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Ketamine and MAG Lipase Inhibitor-Dependent Reversal of Evolving Depressive-Like Behavior During Forced Abstinence From Alcohol Drinking

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Although alcoholism and depression are highly comorbid, treatment options that take this into account are lacking, and mouse models of alcohol (ethanol (EtOH)) intake-induced depressive-like behavior have not been well established. Recent studies utilizing contingent EtOH administration through prolonged two-bottle choice access have demonstrated depression-like behavior following EtOH abstinence in singly housed female C57BL/6J mice. In the present study, we found that depression-like behavior in the forced swim test (FST) is revealed only after a protracted (2 weeks), but not acute (24 h), abstinence period. No effect on anxiety-like behavior in the EPM was observed. Further, we found that, once established, the affective disturbance is long-lasting, as we observed significantly enhanced latencies to approach food even 35 days after ethanol withdrawal in the novelty-suppressed feeding test (NSFT). We were able to reverse affective disturbances measured in the NSFT following EtOH abstinence utilizing the *N*-methyl D-aspartate receptor (NMDAR) antagonist and antidepressant ketamine but not memantine, another NMDAR antagonist. Pretreatment with the monoacylglycerol (MAG) lipase inhibitor JZL-184 also reduced affective disturbances in the NSFT in ethanol withdrawn mice, and this effect was prevented by co-administration of the CB1 inverse agonist rimonabant. Endocannabinoid levels were decreased within the BLA during abstinence compared with during drinking. Finally, we demonstrate that the depressive behaviors observed do not require a sucrose fade and that this drinking paradigm may favor the development of habit-like EtOH consumption. These data could set the stage for developing novel treatment approaches for alcohol-withdrawal-induced mood and anxiety disorders.
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INTRODUCTION

Alcohol use disorders (AUDs) are strongly comorbid with depression (Grant *et al*, 2015), and alcohol withdrawal is associated with affective disturbances (Heilig *et al*, 2010). Developing an understanding of AUD–depression interactions, as well as treatments for these comorbid conditions, represents a clinically crucial research area.

Rodent models of alcohol (ethanol (EtOH)) administration, typically non-contingent, have largely focused on anxiety-like behavior during early withdrawal (Kash *et al*, 2009; Kliethermes, 2005). These and other negative affective behaviors have proven difficult to reliably induce in C57BL/6J mice (Daut *et al*, 2015) frequently utilized in drinking studies (Kash *et al*, 2009; Lovinger and Crabbe, 2005). Recent studies in C57BL/6J mice have modeled depression-like behavior following abstinence from contingent EtOH administration (Lee *et al*, 2015; Pang *et al*, 2013; Stevenson *et al*, 2009). Two weeks of abstinence from EtOH induced a pattern of behavior consistent with onset of a depressive phenotype. This included disruptions in affective measures in the forced swim test (FST), saccharin preference test, and novelty-suppressed feeding test (NSFT) but not in the elevated plus maze (EPM) or light–dark box (Pang *et al*, 2013).

Here we replicated and extended the findings of Pang *et al* (2013). We elucidated a timeline by examining behavior 1, 15–18, and 35 days after EtOH removal. Treatments with typical antidepressants (selective serotonin reuptake inhibitors (SSRIs)) are notoriously troublesome owing to low treatment efficacy and long lag time (weeks to months) before benefits manifest (Gartlehner *et al*, 2012), and such antidepressants can escalate EtOH consumption in rats (Alen *et al*, 2013). We examined alternative pharmacological treatments in reversing depression-like behavior in mice. The *N*-methyl D-aspartate receptor (NMDAR) antagonist

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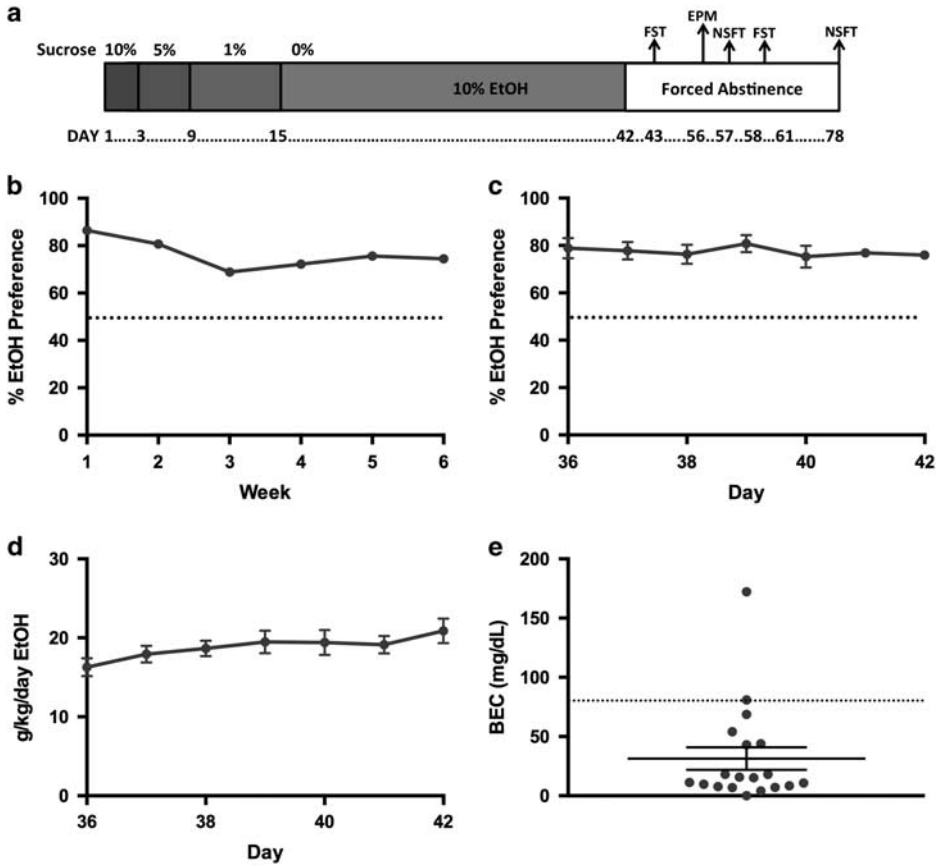

Figure 1 EtOH two-bottle choice with sucrose fade reliably induces significant EtOH preference. (a) Timeline of EtOH two-bottle choice with sucrose fade. 10% EtOH given from day 1 to day 42 with sucrose fade as indicated. (b) Preference for EtOH-containing sipper during 6-week access to EtOH ($N=85$). (c) Daily preference for EtOH-containing sipper during the last week of access in the first cohort ($N=12$). (d) Daily EtOH consumption (g/kg/day) during the last week of access in the first cohort ($N=12$). (e) Individual BECs for mice after 42 days of drinking. Data are represented as mean ± SEM. Dashed line indicates 50% preference in panels (b) and (c); dashed line indicates 80 mg/dl in panel (e). EPM, elevated plus maze; FST, forced swim test; NSFT, novelty-suppressed feeding test.

ketamine has been extensively studied for antidepressant efficacy in humans (Berman *et al*, 2000) and rodents (Autry *et al*, 2011; Li *et al*, 2011). Here we examined the ability of NMDAR antagonists ketamine and memantine to reduce depressive behavior following 2 weeks of EtOH abstinence. We also examined the role of endocannabinoids (eCBs) in this phenotype, as stress-induced affective behaviors can be ameliorated by increasing eCB levels (Sumislawski *et al*, 2011). We tested the monoacylglycerol (MAG) lipase inhibitor JZL-184 in reversing disrupted affective behavior following EtOH abstinence and whether this effect was blocked by the CB1 receptor inverse agonist rimonabant. Further, we examined eCB levels in discrete brain regions during EtOH exposure *vs* abstinence. Finally, we examined whether motivation for EtOH drinking becomes more habit-like following this paradigm.

MATERIALS AND METHODS

Detailed methods are provided in Supplementary Information.

Animals

Two hundred and thirty-two female C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) were used for this study. Females were chosen for two reasons. In humans, females are disproportionately affected by major depressive disorder (MDD; Grigoriadis and Robinson, 2007). Additionally, female C57BL/6J mice develop higher preference for 10% EtOH over water (Middaugh *et al*, 1999). Thus we chose a population that may prove more susceptible to affective disturbances and elevated EtOH preference. Mice were delivered at 7 weeks of age and then singly housed and maintained on 12 h light/dark cycle (lights on at 0600 hours) under controlled temperature (20–25 °C) and humidity (30–50%) levels. Treatments were approved by the Vanderbilt Animal Care and Use Committee.

Two-Bottle Choice EtOH Drinking

Mice had access to two sippers throughout the experiment. Mice in the control groups ($N=69$) received water in both sippers. In EtOH drinking mice ($N=163$), either sucrose fade ($N=85$; Figure 1a) or EtOH ramp ($N=78$; Figure 5a) was used. EtOH was provided during the first 6 weeks. After this, blood and tissue was collected for blood EtOH concentration (BEC; $N=19$) and eCB analysis, respectively, described below. In other mice, following EtOH access, 10% EtOH was replaced with either water for abstinence-related

behavioral studies ($N=144$) or 3% EtOH for the habit-like drinking study ($N=10$).

BEC Analysis

BECs were obtained from blood serum samples of mice on day 42 of EtOH access using a colorometric assay as previously described (Prencipe *et al*, 1987).

Forced Swim Test

Following 1 h acclimation to the testing room, mice were exposed to a 6-min forced swim in a cylinder filled with water (23–25 °C) such that mice could not touch the bottom. Time immobile (no movement except those required to remain afloat) was scored by a blinded observer via video recording during the last 4 min of swim.

Elevated Plus Maze

As described previously (Kash *et al*, 2009), mice were tested for time spent on open and closed arms over 5 min.

Novelty-Suppressed Feeding Test

As previously described (Pang *et al*, 2013) food-deprived mice were tested for latency to first bite of food in a novel arena.

Mass Spectrometry to Determine eCB Metabolites

Tissue punches were obtained from mice either after 42 days of continuous access to EtOH or after 15 days of withdrawal. Samples were then analyzed via mass spectrometry for levels of 2-AG, arachidonic acid (AA), and anandamide (AEA).

Statistics

All data are represented as mean ± SEM. All statistics were run using Prism 6 (Graphpad, La Jolla, CA). BECs were determined by interpolation of concentrations utilizing absorbance values of known EtOH standards. Differences between groups were assessed using *t*-tests, one-way ANOVAs, and two-way ANOVAs, with significance set at $\alpha=0.05$. When significant main effects were obtained using ANOVA tests, appropriate *post-hoc* comparisons between groups were performed.

RESULTS

EtOH Two-Bottle Choice Produces Significant EtOH Preference in Singly Housed Female Mice

Female C57Bl/6J mice were given 24 h access to both 10% EtOH and water for 6 weeks (Figure 1a). Mice preferred 10% EtOH over water (Figure 1b; $P<0.0001$ for each time point; overall preference = 76.39 ± 0.606%), as previously reported (Pang *et al*, 2013). During the last week, preference (Figure 1c) and g/kg/day (Figure 1d) were relatively stable. BECs were sampled on day 42 of EtOH access (31.37 ± 9.47 mg/dl; range 0–172.3 mg/dl; Figures 1e and 5a). Animal weights did not differ between groups ($P>0.05$; results not shown).

Depression-Like Behavior Requires Protracted Forced Abstinence

The FST and NSFT were utilized to measure affective behavior. FST immobility is a well-known proxy for depression-like behavior. Increased NSFT latency is typically attributed to anxiety-like behavior (Bodnoff *et al*, 1988; Dulawa *et al*, 2004). However, other studies have implicated alterations in NSFT latency as indicative of depression-like behavior (Li *et al*, 2011; Pang *et al*, 2013; Santarelli *et al*, 2003). Additionally, in the NSFT, chronic treatment with SSRIs is required to reduce latencies to consume, similar to human depression treatment (Bodnoff *et al*, 1988; Dulawa and Hen, 2005; Santarelli *et al*, 2003). As demonstrated previously (Pang *et al*, 2013), we observed increased FST immobility in EtOH mice following 18 days of forced abstinence (Figure 2a; $P<0.001$, $N=10$–12/group). However, in another cohort, we found no significant difference in FST immobility between EtOH and control mice 24 h following EtOH removal (Figure 2b; $P=0.909$, $N=13$–14/group), suggesting that depression-like behavior in this paradigm requires protracted abstinence.

Forced Abstinence from EtOH does not Affect EPM Behavior

Anxiety-like behavior occurs in C57BL/6J mice following early withdrawal from intermittent vaporized EtOH (Kash *et al*, 2009; Kliethermes, 2005) but not at later time points (Daut *et al*, 2015; Pang *et al*, 2013). We examined whether forced abstinence from EtOH drinking in this paradigm would alter anxiety-like behavior measured by the EPM and found no effect on open arm time (Figure 2c; $P=0.738$, $N=11$/group) or distance traveled (Figure 2c'; $P=0.835$) after 14 days of EtOH forced abstinence.

Forced Abstinence from EtOH Produces Long-Lasting Disruption of Affective Behavior in the NSFT

We aimed to replicate earlier findings on ethanol abstinence actions on NSFT behavior (Pang *et al*, 2013) as well as examine persistence of these alterations. Mice were tested at 15 and 35 days following EtOH removal (Figure 2d). A two-way repeated-measures ANOVA revealed main effects for time ($F(1,31)=9.769$, $P=0.004$), treatment ($F(1,31)=21.40$, $P<0.0001$), and matching subjects ($F(1,31)=2.037$, $P=0.026$) but no interaction ($F(1,31)=0.2576$, $P=0.6153$). *Post-hoc* analysis using Sidak's multiple comparisons tests revealed that EtOH-exposed mice had higher latencies at both 15 ($P<0.001$) and 35 days ($P<0.01$) compared with controls, indicating a persistent affective phenotype in the NSFT following EtOH forced abstinence.

Increased Latency in NSFT Following EtOH Forced Abstinence is Ameliorated by Ketamine and JZL-184 but not by Memantine

Ketamine has rapid and long-lasting antidepressant effects in humans (Berman *et al*, 2000; Zarate *et al*, 2006a, b) and rodents (Louderback *et al*, 2013; Li *et al*, 2011; Autry *et al*, 2011). We postulated that ketamine would ameliorate affective disturbances induced by EtOH abstinence. EtOH

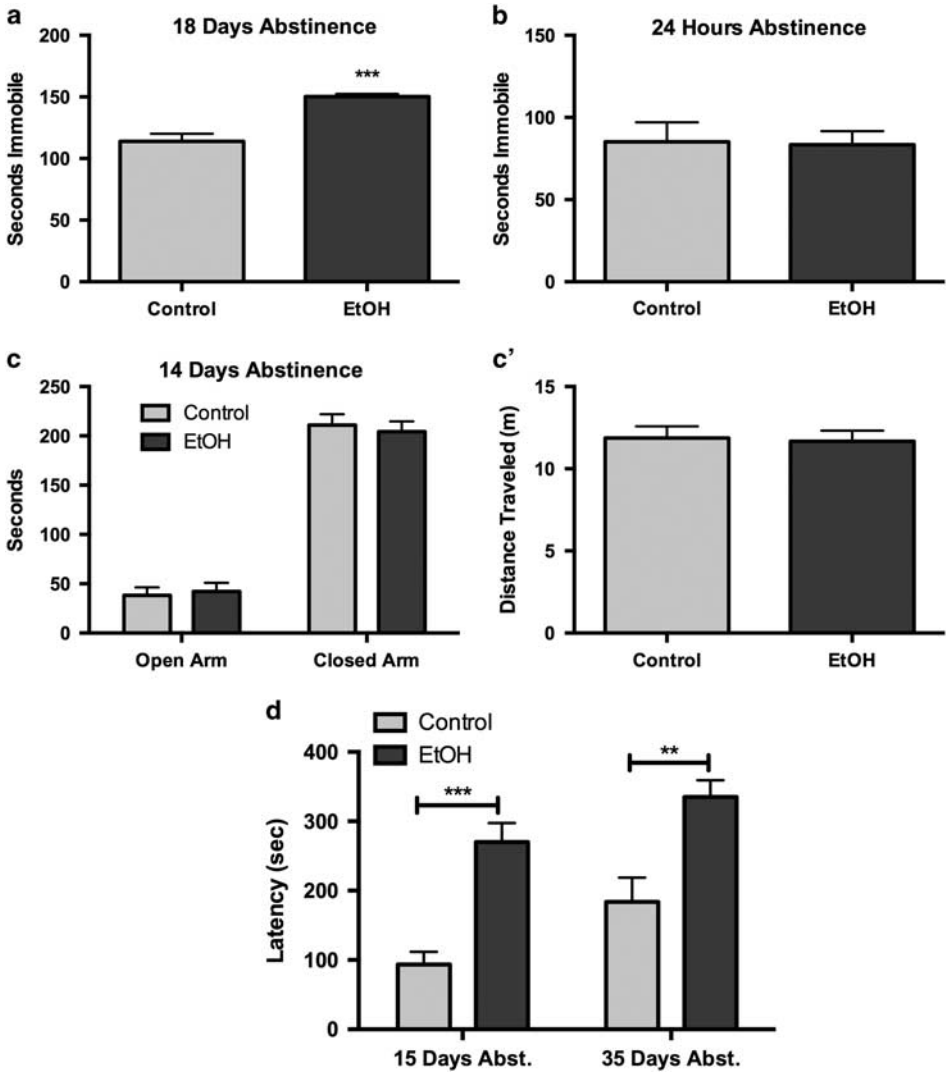

Figure 2 Removal of EtOH induces long-lasting depression-like behavior that requires an abstinence period. (a, b) Abstinence from EtOH significantly increases immobility time in the FST (a) 18 days, but not (b) 1 day, after EtOH removal. (c) Anxiety-like behavior in the EPM is unaffected 14 days after EtOH abstinence as measured by time spent in the open or closed arms of the apparatus. (c') Locomotion, measured by distance traveled in the EPM apparatus, is unaltered after 14 days of EtOH abstinence. (d) Depression-like behavior induced by EtOH abstinence is long-lasting. EtOH-exposed mice demonstrate depression-like behavior—increased latency to consume chow in the NSFT—at both 15 and 35 days following removal of EtOH. Data are represented as mean ± SEM. $^{**}P<0.01$; $^{***}P<0.001$.

and control mice were treated with saline or ketamine (3 mg/kg in saline; $N=6$–7/group) 30 min before the NSFT (Figure 3). We chose a dose that reduces depression-like behavior in rodents at this time point (Autry *et al*, 2011; Louderback *et al*, 2013). Two-way ANOVA revealed main effects of EtOH exposure ($F(1,23)=9.649$, $P=0.005$) and drug ($F(1,23)=10.66$, $P=0.003$) but no interaction ($F(1,23)=3.633$, $P=0.069$). *Post-hoc* analysis using Fisher's LSD revealed that only EtOH+Sal mice significantly differed from Contr+Sal ($P=0.001$) and that ketamine+EtOH mice were indistinguishable from Contr+Sal mice ($P>0.05$). Thus ketamine reversed affective disturbances in EtOH-exposed mice to baseline levels.

Because EtOH and ketamine both inhibit NMDARs, we wondered whether ketamine was eliciting antidepressant-like effects in this model by mimicking EtOH. To test this, we utilized memantine—another NMDAR antagonist. This drug interested us because it has not been shown to reduce depression in either humans (Zarate *et al*, 2006b) or rodents (Gideons *et al*, 2014). We administered memantine (20 mg/kg in saline; $N=11$–12/group) in control and EtOH mice 30 min before the NSFT (Figure 4a) after 15 days of abstinence (main statistics below). We chose this dosage used in a previous study examining memantine for antidepressant efficacy (Gideons *et al*, 2014). *Post-hoc* analysis with Fisher's LSD showed that latencies for both the EtOH+DMSO and EtOH+Mem groups were significantly higher than the Control+DMSO group ($P<0.05$ for both groups), indicating that memantine did not reduce affective disturbances.

The eCB system has been implicated in depression- and anxiety-like behavior (Fowler, 2015; Morena *et al*, 2015). Increasing 2-AG levels through MAG lipase inhibition by JZL-184 reduces stress-induced anxiety-like behavior

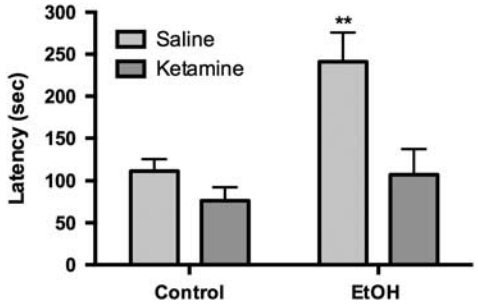

Figure 3 Ketamine (3 mg/kg; 30 min prior to testing) was able to reduce NSFT latency to control levels in EtOH mice 15 days after EtOH removal. Groups were compared with Control-Vehicle for statistics. Data represented as mean ± SEM. $^{**}P<0.01$.

(Sumislawski *et al*, 2011). Additionally, ethanol is not able to substitute for cannabinoids in discrimination tasks in rodents (Jarbe *et al*, 2010; McMahon *et al*, 2008). We administered JZL-184 (8 mg/kg in DMSO; $N=7$–8/group) or vehicle (DMSO; $N=7$–8/group) in control and EtOH mice 15 days after EtOH removal (Figure 4a). Two hours later, mice were tested in the NSFT. Two-way ANOVA revealed a main effect of EtOH ($F(1,45)=10.51$, $P=0.002$) but no main effect of drug ($F(2,45)=1.531$, $P=0.227$) or interaction ($F(2,45)=2.140$, $P=0.130$). *Post-hoc* analysis with Fisher's LSD showed that EtOH+ JZL-184 mice were not significantly different from the Control+DMSO group ($P>0.05$). Similar to ketamine, JZL-184 reversed affective disturbances induced by EtOH abstinence.

To demonstrate that the effect of JZL-184 was mediated by the CB1 receptor, we co-administered JZL-184 with the CB1 inverse agonist rimonabant (1 mg/kg in DMSO, i.p.) *vs* JZL-184 alone (8 mg/kg in DMSO, i.p.) or vehicle ($N=9$–10/group) in control and EtOH mice 15 days after EtOH removal (Figure 4b). Two hours following injection, mice were tested in the NSFT. This rimonabant dosage does not alter affective behavior in a similar task when administered alone (Gamble-George *et al*, 2013). One-way ANOVA revealed a significant effect of treatment ($F(3,34)=6.578$; $P=0.0013$). Fisher's LSD *post-hoc* test revealed significant differences between control-vehicle mice and EtOH-vehicle ($P<0.05$) and EtOH-JZL+Rim ($P<0.001$) mice, between EtOH-vehicle and EtOH-JZL ($P<0.05$) mice, and between EtOH-JZL and EtOH-JZL+Rim ($P<0.01$) mice. No differences were found between control-vehicle and EtOH-JZL ($P>0.05$) mice or between EtOH-vehicle and EtOH-JZL+Rim ($P>0.05$) mice. Thus rimonabant prevented the alleviating effect of JZL-184.

eCB Levels Differ Between EtOH Access and EtOH Abstinence

Given the robust modulation of abstinence-induced affective disturbances through eCB manipulation, we postulated that discrete brain regions may display altered levels of eCBs during drinking *vs* abstinence. Mass spectrometry was used to examine the levels of 2-AG, AEA, and AA in tissue punches from coronal brain sections taken from basolateral amygdala (BLA), central nucleus of the amygdala (CeA), ventral striatum, and bed nucleus of the stria terminalis (BNST). Punches were taken from mice on day 42 of EtOH drinking ($N=6$–7; 2–3 mice/sample) or after 15 days of EtOH abstinence ($N=5$–6; 3 mice/sample). In the BLA, both 2-AG ($P=0.049$) and AEA ($P=0.027$) were higher in mice drinking EtOH than mice in EtOH abstinence, while AA levels were unchanged between groups (Figure 4c). No differences in eCB levels were observed in the CeA, ventral striatum, or BNST (Supplementary Figure S1).

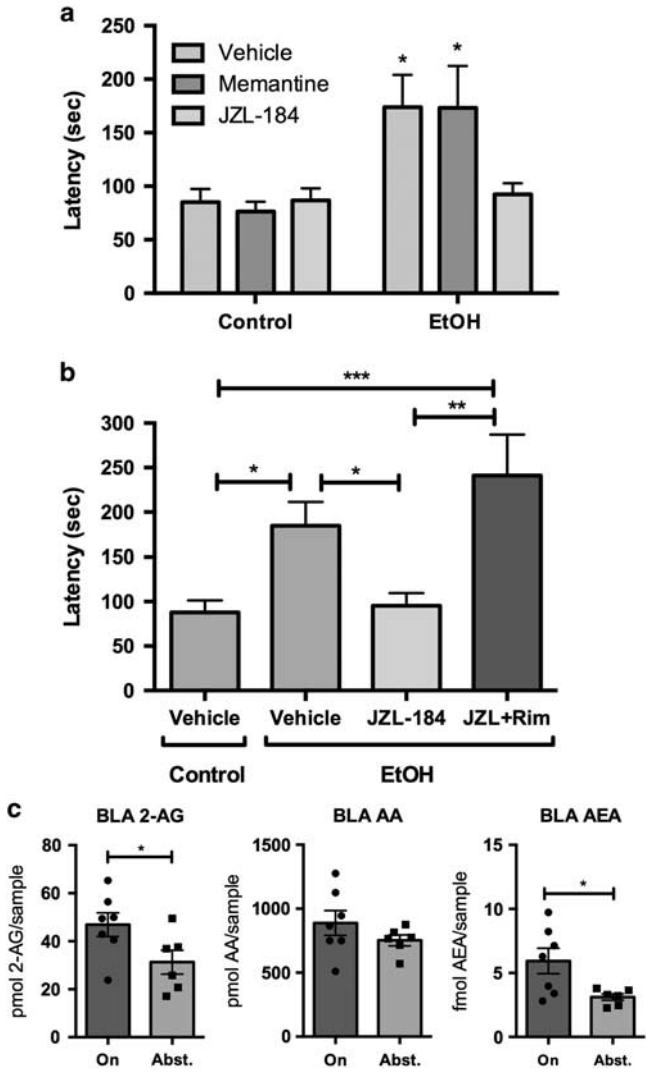

Figure 4 Endocannabinoid modulation of depression-like behavior and eCB levels during two-bottle choice paradigm and EtOH abstinence. (a) JZL-184 (8 mg/kg; 2 h prior to testing) completely reduced NSFT latency to baseline levels in EtOH abstinent mice after 15 days of abstinence, but memantine (20 mg/kg; 30 min prior to testing) had no effect on depression-like behavior. (b) Rimonabant (1 mg/kg; 2 h prior to testing) blocked the effect of JZL-184 in EtOH mice 15 days after EtOH removal. All drugs were administered i.p. In panel (a), groups were compared with Control-Vehicle for statistics. Groups were compared with every other group in panel (b). (c) Levels of 2-AG, AA, and AEA in the BLA after 42 days of drinking (On) or after 15 days of EtOH abstinence (Abst.) Data are represented as mean ± SEM. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

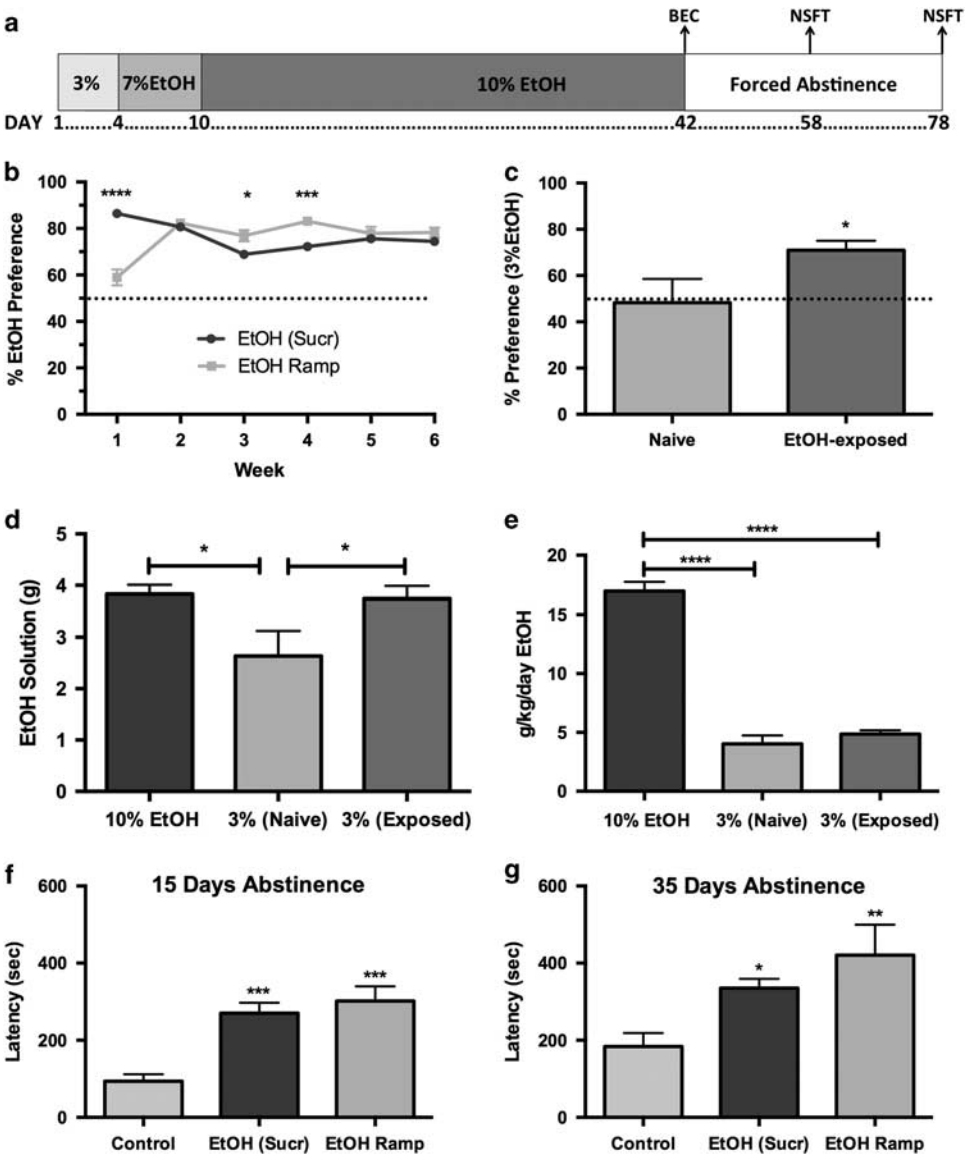

Figure 5 Assessment of factors governing EtOH consumption and depressive responses. (a) Timeline of EtOH Ramp drinking paradigm. EtOH is gradually introduced and no sucrose is used. (b) Comparison of sucrose fade EtOH (EtOH (Sucr), $N=85$) and EtOH Ramp ($N=28$). (c–e) Preference, consumption, and dosage of a 3% EtOH solution over 2 days in naïve (3% (Naïve); $N=9$) or EtOH-exposed (3% (Exposed); $N=10$) mice, compared with mice drinking 10% EtOH at the end of two-bottle choice paradigm (10% EtOH; $N=10$). (c) Preference for 3% EtOH is increased in mice with previous exposure to 6 weeks of 10% EtOH. (d, e) Consumption of 3% EtOH is putatively habit-driven. (d) The same average daily volume of EtOH solution is consumed by 10% EtOH mice and 3% (Exposed) mice. Both groups drink a greater volume than 3% (Naïve) mice. (e) Neither 3% (Naïve) or 3% (Exposed) mice drink sufficient quantities to match dosage (g/kg/day) of 10% EtOH mice. (f, g) The EtOH Ramp procedure induces similar depression-like behavior in the NSFT following (f) 15 and (g) 35 days of abstinence. Data are represented as mean ± SEM. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

Drinking Induction is not Dependent on Sucrose Fade, and Drinking Shifts to Habit-Based Consumption After 6 Weeks of EtOH Exposure

Because sucrose engages reward circuitry, some behaviors observed may be confounded as only EtOH-exposed animals were given sucrose. To address this, we utilized an EtOH ramp ($N=28$) in lieu of sucrose fade (EtOH (Sucr); $N=85$; Figure 5a). Repeated-measures two-way ANOVA of preference utilizing EtOH (Sucr) *vs* EtOH Ramp (Figure 5b) revealed a significant main effect of time ($F(5,555)=7.894$, $P<0.0001$) and significant interaction ($F(5,555)=35.41$; $P<0.0001$) but no main effect of EtOH administration method ($F(1,111)=0.009$; $P=0.9264$). *Post-hoc* analysis revealed significant differences between groups on Week 1 ($P<0.0001$), Week 3 ($P<0.05$), and Week 4 ($P<0.001$).

We postulated that previous exposure to the EtOH paradigm might increase habit-based EtOH consumption. A shift from goal-directed to habit-based consumption is thought to characterize alcohol intake in humans with AUDs (Edwards and Koob, 2013; Everitt and Robbins, 2005). Mice did not show innate preference for 3% EtOH (Figure 5c). We hypothesized that mice previously exposed to 10% EtOH would have increased preference for 3% EtOH. We replaced 10% EtOH with 3% EtOH (and reversed bottle locations to avoid location preference) in mice after 42 days of EtOH

access. Mice with previous EtOH exposure ($N=10$) had significantly increased preference for 3% EtOH over the naïve mice ($N=9$; Figure 5c; $P=0.047$). We also examined both the average amount of EtOH solution consumed (Figure 5d) and the average g/kg/day EtOH (Figure 5e) in three groups: mice during the final week of 10% EtOH exposure (10% EtOH; $N=10$), naïve mice given 3% EtOH (3% (Naïve); $N=9$), or mice with previous 10% EtOH exposure given 3% EtOH (3% (Exposed); $N=10$). We hypothesized that habit-driven consumption would manifest in two ways: first, similar volumes of both 10 and 3% EtOH solution would be consumed. Second, we expected 3% (Exposed) mice would not drink sufficient quantities of EtOH to match dosage (g/kg/day) of 10% EtOH mice. One-way ANOVA of consumption (Figure 5d) revealed a significant main effect of treatment ($F(2,26)=4.225$; $P=0.026$). 3% (Exposed) mice drank a similar volume of EtOH solution as 10% EtOH mice, and the 3% (Naïve) group drank less than either other group ($P<0.05$) using Fisher's LSD *post-hoc* analysis. One-way ANOVA revealed a significant main effect of treatment on g/kg/day EtOH (Figure 5e; $F(2,26)=130.8$; $P<0.0001$). g/kg/day EtOH was significantly higher in the 10% EtOH group compared with either 3% EtOH group ($P<0.0001$ for both groups) using Fisher's LSD *post-hoc* analysis. These data indicate that consumption of 3% EtOH following 6-week exposure to 10% EtOH is possibly habit-driven.

Affective Disturbances Following EtOH Forced Abstinence are not Dependent on Sucrose Fade

EtOH ramp procedure produced high levels of EtOH preference similar to the sucrose fade, so we hypothesized that similar behavior may be induced. As in Figure 2d, we tested for increased latency in the NSFT at both 15 (Figure 5f) and 35 (Figure 5g) days following EtOH removal in three groups: control ($N=10$), EtOH with sucrose fade (EtOH (Sucr); $N=23$), and EtOH Ramp ($N=9$). After 15 or 35 days of forced abstinence, one-way ANOVAs revealed a significant difference between groups (15 days: $F(2,39)=10.32$, $P<0.001$; 35 days: $F(2,39)=6.415$, $P=0.004$), and *post-hoc* analyses using Dunnett's multiple comparison tests showed both EtOH (Sucr) and EtOH Ramp had significantly higher NSFT latencies than control mice (15 days: $P<0.001$ for both groups; 35 days: $P<0.05$ for EtOH (Sucr) and $P<0.01$ for EtOH Ramp).

DISCUSSION

In this study, we replicated and extended previous findings that forced abstinence from EtOH drinking produces long-lasting, reversible affective disturbances in mice. A continuous 6-week two-bottle choice EtOH drinking paradigm induced high preference for 10% EtOH over water in singly housed female mice. BECs after 42 days of 10% EtOH access were relatively low; however, continuous access to EtOH likely limits our ability to observe peak BECs across all animals at any given time. We replicated earlier findings (Pang *et al*, 2013) that subsequent forced abstinence resulted in depression-like behavior and further demonstrated that depression-like behavior requires protracted abstinence and affective disturbances are long-lasting. Both ketamine and JZL-184 reversed affective disturbances in the NSFT to control levels via two disparate pharmacological strategies. eCB levels differed in the BLA of mice currently drinking EtOH *vs* mice in EtOH abstinence. Finally, we provided evidence that this drinking paradigm induces a shift to habit-based consumption of EtOH.

Increased FST immobility is observed after 18 days—but not 1 day—of forced abstinence from EtOH. This is consistent with previous studies (Stevenson *et al*, 2009) and may indicate priming of depression-like behaviors during EtOH administration that require an abstinence incubation period to manifest. In contrast, a recent study utilizing the drinking in the dark (DID) paradigm demonstrated depression-like behavior 1–2 days following EtOH withdrawal (Lee *et al*, 2015). We believe our study may have uncovered an interesting distinction between our paradigm and DID. Because DID is a limited access paradigm, each daily drinking session is followed by an abstinence period. These repeated short withdrawals—not present in the current paradigm—could induce development of depression-like behavior during the course of the DID regimen. Thus, although these two paradigms model a similar component of alcohol withdrawal (depression), subtle discriminating characteristics of DID and continuous two-bottle choice may lead to a more elegant understanding of the development of depression-like behavior during abstinence.

We observe significant effects of EtOH abstinence in the FST and the NSFT, but not in the EPM. Although the NSFT is often thought to relay alterations in anxiety-like behavior, it has also been utilized as a measure of depression-like behavior. Given the outcomes we have observed with distinct tasks in this study, combined with the results from previous studies (Li *et al*, 2011; Pang *et al*, 2013; Santarelli *et al*, 2003), the total data set are most consistent with a depressive behavioral state.

Affective disturbances following EtOH forced abstinence are long-lasting, with increased NSFT latencies 35 days following EtOH removal. This timing agrees with substantial previous literature demonstrating incubation of drug craving during abstinence in rodents for a number of drugs of abuse (Pickens *et al*, 2011), including ethanol (Bienkowski *et al*, 2004). The protracted depression-like behavior outlined here may underlie some of the negative reinforcement thought to drive relapse to alcohol-seeking in abstinent individuals with a history of AUD (Gilpin and Koob, 2008).

Although depression is highly comorbid with alcohol use, specific investigations of drugs to treat individuals for these disorders in tandem have been woefully lacking. Typical clinical strategy regarding comorbid patients has been to treat one disorder prior to treating the other; however, as each of these disorders tend to worsen symptoms of the other, treatments for both disorders at once are needed (Pettinati *et al*, 2013). NMDARs represent potential targets for treating depression, as low doses of the NMDAR antagonist ketamine reduce symptoms of MDD (Berman *et al*, 2000) and treatment-resistant depression (Zarate *et al*, 2006a). Ketamine also reduces depression-like behavior in rodents (Autry *et al*, 2011; Li *et al*, 2011; Louderback *et al*, 2013). Interestingly, family history of alcohol use predicts greater efficacy (Phelps *et al*, 2009) and longer-lasting relief of depression symptoms with ketamine (Niciu *et al*, 2014).

We hypothesized that ketamine would be particularly effective in reversing depression-like behavior following EtOH abstinence. Indeed, ketamine reduced NSFT latency to baseline levels.

We examined another NMDAR antagonist, memantine, in reducing depression-like behaviors. Because ketamine produces dissociative symptoms (Berman *et al*, 2000) and has abuse potential, similar drugs with fewer side effects are needed for the treatment of depression. Memantine has fewer reported side effects than ketamine (Parsons *et al*, 1999). We found that memantine at a relatively high dosage (20 mg/kg) did not reduce depression-like behavior. This is in line with previous data showing that ketamine, but not memantine, is capable of reversing depression-like behavior in mice (Gideons *et al*, 2014), likely because these two ligands differentially affect the NMDAR (Emnett *et al*, 2013; Gilling *et al*, 2009).

One major caveat is that NMDAR antagonists can substitute for ethanol in discriminative stimulus tasks (Kostowski and Bienkowski, 1999; Shelton, 2004). Additionally, ketamine induces alcohol intoxication-like subjective effects in humans (Dickerson *et al*, 2010; Krystal *et al*, 1998). Therefore, future studies investigating specific actions of ketamine in reducing affective behavior following EtOH abstinence will be invaluable in identifying therapeutic compounds lacking ketamine's abuse potential. Our laboratory and others have demonstrated that Ro25-6981, a selective antagonist of the NMDAR GluN2B subunit, mimics the antidepressant effects of ketamine (Li *et al*, 2011; Louderback *et al*, 2013). Although we did not examine subunit specificity of depression-like behavior here, future studies will aim to determine mechanistic actions of NMDAR antagonism in reducing this behavior.

Increasing levels of 2-AG through MAG lipase inhibition by JZL-184 reduces stress-induced affective disturbances (Fowler, 2015; Morena *et al*, 2015; Sumislawski *et al*, 2011). Additionally, unlike ketamine, cannabinoids and ethanol do not share discriminative stimulus profiles in rodents (Jarbe *et al*, 2010; McMahon *et al*, 2008). We sought to determine whether JZL-184 could reduce EtOH abstinence-induced depression-like behavior. Indeed, JZL-184, similar to ketamine, reversed affective disturbances in the NSFT. Additionally, co-administration of the CB1 receptor antagonist rimonabant with JZL-184 completely blocked this effect.

Modulation of the eCB system profoundly affected the affective disturbance in mice following EtOH removal, so we wondered whether the brain eCB levels differed between mice exposed to EtOH and mice 15 days following EtOH removal. We found that 2-AG and AEA were reduced in the BLA during abstinence compared with current EtOH drinking. Because we only examined eCB levels in these two conditions, a caveat is that we could not determine which of these conditions produces aberrant eCB levels when compared with mice naïve to EtOH. Acute stress reduces amygdalar AEA levels (Hill *et al*, 2009), and conversely, enhanced eCB tone reduces the stress response (Patel *et al*, 2004). However, chronic stress has been demonstrated to increase eCB levels in mice (Patel *et al*, 2009). Further investigations are crucial to determine specific contributions of eCBs to both EtOH consumption and abstinence-related behaviors using this paradigm. These findings may indicate cannabinoids as potential therapeutic targets in alcoholism and depression comorbidity.

We were initially concerned with the use of sucrose in this paradigm, as hedonic circuitry altered in a depressed-like state would certainly be engaged by sucrose. However, we found that an EtOH Ramp procedure induced both high preference drinking and similar phenotypes in the NSFT.

To assess potential habit-driven consumption following exposure to our paradigm, we further examined preference for 3% EtOH. Mice did not prefer 3% EtOH over water unless they had previous exposure to 10% EtOH. Mice with previous EtOH exposure drank the same volume of 3% EtOH solution as they had 10% EtOH solution and did not increase consumption to sufficiently match dosage (g/kg/day) of 10% EtOH, lending evidence toward habit-based consumption. Typically, habit *vs* goal-directed consumption is assessed following devaluation of the rewarding stimulus (Colwill and Rescorla, 1990). Animals will decrease effort to acquire a devalued reward if consumption is goal-directed, but habit-driven consumption proves inflexible to devaluation. Here, the 3% EtOH solution could be thought to represent an innately devalued stimulus, as it does not induce preference in naïve mice. 3% EtOH becomes rewarding only in mice that have acquired preference for 10% EtOH. Although it is tempting to interpret these findings as a shift to habit-driven consumption of EtOH, similar to humans with AUDs (Everitt and Robbins, 2005), future studies will be necessary to fully parse out motivation in EtOH consumption.

Here we have shown that depression-like behavior following a two-bottle choice 10% EtOH paradigm requires protracted abstinence and the affective disturbance is long-lasting. Latency in the NSFT is reduced to baseline levels with ketamine but not with memantine. Similarly, JZL-184 reduced latency to baseline levels, and this effect was blocked with co-administration of rimonabant. 2-AG and AEA levels in the BLA were higher in mice currently drinking EtOH compared with mice after 15 days of EtOH abstinence. This paradigm increased putative habit-driven consumption of a 3% EtOH solution. Future studies will aim to uncover alterations in circuit-level plasticity induced by both exposure to and abstinence from EtOH.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)