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MGluR5 activity is required for the induction of ethanol behavioral sensitization and associated changes in ERK MAP kinase phosphorylation in the nucleus accumbens shell and lateral habenula

Rebekah A. Stevenson^{1,2}, Jessica L. Hoffman¹, Antoniette M. Maldonado-Devincci^{1,3}, Sara Faccidomo¹, and Clyde W. Hodge^{1,*}

¹Center for Alcohol Studies, Department of Psychiatry, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

²Current address: Department of Biology, Bucknell University, Lewisburg, PA 17837

³Current address: Department of Psychology, North Carolina A&T State University, Greensboro, NC 27411

Abstract

Metabotropic glutamate receptor subtype-5 (mGluR5) activity regulates a variety of behavioral pathologies associated with alcohol addiction. The main goal of this study was to determine if mGluR5 regulates the induction of ethanol-induced locomotor sensitization, which is a model of experience-dependent plasticity following initial exposure to drugs of abuse. The extracellular signal-regulated kinase (ERK_{1/2}) pathway is downstream of mGluR5 and implicated in alcohol addiction; however, its role in sensitization remains unexplored. We sought to determine if mGluR5-mediated changes in ethanol-induced sensitization are associated with changes in ERK_{1/2} phosphorylation (pERK_{1/2}) in specific brain regions. Adult male DBA/2J mice were tested for acute locomotor response to ethanol (0 or 2 g/kg, IP) followed by a 10-day induction period in which the mGluR5 antagonist MPEP (0 or 30 mg/kg, IP) was administered prior to ethanol (0 or 2.5 g/kg, IP). One day later, ethanol (2 g/kg) produced a robust within- and between-group increase in locomotor activity, indicating sensitization in mice that received MPEP (0 mg/kg) during induction. MPEP (30 mg/kg) treatment during induction resulted in locomotor response to ethanol (2 g/kg) challenge that was equivalent to an acute response, indicating full blockade of sensitization. Sensitization was associated with increased pERK_{1/2} immunoreactivity (IR) in nucleus accumbens shell (AcbSh) and a reduction in lateral habenula (LHb), both of which were blocked by MPEP treatment during induction. Sensitization was also associated with mGluR5-independent increases in pERK_{1/2} IR in the nucleus accumbens core and decreases in the dentate gyrus and lateral septum. These data indicate that mGluR5 activity is required for the induction of

*Correspondence: Clyde W. Hodge, Ph.D., Professor, Department of Psychiatry, Bowles Center for Alcohol Studies, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, Voice: (919) 966-5678, chodge@med.unc.edu.

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ethanol locomotor sensitization and associated changes in ERK_{1/2} phosphorylation in the AcbSh and LHb, which raises the hypothesis that mGluR5-mediated cell signaling in these brain regions may mediate the induction of sensitization. Elucidating mechanisms of sensitization may increase understanding of how ethanol hijacks behavioral functions during the development of addiction.

Keywords

alcohol; ethanol; sensitization; glutamate; mGluR5; ERK; nucleus accumbens; lateral habenula

1. Introduction

Evidence has long indicated that ethanol produces maladaptive changes in glutamate-linked neural processes that support enduring and progressive pathologies associated with alcohol use disorders and addiction [1–5]. Thus, elucidating ethanol-induced alterations in glutamate function that regulate behavioral pathologies is of potential importance for understanding the etiology of alcohol addiction and for development of new pharmacotherapeutic strategies [6–10].

A growing number of preclinical studies have found that metabotropic glutamate receptor 5 (mGluR5) activity regulates an array of ethanol-related behavioral pathologies. For example, systemic administration of the mGluR5 antagonist 2-Methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP) inhibits the reinforcing effects of ethanol in rats and mice [11–13]. MPEP also inhibits the motivation to self-administer ethanol as measured by progressive ratio testing [14], blocks the discriminative stimulus effects of investigator- [15, 16] and self-administered [17] ethanol [14–17], and prevents relapse-like behavior as measured by cue-induced reinstatement [18, 19] and ethanol deprivation procedures [13]. Although these and other findings indicate that mGluR5 regulates a variety of ethanol-related behavioral pathologies, they leave open the question of how mGluR5 might influence the development of alcohol addiction.

Extracellular signal-regulated kinase (ERK) is a downstream mediator of mGluR5 activity [20] that is receiving growing attention in addiction research due to its mechanistic involvement in adaptive processes such as learning, memory, and synaptic plasticity [21, 22] as well as maladaptive forms of plasticity associated with drug use [23, 24]. The two closely related isoforms of ERK (ERK1 and ERK2, or ERK_{1/2}) are activated by dual phosphorylation on both a threonine and tyrosine residue by MEK_{1/2} [25]; reviewed by [26]. Activated ERK_{1/2} phosphorylates cellular targets or translocates to the cell nucleus where it activates specific gene transcription factors [27]. By regulating cellular activities and gene transcription, the ERK_{1/2} cascade transduces the activity of a variety of extracellular and intracellular signals into enduring changes in CNS structure and function [24, 28–31] that may mediate behavioral pathologies in addiction.

Accordingly, we have shown that ERK_{1/2} signaling regulates the discriminative stimulus [32] and positive reinforcing effects of ethanol [33, 34], which are both critical for the development and maintenance of addiction [35, 36]. Moreover, our preclinical research indicates that cue-induced relapse to ethanol-seeking behavior is associated with 4–5 fold

increases in ERK_{1/2} phosphorylation (p-ERK_{1/2}) in the nucleus accumbens and amygdala [19], and that pretreatment with MPEP blocks both relapse-like behavior and cue-induced increases in p-ERK_{1/2} [13, 19]. These findings suggest that mGluR5 may mediate ethanol-related behavioral pathologies in addiction via alterations in the ERK_{1/2} signaling cascade.

At the behavioral level, sensitization to the locomotor stimulant effects of drugs is widely used to model the experience-dependent plasticity associated with the development of addiction [37–39]. Sensitization is typically defined as a progressive increase in locomotor activity following repeated administration of a drug of abuse [40]. The *induction* of sensitization is tested during a series of initial drug exposures, whereas *expression* is tested after a period of withdrawal [41]. Behavioral sensitization to drugs is mediated by an interconnected network of mesocorticolimbic brain regions (e.g., VTA, nucleus accumbens, prefrontal cortex, amygdala, and thalamus) and neurotransmitter systems including glutamate and its signaling pathways [42–44]. For example, cocaine sensitization is associated with an increase of p-ERK_{1/2} in the nucleus accumbens [45] and pharmacological inhibition of ERK_{1/2} prior to each repeated drug treatment blocks the induction of amphetamine and cocaine locomotor sensitization [24, 46]. Further, acute administration of an mGluR5 antagonist fully blocks the expression (tested after a delay) of cocaine [47], morphine [48] and ethanol [49] sensitization. These findings indicate that mGluR5 activity is required for expression of drug sensitization. However, it is not yet known if mGluR5 signaling regulates the induction of ethanol-induced behavioral sensitization.

The present study was designed to determine if: 1) mGluR5 activity is required for the induction, or development, of ethanol-induced locomotor sensitization; 2) ethanol-induced locomotor sensitization is associated with altered p-ERK_{1/2} immunoreactivity (IR) in key brain regions known to mediate sensitization, including the nucleus accumbens and prefrontal cortex; and 3) ethanol-induced changes in p-ERK_{1/2} associated with sensitization might be blocked by MPEP treatment during induction. By addressing the potential involvement of mGluR5 receptor function and ERK_{1/2} signaling in behavioral sensitization, results of this study contribute to understanding neural mechanisms of ethanol-induced neuroadaptation and may influence the development of alcohol addiction.

2. Materials and Methods

2.1 Experimental subjects

Adult (8-week old, N=40) male DBA/2J mice (Jackson Laboratories, Bar Harbor, ME) were group-housed (4 per cage) in standard Plexiglas cages with food (Purina Rodent Chow #3500) and water available *ad libitum*. DBA/2J mice are widely used for ethanol sensitization studies because they show robust and reliable locomotor sensitization as compared to other commonly studied mouse strains, such as C57BL/6J [50–52]. Mice were given 7 days to acclimate to the vivarium. For the next 7 days, mice were weighed daily as a measure of general health. Experimental procedures were conducted during the following 14-day period (10 – 12 weeks of age). Mice were weighed each day within 1-h of experimental procedures to calculate drug dosages relative to body weight and monitor health. Mice showed typical adult body weight during the 14-day procedure (MEAN ± SEM - beginning: 23.31 ± 0.30 g; end: 22.28 ± 0.36 g). The colony was maintained at 21±1°C on

a 12-hour light/dark cycle, with the lights off from 10 AM – 10 PM. All behavioral experiments were conducted during the dark portion of the light cycle to minimize light-associated behavioral and cognitive disruption in mice [53]. Mice were under continuous care and monitoring by the Division of Comparative Medicine at UNC-Chapel Hill (DCM), and all procedures were carried out in accordance with the *NIH Guide to Care and Use of Laboratory Animals* [54] and institutional guidelines.

2.2 Behavioral Apparatus

Locomotor activity (horizontal distance traveled, cm) was measured in eight Plexiglas mouse activity monitors (27.3 × 27.3 × 20.3 cm, Med Associates, Georgia, VT) located in sound attenuating chambers as previously reported by our group [55–57]. Two sets of 16 pulse-modulated infrared photobeams were located on opposite walls to record ambulatory movements in the X-Y (horizontal) plane. The activity chambers were computer-interfaced (Med Associates) for data sampling at 100-ms resolution. All testing was conducted in enclosed chambers under 0 lux conditions.

2.3 Sensitization Procedure

The ethanol sensitization procedure was conducted as previously reported [57] over a 14-d period in four phases: Habituation, Acute Test, Sensitization Induction, and Sensitization Test (Figure 1A). Mice were divided into 5 groups (n=8/group): Control, Repeated EtOH, MPEP Control, MPEP + EtOH and Single EtOH (Figure 1A).

2.3.1 Habituation.—Habituation to injections and locomotor chambers was conducted during the first 3 days of the experiment. On habituation days 1 and 2, all mice were pretreated with an intraperitoneal (IP) injection of saline vehicle (veh) and placed back into their home cages. On habituation day 3, mice received two IP injections (multiple injection procedure required for MPEP × EtOH testing). All mice were transported to the behavioral testing room and allowed to acclimate for 30-min followed by vehicle (IP) administration. Thirty minutes later, mice were given another vehicle injection and were immediately placed in the locomotor chamber for a 10-minute session (Figure 1A, **Habituation / Veh**).

2.3.2 Acute ethanol test.—On the acute ethanol test day, all mice were transported to the behavioral testing room and allowed to acclimate for 30 minutes. Then, all mice were administered veh (IP) followed thirty minutes later by veh (group: Control, MPEP control, and Single EtOH) or ethanol (2 g/kg, IP; group: Repeated EtOH and MPEP + EtOH) and placed immediately in the locomotor chamber for a 10-min test to establish the acute locomotor activating effects of ethanol (Figure 1A, **Acute Test**).

2.3.3 Sensitization Induction: Effect of MPEP Pretreatment.—During the induction of sensitization (days 4 – 13), mice (n=8/group) were pretreated with MPEP (0 or 30 mg/kg) and returned to the home cage. After a 30-minute pretreatment interval, mice were injected with ethanol (0 or 2.5 g/kg) and returned to the home cage. No locomotor testing was performed on these days (Figure 1A, **Sensitization Induction**). Administration of the higher ethanol dose during these days produces robust sensitization in DBA/2J mice when subsequent testing is conducted following a 2.0 g/kg dose of ethanol [57, 58].

2.3.4 Sensitization Induction.—Finally, for the sensitization test (day 14), mice (n=8/group) were transported to the behavioral testing room and allowed to acclimate for 30 minutes. Mice were then given a pretreatment injection of vehicle and returned to the holding cage. Thirty minutes later, mice were injected with ethanol (0 or 2.0 g/kg) and were immediately placed into the locomotor chamber to be tested for locomotor sensitization to ethanol (Figure 1A, **Sensitization Test**). To provide a between-group measurement of ethanol sensitization, a fifth group of mice (n=8) was treated identically to the other groups but received vehicle followed by a single dose of ethanol (2 g/kg) only on the sensitization test day. Importantly, the initial acute locomotor (Day 4) and final test for sensitization, or single EtOH response, on Day 14 were conducted in the absence of MPEP treatment.

2.4 Analysis of p-ERK_{1/2} Immunoreactivity

Analysis of the pERK_{1/2} immunoreactivity was conducted as previously described [19, 32, 34, 59]. Immediately following the locomotor test session on day 14, all mice were deeply anesthetized with pentobarbital (100 mg/kg IP) and transcardially perfused with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde. Brains were fixed overnight, and then rinsed and stored in PBS at 4°C. Brains were sectioned at 40 µm and stored (−20°C) in cryoprotectant until immunohistochemistry processing. Tissue was washed in PBS, the endogenous peroxidase activity was blocked in 0.6% H₂O₂, and antigen retrieval was performed at 70°C for 30 min (Antigen Retrieval Citra, BioGenex). Sections were blocked in PBS/0.1% tritonx/4% horse serum for 30 minutes and incubated at +4°C overnight in primary polyclonal antibody to p-ERK_{1/2} (1:400; Cell Signaling Technology, Inc, Danvers, MA). The following day, sections were washed in PBS and incubated in secondary antibody for one hour (Dako EnVision Kit, Dako). Immunoreactivity was detected with nickel-enhanced diaminobenzidine (Dako EnVision Kit) as a chromagen. Sections were counterstained with toluidine blue, mounted, dried and cover slipped with Cytoseal. For consistency of staining across subjects, brain tissue from all groups was processed simultaneously.

Immunoreactivity was quantified using Bioquant image analysis software (Bioquant, Nova Advanced Image Analysis; R&M Biometric, Nashville, TN) as previously described [19, 57, 60, 61]. The image was background corrected and normalized to preset light levels to ensure consistent data collection. Cell count measurements were calculated using this software for each brain region, divided by the area of the region and expressed as cells/mm². For the dentate gyrus, cells were counted manually and expressed as the average number of cells/dentate. Consistent identification and analysis of the following brain regions were performed with guidance from the mouse brain atlas of Franklin and Paxinos: medial prefrontal cortex (mPFC), Nucleus accumbens core (AcbC) and shell (AcbSh), lateral septum, lateral habenula (LHb), PVN thalamus, central amygdala (CeA), basolateral amygdala (BLA) dentate gyrus (DG), and PVN hypothalamus [62]. Brain regions chosen for analysis showed cellular expression of p-ERK_{1/2} immunoreactivity that was distinguishable from background. Other brain regions were excluded from analysis due to high background staining that precluded reliable detection of individual cells (e.g., hippocampus area CA1) or low intensity of p-ERK_{1/2} immunoreactivity that was below a reliable threshold (e.g., dorsal

striatum). All data was acquired by a researcher blind to group condition from 4 sections/ brain region/animal and averaged to obtain a single value per subject.

2.5 Drugs

Ethanol (95% w/v) was diluted in physiological saline (0.9%) to a concentration of 20% (v/v) and injected (IP) at different volumes to achieve the appropriate dosage (i.e., 2.0 and 2.5 g/kg). The mGluR5 antagonist MPEP [2-methyl-6-(phenylethynyl)-pyridine] (Ellisville, MO, USA) was dissolved in 0.9% saline and administered IP at a dosage of 30 mg/kg 30-min prior to ethanol (2.5 g/kg) during the induction of sensitization. This dose of MPEP enhances the acute sedative hypnotic without altering blood ethanol clearance [63] and reduces the reinforcing effects of ethanol [12] in C57BL/6J mice. Based on evidence indicating that a lower dosage of MPEP (e.g., 10 mg/kg, IP) achieves full receptor occupancy in mouse brain at 5-min and declines to ~75% at 30-min post injection [64], estimated receptor occupancy by MPEP (30 mg/kg) was ~75% at the time of ethanol injection. MPEP and vehicle solutions were administered to mice in a volume of 0.1 ml/10 g body weight.

2.6 Data Analysis

Horizontal distance traveled (cm) during locomotor activity sessions was calculated from the number of photobeam breaks and presented as mean $\pm$ SEM. Locomotor activity data were analyzed statistically by mixed-effects model with between subject factors of 1) pretreatment (MPEP 0 or 30 mg/kg) and 2) ethanol treatment (0 or 2.0 g/kg) and the within subject factor of Treatment Day (Habituation, Acute Test, and Sensitization Test). The Single EtOH group received vehicle treatment throughout induction and acute ethanol on the sensitization test day (day 14) as a between-group measure of ethanol-induced locomotor sensitization. The mixed-effects model was fit using Restricted Maximum Likelihood (REML) with Geisser-Greenhouse correction. Post hoc multiple comparisons were conducted using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, which corrects for multiple comparisons by controlling the false discovery rate [65]. Within-group sensitization was defined as EtOH-induced activity on test day 14 (Sensitization Test) being significantly greater than ethanol-induced activity on day 4 (Acute Test). Between-group sensitization was defined as ethanol-induced activity by the Repeated EtOH group on day 14 being significantly greater than the Single EtOH injection group that received Veh-Veh during induction followed by a single EtOH injection on day 14 and analyzed via one-way ANOVA followed by multiple comparison tests.

pERK_{1/2} immunoreactivity (IR) data were presented as the mean $\pm$ SEM number of cells/mm² and analyzed by 2-Way ANOVA with factors for MPEP (0 or 30 mg/kg) and ethanol (0 or 3 g/kg) followed by multiple comparison tests as described above. pERK_{1/2} IR following the single ethanol injection on the sensitization test day (Single EtOH group) was compared to the Repeated EtOH group as a between-groups measure of sensitization-induced effects via t-test as a planned comparison. All graphs and statistical analyses were prepared using GraphPad Prism v. 8.0.2 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1 Effect of MPEP on the Induction of Ethanol-Induced Locomotor Sensitization

A mixed-effects model analysis revealed significant main effects of Drug [$F(4, 33) = 23.20$; $p < 0.0001$] and Treatment Day [$F(1.952, 59.54) = 53.73$; $P < 0.0001$] as well as a significant Drug $\times$ Treatment Day interaction $F(8, 61) = 13.02$; $P < 0.0001$]. Multiple comparisons analysis showed that locomotor activity did not differ between groups during the first test following Veh+Veh injection (Figure 1B, **Habituation, Day 3**). For the acute EtOH test on Day 4, three groups of mice received Veh+Veh and two groups received Veh+EtOH. The multiple comparison procedure showed that both ethanol treated groups exhibited significantly greater locomotor activity as compared to the corresponding Veh controls (Figure 1B, **Acute Test, Day 4**); these comparisons confirm the acute locomotor activating effect of ethanol. Within-group measures of sensitization showed that the Repeated EtOH group exhibited greater locomotor activity on Day 14 (Sensitization Test) than on Day 4 (Acute Test), confirming within-subject ethanol sensitization (Figure 1B, **Sensitization Test**). By contrast, locomotor activity by the MPEP + EtOH group on Day 14 did not differ within-subjects from Day 4, demonstrating MPEP blockade of ethanol sensitization (Figure 1B, **Sensitization Test**). Between-groups comparisons showed that repeated EtOH increased locomotor activity on the Sensitization Test day by ~90% relative to the Single EtOH treatment group, and that this effect was fully blocked by MPEP treatment during induction (Figure 1C). Importantly, MPEP pretreatment reduced locomotor on the Sensitization Test day to acute levels (both within- and between-subjects) indicating inhibition of the sensitized response only (Figures 1B and C).

3.2 MPEP modulation of Repeated EtOH-induced Changes in pERK_{1/2} Immunoreactivity

3.2.1 Nucleus accumbens shell (AcbSh).—A two-way ANOVA (EtOH $\times$ MPEP) revealed a significant main effect of EtOH on pERK_{1/2} IR [$F(1, 27) = 10.28$, $P = 0.0034$] and a significant EtOH $\times$ MPEP interaction [$F(1, 27) = 5.764$, $P = 0.0235$]. Post hoc multiple comparisons showed that this was due to a significant ethanol-induced increase in pERK_{1/2} IR that was blocked by MPEP pretreatment during induction (Figure 2A and 2B, Table 1). A planned comparison showed that pERK_{1/2} IR following Repeated EtOH was significantly greater than after a Single EtOH injection on the sensitization test day (Figure 2A and 2B).

3.2.2 Lateral Habenula (LHb).—In the LHb, 2-Way ANOVA (EtOH $\times$ MPEP) identified a main effect of ethanol on pERK_{1/2} IR [$F(1, 27) = 7.298$, $P = 0.0118$] and a significant interaction between ethanol and MPEP [$F(1, 27) = 5.106$, $P = 0.0321$]. Multiple comparisons showed this was due to a significant decrease in pERK_{1/2} IR for the ethanol group compared to the Veh group, and MPEP administration restored expression to control levels (Figure 3A and 3B). A planned comparison (unpaired t-test) showed that the Single EtOH treatment was associated with p-ERK_{1/2} IR levels in the LHb that were significantly higher than expression levels in the Repeated EtOH condition (Figure 3A and 3B).

Together, these data indicate that the induction of ethanol sensitization was associated with bidirectional mGluR5-dependent changes in pERK_{1/2} IR in the AcbSh and LHb.

3.3 Repeated EtOH-induced changes in pERK_{1/2} Immunoreactivity

Several brain regions showed ethanol-induced changes in pERK_{1/2} IR that were not modulated by MPEP treatment during induction of sensitization.

3.3.1 Nucleus accumbens core (AcbC).—A 2-way ANOVA (EtOH × MPEP) identified a main effect of ethanol [$F(1, 27) = 5.829$, $P=0.0228$] but no effect of MPEP [$F(1, 27) = 0.1485$, $P=0.7030$] interaction between EtOH and MPEP [$F(1, 27) = 2.100$, $P=0.1588$]. Thus, the main effect of ethanol in the AcbC was due to trends at both doses of MPEP (Table 1).

3.3.2. Dentate gyrus.—A 2-Way ANOVA (EtOH × MPEP) revealed main effect of ethanol on pERK_{1/2} IR in the dentate gyrus [$F(1, 27) = 20.76$, $p<0.0001$] but no significant effect of MPEP [$F(1, 27) = 0.7851$, $P=0.3834$] and no interaction [$F(1, 27) = 0.03605$, $P=0.8508$] (Table 1), which indicates similar trends in the ethanol response at both MPEP doses. A post hoc planned comparison (unpaired t-test) showed that pERK_{1/2} IR following the Single EtOH treatment was significantly lower than expression in the Repeated EtOH group following the ethanol (2 g/kg) injection on the sensitization test day [$t(13)=3.4$, $P=0.005$] (Table 1). It is noteworthy that the number of pERK_{1/2} positive cells in the dentate was relatively low in comparison to all other brain regions evaluated.

3.3.3 Lateral septum.—A 2-Way ANOVA (EtOH × MPEP) identified a main effect of ethanol on pERK_{1/2} IR in the Lateral Septum [$F(1, 27) = 9.294$, $P=0.0051$]. MPEP pre-treatment during induction had no effect on pERK_{1/2} IR [$F(1, 27) = 0.02167$, $P=0.8841$] and there was no EtOH × MPEP interaction [$F(1, 27) = 0.2402$, $P=0.6280$] indicating that the main effect of ethanol was due to trends at each dose of MPEP (Table 1). A post hoc planned comparison showed that the Single EtOH condition was associated no change in pERK_{1/2} IR; however, the high variability in cell counts in this and other brain regions may have precluded detection of statistically significant differences between conditions.

Together, these data indicate that the induction of ethanol sensitization was associated with altered pERK_{1/2} IR in the AcbC, dentate gyrus, and lateral septum that was not dependent on mGluR5 activity.

3.4 Brain regions showing no Repeated EtOH-induced change in pERK_{1/2} IR

Statistical analysis showed that ethanol-induced sensitization was not associated with changes in pERK_{1/2} IR in the medial prefrontal cortex (mPFC), central amygdala (CeA), basolateral amygdala (BLA), PVN thalamus, or PVN hypothalamus. There was also no effect of MPEP on pERK_{1/2} IR in these brain regions (Table 1). However, pERK_{1/2} IR was significantly higher in the central amygdala following the Single EtOH treatment as compared to Repeated EtOH [$t(13)=9.7$, $P<0.0001$] (Table 1), which suggests that ethanol has acute effects on pERK_{1/2} in this brain region that diminish, or adapt, following repeated ethanol treatment.

4. Discussion

Locomotor sensitization is a model of neuronal plasticity and is often used to assess the progressive and enduring pathologies in brain and behavioral functions that characterize drug addiction. The purpose of this study was to test the hypothesis that mGluR5 activity is required for the induction of ethanol-induced sensitization and to determine if this mechanistic regulation of behavior is associated with alterations in downstream pERK_{1/2} IR, which is an intracellular mechanism of neural plasticity [66] and ethanol reward [19, 32–34, 59].

Behavioral results showed that administration of the mGluR5 antagonist MPEP (30 mg/kg, IP) during induction fully blocked ethanol-induced locomotor sensitization to levels seen following an acute response when assessed both within- and between-groups. This indicates that the acute ethanol-induced locomotor activating response remained intact, but the sensitized response was fully blocked. Previous research has demonstrated that the mGluR5 antagonist MTEP also fully blocks the expression of ethanol sensitization in mice in the absence of effects on acute ethanol-induced locomotor activation [49]. In that work, sensitization was initiated by administering ethanol (2.4 g/kg) four times, at 3-day intervals. MTEP was then administered 15-minutes before an expression test for sensitization on day 14, and mice that received MTEP did not display sensitization. The present study differed from the Kotlinska, Bochenski and Danysz [49] study by administering the mGluR5 antagonist MPEP, along with ethanol, on the days when sensitization was developing (days 4 – 13) in order to determine the role of mGluR5 in the induction of sensitization. Importantly, MPEP was not administered prior to the sensitization test (day 14) in the present study, which also dissociates any potential acute antagonist effects of MPEP on motor behavior from the sensitization response to ethanol on the test day. These results are consistent with evidence indicating that glutamatergic transmission is necessary for the induction of sensitization to cocaine, amphetamine, and opioids, indicating a common glutamatergic mechanism for locomotor sensitization to all drugs of abuse (reviewed by [41]). Together, these studies indicate that mGluR5 activity is important to both the induction and the expression of ethanol sensitization.

The induction of locomotor sensitization was associated with bidirectional changes in pERK_{1/2} phosphorylation (e.g., activation) with an increase in pERK_{1/2} IR in the AcbSh and a decrease in the LHb, which were both blocked by MPEP administration during induction. Ethanol sensitization was also associated with changes in pERK_{1/2} IR in the AcbC, dentate gyrus of the hippocampus, and lateral septum that were not altered by mGluR5 blockade during induction. Given that mGluR5 receptor activity increases ERK_{1/2} phosphorylation [20, 67], these data suggest that ethanol-induced locomotor sensitization is modulated by bi-directional mGluR5 activity in a brain region-dependent manner.

The nucleus accumbens has been identified as a region critically important for the induction and expression of ethanol sensitization [40] and plays a key role in behavioral pathologies in alcohol addiction [68–71]. Recently, it has been demonstrated that cocaine sensitization causes an increase in pERK_{1/2} in the nucleus accumbens [72, 73]. Moreover, blockade of ERK_{1/2} has been shown to prevent the induction (or acquisition) of cocaine and

amphetamine sensitization [24]. The present data add to this growing literature showing a role for ERK_{1/2} signaling in the nucleus accumbens in locomotor sensitization to drugs of abuse by showing that mGluR5 inhibition during the induction of ethanol sensitization blocks both the behavioral change and ethanol-induced increase in ERK_{1/2} phosphorylation in the AcbSh. This raises the hypothesis that increased mGluR5 activity in the AcbSh during induction is important for ERK_{1/2} activation that occurs following a sensitized response to ethanol at the end of the induction phase. Future work could directly test the mechanistic significance of this finding by site-specific inhibition of mGluR5 and/or ERK_{1/2} activity in the AcbSh during induction. It would also be interesting to determine if mGluR5 inhibition in the AcbSh during induction would inhibit the expression of sensitization tested after a delay (e.g., [49]).

A novel finding from the present study is that ethanol sensitization is associated with reduced mGluR5-dependent ERK_{1/2} phosphorylation in the LHb. The LHb has numerous afferent and efferent connections with brain regions that regulate reward and other adaptive behavioral functions [74–76], which underscores the importance of understanding its role in addiction [74–76]. Locomotor sensitization to amphetamine [77] and MDMA [78] is associated with increased cFos expression in the LHb. Ethanol enhances GABAergic neurotransmission in the LHb [79] and stimulation of the LHb reduces voluntary ethanol intake in rats [80] indicating that addiction-linked behaviors are regulated by this structure. Although the habenula circuitry is not yet fully defined, it is clear that there is bi-directional communication between the LHb and the VTA [81]. Increased activity in the LHb is associated with a decrease in DA activity in the midbrain [82]. Thus, the ethanol-induced decrease in ERK_{1/2} phosphorylation in the LHb observed in the present study has the potential to promote increased VTA activity via disinhibition, which could drive dopamine release in the nucleus accumbens and promote sensitization. This is consistent with the increase in ERK_{1/2} phosphorylation we observed in the AcbSh following ethanol sensitization. Further, our results showed that mGluR5 inhibition during induction blocked both the ethanol sensitization-induced locomotor response and reduction in pERK_{1/2} IR in the LHb, which suggests that reduced mGluR5 activity in the LHb is involved in ethanol sensitization. Interestingly, recent evidence indicates that LHb neuronal firing is bidirectionally regulated by mGluR-dependent mechanisms [83]. Thus, mGluR5 activity in the LHb may gate ethanol-induced changes in connectivity and influence downstream structures, such as the VTA, that regulate sensitization. Further mechanistic studies are required to evaluate this hypothesis.

Several brain regions evaluated in this study showed no change in ERK_{1/2} phosphorylation following the induction of ethanol behavioral sensitization (e.g., mPFC, amygdala (CeA and BLA), PVN thalamus, and PVN hypothalamus) or ethanol-induced changes that were not modulated by MPEP (e.g., AcbC, dentate gyrus, and lateral septum). However, this finding does not necessarily indicate lack of involvement in the overall neural circuitry of sensitization by these nuclei. Many brain regions have been implicated in behavioral sensitization, which can be divided into induction and expression phases with different brain regions contributing to the neural basis of each [40, 84]. Research has shown that sensitization is mediated by an interconnected network of mesocorticolimbic brain regions (i.e., VTA, nucleus accumbens, prefrontal cortex, amygdala, and thalamus) and

neurotransmitter systems (i.e., dopamine, glutamate, and GABA) that all undergo alterations during drug sensitization [40, 41, 85–87]. Thus, drugs of abuse produce pleiotropic effects on brain systems that regulate behavioral sensitization. Here, we identify mGluR5-mediated changes in ERK_{1/2} signaling in the AcbSh and LHb that are associated with the induction of ethanol sensitization but do not rule out numerous other biochemical and neuroanatomical systems.

For example, although mGlu5 receptors are coupled to ERK_{1/2} activation, evidence indicates that coactivation of mGluR5 and NMDA receptors synergistically increases p-ERK_{1/2} phosphorylation in striatal neurons [20]. Interestingly, the development of ethanol-induced behavioral sensitization is blocked by pretreatment with the NMDA receptor antagonist MK-801 [88] and pretreatment with the mGluR5 antagonist MTEP potentiates the inhibitory effect of MK-801 on the expression ethanol sensitization [49]. This suggests that the expression of ethanol sensitization requires the coordinated action of both mGluR5 and NMDA receptors. We have also shown that MPEP increases the sedative hypnotic effects of ethanol and the NMDA receptor antagonist ketamine [63], which is consistent with coregulation of both low-dose (sensitization) and high-dose (hypnosis) effects of ethanol by mGluR5 and NMDA receptors. Thus, it is plausible that mGluR5 and NMDA receptors interactively modulate changes in ERK_{1/2} signaling associated with ethanol sensitization.

This study has potential limitations that merit consideration. First, MPEP may have direct pharmacological effects on receptors other than mGluR5. In vitro evidence indicates that high concentrations (>20 μ M) of MPEP can inhibit NMDA receptors [89], which regulate expression of ethanol sensitization [90]. Since the NMDA antagonist ketamine shifts ethanol dose-response curve to the left [91], potential MPEP-mediated inhibition of NMDA receptor function could enhance ethanol sedation, which might reduce ethanol-induced locomotor activation during induction and limit sensitization. However, plasma levels in mice 30-minutes after treatment with MPEP (10 mg/kg) were 0.7 μ M, which makes it highly unlikely that levels in the brain following MPEP (30 mg/kg) would be sufficient to affect NMDA receptors directly [64]. In addition, MPEP inhibits norepinephrine uptake in a manner similar to desipramine [92]; however, this effect appears to be mediated through an mGluR5-mediated signaling pathway rather than via direct effects on the norepinephrine transporter [93, 94]. Potential off-target effects could be addressed by evaluating multiple complementary approaches for inhibiting mGluR5 activity including gene knockout or other strategies. Second, results from this study show that ERK_{1/2} phosphorylation was bidirectionally changed in the AcbSh and LHb in a manner correlated with ethanol sensitization and its blockade by MPEP. These data do not address mechanistic regulation of sensitization but raise the hypothesis that inhibition of ERK_{1/2} activation in the AcbSh might block ethanol sensitization. By contrast, ERK_{1/2} inhibition in the LHb might promote sensitization. Finally, this study was conducted with male mice from a single inbred strain, which leaves open the question of generality across a variety of biological variables that might impact the results. Future studies can address generality by evaluating relevant biological variables such as sex, age, and species.

5. Conclusions

In conclusion, locomotor sensitization is a behavioral model of neural plasticity that occurs during initial repeated exposure to drugs of abuse. Here, we show that the induction of ethanol locomotor sensitization requires mGluR5 activity. mGluR5 activity may converge on ERK_{1/2} signaling in the AcbSh and LHB to regulate this maladaptive behavioral response. Future research that evaluates site-specific functions of mGluR5-ERK_{1/2} activity in the AcbSh and LHB is required to evaluate this hypothesis at the mechanistic behavioral level.

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Highlights:

- Repeated alcohol administration produces robust locomotor sensitization in male DBA/2J mice
- Treatment with the mGluR5 antagonist MPEP blunts the induction of sensitization
- Sensitization is associated with changes in ERK1/2 phosphorylation in multiple brain regions
- MPEP administered during induction blocked alcohol-induced changes in ERK1/2 phosphorylation specifically in the nucleus accumbens shell and lateral habenula
- mGluR5-ERK1/2 signaling may be required for the induction of alcohol sensitization

Figure 1. mGluR5 regulation of the induction of Ethanol-induced locomotor sensitization. (A) Timeline of experimental procedures used to test the effects of MPEP pretreatment on the induction of ethanol sensitization. The experiment was conducted in 5 groups of DBA/2J mice over a 14-day period. Treatment was conducted in four phases: Habituation to injections, acute ethanol test, sensitization induction and sensitization test (see Methods for full description of experimental details). Group names are listed on the left and drug injection conditions are shown for each experimental phase. (B) Locomotor response to ethanol (0 or 2 g/kg, IP) administration plotted as a function of test day. * - significantly different from corresponding Veh on Day 4 (between groups), $P < 0.01$; ‡ - significantly different from acute ethanol on Day 4 (within-subject), $P < 0.001$; *ns* – not different from acute ethanol on Day 4 (within subject); † - significantly different from Repeated EtOH (between groups), $P < 0.05$. Data are plotted as $MEAN \pm SEM$ horizontal distance traveled (cm / 10-min, $n = 7 - 8$ mice per group). (C) Ethanol-induced locomotor activity shown as percentage of activity by the Single EtOH treatment group and plotted as a function of treatment condition as a between-groups measure of sensitization. *** - $P = 0.0009$, ** - $P = 0.007$. All symbols and P-values indicate results of multiple comparison procedure of Benjamini, Krieger and Yekutieli.

Figure 2: Effects of the mGluR5 antagonist MPEP on pERK_{1/2} immunoreactivity in the AcbSh. (A) Quantification of pERK_{1/2} immunoreactivity in the AcbSh plotted as the MEAN ($\pm$ SEM) number of pERK_{1/2} immunoreactive cells/mm² in tissue from mice in the 5 treatment conditions on Day 14 (Sensitization Test). * - significant difference between groups, multiple comparison procedure of Benjamini, Krieger and Yekutieli, $P < 0.05$. ** - significant difference via planned comparison $t(10) = 2.52$, $P = 0.03$]. (B) Representative photomicrographs of the cytological pattern of pERK_{1/2} immunoreactivity in AcbSh (20 $\times$ magnification).

Figure 3: Effects of the mGluR5 antagonist MPEP on pERK_{1/2} immunoreactivity in the LHb. (A) Quantification of pERK_{1/2} immunoreactivity in the LHb plotted as the MEAN ($\pm$ SEM) number of pERK_{1/2} immunoreactive cells/mm² in tissue from mice in the four treatment conditions on Day 11 (Sensitization Test). * - indicates statistically significant difference between groups, multiple comparison procedure of Benjamini, Krieger and Yekutieli, $P < 0.05$. ** - significant difference via planned comparison: $t(10) = 5.6$, $P = 0.0002$. (B) Representative photomicrographs of the cytolological pattern of pERK_{1/2} immunoreactivity in LHb (20 $\times$ magnification).

Table 1.

pERK_{1/2} cells / mm² in specific brain regions following the sensitization test on Day 14. Mice received vehicle followed by EtOH (0 or 2 g/kg) as indicated. MPEP was administered as pretreatment to EtOH (0 or 2.5 g/kg) during sensitization induction (Days 5 – 13) but not on the sensitization test day. The Single EtOH group received only vehicle injections throughout the induction procedure and was challenged with EtOH (2 g/kg) on Day 14 as a between group evaluation of sensitization.

Brain Region	MPEP (mg/kg)				
	0		30		Single EtOH (g/kg)
	EtOH(g/kg)		EtOH(g/kg)		
	0	2	0	2	
N. Accumbens (core) [†]	482.9 ± 90.5	814.8 ± 74.5	640.5 ± 83.3	723.4 ± 90.5	546.3 ± 133.3
N. Accumbens (shell) [‡]	637.5 ± 147.2	1331.7 ± 109.4 [*]	805.6 ± 100.1	905.4 ± 128.1	832.7 ± 179
Prefrontal Cortex	338.7 ± 58	426.6 ± 65.6	422.3 ± 94	332.5 ± 24.6	376.7 ± 32.6
Lateral Septum [†]	935 ± 209.8	343.7 ± 99.5	828.5 ± 194.3	401 ± 121.6	723.6 ± 253.6
Dentate Gyrus [†]	16.9 ± 2.3	10 ± 1.3	15.8 ± 1.5	8.3 ± 0.7	4.9 ± 0.5 [#]
Central Amygdala	701.1 ± 209	228.7 ± 34.3	302.7 ± 86.3	311.4 ± 109.2	1624.8 ± 149 [#]
Basolateral Amygdala	277.6 ± 54.5	200.9 ± 100.8	258.4 ± 55.9	158.9 ± 42.2	290 ± 114.5
Lateral Habenula [‡]	1488.3 ± 107.8	608.2 ± 156.6 [*]	1216.2 ± 200.9	1137.8 ± 216.4	1181.4 ± 198 [#]
PVN Thalamus	1299.9 ± 366.1	1114.8 ± 164.1	921.7 ± 164.4	1251.5 ± 161.1	1223.6 ± 122.7
PVN Hypothalamus	1738.4 ± 139.4	2169.8 ± 257.6	1999.7 ± 213	2352.7 ± 228.4	2484.4 ± 382

[†] - significant interaction between ethanol and MPEP.

[‡] - significant main effect of ethanol. Bold text and symbols indicate:

* - significantly different from EtOH (0 g/kg) within MPEP dosage via multiple comparison procedure (interaction), p<0.05

- significantly different from MPEP (0 mg/kg) + EtOH (2 g/kg), planned comparison t-test, p<0.05. Data are shown as MEAN ± SEM.