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CUE-INDUCED REINSTATEMENT OF ALCOHOL-SEEKING BEHAVIOR IS ASSOCIATED WITH INCREASED $ERK_{1/2}$ PHOSPHORYLATION IN SPECIFIC LIMBIC BRAIN REGIONS: BLOCKADE BY THE MGLUR5 ANTAGONIST MPEP

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Abstract

Relapse to alcohol use after periods of abstinence is a hallmark behavioral pathology of alcoholism and a major clinical problem. Emerging evidence indicates that metabotropic glutamate receptor 5 (mGluR5) antagonists attenuate relapse to alcohol-seeking behavior but the molecular mechanisms of this potential therapeutic effect remain unexplored. The extracellular signal-regulated kinase ($ERK_{1/2}$) pathway is downstream of mGluR5 and has been implicated in addiction. We sought to determine if cue-induced reinstatement of alcohol-seeking behavior, and its reduction by an mGluR5 antagonist, is associated with changes in $ERK_{1/2}$ activation in reward-related limbic brain regions. Selectively bred alcohol-preferring (P) rats were trained to lever press on a concurrent schedule of alcohol (15% v/v) vs. water reinforcement. Following 9 days of extinction, rats were given an additional extinction trial or injected with the mGluR5 antagonist MPEP (0, 1, 3, or 10 mg/kg) and tested for cue-induced reinstatement. Brains were removed 90-min later from the rats in the extinction and MPEP (0 or 10 mg/kg) conditions for analysis of p-$ERK_{1/2}$, total $ERK_{1/2}$, and p-ERK_5 immunoreactivity (IR). Cue-induced reinstatement of alcohol-seeking behavior was associated with a 3–5 fold increase in p-$ERK_{1/2}$ IR in the basolateral amygdala and nucleus accumbens shell. MPEP administration blocked both the relapse-like behavior and increase in p-$ERK_{1/2}$ IR. P-$ERK_{1/2}$ IR in the central amygdala and NAcb core was dissociated with the relapse-like behavior and the pharmacological effect of mGluR5 blockade. No changes in total ERK or p-ERK_5 were observed. These results suggest that exposure to cues previously associated with alcohol self-administration is sufficient to produce concomitant increases in relapse-like behavior and $ERK_{1/2}$ activation in specific limbic brain regions. Pharmacological compounds, such as mGluR5 antagonists, that reduce cue-induced $ERK_{1/2}$ activation may be useful for treatment of relapse in alcoholics that is triggered by exposure to environmental events.

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INTRODUCTION

Alcoholism is a complex neuropsychiatric disorder that is characterized by recurring cycles of chronic drinking, abstinence and relapse. Emerging evidence suggests that alcohol and other drugs of abuse may produce maladaptive changes in a variety of molecular and cell signaling pathways that lead to enduring changes in brain structure and function (Crews et al., 1996; Fadda and Rossetti, 1998; Nestler and Aghajanian, 1997). These neuroadaptations are thought to regulate the progressive and recurring behavioral pathologies that occur in alcoholism (Breese et al., 2005; Pandey et al., 2003; Ron and Jurd, 2005). Understanding drug-induced plasticity in brain and behavioral functions is of potential importance for development of new pharmacotherapies for problems associated with alcoholism, such as relapse (Anton et al., 1995; Heinz et al., 2003; Mann, 2004).

A growing number of studies have shown that metabotropic glutamate receptor 5 (mGluR5) activity regulates alcohol-related behavioral processes in animal models. For example, the mGluR5 antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) inhibits the reinforcing (Cowen et al., 2005; Hodge et al., 2006; Schroeder et al., 2005) and discriminative stimulus effects of investigator- (Besheer and Hodge, 2005) and self-administered (Besheer et al., 2006) alcohol in rodents. Similarly, pharmacological blockade of mGluR5 prevents relapse-like behavior as measured by cue-induced reinstatement (Backstrom et al., 2004) and alcohol deprivation procedures (Schroeder et al., 2005). Although these findings indicate mGluR5 blockade attenuates relapse to alcohol-seeking behavior, the molecular or cell signaling mechanisms associated with this potential therapeutic effect remain unexplored.

Extracellular signal-regulated kinase (ERK) is one potential downstream mediator of mGluR5 activity (Yang et al., 2004) that is also known to modulate adaptive processes such as learning, memory, and synaptic plasticity (Sweatt, 2004; Thomas and Huganir, 2004) as well as maladaptive forms of plasticity associated with drug use (Girault et al., 2007; Valjent et al., 2006). The two closely related isoforms of ERK (ERK_1 and ERK_2, or $ERK_{1/2}$) are activated by dual phosphorylation on both a threonine and tyrosine residue by $MEK_{1/2}$ (Anderson et al., 1990); reviewed by (Cobb, 1999). Activated $ERK_{1/2}$ phosphorylates cellular targets or translocates to the nucleus where it activates specific gene transcription factors (Grewal et al., 1999). 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 (Pearson et al., 2001; Qi and Elion, 2005; Treisman, 1996; Valjent et al., 2000; Valjent et al., 2006).

Alcohol exposure produces changes in the active (i.e., phosphorylated) form of $ERK_{1/2}$ (p-$ERK_{1/2}$) in brain regions that are of significance to behavioral pathologies associated with addiction (Chandler and Sutton, 2005; Davis et al., 1999; Kalluri and Ticku, 2002a; Sanna et al., 2002; Tsuji et al., 2003). For example, acute ethanol has been shown to produce a dose- and time-dependent decrease in p-$ERK_{1/2}$ levels in mouse and rat cerebral cortex (Chandler and Sutton, 2005; Kalluri and Ticku, 2002b; Tsuji et al., 2003); but other studies have shown that acute ethanol increases $ERK_{1/2}$ phosphorylation in the Edinger-Westphal nucleus (Bachtell et al., 2002) and amygdala (Pandey et al., 2008). Forced exposure to chronic ethanol and withdrawal alter $ERK_{1/2}$ phosphorylation in brain regions that regulate alcohol self-

administration and relapse-like behavior, including the amygdala (Pandey et al., 2008; Sanna et al., 2002).

Although the mechanisms by which alcohol alters $ERK_{1/2}$ phosphorylation are not fully characterized, converging evidence indicates that mGluR5 may regulate both $ERK_{1/2}$ activation and the neural effects of alcohol. For example, pharmacological activation of Group I metabotropic glutamate receptors (mGluR1 and mGluR5), via site-specific infusion of the agonist DHPG, increases $ERK_{1/2}$ phosphorylation in rat striatum (Choe and Wang, 2001). This effect of the nonselective agonist is fully blocked by the mGluR5 antagonist MPEP in cultured striatal neurons but not altered by an mGluR1 antagonist (Yang et al., 2004), which suggests mGluR5 specificity in $ERK_{1/2}$ activation. Other data show that ethanol inhibits mGluR5 activity *in vitro* (Minami et al., 1998), which could lead to reduced $ERK_{1/2}$ activation as noted above. When taken together with evidence that cue-induced reinstatement of alcohol-seeking behavior has recently been linked to increase $ERK_{1/2}$ phosphorylation in the amygdala (Radwanska et al., 2008), these findings suggest that mGluR5 antagonist-induced effects on relapse-like behavior may be associated inhibition of $ERK_{1/2}$ activation in specific brain regions.

To address this hypothesis, the present study was designed to first determine if the mGluR5 antagonist MPEP would inhibit cue-induced reinstatement of alcohol-seeking behavior in selectively-bred alcohol-preferring (P) rats (Li et al., 1979). Second, we examined p-$ERK_{1/2}$ immunoreactivity (IR) in the nucleus accumbens and amygdala following extinction, reinstatement, and MPEP treatment to determine if the behavioral effects of MPEP are associated with altered $ERK_{1/2}$ activation. These brain regions were chosen for study because they are key elements in the neurobiological regulation of associative learning processes in drug addiction and reward (Everitt et al., 1999), are known to mediate the reinforcing and subjective effects of alcohol (Besheer et al., 2003; Hodge et al., 1995; Hodge and Cox, 1998; Schroeder et al., 2003), and have recently been linked to alcohol relapse-like behavior in rodents (Dayas et al., 2007; Zhao et al., 2006) and cue-induced craving in abstinent human alcoholics (Schneider et al., 2001). Third, we examined total $ERK_{1/2}$ IR to establish if changes in p-$ERK_{1/2}$ IR were associated with altered abundance of the kinase. Finally, adjacent brain sections were processed for p-ERK_5 IR in an effort to address potential specificity of $ERK_{1/2}$ activation in cue-induced reinstatement of alcohol-seeking behavior and its blockade by MPEP.

MATERIALS AND METHODS

Animals

Male alcohol-preferring (P) rats (N=31 total) were bred from a line provided by Indiana University (courtesy of Dr. T.K. Li) and housed two per cage in Plexiglas cages. This rat strain was chosen for study because it has been found to fulfill the requirements of an animal model of alcoholism (Cicero, 1979; Lester and Freed, 1973), including voluntarily consumption of alcohol in quantities that produce significant blood alcohol concentrations (50–200 mg%), self-administration of alcohol for its pharmacological rather than the sensory effects and development of tolerance and dependence through voluntary drinking (Kampov-Polevoy et al., 2000; Li et al., 1987; Murphy et al., 2002). The animal colony room was maintained on a 12L: 12D cycle with the lights on at 07:00. All experimental procedures were conducted under institutional and NIH guidelines.

Self-Administration Apparatus

Self-administration, extinction, and reinstatement sessions were conducted in Plexiglas operant chambers for rats (Med Associates, Georgia, VT). Each chamber was housed in a sound-

attenuating cubicle equipped with a fan that provided ventilation and helped to mask external noise. The left and right wall of each operant chamber was equipped with one ultra-sensitive stainless steel response lever, a cue-light, and a liquid delivery system. Liquid solutions (ethanol or water) were maintained in 60 ml syringes mounted on a programmable pump (PHM-100, Med Associates), which delivered 0.1 ml per activation into a stainless steel cup located to the left of the associated response lever. Head entries into the drinking cup were recorded when an infrared photo beam was broken. Each chamber also contained a house light. The chambers were interfaced (Med Associates) to an IBM-PC compatible computer.

Self-Administration, Extinction, and Reinstatement Procedure

After 2 weeks of adaptation to laboratory housing conditions, fluid access was restricted to 1-h per day (for 2 days only) and the rats were trained to lever press by reinforcement of successive approximations with sucrose (10% w/v) vs. water available as reinforcers on a concurrent fixed ratio-1 (CONC FR1-FR1) schedule of reinforcement during two 16-h overnight sessions. Daily (M-F) 30-min sessions were conducted with sucrose (10% w/v) vs. water available concurrently. When sucrose and water responding stabilized (i.e., <10% daily variation for 5 days), the rats were trained to self-administer concurrent ethanol (15% v/v) vs. water using a sucrose fading procedure (Samson, 1986) as previously described (Hodge et al., 1993). Briefly, ethanol was added to the sucrose solution over a 4-week period and then the sucrose was faded out of the solution until P-rats were self-administering 15% ethanol vs. water. During operant training sessions, responses on the ethanol-paired lever resulted in the presentation of a compound stimulus consisting of a visual cue light and the auditory stimulus of pump activation from a fixed spatial location. Responses on the water-paired lever resulted in the presentation of a similar compound stimulus, but from an alternate fixed spatial location.

Following a 1-month period of baseline responding, operant responding was extinguished by removing all consequences of lever responding (i.e., cue-light, pump sound, and ethanol or water delivery). Following 9 sequential days of extinction training, P-rats responded upon the formerly alcohol and water paired levers equivalently, indicating an extinction of selective responding on the alcohol-paired lever. On day 10, the P-rats were given a reinstatement test in which lever press responses were followed by presentation of the cue light and auditory cue (as during training) *in the absence of ethanol or water reinforcement* to examine cue-induced ethanol-seeking behavior. One hour prior to the reinstatement session 4 separate groups of P-rats were injected with the mGluR5 antagonist MPEP (0, 1, 3 or 10 mg/kg) to determine if MPEP alters cue-induced reinstatement of alcohol-seeking behavior in this genetic model of alcoholism. Each drug dose group consisted of n=6 rats with the exception of the MPEP (1 mg/kg) group, which was n=7 rats. A fifth group of P-rats (n=6) was given another day of extinction training to serve as a control for the reinstatement conditions. Groups were matched on body weight, baseline response totals, and extinction performance prior to drug testing.

Drugs

Ethanol solutions were prepared by mixing appropriate volumes of ethanol (95% v/v) and distilled water. Sucrose (w/v) solutions consisted of granulated cane sugar dissolved in distilled water. Ethanol, sucrose, and water solutions were presented at room temperature. MPEP (Tocris Bioscience, Ellisville, Missouri) was dissolved in physiological saline and injected in a volume of 1 ml/kg body weight.

Immunohistochemistry

Ninety minutes after the reinstatement test session, rats were deeply anesthetized with pentobarbital (100 mg/kg IP) and transcardially perfused with ice cold 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde. The intact skulls were postfixed overnight, and then rinsed with PBS. Brains were removed from the skull and stored

in PBS at 4°C. Coronal vibratome sections (40 μm) were collected and stored (−20°C) in cryoprotectant until immunohistochemistry processing. Endogenous peroxidase was blocked by incubating the free floating sections for 10 min in 0.6% H_2O_2 followed by citra buffer antigen retrieval performed at 70°C for 30 min (Antigen Retrieval Citra, BioGenex, San Ramon, CA). Sections were blocked in PBS/0.1%triton-x/4% horse serum for 30 minutes and incubated at +4°C overnight in primary polyclonal antibody to p-$ERK_{1/2}$ (1:400), $ERK_{1/2}$ (1:500), or p-ERK_5 (1:1000) from Cell Signaling Technology (Danvers, MA). The sections were then incubated in secondary antibody for one hour using the Dako EnVision Kit (Dako, Carpinteria, CA). Immunoreactivity was detected with nickel-enhanced diaminobenzidine (Dako EnVision Kit) as a chromagen. Sections were then counterstained with toluidine blue, mounted, dried and coverslipped with Cytoseal. For consistency of staining across subjects, brain tissue from all groups was processed simultaneously.

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita model, QImaging, Burnaby, BC) interfaced to a desktop computer (Dell, Round Rock, TX). Image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, TN) was used to quantify immunoreactivity. The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition. Pixel density and cell count measurements were calculated from a circumscribed field (e.g., brain region) and divided by the area of the region and expressed as pixels/mm^2. Immunoreactivity data were collected by a researcher blind to treatment conditions. Data for p-$ERK_{1/2}$ IR data were acquired from a minimum of 4 sections/brain region/animal and averaged to obtain a single value per subject. Total $ERK_{1/2}$ and p-ERK_5 IR were evaluated in a single section adjacent to those used for p-$ERK_{1/2}$ from each animal. The brain regions examined were the nucleus accumbens (core and shell; AP +1.7 to 1.0 mm; see Figure 2B) and the amygdala (basolateral and central nucleus; AP −2.8 to 3.3 mm; see Figure 2C).

Data Analysis

The principal behavioral parameters of operant ethanol self-administration analyzed were number of ethanol and water lever presses, volume of ethanol consumed (mls), and response latency (i.e., temporal delay to the first ethanol response). Ethanol intake (g/kg) was determined using each animal's daily body weight and the volume of ethanol delivered per reinforcer (0.1 ml of 15% v/v ethanol). Extinction data were analyzed statistically by two-way repeated measures analysis of variance (RM ANOVA) with factors for lever (ethanol vs. water) and day. MPEP dose effects on reinstatement were analyzed by two-way RM ANOVA with factors for drug dose (between subjects) and response lever (within subject). Immunohistochemistry data were analyzed statistically by one-way ANOVA. Significant main effects and interactions were followed by post hoc multiple comparisons (Tukey test) test using SigmaStat v. 3.0 (Systat Software, La Jolla, CA)

RESULTS

Baseline Performance

On the last day of baseline P-rats from all treatment groups combined (N=31 total) responded significantly more on the ethanol lever as compared to the concurrently available water lever ($F(1,30)=66.8$, $p<0.0001$) (Figure 1A, left). This resulted in an average ethanol dose of 0.81 ± 0.06 g/kg/30-min with a strong preference for ethanol over water of 87.5%, which is typical for this genetically selected rat line (Besheer et al., 2008; Schroeder et al., 2005). A two-way ANOVA (Reinforcer X Treatment Condition) conducted on data from the last day of baseline showed that there were no differences in total ethanol and water reinforced responding among the groups of rats that went on to receive extinction only or extinction followed by MPEP (0,

1, 3, or 10 mg/kg, IP) administration prior to cue-induced reinstatement ($F(4, 52)=0.32$, $p = 0.86$, ns). Thus, any subsequent differences seen on reinstatement tests were not likely related to differential baseline performance.

Extinction of Alcohol Self-Administration

During the nine days of extinction, rats from all treatment groups (N=31 total) emitted significantly more responses on the lever that previously produced ethanol reinforcement ($F(1,30) = 155$, $p < 0.001$) (Figure 1A, right). Although responding on both ethanol and water levers decreased as a function of time ($F(8,240) = 58$, $p <0.001$), the magnitude of change was greater for responses on the ethanol lever as indicated by the significant statistical interaction between reinforcement condition (ethanol × water) and time ($F(8,240) = 44.5$, $p < 0.001$). Multiple comparison procedures (Tukey test) showed that responses on the ethanol-associated lever decreased as a function of time and were not different from water responding on the last two days (8 and 9) of extinction (Figure 1B, right). Additional analysis of extinction performance using separate two-way ANOVAs showed that there were no differences in total ethanol ($F(4,208) = 0.32$, ns) or water ($F(4,208) = 0.67$, $p = 0.62$, ns) lever responses during the nine days of extinction between the five treatment groups that went on to receive EXTINCTION or EXTINCTION followed by MPEP (0, 1, 3, or 10 mg/kg). These data show extinction of ethanol reinforced responding (i.e., equivalent responding on levers that previously produced ethanol and water reinforcement) in alcohol preferring P-rats when all consequences of lever pressing were removed for 9 days. In addition, subsequent group differences on reinstatement tests were not a function of differential between-group rates of extinction.

Cue-induced Reinstatement of Alcohol-Seeking Behavior: Blockade by MPEP

On day 10 of the procedure, one group of rats was administered a saline pretreatment injection and continued under the extinction condition. The other four groups of rats were pretreated with MPEP (0, 1, 3, or 10 mg/kg) and underwent reinstatement tests in which responses on either the ethanol- or water-associated lever resulted in presentation of discrete cues (stimulus light above the lever and pump sound) that were previously paired with reinforcer delivery during baseline. Overall, the behavioral data demonstrate cue-induced reinstatement of alcohol-seeking behavior that was blocked by the mGluR5 antagonist MPEP (Figure 1B).

Statistical analysis using a two-way repeated measures ANOVA with one factor repetition revealed a significant main effect of reinforcement condition ($F (1, 4) = 34.7$), $p < 0.001$ due to an overall increase in responding on the ethanol-associated lever across the 5 treatment conditions (Figure 1B). There was also a significant main effect of treatment condition ($F (4, 26) = 4.9$, $p = 0.004$) and interaction between treatment and reinforcement conditions ($F (4, 26) = 4.4$, $p = 0.007$), which indicates that the increases in responding on the ethanol lever were dependent on the treatment condition (i.e., Extinction or MPEP dose). Multiple comparison procedures (Tukey test) comparing extinction performance to MPEP (0 mg/kg) showed significant cue-induced reinstatement of ethanol-seeking behavior with no change in responding on the concurrently available water lever (Figure 1B). Moreover, MPEP (1 and 10 mg/kg) selectively blocked cue-induced reinstatement of ethanol-seeking behavior with no significant suppression of water responding (Figure 1B). However, the effect of the middle dose of MPEP (3 mg/kg) did not reach statistical significance due to variability in performance of one rat that responded on the ethanol-associated lever at a rate that was 3 standard errors above the group mean. Otherwise, the response totals following MPEP (3 mg/kg) were indistinguishable from those following the low dose.

$ERK_{1/2}$ Activation

Nucleus Accumbens (shell and core)—Cue-induced reinstatement of alcohol-seeking behavior and its blockade by MPEP (10 mg/kg) was associated with an overall significant change in p-$ERK_{1/2}$ IR in the shell of the nucleus accumbens ($F(2, 15) = 45.5$, $p < 0.0001$). Multiple comparison procedures showed that cue-induced reinstatement of alcohol-seeking was associated with a 5-fold increase in p-$ERK_{1/2}$ IR as compared to the extinction condition (Tukey, $p<0.05$; Figure 2A). Administration of a dose of the mGluR5 antagonist MPEP (10 mg/kg) that blocked cue-induced reinstatement of alcohol-seeking behavior also completely blocked the increase in p-$ERK_{1/2}$ IR that occurred following saline administration (Tukey, $p<0.05$; Figure 2A). Additional analysis showed that the increase in p-$ERK_{1/2}$ IR in the nucleus accumbens shell was the result of a concomitant 5-fold increase ($F(2, 15) =61$, $p < 0.0001$) in the number of p-$ERK_{1/2}$ positive cells / mm^2: mean±SEM cells: Ext – (53.25 ±11.48); saline – (281.5±29.86); MPEP 10 mg/kg – (10.40±4.776) indicating a cellular pattern of $ERK_{1/2}$ activation. No significant ($F(2, 15) = 0.84$, $p = 0.4$, ns) changes were observed in total $ERK_{1/2}$ IR (Table 1) or p-ERK_5 IR (Table 2), suggesting that the observed changes were specific to $ERK_{1/2}$ phosphorylation and not related to altered abundance of the kinase or general activation of mitogen activated protein kinase (MAPK) pathways. Representative photomicrographs illustrating the cytological pattern of p-$ERK_{1/2}$ IR in the nucleus accumbens shell following extinction and MPEP (0 or 10 mg/kg) are shown in Figure 2C.

In the nucleus accumbens core, there was an overall trend toward a significant change in p-$ERK_{1/2}$ IR associated with cue-induced reinstatement but it failed to reach statistical significance ($F(2, 16) = 3.5$, $p = 0.054$). Pixel density values (mean±SEM: extinction – 1310 ±117.7; saline – 1801±207.8; MPEP 10 mg/kg – 1224±151.8) suggested that p-$ERK_{1/2}$ IR following the saline-reinstatement condition may have been greater than following extinction but a planned comparison (t-test) between these two conditions also failed to reach statistical significance ($t(11) = 1.9$, $p = 0.07$), which further confirms the lack of significant change in p-$ERK_{1/2}$ IR in the nucleus accumbens core. There was also no change in total-$ERK_{1/2}$ IR (Table 1) or p-ERK_5 IR (Table 2) in the nucleus accumbens core.

Amygdala (basolateral and central)—In the basolateral amygdala, cue-induced reinstatement of alcohol-seeking behavior was associated with a significant change in p-$ERK_{1/2}$ IR ($F(2, 15)=26.4$, $p < 0.0001$). Multiple comparison procedures indicated that the overall significant difference was due to a 3-fold increase in p-$ERK_{1/2}$ IR following the saline-reinstatement condition as compared to extinction (Tukey, $p<0.05$; Figure 3A). Administration of MPEP (10 mg/kg) prior to the reinstatement test session completely blocked the increase in p-$ERK_{1/2}$ IR that followed saline administration (Tukey, $p<0.05$; Figure 3A). Cell count analysis showed that the increase in p-$ERK_{1/2}$ IR in the basolateral amygdala was associated with a 59% increase ($F(2, 15) = 7.2$, $p = 0.006$) in the number of p-$ERK_{1/2}$ positive cells / mm^2: mean±SEM cells: Ext – (153±42); saline – (244±36); MPEP 10 mg/kg – (49±18.7) that the increase in immunoreactivity was partially influenced by an increase in the number of p-$ERK_{1/2}$ positive cells. No changes were observed in total-$ERK_{1/2}$ (Table 1) or p-ERK_5 IR (Table 2) indicating that observed changes in $ERK_{1/2}$ phosphorylation were not related to an overall change in kinase abundance or nonspecific activation of MAPK pathways. Figure 3C shows representative photomicrographs of the basolateral amygdala illustrate the cytological pattern of p-$ERK_{1/2}$ IR under extinction and reinstatement conditions.

By contrast, cue-induced reinstatement of alcohol-seeking behavior was not associated with changes in p-$ERK_{1/2}$ IR in the central amygdala (i.e., saline-reinstatement not different from extinction). However, one-way ANOVA identified an overall significant $F(2,15) = 3.9$, $p = 0.04$) change in p-$ERK_{1/2}$ IR, which was due primarily to a significant reduction following MPEP (10 mg/kg) during reinstatement as compared to extinction (Tukey, $p < 0.05$; mean

±SEM pixel density values: extinction – 3343±570; saline – 2522±513 ; MPEP 10 mg/kg – 1555±118). This MPEP-induced inhibition of p-$ERK_{1/2}$ IR was associated with a trend toward a reduction in p-$ERK_{1/2}$ positive cells (data not shown). No changes were observed in total $ERK_{1/2}$ IR in the central amygdala (Table 1) indicating that the effects of MPEP (10 mg/kg) were associated with a specific change in $ERK_{1/2}$ phosphorylation and not abundance of the kinase. There was also no change in p-ERK_5 IR in the central amygdala (Table 2).

DISCUSSION

The results of this study show that the mGluR5 antagonist MPEP blocks relapse-like behavior and an associated upregulation of $ERK_{1/2}$ activation. Using a rat genetic model of alcoholism, we found that reinstatement of alcohol-seeking behavior, produced by response contingent presentation of alcohol-associated cues, is associated with a profound 3 – 5 fold increase in $ERK_{1/2}$ phosphorylation specifically in the nucleus accumbens shell and basolateral nucleus of the amygdala. This upregulation of $ERK_{1/2}$ phosphorylation was solely attributable to cue exposure and the subsequent alcohol-seeking behavior since no alcohol was presented on the test day. Both the increase in $ERK_{1/2}$ phosphorylation and relapse-like behavior was completely blocked by the mGluR5 antagonist MPEP. We found no changes in total $ERK_{1/2}$ or in ERK_5 phosphorylation, suggesting some degree of specificity for $ERK_{1/2}$ activation in cue-induced reinstatement. These findings indicate that cue-induced relapse to alcohol-seeking behavior is associated with an upregulation of $ERK_{1/2}$ phosphorylation (i.e., activation) specifically in sub-nuclei of the amygdala-ventral striatal pathway.

Several recent studies have shown that alcohol exposure alters $ERK_{1/2}$ phosphorylation. Moderate doses of acute alcohol (1.5 – 3.5 g/kg) produce a dose- and time-dependent decrease in p-$ERK_{1/2}$ in mouse cortex (Kalluri and Ticku, 2002a). Similarly, another recent study extended these findings by showing that acute ethanol (3.5 g/kg) reduced p-$ERK_{1/2}$ in the cerebral cortex and hippocampus in rat pups ranging from PND 5 to adulthood (Chandler and Sutton, 2005). Evidence also indicates that the ERK/MAPK system may regulate adaptive changes associated with chronic ethanol exposure and abstinence (Pandey et al., 2008). Forced exposure to chronic ethanol vapor (blood alcohol levels of approximately 200 mg%) suppressed p-$ERK_{1/2}$ immunoreactivity in the amygdala, cortex, cerebellum, and dorsal striatum in rats (Sanna et al., 2002). Conversely, the same study showed that withdrawal from ethanol vapor resulted in a time-dependent increase in p-$ERK_{1/2}$ levels that peaked at 13-h post withdrawal and remained elevated at 24-h (Sanna et al., 2002). Our results show that response-contingent exposure to stimuli (i.e., cues) that were previously paired with moderate levels of self-administered alcohol is sufficient to induce a 5-fold increase in $ERK_{1/2}$ phosphorylation specifically in the nucleus accumbens shell and a 3-fold increase in the basolateral amygdala in alcohol preferring P-rats, and a 3-fold increase in alcohol-seeking behavior. These data in alcohol preferring P-rats confirm and extend prior results showing that non-contingent exposure to alcohol paired stimuli increases p-$ERK_{1/2}$ IR in the lateral amygdala of Wistar rats (Radwanska et al., 2008). Together, these studies demonstrate that alcohol exposure, withdrawal, and cue-induced reinstatement are associated with adaptive changes in $ERK_{1/2}$ activation in the mammalian brain.

In the present study, we also found that systemic administration of the mGluR5 antagonist MPEP completely blocked cue-induced increases in both $ERK_{1/2}$ phosphorylation and alcohol-seeking behavior, which raises the hypothesis that an mGluR5-$ERK_{1/2}$ pathway may regulate relapse to alcohol-seeking behavior. This interpretation is consistent with evidence showing that presentation of cues previously paired with ethanol injection can induce glutamate release (De Witte, 2004) and that activation of Group I metabotropic glutamate receptors (mGluR1 and 5) increases $ERK_{1/2}$ phosphorylation in striatal neurons *in vivo* (Choe and Wang, 2001). Thus, we propose that exposure to alcohol-associated cues may upregulate $ERK_{1/2}$

phosphorylation via increased glutamate activity at mGluR5 receptors in the nucleus accumbens shell and basolateral amygdala, which in turn mediates relapse-like behavior. However, increases in $ERK_{1/2}$ phosphorylation are modulated by activity of a variety of cell surface proteins, including NMDA receptors (Valjent et al., 2000), which are coupled to mGluR5 and also regulate relapse-like behavior (Backstrom and Hyytia, 2006). For this reason, it remains to be determined whether mGluR5 blockade inhibited cue-induced reinstatement of alcohol-seeking behavior via $ERK_{1/2}$ inhibition or whether the two effects of the mGluR5 antagonist were independent processes. Functional involvement of $ERK_{1/2}$ activity in specific brain regions can be evaluated using a variety of approaches such as microinjection of a specific $MEK/ERK_{1/2}$ inhibitor in specific brain regions, which has been shown to inhibit reinstatement of cocaine-seeking behavior (Lu et al., 2005).

The results of this study complement and extend a growing body of preclinical studies showing functional regulation of ethanol self-administration and relapse by metabotropic glutamate receptor activity. For instance, the mGluR5 antagonist MPEP reduces maintenance of operant alcohol self-administration as well as abstinence- and cue-induced relapse in rats (Backstrom et al., 2004; Schroeder et al., 2005) in the dose range of 2.5–10 mg/kg which has no effect on exploratory locomotor behavior in rodents (Henry et al., 2002; Tatarczynska et al., 2001). Other studies have shown that MPEP, or the more recently available mGluR5 antagonist MTEP, reduce alcohol self-administration in mice and rats under a variety of experimental conditions (Besheer et al., 2008; Cowen et al., 2005; Cowen et al., 2007; Hodge et al., 2006), which demonstrates interspecies generality and further supports specific involvement of mGluR5. In addition, *agonism* of presynaptic mGluR2/3 attenuates the ability of formerly drug-paired cues to reinstate responding (Zhao et al., 2006). Thus, it appears that inhibition of glutamate activity by blockade of postsynaptic mGluR5, or activation of presynaptic mGluR2/3, is sufficient to reduce alcohol self-administration and relapse-like behavior. It will be of interest to determine if reduced $ERK_{1/2}$ activation is a common downstream mechanism by which mGluR5 antagonists or mGluR2/3 agonists prevent cue-induced reinstatement of alcohol-seeking behavior via inhibition of glutamate transmission

The results of this study are also consistent with numerous other studies implicating the basolateral amygdala and nucleus accumbens shell in relapse-like behavior. For example, evidence suggests that the basolateral nucleus of the amygdala regulates cue-induced reinstatement of cocaine- and food-seeking behavior (i.e., (Fuchs et al., 2006; Ghitza et al., 2003; Ledford et al., 2003; McLaughlin and Floresco, 2007; See et al., 2003). Functional inactivation of the BLA by excitotoxic lesions (Meil and See, 1997), microinjection of tetrodotoxin (Grimm and See, 2000) or lidocaine (Kantak et al., 2002) have all been shown to inhibit reinstatement of cocaine-seeking behavior following extinction (reviewed by (Everitt and Wolf, 2002). Other evidence indicates that activation of dopamine (Schmidt et al., 2006; Schmidt and Pierce, 2006) or Group II mGluRs (Bossert et al., 2006) in the nucleus accumbens shell regulates reinstatement of cocaine and heroin seeking-behavior. Overall, the results from these and numerous other studies suggest that the nucleus accumbens shell and basolateral amygdala are part of a mesocorticolimbic neural circuit that regulates cue-induced reinstatement of alcohol, drug, and food-seeking behavior after extinction.

Regulation of cue-induced reinstatement of alcohol-seeking behavior by an mGluR5 – $ERK_{1/2}$ pathway in the basolateral amygdala and nucleus accumbens shell is consistent with the more general role of these proteins and brain regions in learning and memory. Considerable evidence indicates that the basolateral amygdala modulates memory consolidation in a number of tasks (Fuchs et al., 2006; Hatfield and McGaugh, 1999; Hsu et al., 2002; Lalumiere et al., 2004; Miranda et al., 2003; Schroeder and Packard, 2002, 2003, 2004) and growing evidence implicates ERK/MAPK and mGluR5 in these effects. For example, $ERK_{1/2}$ is activated (i.e., phosphorylated) specifically in the lateral amygdala 60 min after Pavlovian fear conditioning

in a manner that is dependent on tone-shock pairings, and site-specific infusion of the MEK/ERK inhibitor U0126 in the lateral amygdala impaired both long-term memory of Pavlovian fear conditioning and long-term potentiation in the lateral amygdala. This suggests that activation of the ERK/MAPK pathway is required for memory consolidation and synaptic plasticity in the amygdala (Schafe et al., 2000). Similarly, infusion of the mGluR5 antagonist MPEP in the amygdala blocks acquisition of contextual fear conditioning and long-term potentiation at thalamic input synapses to the lateral amygdala (Rodrigues et al., 2002). Moreover, the BLA has a direct glutamatergic projection to the nucleus accumbens (Kelley et al., 1982; Robinson and Beart, 1988) and evidence indicates that the basolateral amygdala and nucleus accumbens shell (but not the core) interactively modulate memory consolidation (LaLumiere et al., 2005). Thus, it is plausible that increased $ERK_{1/2}$ phosphorylation in the basolateral amygdala and nucleus accumbens shell in the present study may reflect activation of a memory circuit that processes information about stimuli that are paired with alcohol during voluntary self-administration. Together, these results underscore the many conceptual and mechanistic overlaps between the neurobiology of addiction and memory (Kelley, 2004).

Although the present data raise the possibility that that the mGluR5 antagonist MPEP blocked cue-induced reinstatement of alcohol-seeking by inhibiting the associated increase in $ERK_{1/2}$ phosphorylation, a number of potential alternative explanations and limitations to this study should be discussed. First, an alternative to the interpretation that MPEP specifically reduced cue-induced reinstatement is that injection of the mGluR5 antagonist may have reduced general locomotor activity. However, evidence indicates that the highest dose of MPEP (10 mg/kg) tested in the present study has no effect on exploratory locomotor behavior in P-rats with a history of alcohol self-administration (Besheer et al., 2008). Second, MPEP may have reduced cue-induced reinstatement by producing a negative affective state in the subjects. This possibility also appears unlikely as previous research indicates that MPEP does not produce a conditioned place preference or conditioned place aversion (McGeehan and Olive, 2003; Popik and Wrobel, 2002). Moreover, MPEP alone does not produce a subjective (i.e., discriminative) stimulus effect that resembles alcohol (Besheer and Hodge, 2005), which suggests that the compound did not act as an alcohol substitute. Third, a limitation of the present study is that these results show an association between the behavioral effects of MPEP and the changes in $ERK_{1/2}$ activation but do not demonstrate functional involvement of $ERK_{1/2}$ in reinstatement. Studies that directly manipulate $ERK_{1/2}$ activation either systemically or in specific brain regions are required to show functional involvement. Fourth, it will be important in future studies to dissociate the effects of alcohol-seeking from cue presentation on $ERK_{1/2}$ activation to rule out any potential interactions between lever press behavior and subsequent changes in $ERK_{1/2}$ phosphorylation. This can be addressed by including a cue-only control or conducting studies examining effects of contextual cues, which may alter $ERK_{1/2}$ activation. Finally, one of the main goals of this study was to examine cue-induced changes in $ERK_{1/2}$ activity after extinction of alcohol-seeking behavior; however, this approach did not address potential effects of extinction on $ERK_{1/2}$ activity, which might be expected since extinction is an active learning process (Schroeder and Packard, 2003).

Similar to $ERK_{1/2}$, extracellular-signal-regulated kinase 5 (ERK_5) is a member of the multigene MAP kinase family that is activated (i.e., phosphorylated) by MEK5. Although $ERK_{1/2}$ is activated by the separate $MEK_{1/2}$ pathway, these two MAP kinases share a number of structural and functional properties that suggest common activities. For example, both $ERK_{1/2}$ and ERK_5 have a Thr–Glu–Tyr activation motif and show 50% homology of the N-terminal catalytic domain (Nishimoto and Nishida, 2006), are activated by platelet-derived growth factor (Abe et al., 1996), and are inhibited by the commonly used $MEK_{1/2}$ inhibitors, PD98059, U0126, and PD184352 (Kamakura et al., 1999; Mody et al., 2001), which are commonly thought to specifically inhibit $ERK_{1/2}$ activation. For these reasons, we also examined immunoreactivity of the phosphorylated (i.e., active) form of ERK_5 (p-ERK_5) in the nucleus

accumbens and amygdala as a measure of $ERK_{1/2}$ specificity. P-ERK_5 IR was detected at high levels in all subnuclei of the nucleus accumbens and amygdala. However, no changes were observed in p-ERK_5 IR following cue-induced reinstatement of alcohol-seeking behavior or administration of MPEP. These data indicate that the observed effects on $ERK_{1/2}$ phosphorylation were not associated with nonspecific activation of MAPK pathways. It remains to be determined if phosphorylation of other MAPKs, such as JNK or p38, is altered during relapse-like behavior.

In conclusion, the MAP kinases have been associated with many of the negative health effects of alcohol including liver disease, pancreatitis, cancer risk, neurotoxicity, and cardiovascular disease (Aroor and Shukla, 2004). Results of this study show for the first time that $ERK_{1/2}$ is activated in specific sub-nuclei of the amygdala-ventral striatal pathway by response-contingent presentation of alcohol-associated stimuli. Concomitant blockade of the $ERK_{1/2}$ activation and relapse-like behavior by the mGluR5 antagonist MPEP suggests that cue-induced reinstatement of alcohol-seeking may be functionally regulated by an mGluR5 – $ERK_{1/2}$ pathway. Overall, these results provide further support for the potential utility of mGluR5 antagonists in the treatment of problems associated with alcoholism such as relapse, and provide a novel molecular mechanism by which these compounds may change brain and behavioral function.

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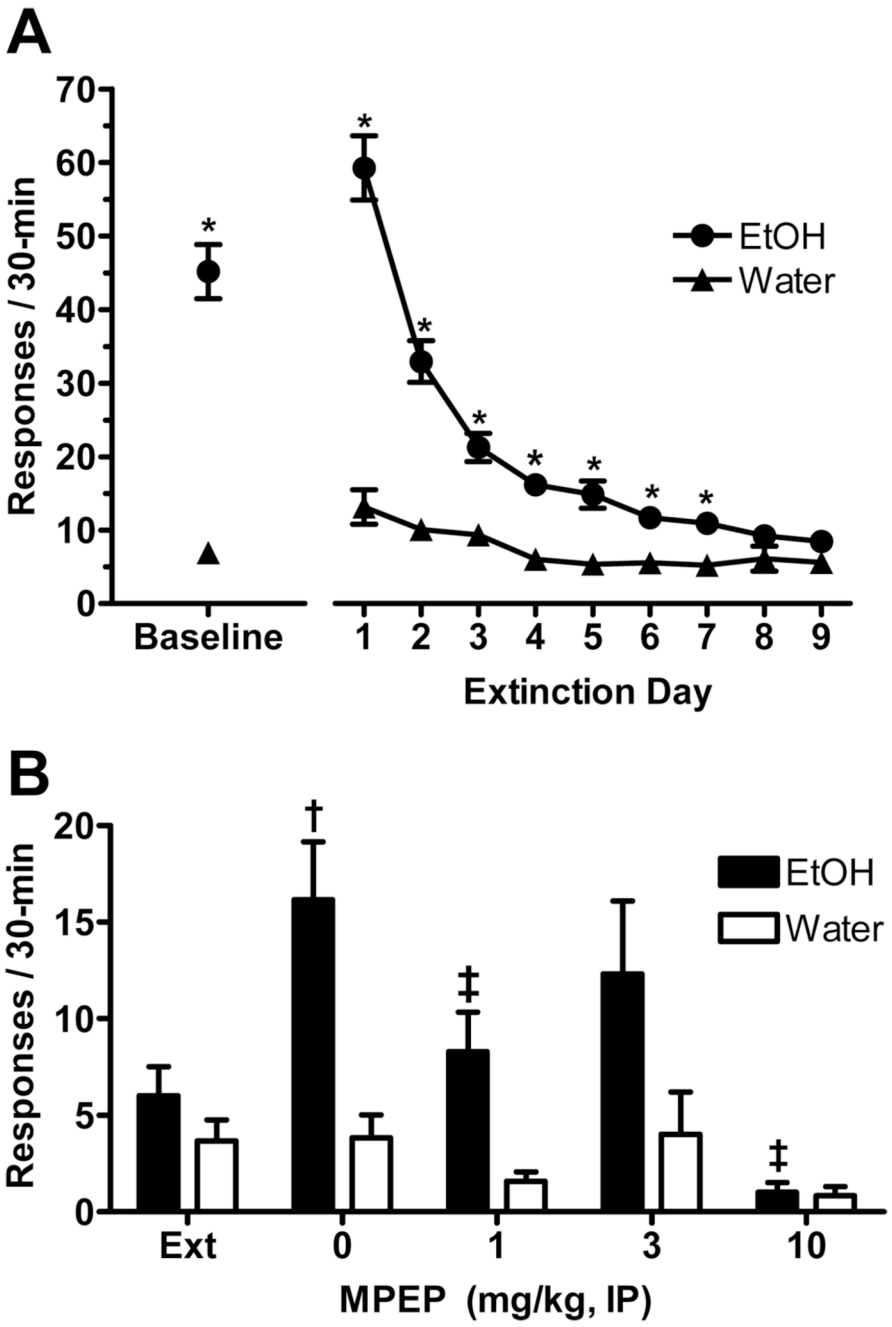

Figure 1.

(A) Extinction of alcohol-reinforced lever pressing. Data are plotted as mean (± SEM) total responses per session on the ethanol and water levers on the last day of baseline (left) and through the 9 days of extinction training (right). Data represent an average of 5 treatment groups (N=31) shown in the lower panel. (**B) Effect of the mGluR5 antagonist on cue-induced reinstatement of alcohol-seeking behavior**. Data are shown as mean (± SEM) total responses on day 10 of the procedure. Bars show performance of a group that continued on extinction training (Ext) as compared to groups that received MPEP (0 – 10 mg/kg) prior to a cue-induced reinstatement test session. Data represent mean of n=6–7 rats from each drug dose / condition. * - significantly different from water on the same day; † - significantly different from Ext within

the same reinforcement condition; ‡ - significantly different from MPEP (0 mg/kg) within the same reinforcement condition, $p < 0.05$, Tukey test.

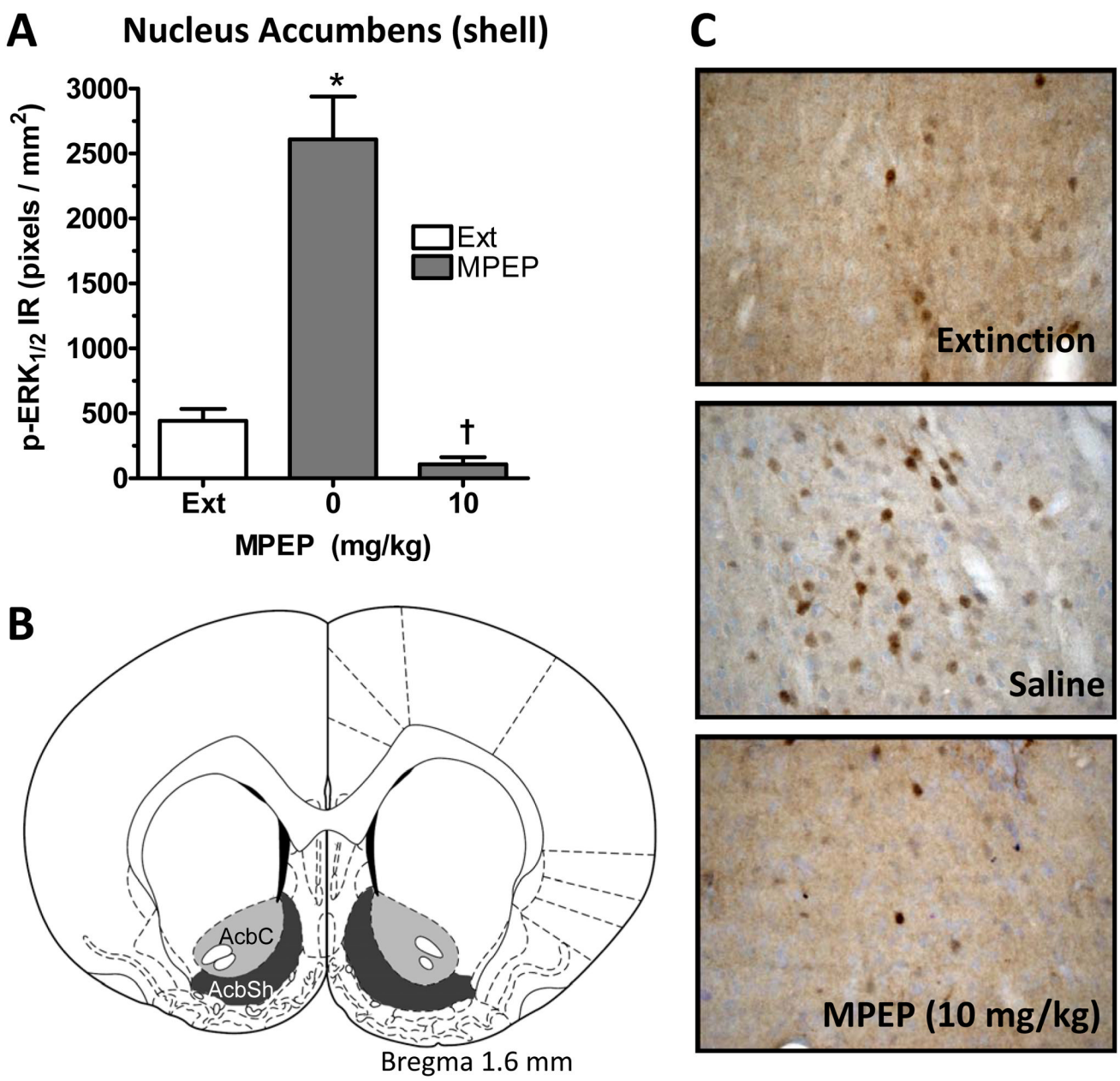

Figure 2. p-ERK$_{1/2}$ IR in nucleus accumbens shell

(**A**) Mean (± S.E.M.) p-ERK$_{1/2}$ immunoreactivity (pixels/mm^2) in the nucleus accumbens shell following extinction (Ext) or cue-induced reinstatement with MPEP (0 or 10 mg/kg) pretreatment. (**B**) Illustration of brain regions analyzed (adapted from Paxinos and Watson, 1998; nucleus accumbens core – AcbC; nucleus accumbens shell – AcbSh). (**C**) Representative photomicrographs of the cytological pattern of p-ERK$_{1/2}$ IR in the nucleus accumbens shell following Extinction or cue-induced reinstatement with MPEP (0 or 10 mg/kg) pretreatment. Data represent means of multiple brain sections from n=6 rats per condition. * - indicates significantly different from Ext; † - significantly different from MPEP (0 mg/kg), $p<0.05$, Tukey test.

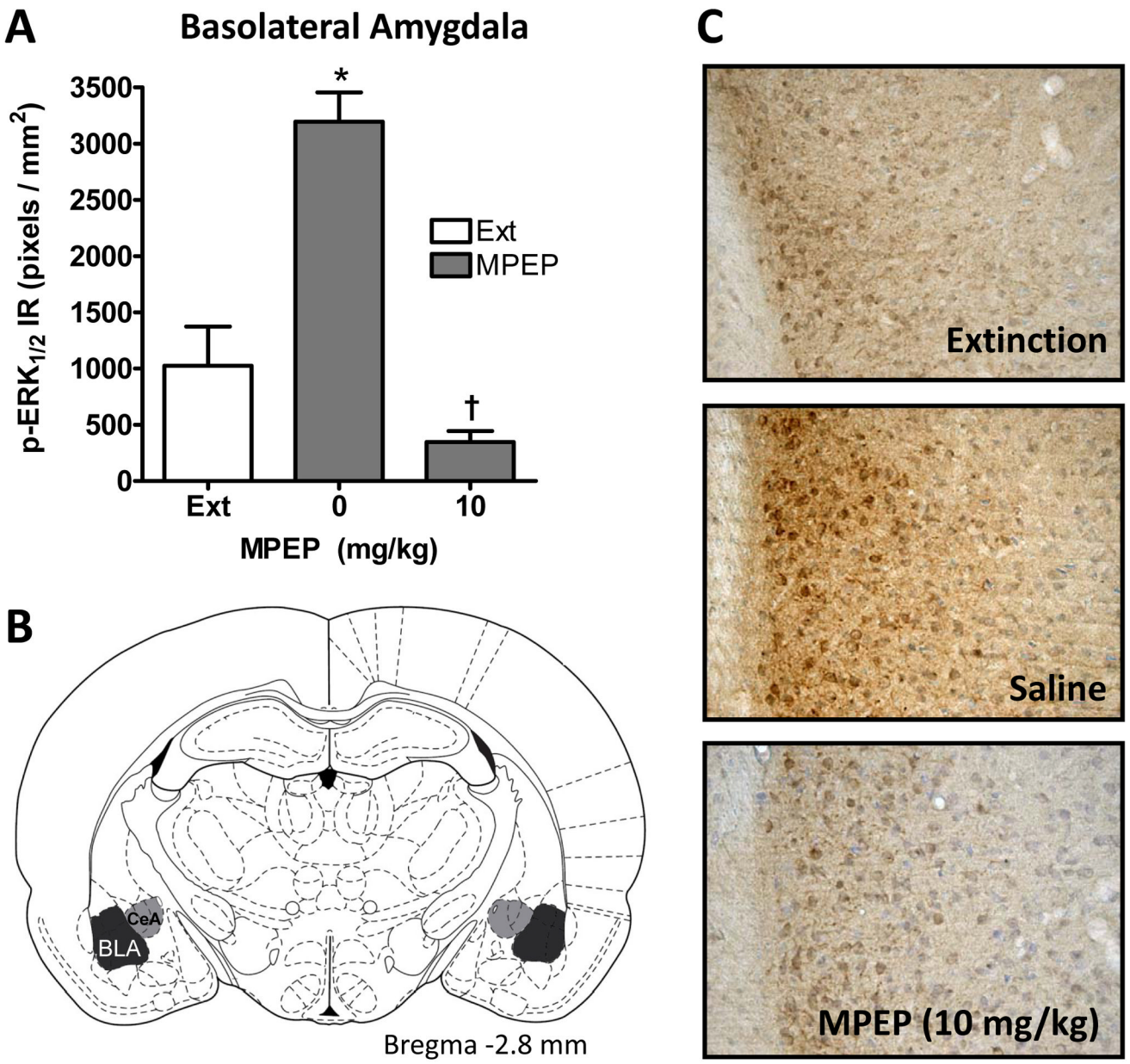

Figure 3. p-ERK$_{1/2}$ IR in basolateral amygdala

(**A**) Mean (± S.E.M.) p-ERK$_{1/2}$ IR (pixels/mm^2) in the basolateral amygdala following extinction (Ext) or cue-induced reinstatement with MPEP (0 or 10 mg/kg) pretreatment. (**B**) Illustration of brain regions analyzed (adapted from Paxinos and Watson, 1998; basolateral amygdala - BLA; central nucleus of the amygdala – CeA. (**C**) Representative photomicrographs of the cytological pattern of p-ERK$_{1/2}$ IR in the basolateral amygdala following Extinction or cue-induced reinstatement with MPEP (0 or 10 mg/kg) pretreatment. Data represent means of multiple brain sections from n=6 rats per condition. * - indicates significantly different from Ext; † - significantly different from MPEP (0 mg/kg), $p<0.05$, Tukey test.

Table 1

Measures of total $ERK_{1/2}$ immunoreactivity in the N. Accumbens and Amygdala.

Brain Region	Treatment	Total $ERK_{1/2}$ IR (pixels / mm^2)
Nucleus Accumbens		
Shell	Ext	12403 ± 1931
	Veh	9247 ± 1342
	MPEP (10 mg/kg)	9770 ± 1995
Core	Ext	9867± 991.8
	Veh	11823 ± 1488
	MPEP (10 mg/kg)	12417 ± 2136
Amygdala		
Basolateral	Ext	8679 ± 1456
	Veh	7025 ± 829
	MPEP (10 mg/kg)	10248 ± 1472
Central	Ext	10650 ± 1238
	Veh	10379 ± 488.8
	MPEP (10 mg/kg)	7956 ± 905

Abbreviations – Extinction (Ext); Vehicle (Veh)

Table 2

Measures of p-ERK$_5$ immunoreactivity in the N. Accumbens and Amygdala.

Brain Region	Treatment	p-ERK$_5$ IR (pixels / mm^2)	p-ERK$_5$ Positive Cells (cells / mm^2)
Nucleus Accumbens			
Shell	Ext	18824 ± 3967	1709 ± 249.4
	Veh	14675 ± 2488	1591 ± 194.6
	MPEP (10 mg/kg)	15869 ± 2490	1622 ± 167.8
Core	Ext	5626 ± 1211	864.2 ± 149.4
	Veh	6336 ± 2246	990.0 ± 293.2
	MPEP (10 mg/kg)	5902 ± 719.1	958.6 ± 90.10
Amygdala			
Basolateral	Ext	6116 ± 1506	741.3 ± 113.9
	Veh	3870 ± 699.6	548.4 ± 72.78
	MPEP (10 mg/kg)	5471 ± 1695	619.4 ± 135.5
Central	Ext	19226 ± 2360	1749 ± 50.48
	Veh	19046 ± 2941	1717 ± 86.25
	MPEP (10 mg/kg)	21025 ± 3773	1636 ± 81.66

Abbreviations – Extinction (Ext); Vehicle (Veh)