohimbine (Sigma-Aldrich, St Louis, MO) was dissolved in distilled water and administered by intraperitoneal (i.p.) injection at a dose of 2 mg/kg in a volume of 0.5 ml/kg, in accordance with previous reinstatement studies (Ghitza *et al*, 2006; Le *et al*, 2005; Richards *et al*, 2008, 2009; Shepard *et al*, 2004). Vehicle injections were administered using the same volume. Ethanol (v/v) solutions were prepared using filtered water and 95% ethyl alcohol (Gold Shield Chemical, Hayward, CA).

Operant Self-Administration Apparatus

Self-administration testing was conducted in standard operant conditioning chambers (Coulbourn Instruments, Allentown, PA). Details regarding the apparatus have been extensively described elsewhere (Richards *et al*, 2008; Steensland *et al*, 2007).

10% Ethanol Self-Administration with Sucrose Fade

For the traditional 10% ethanol operant self-administration paradigm, Long-Evans rats ($n = 30$) were initially exposed to 10% ethanol as the only liquid source in their home cages for 4 days. Following the fourth day of forced ethanol exposure, rats were placed in the operant chambers for a 14 h overnight session on an FR1 schedule of reinforcement (0.1 ml reward after a single lever press). The start of the training session was signaled by the illumination of the house light and extension of the active lever. During this phase, only the active lever was available for the rat to press, to facilitate learning. Rats were trained to respond for 10% sucrose in overnight sessions (1–3 nights) and continued on 10% sucrose until they reached the FR3 stage of training. Initial daily training consisted of 45 min FR1 sessions and 1-h daily water access, with water access immediately following the training sessions. Once responding was established (2–4 days), rats were given free access to water in the home cage and continued on a 45 min FR1 schedule for an additional 3–4 days. Subsequently, training sessions were reduced to 30 min and the work ratio was increased to an FR3 schedule of reinforcement (3 active lever presses required for 0.1 ml reward). A second, inactive lever was also introduced at this time. On pressing the inactive lever, no reinforcer, visual (light), or auditory stimuli were presented and the event was merely recorded as a measure of nonspecific behavioral activity. Following three sessions of FR3 training with 10% sucrose as the reinforcer, a modified sucrose fade technique (Samson, 1986) was initiated. Ten percent ethanol was added to the 10% sucrose solution, and over the next 12 sessions the sucrose concentration was gradually decreased (10, 5, 3, and 1.5%, respectively) until rats responded on an FR3 schedule for 10% ethanol alone. Rats continued on the FR3 protocol with 10% ethanol as the reinforcer for a minimum of 20 sessions. Any animals not reaching 0.3 g/kg ethanol intake per session were excluded from further study.

Intermittent 20% Ethanol Self-Administration

After the period of acclimatization, intermittent 20% ethanol self-administration was initiated in a separate group of Long-Evans rats ($n = 20$). Importantly, food and water were available *ad libitum* at all times in the home cage throughout the training. On the first day of training, animals were placed in the operant conditioning chambers for a 14-h overnight session on an FR1 schedule of reinforcement (0.1 ml after a single lever press) with 20% ethanol solution as the reinforcer. These FR1 overnight sessions were performed three times a week (Monday (M), Wednesday (W), and Friday (F)) for 4 consecutive weeks (12 total sessions). During the overnight sessions, only the active lever was available for the rat to press, to facilitate learning. Following the completion of these sessions, rats were then exposed to 45-min FR1 sessions three times a week (MWF) for 2 consecutive weeks (6 sessions). Subsequently, intermittent training sessions (MWF) were reduced to 30 min and the work ratio was increased to an FR3 schedule of reinforcement (three active lever presses required for 0.1 ml reward). The second (inactive) lever was also introduced at this time. On pressing the inactive lever, no reinforcer, cue light, or auditory stimuli were presented and the event was merely recorded as a measure of nonspecific behavior. Rats continued on the FR3 protocol with 20% ethanol as the reinforcer for a minimum of

20 sessions. Any animals not reaching 0.3 g/kg ethanol intake per session were excluded from further study.

Daily 20% Ethanol Self-Administration

A separate group of Long-Evans rats ($n = 15$) were trained as described above for intermittent 20% ethanol self-administration, but ethanol was presented daily, Monday through Friday. The animals received the same number of total drinking sessions at each stage of the protocol (ie, twelve 14 h overnights, six 45 min FR1 sessions and at least twenty 30 min FR3 sessions). Any animals not reaching 0.3 g/kg ethanol intake per session were excluded from further study.

Blood Ethanol Concentration Analysis

When the rats had maintained a stable baseline (>20 sessions) in each of the self-administration paradigms described above, blood samples were collected from the lateral tail vein immediately following the 30-min FR3 session. The samples were centrifuged at 4°C for 13 min at 8000 r.p.m. and blood ethanol concentrations (BECs) were determined from the plasma using gas chromatography (Doyon *et al*, 2003). The BECs were then correlated with the ethanol consumed (g/kg every 30 min) before the blood sampling.

20% Ethanol Challenge for Sucrose-Trained Animals

One group of animals ($n = 14$) trained to self-administer 10% ethanol with the use of a sucrose-fading procedure was subsequently challenged with 20% ethanol as the reinforcer. Following 25 sessions of 10% ethanol self-administration on an FR3 schedule, the group was switched to 20% ethanol for five consecutive sessions. Ethanol consumption was measured and blood samples were collected from the lateral tail vein immediately following the final three 30-min FR3 sessions at each concentration (one sample per rat with all samples collected over 3 days, days 23–25 for 10% ethanol and days 28–30 for 20% ethanol) for determination of BECs.

Ethanol Dose–Response Challenge

To directly compare the effect of the training history on subsequent ethanol self-administration, one group of rats ($n = 14$) trained to self-administer 10% ethanol with the use of a sucrose-fading procedure and one group ($n = 13$) trained to self-administer 20% ethanol using the daily access schedule were each challenged with the same five concentrations of ethanol (method adapted from Carnicella *et al*, submitted). Following 40 sessions of ethanol self-administration on an FR3 schedule with their respective solutions, the ethanol concentration for both groups was changed to 5% and presented for 5 consecutive days (M–F). This procedure continued for 4 more weeks, with 1 week each at 10, 20, 30, and 40% ethanol, respectively. Ethanol consumption was measured and blood samples were collected from the lateral tail vein immediately following the third and fourth 30-min FR3 sessions at each concentration (one sample per rat with all samples collected over 2 days each week) for determination of BECs.

Yohimbine Stress-Induced Reinstatement

To compare the levels of reinstatement in each of the methods described, lever-pressing behavior was extinguished in rats trained to self-administer ethanol under FR3 conditions. Extinction sessions were conducted on Monday, Wednesday, and Friday for the intermittent 20% ethanol group and Monday through Friday for the 10% ethanol and daily 20% ethanol group. During extinction training, active lever pressing resulted in presentations of both the light and tone cues but without the associated reward delivery. The ethanol solution was not available throughout the extinction procedure. Extinction training continued until the rats responded with less than 10 active lever presses per session or less than 10% of their previous baseline pressing on the active lever for two consecutive sessions. Once extinction criteria were achieved, rats were tested over two sessions, 7 days apart; on the first test session, all the rats were administered an acute injection of vehicle (distilled water), and on the second, they all received yohimbine (2 mg/kg, i.p.). Regular extinction sessions were run on the days between the vehicle and yohimbine challenges.

Statistics

All statistical analyses were performed using SigmaStat version 3.5 (Systat Software, San Jose, CA). Ethanol intake (g/kg) and active lever presses for the overnight sessions were analyzed using two-way analysis of variance (ANOVA), followed by Newman–Keuls *post hoc* analysis. Ethanol intake (g/kg), active lever presses, and inactive lever presses in the 30-min operant sessions were analyzed using two-way ANOVA comparing each of the 20% ethanol groups with the 10% ethanol group individually, followed by Newman–Keuls *post hoc* analysis when a significant overall main effect was found ($p < 0.05$). The correlation between the ethanol consumption and the BEC data was analyzed using linear regression. In addition, one-way ANOVA was used to compare the BECs between groups followed by Newman–Keuls *post hoc* analysis when an overall effect was found ($p < 0.05$). Ethanol consumption (g/kg), active lever presses, and inactive lever presses before and after the 20% ethanol challenge were compared with one-way ANOVA with repeated measures, whereas a paired *t*-test was used to compare the BECs. Ethanol consumption (g/kg), active lever presses, and BECs for the dose–response challenge were analyzed using two-way ANOVA with repeated measures followed by Newman–Keuls *post hoc* analysis when an overall effect was found ($p < 0.05$). Active lever presses for the reinstatement were analyzed by two-way ANOVA, followed by Newman–Keuls *post hoc* analysis when a significant overall main effect was found ($p < 0.05$).

RESULTS

Acquisition Characteristics of 10% Ethanol and Intermittent and Daily 20% Ethanol Self-Administration Groups

One group of rats ($n = 30$) was trained to self-administer 10% ethanol using a modified sucrose-fading procedure

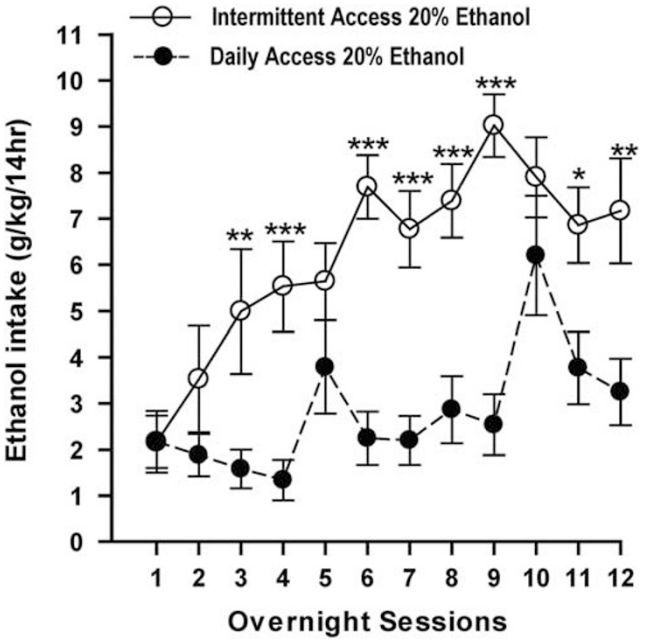

Figure 1 Ethanol consumption was significantly higher for animals trained on the intermittent 20% ethanol group than the daily 20% ethanol group during the 12 overnight operant training sessions. The values are expressed as mean ethanol intake g/kg ± SEM (two-way ANOVA followed by Newman–Keuls *post hoc* test). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $n=15$ for the intermittent group and $n=13$ for the daily group.

(Samson, 1986) (data not shown). To determine if rats would self-administer ethanol without the use of a sucrose fade, two separate groups of rats were introduced to either an intermittent ($n=20$) or daily ($n=15$) 20% ethanol self-administration schedule for 12 overnight acquisition sessions. During the overnight acquisition sessions, there was a steady increase in ethanol consumption and active lever presses for both 20% ethanol groups. Two-way ANOVA analysis comparing the daily ethanol consumption (g/kg every 14 h) of the two 20% ethanol groups revealed an overall main effect of group ($F(1,335)=88.46$, $p<0.001$), an overall main effect of day ($F(11,335)=6.770$, $p<0.001$), and an overall significant interaction (group × day) ($F(11,335)=2.651$, $p<0.01$). *Post hoc* analysis revealed significant differences in consumption between the groups (Figure 1). Two-way ANOVA analysis comparing the active lever presses of the two groups during the overnight acquisition sessions revealed an overall main effect of group ($F(1,335)=78.607$, $p<0.001$), an overall main effect of day ($F(11,335)=7.566$, $p<0.001$), and an overall significant interaction (group × day) ($F(11,335)=3.136$, $p<0.01$). *Post hoc* analysis revealed significant differences in active lever responding between the groups (data not shown).

Following the overnight acquisition sessions, the animals were switched to 45 min FR1 sessions. During the six 45 min sessions, there was an increase in ethanol consumption and active lever presses for both the intermittent and daily 20% ethanol groups. The ethanol consumption on the last 45 min FR1 session for the intermittent group (2.26 ± 0.23 g/kg every 45 min) was significantly higher than that for the daily 20% ethanol group (1.15 ± 0.24 g/kg every 45 min) (*t*-test, $p<0.01$; data not shown).

After the rats from both the 20% ethanol groups had been trained to acquire ethanol self-administration over the 12 overnight and six 45 min FR1 sessions, beginning with the 19th training day they were switched to the 30 min FR3 reinforcement schedule. The total ethanol consumption (g/kg) over the 18 acquisition session for the intermittent and daily access groups was 85.55 ± 8.30 and 43.13 ± 7.64 g/kg, respectively. The group of rats trained to self-administer 10% ethanol with the use of a sucrose-fading procedure reached the 30 min FR3 reinforcement schedule following 4 days of forced 10% ethanol in the home cage, 1–3 overnight sessions (data not shown) and seven 45 min FR1 sessions (data not shown). They then had 12 sessions in which the sucrose was faded from their solution until they were responding to unsweetened 10% ethanol on ~25th training day. The total ethanol consumption (g/kg) over the acquisition period (including forced ethanol days and the sucrose fade sessions) for the 10% ethanol group was 45.63 ± 2.02 g/kg. To determine their ethanol consumption and seeking behavior, all three groups of rats were kept on the 30 min FR3 reinforcement schedule for at least 20 drinking sessions (at least 20 sessions with unsweetened 10% ethanol for the animals trained with a sucrose-fading procedure).

Baseline Drinking Characteristics of 10% Ethanol and Intermittent and Daily 20% Ethanol Self-Administration Groups

A total of 65 Long-Evans rats were trained to acquire ethanol self-administration; however, only 55 met the acquisition criteria of greater than 0.3 g/kg ethanol in the 30 min FR3 sessions (90% (27/30) of the 10% ethanol group; 75% (15/20) of the intermittent 20% ethanol group; 86.7% (13/15) of the daily 20% group). Two-way ANOVA analysis comparing the daily consumption (g/kg every 30 min) of the intermittent 20% ethanol group *vs* the 10% ethanol group revealed an overall main effect of group ($F(1,839)=520.443$, $p<0.001$). There was no overall main effect of day ($F(19,839)=1.323$, NS); however, there was an overall significant interaction (group × day) ($F(19,839)=1.685$, $p<0.05$). *Post hoc* analysis found significant differences for all 20 baseline days (Figure 2a). Two-way ANOVA analysis between the daily 20% ethanol and the 10% ethanol groups also revealed an overall main effect of group ($F(1,799)=331.965$, $p<0.001$), an overall main effect of day ($F(19,799)=3.013$, $p<0.001$), and an overall significant interaction (group × day) ($F(19,799)=3.495$, $p<0.001$). *Post hoc* analysis revealed significant differences for all but 3 of the 20 baseline days (Figure 2b). However, unlike the acquisition phase, the 20% ethanol consumption for intermittent and daily groups did not differ during their 30 min baseline drinking sessions using the FR3 reinforcement schedule. Two-way ANOVA revealed no significant effect of group ($F(1,559)=1.748$, NS). There was an overall effect of day ($F(19,559)=2.555$, $p<0.001$); however, there was no significant overall interaction (treatment × day) ($F(19,559)=0.918$, NS). *Post hoc* analysis found no significant differences between the groups.

The amount of ethanol self-administered in each group correlated significantly with the BECs. The BECs were higher in the 20% ethanol groups in comparison with the

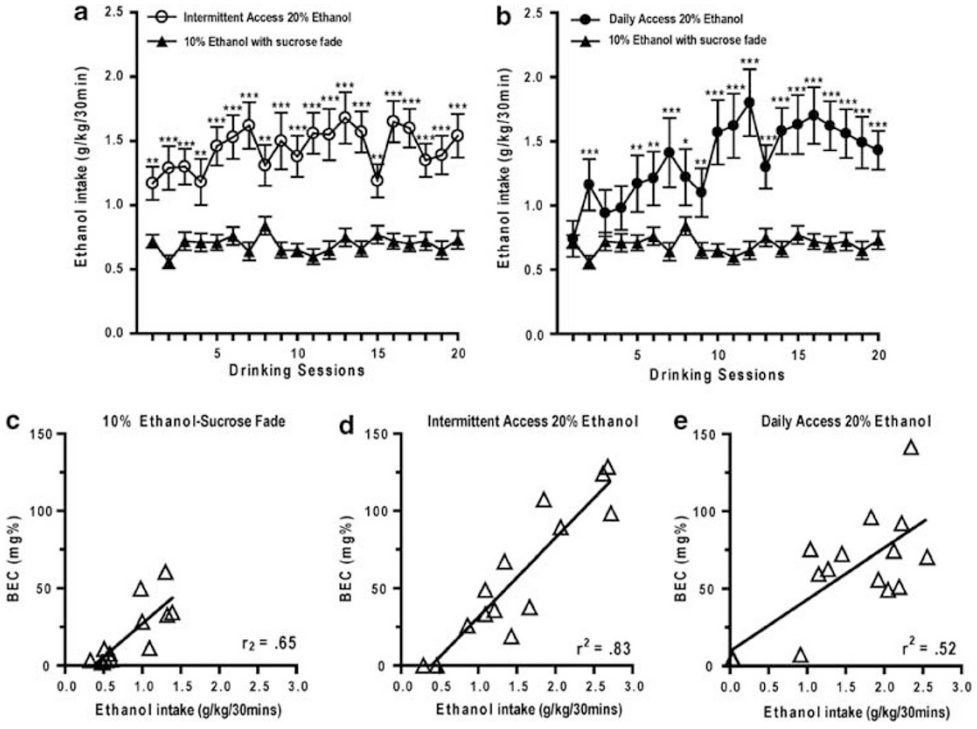

Figure 2 Ethanol consumption (g/kg) and blood ethanol concentrations (BECs) were significantly higher for both groups trained with 20% ethanol compared with the group trained to consume 10% ethanol with a sucrose-fading procedure. Both the intermittent 20% ethanol (a) and daily 20% ethanol (b) models yielded significantly higher baseline consumption than did the 10% ethanol group. The values are expressed as mean ethanol intake (g/kg every 30 min) ± SEM (two-way ANOVA followed by Newman–Keuls *post hoc* test). $*p<0.05$, $**p<0.01$, $***p<0.001$ compares ethanol consumption within each day for the 20% ethanol intermittent group and the 10% ethanol group in (a) and the 20% ethanol daily group and the 10% ethanol group in (b). Blood samples were taken immediately following an operant session (one sample per rat collected between sessions 23 and 25) to analyze and calculate blood ethanol concentrations (BECs). The amount of ethanol consumed correlated significantly with the measured BECs (linear regression): 10% ethanol (c): $r^2=0.65$, $p<0.001$, $n=13$; intermittent 20% ethanol (d): $r^2=0.83$, $p<0.0001$, $n=15$; daily 20% ethanol (e): $r^2=0.52$, $p<0.01$, $n=13$.

10% ethanol group. There was an overall main effect of the group on BEC ($F(2,41)=5.912$, $p<0.01$). *Post hoc* analysis revealed that both 20% ethanol groups attained significantly higher BECs than did the group consuming 10% ethanol (intermittent 20% ethanol group, $p<0.01$; daily 20% ethanol group, $p<0.01$). In the 10% ethanol rats, the BECs ranged from 1.9 mg% to 60.7 mg% with a mean of 19.2 ± 5.8 mg per 100 ml (Figure 2c). In the intermittent 20% ethanol rats, the BECs ranged from 0 to 128.5 mg% with a mean of 58.3 ± 12.3 mg% (Figure 2d), and in the group consuming 20% ethanol daily, the BECs ranged from 4.0 to 141.6 mg% with a mean of 61.2 ± 9.8 mg% (Figure 2e). Linear regression analysis shows a significant correlation between the ethanol consumed (g/kg) and the BECs attained in all three groups (Figure 2c, d, and e).

Although the amount of ethanol consumed (g/kg) was higher in each of the animals in the 20% ethanol group, counterintuitively, there was no difference between the active lever presses of the 20% ethanol groups and the 10% ethanol groups (data not shown). This discrepancy can be explained by the difference in ethanol concentration (ie, animals in the 20% ethanol groups receive twice the amount of ethanol (g/kg) at each reward presentation). We did find that the inactive lever presses between the 20% ethanol groups and the 10% ethanol groups were significantly different. The difference in inactive lever pressing can be explained by the fact that the inactive lever is novel to the 20% ethanol groups for the first few 30-min FR3 sessions, whereas the 10% ethanol group has seen the inactive lever throughout the sucrose-fading procedure. These differences are transient and are not seen after the 12th 30 min FR3 session in either of the 20% ethanol groups (data not shown).

20% Ethanol Challenge for Sucrose-Trained Animals

Animals trained to self-administer 10% ethanol with the use of the sucrose-fading procedure consume significantly more ethanol when challenged with 20% ethanol (Figure 3a). The ethanol concentration (10 *vs* 20%) had an overall effect on consumption ($F(9,109)=13.23$, $p<0.001$). *Post hoc* analysis showed that all 5 days of 20% ethanol self-administration yielded significantly higher ethanol intake when compared with the last day of 10% ethanol self-administration (Figure 3a). The BECs attained following the 20% ethanol challenge were significantly greater than those attained with 10% ethanol (paired *t*-test, $p<0.05$, Figure 3b). The BECs ranged from 13 to 143 mg% with a mean of 50.3 ± 11.46 mg%. In addition, the amount of 20% ethanol consumed during the 30 min operant session correlated significantly with the measured BECs ($r^2=0.70$, $p<0.001$, $n=11$; Figure 3c). Two animals were excluded from both the consumption and BEC analysis because their BEC was well below what would be expected for the amount of ethanol they pressed for, indicating that the animals were not drinking the full 0.1 ml at each reward presentation.

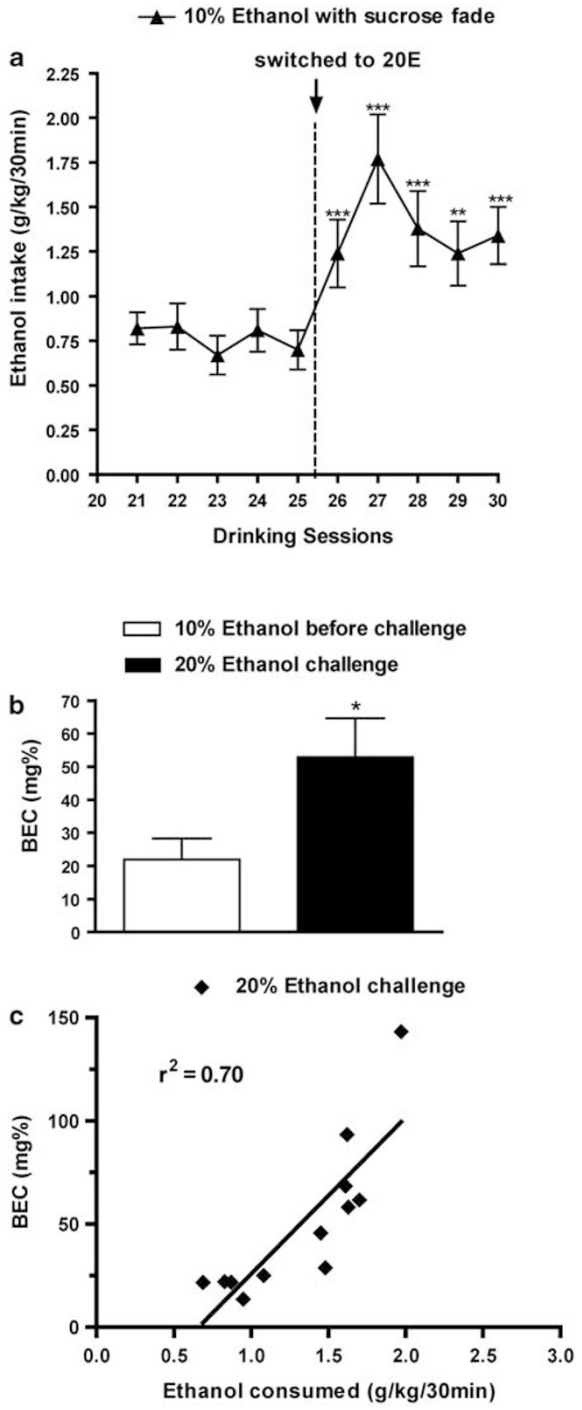

Figure 3 A 20% ethanol challenge in animals trained in the traditional 10% ethanol model with sucrose fading yielded significantly greater ethanol intake (a) and BECs (b). The values are expressed as mean ethanol intake g/kg every 30 min ± SEM (repeated measures ANOVA followed by Newman–Keuls *post hoc* test). $^{**}p<0.01$, $^{***}p<0.001$ compares each of the 20% ethanol days (26–30) with the last 10% ethanol day (25). Blood samples were collected from the lateral tail vein immediately following the final three 30-min FR3 sessions at each concentration (one sample per rat with all samples collected over 3 days, days 23–25 for 10% ethanol and days 28–30 for 20% ethanol) for determination of BECs for 10% ethanol and 20% ethanol, respectively. The BECs following the 20% ethanol challenge were significantly greater than those seen with 10% ethanol (b). The values are expressed as mean blood ethanol concentration, mg% ± SEM (paired *t*-test), $^{*}p<0.05$. Linear regression analysis revealed that the amount of 20% ethanol consumed correlated significantly with the measured BECs (c): $r^2=0.70$, $p<0.001$.

Active and inactive lever responding were unaffected by the 20% ethanol challenge (data not shown).

Ethanol Dose–Response Challenge

To directly compare ethanol self-administration and consumption between the two groups with different training histories, we challenged one group of rats trained to self-administer 10% ethanol with a sucrose fade and one group trained to self-administer 20% ethanol on the daily access schedule to respond to five different concentrations of ethanol to examine dose–response effects. We found that animals trained using the daily access 20% ethanol model responded more and consumed significantly higher amounts of ethanol when high concentrations were presented. Two-way ANOVA analysis of active lever pressing revealed a significant effect of training history (10 *vs* 20%) ($F(1,129)=5.81$, $p<0.05$) and concentration ($F(4,129)=11.84$, $p<0.001$) but no interaction (training history × concentration) ($F(4,129)=1.49$, $p>0.05$, NS). *Post hoc* analysis showed differences between the groups at 10, 20, and 30% ethanol (Figure 4a). Two-way ANOVA analysis of ethanol consumption (g/kg) revealed a significant effect of training history (10 *vs* 20%) ($F(1,129)=5.10$, $p<0.05$) and concentration ($F(4,129)=94.64$, $p<0.001$) but no interaction (training history × concentration) ($F(4,129)=2.41$, $p=0.055$, NS). *Post hoc* analysis showed differences between the groups at 30 and 40% ethanol (Figure 4b). Analysis of the BECs revealed a significant effect of training history (10 *vs* 20%) ($F(1,129)=7.529$, $p<0.05$), concentration ($F(4,129)=53.63$, $p<0.001$), and an interaction (training history × concentration) ($F(4,129)=5.78$, $p<0.001$). *Post hoc* analysis showed differences between the groups at 30 and 40% ethanol (Figure 4c). One animal was excluded from the 10% ethanol-trained group because the BEC measured was well below what would be expected for the amount of ethanol the animal pressed for at several of the concentrations, indicating that the animal was not drinking the full 0.1 ml at each reward presentation.

Reinstatement of Ethanol-Seeking Behavior

The study of reinstatement to ethanol-seeking behavior is critical to the development of new treatments for AUDs. We, therefore, examined the ability of the pharmacological stressor yohimbine to reinstate ethanol seeking and found that both the 20% ethanol models are amenable to the study of reinstatement (Figure 5). During the first extinction session, the rats averaged 62.6 ± 7.9 (10% ethanol), 78.4 ± 13.6 (intermittent 20% ethanol), and 79.0 ± 10.6 (daily 20% ethanol) active lever presses. Before the reinstatement test, the lever pressing had decreased to 8.2 ± 1.5, 9.0 ± 2.4, and 15.2 ± 3.6, respectively. For the reinstatement test, an acute injection of yohimbine was administered, which caused a significant increase in the active lever responding in all the groups. Two-way ANOVA analysis of active lever presses revealed an overall effect of treatment (vehicle or yohimbine) ($F(1,63)=47.891$, $p<0.001$). There was no effect of group (10% ethanol, intermittent 20% ethanol, or daily 20% ethanol) on yohimbine-induced reinstatement of ethanol seeking ($F(2,63)=1.183$, NS) and no interaction (treatment × group) ($F(2,63)=1.714$, NS). *Post hoc* analysis

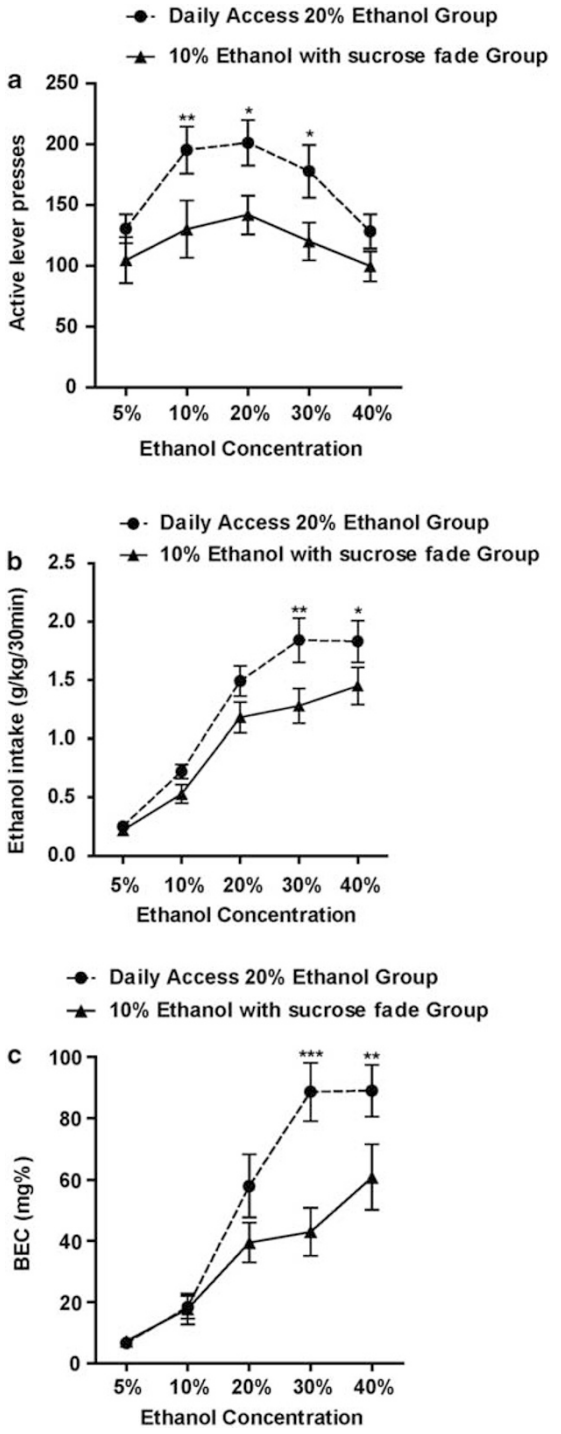

Figure 4 Animals trained using the daily-access 20% ethanol model exhibited significantly higher levels of active lever responding (a), ethanol consumption (b), and BECs (c) compared with animals trained to self-administer 10% ethanol with a sucrose-fading procedure when each group was challenged with the same five concentrations of ethanol. The values are expressed as mean active lever presses, ethanol consumption (g/kg), BEC (mg%) ± SEM measured on the third and fourth sessions at each concentration (repeated measures two-way ANOVA followed by Newman–Keuls *post hoc* test). $*p<0.05$, $**p<0.01$, $***p<0.001$, $n=13$ for each group.

further revealed a significant increase in active lever responding between the vehicle and the corresponding yohimbine response for each of the three groups (Figure 5).

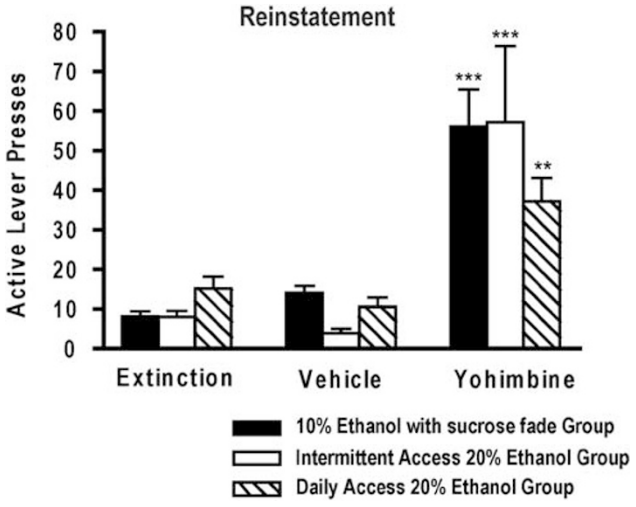

Figure 5 Following a period of extinction, yohimbine significantly reinstated ethanol seeking in animals from all three groups. Extinction levels are from the last three extinction sessions before the reinstatement test. Rats were pretreated with yohimbine (2 mg/kg, i.p.) or its vehicle 30 min before the operant session. Vehicle tests were performed 1 week preceding the yohimbine tests. The extinction, vehicle, and yohimbine values are expressed as the average number of active lever presses ± SEM (two-way ANOVA followed by Newman–Keuls *post hoc* test). $**p<0.01$, $***p<0.001$ compares the yohimbine challenge for each group with their corresponding vehicle response, $n=12$ for the 10% ethanol group, $n=7$ for the intermittent 20% ethanol group, and $n=13$ for the daily 20% ethanol group.

It is important to note that all the groups reinstated to approximately the same level, which could be attributed to the fact that the baseline active lever responding during the maintenance phase was similar for each of the three training groups.

DISCUSSION

We show that Long-Evans rats will acquire operant self-administration of 20% ethanol without the use of sucrose fading. Animals trained with 20% ethanol exhibited significantly greater consumption when compared with animals trained to consume 10% ethanol with a sucrose fade. These high levels of consumption were maintained for several weeks. In addition, following extinction, the 20% ethanol self-administration paradigms have proven to be an effective means of studying yohimbine-induced reinstatement.

The consumption levels attained in this study are some of the highest reported in the literature with the mean ethanol intake being 1.5 g/kg every 30 min and ranging up to 2.7 g/kg every 30 min. These high levels were maintained in both groups of animals self-administering 20% ethanol, using either an intermittent schedule or a 5 days per week schedule. Although animals trained on the intermittent schedule consumed significantly more ethanol during the acquisition phase of the experiment, their consumption was identical to those trained 5 days per week once they reached the 30 min FR3 sessions. Both groups outperformed the group trained to self-administer 10% ethanol with a sucrose fade (1.5 *vs* 0.7 g/kg every 30 min). Although the high intake levels for the 20% ethanol groups were somewhat

unexpected, there is some evidence in the literature that suggests that outbred rats may consume greater amounts of ethanol when higher concentrations of ethanol are presented (Samson *et al*, 1988, 1999). In agreement, the 20% ethanol challenge in sucrose-trained animals in this study caused both the consumption and BEC levels to nearly double when 20% ethanol was substituted for 10%. Our data suggest that rats can easily be trained to respond to 20% ethanol as the reinforcer, without the need for the traditional sucrose-fading procedures, yielding notably high levels of ethanol consumption. Importantly, the animals in this study and in the two-bottle choice setting (Simms *et al*, 2008) have consistently initiated and maintained consumption of a more concentrated ethanol solution (20%).

In correlation with the high intake levels, rats in the 20% ethanol self-administration groups exhibited BECs that are considered pharmacologically relevant, with a mean concentration of 60 mg%, ranging up to 142 mg% (Bell *et al*, 2006; Myers *et al*, 1998). In fact, more than half of the Long-Evans rats in the 20% ethanol groups reached and, in some cases, exceeded the BECs seen in rat strains selectively bred for alcohol preference following 30 min operant self-administration sessions (Gilpin *et al*, 2008c; Vacca *et al*, 2002). In agreement with our data, a recent study has also reported BECs at around 60 mg% when high concentrations of ethanol are presented to outbred Long-Evans rats during a dose–response challenge in the operant setting (Carnicella *et al*, submitted). To the best of our knowledge, only one other study has shown similar BECs in sucrose-faded, outbred animals using a sipper tube model of self-administration (Czachowski *et al*, 2002). Interestingly, the mean BECs for the 20% ethanol self-administration groups in this study are well within the range reported by others using ethanol vapor chambers (Gilpin *et al*, 2008c; Roberts *et al*, 2000) or ethanol vapor-exposed alcohol-preferring rats (Gilpin *et al*, 2008c) to increase operant self-administration.

Since its introduction in the 1980s, the sucrose-fading procedure has been the most widely used technique for inducing operant self-administration of ethanol in rats. This method has high face validity as most humans consume sweetened ethanol solutions when they first drink alcohol. The study of the relationship between the consumption of these sweetened ethanol solutions in the early stages and the development of pathological ethanol consumption will continue to be a vital tool in preclinical research. As it pertains to rodents, sucrose fading has been shown to help initiate ethanol consumption in animals with a low natural preference for ethanol and was an effective means of inducing lever responding in low alcohol-preferring strains (NP rats, LAD1, and LAD2) (Samson *et al*, 1998). The long-standing justification for using sucrose to initiate ethanol intake is that rats find any ethanol solution greater than 10% aversive (Richter and Campbell, 1940; Samson *et al*, 1988); however, the data from this study, combined with our previous studies using the two-bottle choice drinking protocol (Nielsen *et al*, 2008; Simms *et al*, 2008; Steensland *et al*, 2007) and the ethanol dose–response study (Carnicella *et al*, submitted), suggest that rats do not find 20% ethanol aversive. Although we found a higher rate of attrition (animals with ethanol intake levels below 0.3 g/kg every 30 min) in the intermittent 20% ethanol group compared with the 10% ethanol group trained with sucrose fade (25 *vs* 10% attrition, respectively), the difference in the attrition rates was negligible when comparing the daily 20% ethanol group with the 10% ethanol group (13 *vs* 10% attrition, respectively). Sucrose may be helpful for the acquisition of ethanol self-administration in some animals with a low natural preference for ethanol.

The importance of simplifying animal models to evaluate the effects produced by ethanol alone is further highlighted in the dose–response challenge study that allowed for a direct comparison of ethanol self-administration behavior between two groups with different training histories. Although both groups (10% ethanol with sucrose fade and 20% ethanol daily access) exhibited a typical inverted U-shaped dose–response curve with increased consumption at higher ethanol concentrations (as shown by Carnicella *et al*, submitted); the group trained using the 20% ethanol protocol consumed significantly more ethanol at higher concentrations than those trained with 10% ethanol. In addition, it was well reflected in their corresponding BECs. The primary difference between these two groups is the training history, which includes longer overnight access to ethanol in the 20% ethanol group during the training phase and sucrose fading for the 10% ethanol group. We hypothesize that the longer ethanol access conditions over the 12 overnight sessions in combination with the higher daily intake throughout the experiment in the 20% ethanol group could lead to an upward shift in the dose–response curve, which could be attributed to a change in the hedonic set point, as seen in cocaine-treated animals (Ahmed and Koob, 1998). It is this shift in the set point that may cause animals to seek a higher intoxication state; however, the precise molecular mechanism remains to be determined.

Sucrose exposure can cause several stages of addiction in rats, including bingeing, withdrawal, and craving and sensitization (for a review, see Avena *et al* (2008), and withdrawal symptoms can be induced by administration of an opioid antagonist suggesting the formation of dependence on the endogenous opioid release caused by excessive sugar intake (Colantuoni *et al*, 2002). Furthermore, the nucleus accumbens, an area of the brain that is known to be critical in the reinforcing effects of drugs of abuse (including ethanol), has been shown to exhibit opiate-like activation following excessive sugar intake (Spangler *et al*, 2004). Finally, sweetened solutions can serve as highly potent reinforcers to rodents even superseding the choice for the highly addictive drug, cocaine, in a concurrent choice paradigm (Lenoir *et al*, 2007). Our data suggest that the use of sweetened solutions to initiate ethanol consumption and self-administration may be a potential confound in the study of ethanol-mediated behaviors. In addition, the removal of these sweetened solutions from our operant protocols allows for unambiguous interpretation of our results. Hence, we have eliminated sucrose from the operant paradigms and developed an animal model of excessive ethanol intake.

In addition to sucrose fading, several other procedures have been used to increase ethanol intake in the operant setting, including the use of alcohol deprivation (Heyser *et al*, 1997; Holter *et al*, 2000; Holter and Spanagel, 1999), ethanol vapor exposure (Rimondini *et al*, 2002; Roberts *et al*, 1996; Walker and Koob, 2007; Walker *et al*, 2008), and

using various rat strains selectively bred for high preference to ethanol (Samson *et al*, 1998; Vacca *et al*, 2002). The effects of alcohol deprivation on ethanol consumption are transient and fail to persist beyond 2–3 sessions (Heyser *et al*, 1997). The effect can be strengthened when multiple cycles of consumption followed by deprivation are applied to alcohol-preferring rats; however, even these animals return to baseline consumption following the 4th or 5th re-exposure session (Oster *et al*, 2006). In comparison to the transient increases seen with alcohol deprivation, ethanol vapor exposure has been shown to cause persistent increases in operant self-administration (out to 8 weeks post-vapor exposure), particularly when exposure is combined with periods of deprivation (Roberts *et al*, 2000). The drinking levels in this study compare favorably with the ethanol intake of the ethanol-deprived, vapor-exposed animals (1.5 *vs* 1.5 g/kg every 30 min, respectively) in Roberts's study. However, it is important to highlight that there is a fundamental difference between the drinking pattern and pharmacological regulation of the drinking seen in dependent, vapor-exposed animals *vs* non-dependent animals. The dependence-induced increases in ethanol intake have been shown to be more sensitive to various pharmacological manipulations, including corticotrophin-releasing factor and neuropeptide Y receptor antagonists (Gilpin *et al*, 2008a, b; Rimondini *et al*, 2005; Sommer *et al*, 2008; Valdez *et al*, 2002). More research is needed to uncover potential differences between the groups described here. Other researchers have used P rats, HAD1, and HAD2 strains that are selectively bred for ethanol preference to increase operant self-administration, but, following a sucrose fade, self-administration levels are $\sim$1 g/kg every 30 min (Samson *et al*, 1998), well below the levels described here. Some investigators have examined the effect of ethanol vapor exposure on self-administration in the preferring strains. It has been reported that intermittent vapor exposure causes an increase (from 0.8 to 1.1 g/kg every 30 min) in ethanol self-administration in the Sardinian alcohol-preferring lines (Sabino *et al*, 2006) and in P rats (from 1 to 1.4 g/kg every 30 min) (Gilpin *et al*, 2008c). Again, the results described here are well within the range of those found by researchers using initiation procedures, including alcohol deprivation, vapor chambers, and selective breeding.

Another critical need in the development of medications to treat AUDs is relapse prevention. The high rate of recidivism, usually triggered by stressful events, is a major problem in treating the disease. An effective preclinical drinking model should ideally be amenable to the study of relapse to alcohol seeking and consumption. The protocol developed for studying reinstatement of drug seeking in animals has been shown to have validity for studying relapse to drug addiction in humans (Epstein *et al*, 2006; Katz and Higgins, 2003; Spanagel, 2003). Stress and re-exposure to cues or to the context previously associated with drug availability are common reasons for relapse to drug seeking in humans and induce reinstatement of drug seeking in rodents (Liu and Weiss, 2003; Shaham *et al*, 2000; Zironi *et al*, 2006). A 'stress response' is generally believed to involve the CRF system and activation of the HPA axis (for a review, see Koob (1999)). Footshock has been the most commonly used method of stress-induced reinstatement in rodents. However, it has recently been shown that the pharmacological stressor, yohimbine, is a viable alternative, not only in its ability to reinstate drug seeking but also in its effects on CRF production and activation of the same reward circuitry as footshock (Funk *et al*, 2006). Yohimbine is an alkaloid that acts as an α-2 adrenoceptor antagonist, leading to the release of noradrenaline, which stimulates the sympathetic nervous system. Stress responses, whether triggered by footshock or yohimbine administration, have been shown to induce reinstatement of ethanol seeking in animals trained to self-administer ethanol with a sucrose fade (Bremner *et al*, 1996; Gass and Olive, 2007; Le *et al*, 2000, 2005; Liu and Weiss, 2002, 2003). This study shows that both schedules of 20% ethanol self-administration can also be used in the study of yohimbine-induced reinstatement. In addition, as no sucrose-fading procedure was used, the animals are unequivocally reinstating for ethanol.

In summary, the present experiments illustrate that Long-Evans rats will acquire operant self-administration of 20% ethanol without the use of sucrose fading or other initiation procedures. The training methods described result in high ethanol consumption that is maintained for several weeks. This increase in consumption leads to a greater signal-to-noise ratio, which makes more subtle changes in ethanol consumption more apparent, and results in pharmacologically relevant BECs. Furthermore, animals trained to self-administer 20% ethanol consume significantly more ethanol and reach significantly higher BECs when higher ethanol concentrations are presented than animals trained to self-administer 10% ethanol with a sucrose-fading procedure. Both the 20% ethanol self-administration paradigms hold promise as simple and straightforward models of operant self-administration in rats and are amenable to the study of maintenance, motivation, and reinstatement.

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DISCLOSURE

The authors declare that, except for income received from her primary employer, S.E.B. has received financial support for research for an unrelated clinical study, but has not received compensation from any individual or corporate entity over the past 3 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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