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Enhanced Sensitivity to Attenuation of Conditioned Reinstatement by the $mGluR_{2/3}$ Agonist LY379268 and Increased Functional Activity of $mGluR_{2/3}$ in Rats with a History of Ethanol Dependence

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Recent findings implicate group II metabotropic glutamate receptors ($mGluR_{2/3}$) in the reinforcing and dependence-inducing actions of ethanol and identify these receptors as treatment targets for alcoholism. Here, we investigated the effects of $mGLuR_{2/3}$ activation on conditioned reinstatement in rats with different ethanol-dependence histories and examined dependence-associated changes in the functional activity of $mGluR_{2/3}$. Following ethanol self-administration training and conditioning procedures, rats were made ethanol dependent, using ethanol vapor inhalation, under three conditions: a single intoxication and withdrawal episode (SW), repeated cycles of intoxication and withdrawal (RW), or no intoxication (CTRL). At 1 week after removal from ethanol vapor, self-administration resumed until stable baseline performance was reached, followed by extinction of operant responding and reinstatement tests. Post-withdrawal self-administration was increased in the RW group, but all groups showed conditioned reinstatement. The $mGluR_{2/3}$ agonist LY379268 dose -dependently reduced reinstatement in all groups, but was more effective at low doses in the SW and RW groups. The highest dose of LY379268 tested reduced spontaneous locomotor activity and operant responding maintained by a non-drug reinforcer, without differences among groups. The heightened sensitivity to the effects of LY379268 in rats with an ethanol-dependence history was therefore specific to behavior motivated by ethanol-related stimuli. Both the SW and RW groups showed elevated [^{35}S]GTPγS binding in the central nucleus of the amygdala (CeA) and bed nucleus of stria terminalis (BNST), relative to the CTRL group. The findings implicate changes in $mGluR_{2/3}$ functional activity as a factor in ethanol dependence and support treatment target potential of $mGlu_{2/3}$ receptors for craving and relapse prevention.

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INTRODUCTION

Ethanol abuse and dependence remain the most significant substance abuse problems in the United States and worldwide (Harwood *et al*, 1999). Recent findings have implicated metabotropic glutamate receptors (mGluR), and in particular, the group II family of mGluRs ($mGluR_{2/3}$), as substrates mediating several aspects of the reinforcing and dependence-inducing actions of ethanol and have identified these receptors as possible treatment targets for alcoholism (see, eg, Heilig and Egli, 2006). $mGluR_{2/3}$ are located pre-, peri-, and post-synaptically (Benarroch, 2008), and negatively modulate glutamate transmission by inhibiting glutamate release and reducing neural excitability at the postsynaptic level (Ferraguti and Shigemoto, 2006; Pinheiro and Mulle, 2008; Schoepp, 2001). $mGluR_{2/3}$ are widely expressed throughout brain circuits that mediate ethanol-associated conditioned reinforcement, incentive motivation and reward (Dayas *et al*, 2007; Kenny and Markou, 2004; Tzschentke and Schmidt, 2003; Zhao *et al*, 2006), as well as circuits mediating stress and anxiety responses (Benarroch, 2008; Dayas *et al*, 2007; Zhao *et al*, 2006), the latter representing important risk factors for ethanol abuse and relapse (Brown *et al*, 1995; Fox *et al*, 2007, 2008; Kreek and Koob, 1998; Sinha, 2000, 2001; Sinha *et al*, 2009). Recent findings provide strong evidence that $mGluR_{2/3}$ participate in mediating many addiction-relevant actions of ethanol. Specifically, $mGluR_{2/3}$ have been shown to play a role in the reinforcing effects of ethanol (Backstrom *et al*, 2004; Backstrom and Hyytia, 2005; Besheer *et al*, 2010; Gass and Olive, 2009; Olive *et al*, 2005; Sidhpura *et al*, 2010),

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withdrawal states (Blednov and Harris, 2008; Sidhpura *et al*, 2010), and processes that convey vulnerability to relapse (Adams *et al*, 2010; Backstrom *et al*, 2004; Rodd *et al*, 2006; Schroeder *et al*, 2008; Sidhpura *et al*, 2010; Zhao *et al*, 2006). The effects of mGluR$_{2/3}$ manipulation in animal models of these aspects of ethanol addiction are suggestive of significant treatment target potential (Heilig and Egli, 2006).

Recent findings suggest that among the roles of mGluR$_{2/3}$ in various neurobehavioral effects of ethanol, mGluR$_{2/3}$ have a prominent function in the regulation of ethanol-seeking behavior as measured in animal models of relapse. Activation of mGluR$_{2/3}$ dose-dependently reverses the effects of ethanol-related environmental stimuli as well as footshock stress on reinstatement of extinguished ethanol seeking (Sidhpura *et al*, 2010; Zhao *et al*, 2006). These effects occurred in the absence of nonspecific sedative or motor effects and appear not to interfere with the reinforcing effects of highly palatable natural reward (Baptista *et al*, 2004; Bossert *et al*, 2006), although the lack of such side effects has not been consistently observed (Backstrom and Hyytia, 2005) and therefore represents an issue addressed in the present study. Recent findings have also revealed that dependence-inducing exposure to ethanol increases the efficacy of mGluR$_{2/3}$ activation to attenuate stress-induced reinstatement of ethanol seeking (Sidhpura *et al*, 2010). These findings identify ethanol-induced alteration in the functioning of mGluR$_{2/3}$ as a possible mechanism contributing to motivating effects of ethanol in dependent subjects. Moreover, changes in mGluR$_{2/3}$ associated with a history of ethanol dependence may have implications for the treatment target potential of these receptors.

The purpose of this study was to investigate whether the effects of a selective mGluR$_{2/3}$ agonist, LY379268, on conditioned reinstatement of ethanol seeking differ in ethanol-nondependent *vs* ethanol-dependent rats and whether such differences are exacerbated in rats subjected not on a single occasion, but repeatedly to ethanol intoxication and withdrawal. The latter question was addressed by employing the 'kindling' model of ethanol withdrawal (Becker and Hale, 1993). A second objective was to examine whether dependence-associated alterations in the behavioral effects of the mGluR$_{2/3}$ agonists are accompanied by corresponding changes in mGluR$_{2/3}$ G-protein coupling within specific brain regions, determined by LY379268-stimulated [^{35}S]GTPγS binding. This assay has been used to find changes in functional activity of mGluR$_{2/3}$ receptors as a consequence of chronic cocaine and nicotine exposure (Hao *et al*, 2010; Liechti *et al*, 2007; Xi *et al*, 2002), as well as the induction of cocaine dependence (Hao *et al*, 2010). Additionally, to establish whether or not the dependence-associated changes in the effects of the mGluR$_{2/3}$ agonist on conditioned reinstatement are the result of sedation or motor impairment, LY379268 was examined for its effects on spontaneous locomotor activity and operant responding maintained by a non-alcoholic sweet solution.

MATERIALS AND METHODS

Animals

A total of 126 male Wistar rats were pair-housed and maintained on a 12 h/12 h reverse light/dark cycle, and all training and testing were conducted without food or water restriction during the dark phase of the cycle. All procedures were conducted in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute.

Drugs

Ethyl alcohol was dissolved in tap water to a concentration of 10% w/v for self-administration. LY379268 ((1R, 4R, 5S, 6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarbolic acid; Eli Lilly Research Laboratories, Indianapolis, IN) was dissolved in sterile water and administered subcutaneously (SC) in a volume of 1 ml/kg, 30 min before behavioral testing.

Operant Ethanol Self-Administration

Following initiation of ethanol self-administration by a supersac (Walker and Koob, 2008) sweet solution fading procedure (see Supplementary Methods), rats ($N = 96$) self-administered ethanol (10% w/v) in daily sessions under a regimen in which responding at the active lever was differentially reinforced in the presence of discriminative stimuli (SD) signaling the availability (S^+) or nonavailability (S^-) of ethanol. Each SD consisted of a compound olfactory (banana or anise extract; McCormick, Hunt Valley, MD) plus auditory stimulus, using continuous white noise (70 dB) or a continuous tone (7 kHz, 70 dB). The stimuli were introduced 30 s before onset of self-administration sessions by depositing five drops of olfactory extract into the bedding of the operant chamber, paired with one of the auditory stimuli. Banana/white noise and anise/tone stimulus combinations served as either the S^+ or S^- for ethanol availability in a counterbalanced fashion. The first two conditioning sessions were conducted in the S^+ condition, followed on the third day by an S^- session. Thereafter, the sequence of S^+ and S^- conditions was determined randomly and daily training continued for a total of 20 (10 S^+ and 10 S^-) sessions. During the S^+ sessions, ethanol was available on a continuous reinforcement (FR1) schedule.

Ethanol-Dependence Induction

After completion of the conditioning phase, rats were randomly assigned to three ethanol-dependence induction and withdrawal conditions as shown in Figure 1. (1) Repeated withdrawal (RW), consisting of four 3-day cycles of ethanol vapor inhalation (14 h on/10 h off, 0.25% air/ethanol mixture, blood alcohol levels (BALs) maintained between 225 and 250 mg/dl), with each cycle separated by 5 days of withdrawal during which the rats were returned to the vivarium. Following the final cycle, rats remained in the vivarium for seven days of recovery. (2) Single withdrawal (SW), consisting of 12 consecutive days of ethanol vapor inhalation (under the same daily conditions as in the RW group) followed by a single 7-day period of withdrawal and recovery in the vivarium. (3) A non-intoxication control (CTRL) condition. These rats remained on 'control vapor'

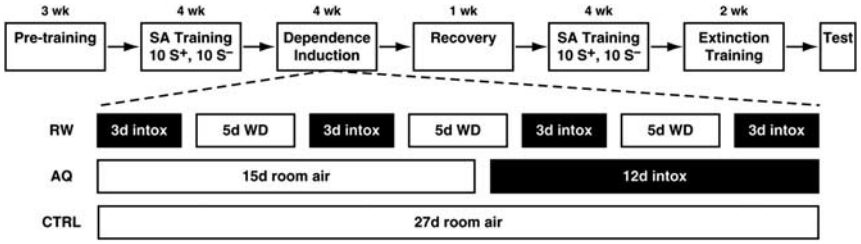

Figure 1 Diagram illustrating the experimental procedure and intoxication protocol for the RW, SW, and CTRL conditions.

(ie, room air) and served as nondependent controls. BALs were determined from tail blood samples obtained on the initial 3 days and subsequently, every third day throughout the vapor inhalation procedure.

Reacquisition of Ethanol Responding and Extinction Training

At 1 week after completion of the SW, RW, or CTRL procedures, rats were again given daily 30-min access to 10% ethanol for a total of 20 (10 S^+ and 10 S^-) sessions, scheduled in random order, to confirm or reestablish stable ethanol self-administration. Daily 30-min sessions then continued under extinction conditions until the criterion of $\leqslant 5$ responses was reached. During this time, levers were presented without the SD, and operant responses resulted in no scheduled consequences.

Effects of LY379268 on Conditioned Reinstatement

Reinstatement tests began 1 day after the final extinction session. These tests lasted 30 min and were conducted under conditions identical to those during the conditioning phase, except that ethanol was not made available, as previously described (Liu and Weiss, 2002b). LY379268 or vehicle was administered 30 min before reinstatement tests. All rats were tested first under S^- conditions, following vehicle injection, to ascertain whether reinstatement occurred selectively under S^+ conditions. After the S^- test, rats remained in their home cages for 2 days, and then were randomly selected for injection with one of four LY379268 doses (0, 0.3, 1.0, or 3.0 mg/kg; SC) and tested for reinstatement in the presence of the S^+ ($n = 7$–10).

Effects of LY379268 on Spontaneous Locomotor Activity

To evaluate nonspecific sedative effects of LY379268 at the doses used for the reinstatement tests, the mGluR$_{2/3}$ agonist was tested on spontaneous locomotor activity in two separate groups of rats ($n = 6$ per group). Rats in one group (INTOX) had a history of ethanol vapor exposure, withdrawal, withdrawal ratings, and recovery identical to that in the RW condition described above. The intoxication and withdrawal procedure of the RW group was chosen because it was associated with greater scores of overt withdrawal signs. Rats in the other group (CTRL) had a history identical to that in the CTRL group above. Neither group had been trained to self-administer ethanol. Following withdrawal and recovery, rats were first habituated to the activity cages in three 30 min sessions, separated by 2 days. After 3 days, rats were given a vehicle injection (1 ml/kg, SC), left in their home cages for 30 min, and placed in the locomotor chambers for 30 min to habituate to the testing procedure. Starting 5 days later, the test sessions were performed twice a week, with the rats given a test dose of LY379268 (0, 0.3, 1.0, or 3.0 mg/kg; SC) 30 min before the locomotor test. The test doses were given according to a Latin square design.

Effects of LY379268 on Responding Maintained by an Ethanol-Free Sweet Solution

To control for LY379268-induced motor impairment, the effects of the mGluR$_{2/3}$ agonist were tested on supersac-reinforced responding in ethanol-naive rats. Rats were trained to respond for the sweet solution in 10 daily 30-min sessions as above (see Supplementary Methods), and then randomly divided into two groups. Rats in one group (INTOX, $n = 8$) were subjected to ethanol vapor, withdrawal, withdrawal ratings and recovery identical to the RW condition above. The remaining rats (CTRL, $n = 8$) remained on room air. Following withdrawal and recovery, stable supersac-maintained responding was reestablished in five daily 30-min sessions. At 30 min before the final session, rats received a habituating vehicle injection (1 ml/kg; SC). Thereafter, the effects of LY379268 were tested twice a week in rats treated with one of four LY379268 doses (0, 0.3, 1.0, or 3.0 mg/kg; SC) according to a Latin square design. Each test was followed by 2 days of 'baseline' supersac self-administration without drug or vehicle administration.

Brain Dissection and Tissue Sampling

At 24 h after the final reinstatement test, all vehicle-treated rats were deeply anesthetized and decapitated, and their brains rapidly extracted, snap frozen in methylbutane, and stored at $-80\,^{\circ}C$. Brains were subsequently dissected into coronal sections, and brain regions of interest were located by atlas (Paxinos and Watson, 1998) and collected by 15-gauge tissue punches, resulting in two 780 µl samples per region (Figure 2). Sampled regions included the medial prefrontal cortex (mPFC, 3.20–1.80 mm, relative to bregma), nucleus accumbens (NAc, 1.70–0.70 mm), bed nucleus of stria terminalis (BNST, 0.12 to −0.96 mm), central nucleus of the amygdala (CeA, −1.44 to −2.70 mm), dorsal hippocampus (dorsal Hipp, G, −1.90 to −3.12 mm), and ventral tegmental area (VTA, −5.20 to −6.30 mm). Brain punches were frozen in dry ice and stored at $-80\,^{\circ}C$.

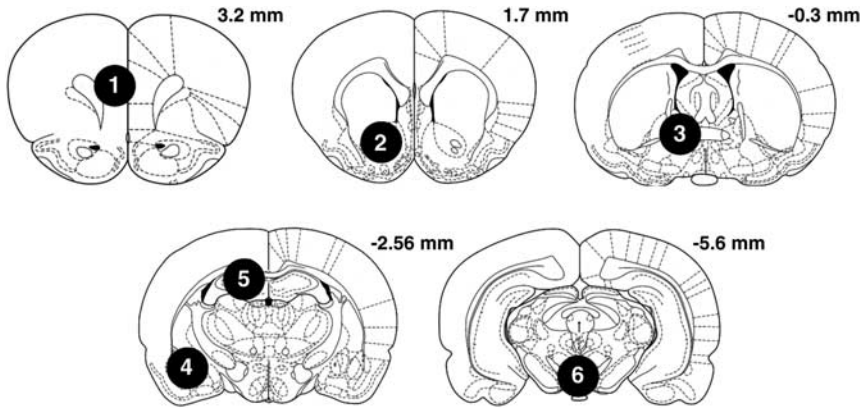

Figure 2 Diagram of brain regions sampled by tissue punch for [^{35}S]GTPγS binding assays. Samples were taken bilaterally from the following regions: (1) medial PFC, (2) NAc, (3) BNST, (4) CeA, (5) dorsal hippocampus, and (6) VTA. The diagrams represent anterioposterior coordinates relative to bregma (Paxinos and Watson, 1998).

Membrane Preparation and [^{35}S]GTPγS Binding Assay

For [^{35}S]GTPγS binding assays, 10 μg of membrane proteins (see Supplementary Methods) were incubated for 1 h at 37 °C in assay buffer (20 mM HEPES, 10 mM MgCl, 0.25 mM EGTA, 100 mM NaCl, protease inhibitor, 10 μg GDP (Sigma, St Louis, MO), 0.5 mM DTT, and 0.5 U/ml type X adenosine deaminase) in the presence of various concentrations or absence of the agonist (LY379268, 0.01–100 μM). The reaction was terminated by centrifugation at 14 500 r.p.m. at 4 °C for 24 min. Pellets were washed three times in a wash buffer (20 mM HEPES and 0.01% Triton-X, pH 7.4). The final pellet was dissolved in 100 μl of 2 mM sodium hydroxide. Radioactivity was counted by liquid scintillation spectrophotometry. Basal binding was assessed in the absence of the agonist, and nonspecific binding of [^{35}S]GTPγS was measured in the presence of a non-limiting concentration of unlabeled GTPγS.

Statistical Analysis

Average ethanol-reinforced responses were analyzed by 3×2 mixed factorial analysis of variance (ANOVA) with *ethanol history* (RW, SW, or CTRL) as a between-subject factor and *intoxication phase* (preintoxication or postintoxication) as a within-subject factor. Extinction responses were analyzed by 3×2 mixed factorial ANOVA, using *ethanol history* as a between-subject factor and *extinction day* (first or final) as a within-subject factor. Withdrawal rating scores (see Supplementary Methods) were analyzed using Mann–Whitney *U*-test. The effects of LY379268 on reinstatement were analyzed by $4 \times 3 \times 3$ mixed factorial ANOVA with *LY379268 dose* (0, 0.3, 1.0, or 3.0 mg/kg) and *ethanol history* as between-subject factors and *cue condition* (extinction, S^-, or S^+) as a within-subject factor. Responses at the active and inactive levers were analyzed separately. The effects of LY379268 on locomotor activity were analyzed by 2×4 mixed factorial ANOVA with *ethanol history* (INTOX or CTRL) as a between-subject factor and *LY379268 dose* as a within-subject factor. The effects of LY379268 on supersac-reinforced responding were analyzed by $2 \times 4 \times 2$ mixed factorial ANOVA with *ethanol history* as a between-subject factor, and *LY379268 dose* and *lever* (active or inactive) as within-subject factors. The [^{35}S]GTPγS binding data were analyzed after normalizing the data as a percentage change from specific basal [^{35}S]GTPγS binding rate by 6×3 mixed-factorial ANOVA with *LY379268 concentration* as a within-subject factor and *ethanol history* as a between-subject factor. The [^{35}S]GTPγS binding was analyzed separately for each brain region. Significant main effects or interactions were followed by reduced ANOVA models, Fisher's LSD tests, or simple effects ANOVAs.

RESULTS

Ethanol BALs and Withdrawal Ratings

The BALs (mean ± SEM) of the RW groups were consistent across intoxication cycles (cycle 1 BAL = 210 ± 25 mg/dl, cycle 2 BAL = 225 ± 10 mg/dl, cycle 3 BAL = 223 ± 15 mg/dl, and cycle 4 BAL = 215 ± 19 mg/dl) and with the BALs of the SW group (221 ± 16 mg/dl).

Compared with nondependent controls (CTRL-SW and CTRL-RW), withdrawal ratings were significantly increased in both the SW and RW groups 12 h after termination of ethanol vapor exposure on day 12 (see Figure 3; SW *vs* CTRL-SW: $U_{36,8} = 224$, $p < 0.005$; RW *vs* CTRL-RW: $U_{33,8} = 224$, $p < 0.005$), and higher in the RW than SW group ($U_{33,36} = 364$, $p < 0.01$; Figure 3). Additionally, increased withdrawal signs were recorded in the RW group 12 h after intermittent removal from ethanol vapor on day 3 ($U_{33,8} = 194$, $p < 0.01$), day 6 ($U_{33,8} = 208$, $p < 0.005$), and day 9 ($U_{33,8} = 211$, $p < 0.005$).

Ethanol Self-Administration and Extinction

All groups of rats showed stable ethanol-reinforced responding during both the preintoxication and postintoxication self-administration phases. The mean (± SEM) numbers of responses on the last day of the respective phases were: preintoxication RW = 33.0 ± 2.2, SW = 35.7 ± 2.5, CTRL = 33.7 ± 2.4; postintoxication, RW = 54.4 ± 3.3, SW = 48.7 ± 2.5, CTRL = 45.1 ± 2.1. These totals correspond to a mean (± SEM) last-day ethanol intake (g/kg) of: preintoxication RW = 0.56 ± 0.06, SW = 0.57 ± 0.05,

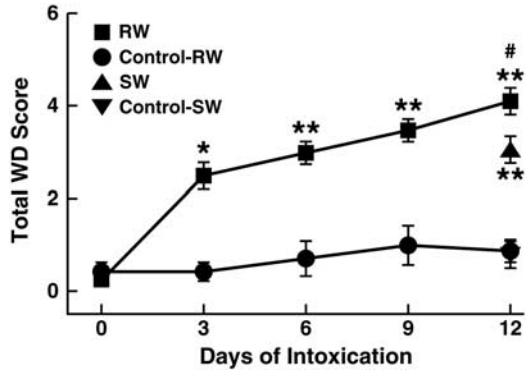

Figure 3 Total withdrawal rating scores determined 12 h after removal from the ethanol vapor chambers, following 0, 3, 6, 9, or 12 days of intoxication. $^{*}p<0.05$, $^{**}p<0.005$ *vs* CTRL (Mann–Whitney *U*-tests); $^{\#}p<0.05$ *vs* SW (sample sizes (*N*): RW = 31, SW = 33, RW Control condition = 12, and SW Control Condition = 12).

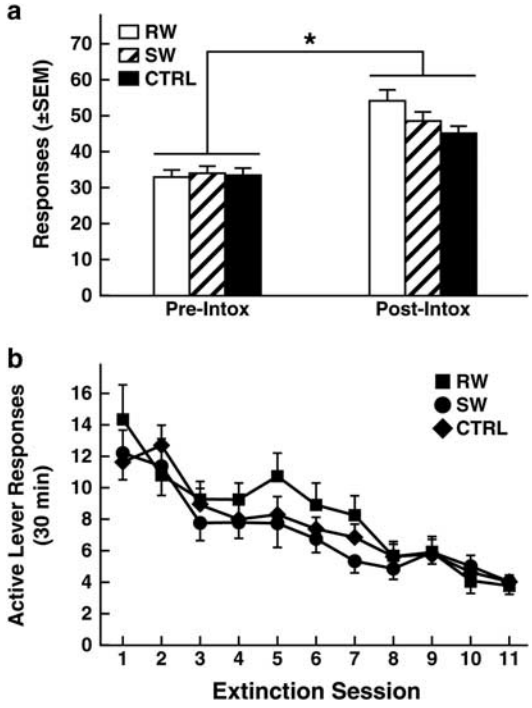

Figure 4 Effects of chronic ethanol exposure on self-administration and extinction responses in rats of the RW (repeated withdrawal), SW (single withdrawal), and CTRL (nondependent control) conditions. (a) Mean (± SEM) ethanol-reinforced responses (active lever) per 30 min session before (Pre-Intox) and after (Post-Intox) dependence induction. $^{*}p<0.01$. (b) Mean (± SEM) active lever responses per extinction session (sample sizes (*N*): RW = 31, SW = 33, and CTRL = 44).

CTRL = 0.62 ± 0.06; postintoxication RW = 0.66 ± 0.06, SW = 0.58 ± 0.06, CTRL = 0.54 ± 0.06. The 10-session average rate of ethanol-reinforced responding was significantly increased during the postintoxication phase in all groups (Figure 4a), as reflected by a significant main effect of *intoxication phase* ($F_{1,105}=121$, $p<0.0005$) and an *intoxication phase* × *ethanol history* interaction ($F_{2,105}=4.0$, $p<0.05$). RW rats demonstrated greater responding for ethanol ($t_{29}=6.9$, $p<0.0001$) as well as ethanol intake in the postintoxication phase ($t_{29}=2.8$, $p<0.01$), whereas the SW and CTRL rats exhibited greater responding for ethanol (SW: $t_{31}=6.6$, $p<0.0001$; CTRL $t_{42}=5.4$, $p<0.0001$) but not greater intake, compared with average preintoxication levels. In contrast, responding levels during the S$^-$ sessions remained virtually unaffected by intoxication phase or ethanol history. The mean (± SEM) numbers of responses during the last S$^-$ session of the two phases were: preintoxication RW = 14.6 ± 9.2, SW = 12.9 ± 9.7, CTRL = 14.7 ± 10.2; postintoxication RW = 11.6 ± 8.0, SW = 14.8 ± 18.6, CTRL = 14.3 ± 11.8. In contrast, inactive lever pressing remained unchanged by intoxication phase during the S$^+$ and S$^-$ sessions. During the extinction phase, responding at the active lever progressively decreased (Figure 4b) as confirmed by a main effect of *extinction day* ($F_{10,1050}=28.3$, $p<0.0005$), with a mean (± SEM) of 12.7 ± 0.9 responses on the first day of training and 3.9 ± 0.3 responses on the final day. All groups underwent 11 days of extinction training to reach criterion. This progressive decrease in extinction responding was consistent across groups of rats.

Effects of LY379268 on Reinstatement of Ethanol-Seeking Behavior

When tested in the S$^+$ (but not the S$^-$) condition, vehicle-treated rats in all ethanol history conditions showed robust recovery of responding (Figure 5a). This effect was confirmed by a main effect of *cue condition* ($F_{2,192}=67.5$, $p<0.0005$) in the overall ANOVA that also revealed significant main effects of *ethanol history* ($F_{2,96}=3.05$, $p<0.05$), *LY379268 dose* ($F_{3,96}=19.8$, $p<0.0005$), and significant interactions for *LY379268 dose* × *cue condition* ($F_{6,192}=29.3$, $p<0.0005$) and *ethanol history* × *cue condition* ($F_{4,192}=4.1$, $p<0.005$).

LY379268 dose dependently reduced reinstatement behavior in the S$^+$ condition in all intoxication groups, but significant differences were observed across the *ethanol history* conditions in the reduction of reinstatement by individual LY379268 doses as revealed by significant *LY379268 dose* × *cue condition* interactions (Figure 5a, CTRL: $F_{6,80}=10.3$, $p<0.0005$, SW: $F_{6,58}=23.3$, $p<0.0005$, RW: $F_{6,54}=8.1$, $p<0.0005$). Specifically, no significant effects were obtained with the 0.3 mg/kg dose in the CTRL group, whereas this dose significantly reduced reinstatement in the SW (Fisher's LSD, $p<0.001$) and RW (Fisher's LSD, $p<0.05$) groups. Moreover, the suppression of reinstatement at this dose was significantly greater in the SW (Fisher's LSD, $p<0.05$) and RW (Fisher's LSD, $p<0.05$) groups than in the CTRL group. The responding was significantly higher during the S$^+$ test than the S$^-$ test in the RW group at 0 (Fisher's LSD, $p<0.005$) and 0.3 mg/kg LY379268 (Fisher's LSD, $p<0.01$), in the SW group at 0 (Fisher's LSD, $p<0.0005$) and 0.3 mg/kg LY379268 (Fisher's LSD, $p<0.05$), and in the CTRL group at 0 (Fisher's LSD, $p<0.005$), 0.3 mg/kg (Fisher's LSD, $p<0.005$), and 1.0 mg/kg LY379268 (Fisher's LSD, $p<0.05$), indicating that responding during the S$^+$ test decreased to levels exhibited during the S$^-$ test at higher doses in the post-dependent rats than in the nondependent rats. However, the overall effect of LY379268 on reinstatement in all *ethanol history* groups was confirmed by significant main effects of *LY379268 dose* (Figure 5a, CTRL: $F_{3,40}=8.3$, $p<0.0005$, SW: $F_{3,29}=7.7$, $p<0.001$, RW: $F_{3,27}=6.8$, $p<0.001$) and *cue*

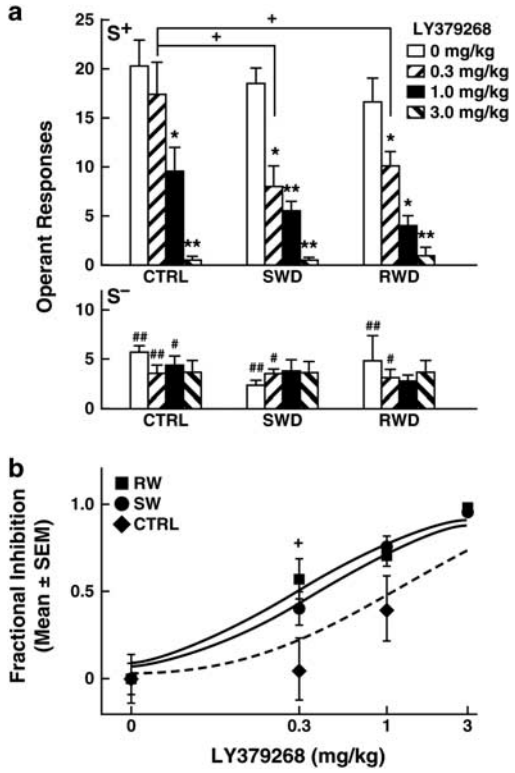

Figure 5 Effects of the mGluR$_{2/3}$ agonist LY379268 on conditioned reinstatement in rats of the RW (repeated withdrawal), SW (single withdrawal), and CTRL (nondependent control) conditions. (a) Mean (±SEM) active lever presses in the S^+ condition (S^+, upper panel), and mean (±SEM) active lever presses in the S^- condition, treated with vehicle only (S^-, lower panel). (b) Active lever responses expressed as the fractional inhibition of responding relative to mean baseline reinstatement response in vehicle-treated rats of the respective intoxication group. Fitted curves are the results of nonlinear regression to an ordinary sigmoid pharmacokinetic model. $^*p<0.05$, $^{**}p<0.005$, different from vehicle-treated rats; $^+p<0.05$, SW *vs* CTRL; $^{\#}p<0.05$, $^{\#\#}p<0.005$, less than corresponding S^+ responses (*post hoc* Fisher's LSD tests). The *n* for each dose group (0, 0.3, 1, and 3 mg/kg LY379268, respectively) was 13, 9, 13, and 9 for the CTRL condition, 8, 8, 9, and 8 for the SW condition, and 7, 10, 7, and 7 for the RW condition.

condition (CTRL: $F_{2,80}=38.6$, $p<0.0005$, SW: $F_{2,58}=30.6$, $p<0.0005$, RW: $F_{2,54}=13.2$, $p<0.0005$).

To better illustrate and evaluate the apparent differences in LY379268 potency across the ethanol history conditions, the responses recorded during the S^+ tests were converted to scores representing the fractional inhibition compared with the responses of vehicle-treated rats (see Supplementary Methods) and fit to a sigmoid curve (Figure 5b). Comparisons between groups at each data point further confirmed that the dose-response curve for the effects of LY379268 differed across ethanol history conditions, in that the dose-response functions appeared to be shifted leftward in the SW and RW groups as they exhibited effects at the 0.3 mg/kg dose when compared with the CTRL group (SW *vs* CTRL: $t_{16}=2.7$, $p<0.05$, RW *vs* CTRL: $t_{18}=2.1$, $p<0.05$), but with no change in efficacy at the maximal dose (3.0 mg/kg) and no differences between the SW and RW conditions. Responding during S^- tests was negligible, did not differ across ethanol history conditions, and was not altered by LY379268. Similarly, inactive lever responses were negligible and not modified by LY379268.

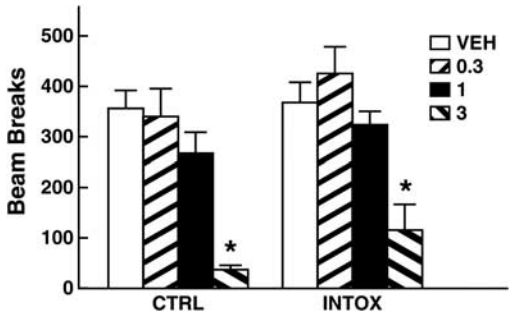

Figure 6 Effects of LY379268 on spontaneous locomotor activity in rats subjected to chronic ethanol intoxication and withdrawal (INTOX, $n=6$) *vs* nondependent controls (CTRL, $n=6$). $^*p<0.05$, different from vehicle controls (VEH; *post hoc* Fisher's LSD tests).

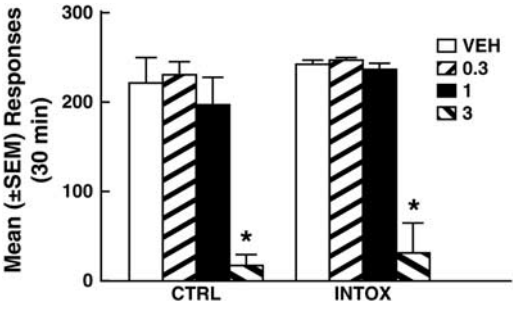

Figure 7 Effects of LY379268 on responding reinforced by a non-alcoholic sweet solution (supersac) in rats subjected to chronic ethanol intoxication and withdrawal (INTOX, $n=8$) and nondependent controls (CTRL, $n=8$). $^*p<0.05$, different from vehicle controls (VEH; *post hoc* Fisher's LSD tests).

Effects of LY379268 on Spontaneous Locomotor Activity

In both post-dependent and nondependent rats, spontaneous locomotor activity was reduced at the highest (3.0 mg/kg) dose of LY379268 compared with all other doses ($p<0.005$, Fisher's LSD tests after main effect of *LY379268 dose*: $F_{1,10}=62.1$, $p<0.0005$; Figure 6).

Effects of LY379268 on Supersac-Reinforced Responding

Similar to locomotor behavior, supersac self-administration was attenuated at the highest LY379268 dose (3.0 mg/kg) relative to all other doses in both post-dependent ($p<0.05$) and nondependent ($p<0.05$) rats without differences between groups (Fisher's LSD tests following main effect of *LY379268 dose*: $F_{1,14}=133.3$, $p<0.0005$; Figure 7).

Effect of Ethanol Intoxication Histories on LY379268-Stimulated [^{35}S]GTPγS Binding

LY379268 concentration dependently stimulated specific [^{35}S]GTPγS binding in all brain regions examined (Figure 8), reflected by significant main effects of *LY379268 dose*. Specifically, increased [^{35}S]GTPγS binding was found in ethanol vapor-exposed rats in the BNST ($F_{2,21}=6.9$, $p<0.005$) and CeA ($F_{2,21}=5.9$, $p<0.01$). In the BNST, the [^{35}S]GTPγS binding was significantly increased at 10 μM

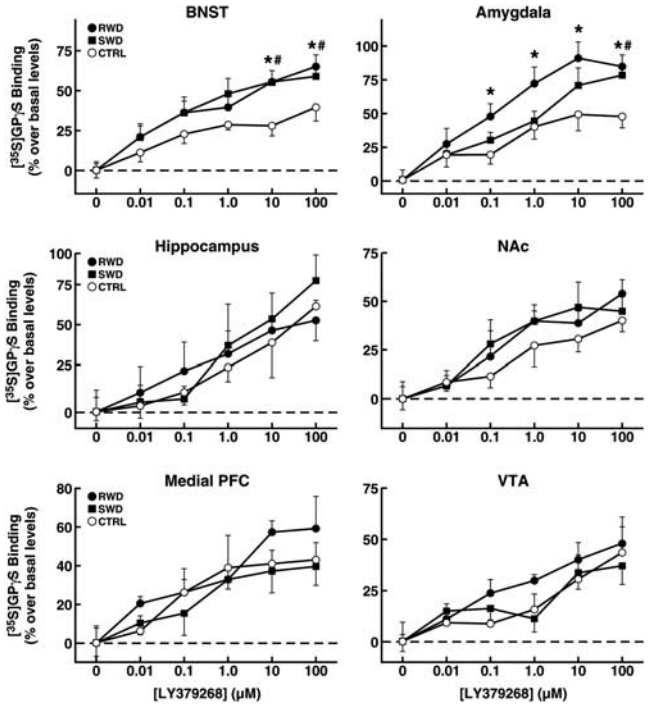

Figure 8 LY379268-stimulated [^{35}S]GTPγS binding in rats of the SW (single withdrawal), RW (repeated withdrawal), and CTRL (nondependent control) groups. The mGluR$_{2/3}$ agonist LY379268 concentration dependently stimulated [^{35}S]GTPγS binding in all examined brain regions, but with differential effects as a function of ethanol history in the CeA and BNST. Data are expressed as the mean (±SEM) of four binding experiments, each performed in duplicate ($n=8$). *$p<0.05$, RW *vs* CTRL; $^{\#}p<0.05$, SW *vs* CTRL (*post hoc* Fisher's LSD tests).

(Fisher's LSD, $p<0.01$) and 100 μM ($p<0.05$) LY379268 in both RW and SW groups. Additionally, [^{35}S]GTPγS binding in the BNST was significantly greater in RW and SW groups when compared with CTRL rats ($p<0.05$). In the CeA, the [^{35}S]GTPγS binding rate was significantly greater relative to the CTRL group at 0.1 μM (Fisher's LSD, $p<0.05$), 1.0 μM ($p<0.05$), 10 μM ($p<0.05$), and 100 μM LY379268 ($p<0.05$) in the RW group, and at 100 μM LY379268 in the SW group ($p<0.05$). Additionally, the mean overall [^{35}S]GTPγS binding rates in the CeA were significantly greater in the RW group than in both SW and CTRL groups ($p<0.05$). No significant differences in [^{35}S]GTPγS binding rates were found in the dorsal Hipp, NAc, medial PFC, and VTA. The kinetic parameters of the [^{35}S]GTPγS binding rates were found for each region after a nonlinear regression to a single-compartment model for specific binding (see Supplementary Methods). The maximum specific binding was elevated in the RW and SW [^{35}S]GTPγS binding rates, relative to CTRL [^{35}S]GTPγS binding in the CeA and BNST (*t*-tests, $p<0.05$), but significant differences were not found in the [^{35}S]GTPγS binding data of the other regions (see Supplementary Table).

DISCUSSION

Activation of mGlu$_{2/3}$ receptors by the selective group II agonist LY379268 dose-dependently reduced cue-induced reinstatement of ethanol seeking, and this effect was characterized by increased potency of low LY379268 doses to attenuate reinstatement in rats with histories of ethanol dependence compared with ethanol-nondependent rats. This change in the pharmacological profile of LY379268 was accompanied by regionally specific increases in mGluR$_{2/3}$ G-protein coupling, suggesting the presence of a neuroadaptive change in the functional activity of these receptors in rats with a history of ethanol dependence and withdrawal. At the highest dose tested, LY379268 reduced spontaneous locomotor activity and operant responding maintained by a non-drug reinforcer without differences as a function of ethanol history. At intermediate doses of LY379268, ethanol-dependent rats demonstrated reduced drug seeking during reinstatement tests but no motor impairment in the locomotor and supersac self-administration tasks. These data suggest that at intermediate doses, the increased sensitivity to the behavioral effects of LY379268 in rats with histories of ethanol dependence is specific to tasks motivated by ethanol-related stimuli. These findings have possible implications for the neural basis of alcohol dependence, and provide further evidence supporting treatment target potential for mGluR$_{2/3}$ for ethanol craving and relapse prevention.

Behavioral Changes Precipitated by Repeated Withdrawal Cycles

The RW group was exposed to several cycles of intoxication and withdrawal in order to model the histories of repeated intoxication and withdrawal in severely alcohol-dependent individuals. Overt withdrawal signs in the RW group increased with each intoxication cycle to a level beyond that of the SW group. This observation suggests that sensitization of behavioral withdrawal symptoms developed, similar to the increasing withdrawal severity in kindling studies (Becker, 2000; Becker and Hale, 1993; Clemmesen and Hemmingsen, 1984) and consistent with the clinical literature (Brown *et al*, 1998). RW rats also showed higher levels of ethanol intake during post-withdrawal self-administration sessions, whereas rats in the SW and CTRL groups maintained predependence rates of consumption. This too was consistent with previous observations of elevated alcohol intake in some (Roberts *et al*, 1996, 2000) but not all (Ciccocioppo *et al*, 2003b) experiments that used ethanol vapor inhalation to induce dependence in outbred Wistar rats. However, irrespective of ethanol history, all rats showed the same levels of reinstatement in the S$^+$ context, suggesting that increased withdrawal severity and ethanol consumption did not translate into enhanced ethanol seeking. This outcome is a likely consequence of the absence of ethanol availability via self-administration during acute withdrawal, precluding learning about negative reinforcement as an important aspect of ethanol's subjective effects and consequently enhancing the incentive salience of ethanol-associated contextual stimuli (Ciccocioppo *et al*, 2003b; Liu and Weiss, 2002b). The consistency between intoxication groups in both the peak and the rate of progressive decrease of responding during extinction training also suggests an absence of learning effects, despite the increased ethanol intake by the RW rats during the postintoxication phase of self-administration. For the present purposes, this outcome

was desired in order to permit examination of changes in mGluR$_{2/3}$ sensitivity and function independent of learning factors that would alter baseline levels of reinstatement. Thus, the differential pharmacologic profile of LY379268 in rats with *vs* without an ethanol-dependence history cannot be explained by differences in the incentive salience of the ethanol-paired contextual stimuli.

Attenuation of Conditioned Reinstatement by LY379268

The effects of LY379268 on reinstatement in nondependent rats were significant at the 1.0 and 3.0 mg/kg doses, replicating earlier findings (Zhao *et al*, 2006). However, post-dependent rats were notably more sensitive to the 'anti-reinstatement' actions of LY379268 with significant effects occurring already at the lowest (0.3 mg/kg) dose, suggestive of a leftward shift in dose-response function of the mGluR$_{2/3}$ agonist (Figure 5). This finding extends previous observations showing that LY379268 is more effective in inhibiting stress-induced reinstatement and alcohol self-administration at low doses in post-dependent rats (Sidhpura *et al*, 2010). In conjunction with this evidence, the present results suggest that mGluR$_{2/3}$ receptors participate in the regulation of both conditioned and stress-induced reinstatement as well as ethanol reinforcement because LY379268 inhibits all of these behaviors at the same dose range in nondependent rats (Rodd *et al*, 2006; Zhao *et al*, 2006) and, with the same apparent leftward shift in the effective dose range, in post-dependent rats (Sidhpura *et al*, 2010).

Effects of LY379268 on Locomotor Activity and Responding Maintained by Non-Drug Reward

To understand whether nonspecific effects of LY379268 exist at doses required to reduce conditioned reinstatement of ethanol seeking, the mGluR$_{2/3}$ agonist was examined for its effects on locomotor activity and operant responding maintained by a non-drug reinforcer (supersac). These tests were implemented with the sole purpose to dissociate possible inhibitory effects on expression of locomotor activity behavior from motor effects that would impair the ability of rats to engage in tasks that require substantial motoric demands (ie, high rates of lever press responses maintained by a palatable reinforcer). This aspect of the study was dedicated to the question as to whether or not rats treated with LY379268 could engage in demanding motor tasks if they were motivated to do so, and whether this capability changed with a history of physical dependence on ethanol. LY379268 treatment suppressed locomotor activity and operant responding, but both of these effects required a 3.0 mg/kg dose whereas significant effects on conditioned reinstatement occurred at lower doses. Moreover, the greater sensitivity to reversal of ethanol seeking by LY379268 in post-dependent rats was not matched by corresponding differences in either locomotor activity or supersac-reinforced behavior. Thus, at moderate doses, the greater sensitivity to the behavioral effects of LY379268 in rats with an ethanol-dependence history is specific to tasks motivated by ethanol-related stimuli and does not extend to behavior reflective of sedative effects or motor impairment. The issue of nonselective sedative and motor effects of LY379268 remains to be more systematically examined because, in contrast to the present findings, it has been shown that a 3.0 mg/kg dose of this agent did not interfere with responding reinforced by sweetened condensed milk (SCM) in freely feeding rats (Baptista *et al*, 2004) or with responding reinforced by food pellets in nutritionally deprived rats (Zhao *et al*, 2006), but has been found to attenuate self-administration of food pellets in nondeprived rats (Liechti *et al*, 2007) and reinstatement of extinguished responding by SCM-paired cues (Baptista *et al*, 2004).

Neuroplasticity in mGluR$_{2/3}$ Function in Rats with a History of Alcohol Dependence

Stimulation of [^{35}S]GTPγS binding by LY379268 was significantly increased in the CeA and BNST in post-dependent rats of both the SW and RW groups, suggesting that these brain regions are important loci of neuroadaptation in mGluR$_{2/3}$ function associated with an ethanol-dependence history. Functional coupling of mGluR$_{2/3}$ was measured by the [^{35}S]GTPγS assay, which assessed the initial step in receptor-mediated G-protein activation in membranes extracted from brain micropunches (Beresford *et al*, 1998; Harrison and Traynor, 2003). Hence, the increased agonist-stimulated binding in the BNST and CeA revealed neuroadaptive changes resulting in elevated G-protein coupling and, presumably, resulting in greater sensitivity to LY379268. Although to our knowledge there have been no previous published reports of increased mGluR$_{2/3}$ expression or function as a result of ethanol dependence, increased G-protein coupling to mGluR$_{2/3}$ has been identified in rats with a history of dependence-inducing, long-access cocaine self-administration session, leading to escalated cocaine intake (Hao *et al*, 2010). This finding contrasts with reports of decreased mGluR$_{2/3}$ functional activity following chronic involuntary cocaine exposure (Ghasemzadeh *et al*, 2009; Xi *et al*, 2002), and decreased [^{35}S]GTPγS binding in the CeA of rats with a history of short-access cocaine self-administration typically not associated with the development of physical dependence (Hao *et al*, 2010). The molecule AGS3, a modulator of G-protein signaling that sequesters Giα and therefore inhibits functional coupling of mGluR$_{2/3}$, has been shown to be upregulated in the PFC and NAc of rats with a history of several weeks of home-cage ethanol self-administration (Bowers *et al*, 2008). The present data revealed no dependence-associated effects in [^{35}S]GTPγS binding of the PFC or NAc tissue samples, indicating that the induction of ethanol dependence by vapor inhalation produced physiological changes distinct from those obtained in models of home-cage consumption that do not induce physical dependence and overt signs of withdrawal. Overall, these findings suggest that increased sensitivity to the behavioral effects of LY379268 in animals with a history of ethanol (or cocaine) dependence is associated with a functional upregulation of mGluR$_{2/3}$ receptor signaling.

The BNST and CeA are common sites of *c-fos* activation associated with both conditioned and stress-induced reinstatement of ethanol seeking in nondependent rats (Dayas *et al*, 2007; Zhao *et al*, 2006). In the present study, the BNST and CeA were the only brain regions in which

post-dependent rats showed enhanced mGluR$_{2/3}$ agonist-stimulated [^{35}S]GTPγS binding. Thus, neuroadaptive changes in mGluR$_{2/3}$ functional activity associated with ethanol dependence appear to be confined to these sites. These brain regions are components of neurocircuitry that mediates anxiety-like behavior and behavioral responses to stress as well as drug reinforcement and incentive motivation and, thus, represent key sites for the control of behavior associated with drug and alcohol dependence (Koob and Volkow, 2009). In particular, the enhanced [^{35}S]GTPγS binding in the BNST may be relevant for possible enhanced effects of LY379268 on stress or anxiety-like effects of ethanol cues, consistent with the suggestion that the absence of ethanol reward in an ethanol-predictive stimulus environment may elicit an aversive motivational state resulting in stress (Topple *et al*, 1998) and findings showing that craving states associated with drug cue exposure are accompanied by anxiety and stress-like reactions in alcoholics (Fox *et al*, 2005; Sinha *et al*, 2003).

In addition to their roles in mediating stress responses, both the BNST and CeA are important substrates of positive drug reinforcement and incentive motivation (Koob, 2003). Several drugs of abuse, including alcohol (Carboni *et al*, 2000) and cocaine (Epping-Jordan *et al*, 1998), increase extracellular dopamine levels in the BNST. In addition, intracerebral self-stimulation (ICSS) in the BNST supports operant behavior in alcohol-preferring (P) but not non-alcohol-preferring (NP) rats (Eiler *et al*, 2007) and is a substrate for heightened sensitivity to the anxiogenic effects of corticotropin-releasing factor in Marchigian Alcohol-Preferring (msP) rats (Ciccocioppo *et al*, 2003a). Additionally, elevations in BNST excitatory transmission have been linked to behavioral responses reinforced by cocaine and food, but not to either reward when given passively (Dumont *et al*, 2005). Within the amygdala, the CeA supports the highest rates of ICSS (Wurtz and Olds, 1963) and is an important substrate of oral ethanol reinforcement (Heyser *et al*, 1999; Hyytia and Koob, 1995; Moller *et al*, 1997). The CeA and BNST are among the targets of glutamatergic projections originating from the hippocampus, a region associated with context-driven reinforcement processes, including conditioned reinstatement of drug seeking (Fuchs *et al*, 2005; Holland and Bouton, 1999). As has been argued previously (Zhao *et al*, 2006), the motivational impact of ethanol-paired cues may be particularly sensitive to manipulation of mGluR$_{2/3}$, in a manner analogous to the attenuation of conditioned anxiety responses by the mGluR$_{2/3}$ agonist LY354740 (Helton *et al*, 1998). Stimulation of mGluR$_{2/3}$ that reduces presynaptic glutamate release and exerts inhibitory control over postsynaptic excitability (Schoepp, 2001; Swanson *et al*, 2005) may therefore exert a greater dampening effect on BNST- and CeA-mediated reinforcement in post-dependent than nondependent rats because of increased agonist sensitivity of mGluR$_{2/3}$ in these sites.

Implications for the Potential of mGluR$_{2/3}$ as Therapeutic Targets

The results document that attenuation of glutamatergic transmission by mGluR$_{2/3}$ activation attenuates reinstatement induced by ethanol cues in ethanol-nondependent rats, confirming earlier reports (Backstrom and Hyytia, 2005; Rodd *et al*, 2006; Sidhpura *et al*, 2010; Zhao *et al*, 2006). More importantly, the inhibitory actions of the mGluR$_{2/3}$ agonist on conditioned reinstatement were enhanced in ethanol post-dependent rats, regardless of the withdrawal pattern experienced by the post-dependent rats. This observation may have important implications from a treatment target potential perspective because interference with conditioned reinstatement in post-dependent rats occurred at a dose well outside the range of undesirable side effects such as locomotor impairment or general suppression of operant responding in this and previous work (see, eg, Baptista *et al*, 2004; Bossert *et al*, 2006; Cartmell *et al*, 1999, 2000). Moreover, the effects of mGluR$_{2/3}$ activation extend to attenuation of stress-induced reinstatement of ethanol seeking and, similar to the present findings, these effects were characterized by an apparent leftward shift in the dose-response effects of LY379268 in ethanol post-dependent rats (Sidhpura *et al*, 2010). Thus, dependence-associated changes in mGluR$_{2/3}$ function appear accompanied by increased potency of LY379268, particularly at low doses, and consequently potentially a wider 'therapeutic' window of mGluR$_{2/3}$ agonists for the prevention of drug seeking and relapse. Overall, therefore, mGlu$_{2/3}$ receptors provide a potentially highly effective target for the prevention of craving relapse associated not only with exposure to ethanol-associated environmental stimuli but also stress. Importantly as well, the effects of mGluR$_{2/3}$ activation extend to conditioned reinstatement of cocaine- (Baptista *et al*, 2004), nicotine- (Liechti *et al*, 2007), and heroin-seeking (Bossert *et al*, 2005) and may be enhanced in subjects with histories of drug dependence as suggested by recent findings with cocaine (Hao *et al*, 2010). The capability of mGluR$_{2/3}$ agonists to reduce conditioned and stress-induced reinstatement has been credited to this receptor family's role in reducing neuronal excitability by glutamate (Knopfel *et al*, 1995; Sidhpura *et al*, 2010; Zhao *et al*, 2006). The present findings suggest that this regulatory mechanism may be particularly important in ethanol-dependent subjects, who may experience long-lasting dysregulation of glutamatergic transmission. This therapeutic advantage is not shared by the opioid antagonist naltrexone—a medication currently in use for relapse prevention in alcoholics—in that this agent was less effective in reducing reinstatement in ethanol post-dependent compared with nondependent rats, and even less so in rats with a history of repeated ethanol intoxication and withdrawal (Ciccocioppo *et al*, 2003b; Liu and Weiss, 2002a).

Conclusion

The findings revealed that a history of ethanol dependence is associated with increased sensitivity to the anti-reinstatement actions of the mGluR$_{2/3}$ agonist LY379268 and that this effect is accompanied by upregulated G-protein coupling in the BNST and CeA of post-dependent rats, linking neuroadaptive changes in these brain regions to increased sensitivity to the 'anti-reinstatement' actions of LY379268. These findings have encouraging implications for the target treatment potential of mGluR$_{2/3}$ in alcohol craving and relapse prevention. What remains for

future research is to determine whether neuroadaptation in mGluR$_{2/3}$ function is linked to dependence-inducing exposure to ethanol, or represents a specific consequence of ethanol withdrawal. A further remaining issue is a better understanding of the mGluR$_{2/3}$ neuroadaptive changes within specific subregions of the CeA or BNST responsible for the altered pharmacological profile of LY379268 in post-dependent rats.

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DISCLOSURE

The authors declare no conflict of interest.

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