0026-895X/05/6702-349–355$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 67:349–355, 2005

Vol. 67, No. 2
3319/1193675
Printed in U.S.A.

ACCELERATED COMMUNICATION

The mGluR5 Antagonist 6-Methyl-2-(phenylethynyl)pyridine Decreases Ethanol Consumption via a Protein Kinase Cε-Dependent Mechanism

M. Foster Olive, Andrew J. Mcgeehan, Jennifer R. Kinder, Thomas McMahon, Clyde W. Hodge, Patricia H. Janak, and Robert O. Messing

Ernest Gallo Clinic and Research Center, Department of Neurology, University of California at San Francisco, Emeryville, California (M.F.O., A.J.M., J.R.K., T.M., P.H.J., R.O.M.); and Bowles Center for Alcohol Studies, Departments of Psychiatry and Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (C.W.H.)

Received May 28, 2004; accepted November 5, 2004

ABSTRACT

Glutamatergic neurotransmission plays a critical role in addictive behaviors, and recent evidence indicates that genetic or pharmacological inactivation of the type 5 metabotropic glutamate receptor (mGluR5) reduces the self-administration of cocaine, nicotine, and alcohol. Because mGluR5 is coupled to activation of protein kinase C (PKC), and targeted deletion of the epsilon isoform (PKCε) in mice reduces ethanol self-administration, we investigated whether there is a functional link between mGluR5 and PKCε. Here, we show that acute administration of the mGluR5 agonist (*R*,*S*)-2-chloro-5-hydroxyphenylglycine to mice increases phosphorylation of PKCε in its activation loop (T566) as well as in its C-terminal region (S729). Increases in phospho-PKCε are dependent not only on mGluR5 stimulation but also on phosphatidylinositol-3 kinase (PI3K). In addition, the selective mGluR5 antagonist 6-methyl-2-(phenylethynyl)pyridine (MPEP) reduced basal levels of phosphorylation of PKCε at S729. We also show that MPEP dose dependently reduced ethanol consumption in wild-type but not in PKCε-null mice, suggesting that PKCε is an important signaling target for modulation of ethanol consumption by mGluR5 antagonists. Radioligand binding experiments using [^{3}H]MPEP revealed that these genotypic differences in response to MPEP were not a result of altered mGluR5 levels or binding in PKCε-null mice. Our data indicate that mGluR5 is coupled to PKCε via a PI3K-dependent pathway and that PKCε is required for the ability of the mGluR5 antagonist MPEP to reduce ethanol consumption.

Glutamatergic neurotransmission plays an important role in the behavioral and neurochemical effects of drugs of abuse as well as in drug self-administration. Although most studies have examined the role of ionotropic glutamate receptor subtypes (Tzschentke and Schmidt, 2003; Kalivas, 2004), there is increasing evidence that metabotropic glutamate receptors (mGluRs) also play an important role in drug-related behaviors (Kenny and Markou, 2004; Olive, 2005). For example, mice lacking the type 5 mGluR (mGluR5) show dramatically reduced levels of cocaine self-administration and cocaine-induced hyperactivity (Chiamulera et al., 2001). In addition, studies using selective mGluR5 antagonists have confirmed a role for mGluR5 in cocaine-related behaviors such as conditioned place preference (Mcgeehan and Olive, 2003), hyperlocomotion (Mcgeehan et al., 2004), and self-administration (Chiamulera et al., 2001; Kenny et al., 2003b; Tessari et al., 2004). Self-administration of nicotine (Kenny et al., 2003b; Paterson et al., 2003; Tessari et al., 2004) and ethanol (Sharko et al., 2002; Backstrom et al., 2004) are also reduced

This work was supported by Public Health Service Grants AA13276 and AA013852 (to M.F.O.), AA08117 and AA13588 (to R.O.M.), the Bowles Center for Alcohol Studies, and funds provided by the State of California for medical research on alcohol and substance abuse through the University of California at San Francisco.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.003319.

ABBREVIATIONS: mGluR5, type 5 metabotropic glutamate receptor; DAG, diacylglycerol; PKCε, protein kinase Cε; CHPG, (*R*,*S*)-2-chloro-5-hydroxyphenylglycine; MPEP, 6-methyl-2-(phenylethynyl)pyridine; ANOVA, analysis of variance; PDK1, phospholipids-dependent kinase 1; PI3K, phosphatidylinositol-3 kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride.

by mGluR5 antagonists. Finally, blockade of mGluR5 increases threshold current levels for intracranial self-stimulation (Harrison et al., 2002; Kenny et al., 2003a), suggesting that these receptors are centrally involved in primary reward mechanisms.

Activation of group I mGluRs, which include mGluR1 and mGluR5, stimulates the activity of phospholipase C, thereby generating diacylglycerol (DAG), which in turn can activate PKC (Hermans and Challiss, 2001). Nine different PKC genes have been identified, seven of which encode isozymes activated by DAG. It is currently not known which PKC isozyme(s) is activated by mGluR5. One candidate is PKCε, which, like mGluR5, is enriched in regions of the brain involved in drug self-administration and addiction, including the frontal cortex, striatum, nucleus accumbens, and hippocampus (Saito et al., 1993; Minami et al., 2001). Moreover, there is considerable anatomical overlap in the expression of mGluR5 and PKCε in the brain (Saito et al., 1993; Shigemoto et al., 1993; Romano et al., 1995; Minami et al., 2001), suggesting that this PKC may be a downstream signaling target of mGluR5. It is interesting to note that our research has shown that similar to pharmacological blockade of mGluR5, genetic deletion of PKCε reduces ethanol consumption in mice (Hodge et al., 1999; Olive et al., 2000; Choi et al., 2002). Here, we sought to establish biochemical link between mGluR5 and PKCε and to determine whether PKCε is necessary for the ability of mGluR5 antagonists to reduce ethanol consumption.

Materials and Methods

Animals. Male mice (age 2–4 months) were used for all experiments and were housed in temperature- and humidity-controlled Plexiglas cages (BioZone, Fort Mill, SC) under a 12-h/12-h light/dark cycle with lights on at 6:00 AM. Food and water were available ad libitum except where noted. Male wild-type C57BL/6J × 129SvJae littermates (F1 or F2 generation) were used for all studies except ethanol consumption experiments (see below). All procedures were conducted in accordance with Institutional Animal Care and Use Committee procedures and the Society for Neuroscience's Policy on the Use of Animals in Neuroscience Research.

Drugs and Reagents. (*R*,*S*)-2-Chloro-5-hydroxyphenylglycine (CHPG, Tocris Cookson Inc., Ellisville, MO) was dissolved in 110 mM NaOH, and LY294002 (Calbiochem, San Diego, CA) was suspended in dimethyl sulfoxide before dilution in artificial cerebrospinal fluid (Harvard Apparatus Inc., Holliston, MA) for intracerebroventricular (i.c.v.) administration. 6-Methyl-2-(phenylethynyl)pyridine (MPEP, Sigma-Aldrich, St. Louis, MO) was dissolved in physiological saline before intraperitoneal (i.p.) administration. All other chemical reagents were obtained from Fisher Scientific (Santa Clara, CA) or Sigma-Aldrich.

Intracerebroventricular Cannula Implantation and Drug Treatments. For experiments involving administration of CHPG, mice were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (7 mg/kg i.p.) and placed in a stereotaxic frame equipped with a mouse adapter (Harvard Apparatus Inc.). A plastic guide cannula (26-gauge outer diameter; Plastics One, Roanoke, VA) aimed at the lateral ventricle was unilaterally implanted using the following coordinates with bregma and skull surface as reference points (Franklin and Paxinos, 2001): posterior, −0.3 mm; lateral, +1.0 mm; and ventral, −2.0 mm. The cannula was secured with skull screws and dental cement, and mice were allowed at least 5 days of postsurgical recovery before experiments.

A 33-gauge outside diameter microinjection needle (Plastics One) that extended 1 mm beyond the ventral tip of the guide cannula was used for i.c.v. drug administration. Microinjection needles were connected to gastight Hamilton syringes via FEP tubing (CMA/Microdialysis, North Chelmsford, MA). Syringes were driven by a Harvard syringe pump at a flow rate of 1 μl/min for a total infusion of 1 μl of fluid. The microinjection needle was left in place for an additional 1 min after each infusion to allow for diffusion of the drug into the ventricle.

Separate experimental groups of mice were treated as follows: saline or MPEP (20 mg/kg i.p.) 15 min before the administration of CHPG (3 μg/μl i.c.v.) or its corresponding vehicle (dilute NaOH); LY294002 (30 μg/μl i.c.v.) or its corresponding vehicle (dilute dimethyl sulfoxide) 15 min before the administration of CHPG (3 μg/μl i.c.v.); and saline or MPEP (20 mg/kg i.p.). Fifteen minutes after the final drug treatment, mice were euthanized, and brains were removed and placed into ice-cold phosphate-buffered saline (pH 7.2) for dissection of striatal tissue.

Striatal Homogenate Preparation and Immunoblot Analysis. Striatal tissue was placed in 10 volumes of homogenization buffer (pH 7.5) consisting of 20 mM Tris, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, and a 1:100 dilution of a protease inhibitor cocktail (Sigma-Aldrich) and a serine/threonine phosphatase inhibitor cocktail (Sigma-Aldrich). Tissue was homogenized with 10 to 12 strokes of a motorized homogenizer at 5000 rpm and centrifuged at 10,000*g* at 4°C for 10 min. The supernatant was then stored at −70°C until Western analysis.

For Western analysis, samples were split into two equal aliquots (20 μg of protein each) and subjected to SDS-polyacrylamide gel electrophoresis using separate 10% Bis-Tris gels (Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes, preblocked with phosphate-buffered saline containing 0.1% (v/v) Tween 20 and either 5% (w/v) bovine serum albumin (for phospho-PKC) or 5% (w/v) nonfat dried milk powder (for total PKC) for 1 h before overnight incubation with primary antibodies. We used the following rabbit polyclonal antibodies: anti-phospho-PKCε T566 (Parekh et al., 1999; 1:5000 dilution), anti-phospho-PKCe S729 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:5000 dilution), anti-phospho-PKCα T638/PKCβ T641 (Cell Signaling Technology Inc., Beverly, MA; 1:1000 dilution), anti-PKCε (Choi et al., 2002; 1:5000 dilution), and anti-PKCβII (Santa Cruz Biotechnology, Inc., 1:500 dilution). Membranes were then washed and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antisera (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 1:10,000 dilution) for 90 min, and immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus; Amersham Biosciences Inc., Piscataway, NJ). Protein bands corresponding to a molecular mass of approximately 90 kDa were visualized on a Storm 860 PhosphorImager (Amersham Biosciences Inc.) at 100-μm resolution using ImageQuant software (Amersham Biosciences Inc.). The optical density of the phospho-PKC band was divided by that of the corresponding total PKC band to yield a phospho-PKC/total PKC ratio.

Radioligand Binding. Cerebral cortex and striatum tissue from naive wild-type and PKCε-null mice (Hodge et al., 1999; Khasar et al., 1999) were homogenized in 10 volumes of ice-cold assay buffer consisting of 50 mM Tris-HCl, 0.9% (w/v) NaCl, pH 7.5, and the homogenate was centrifuged at 39,800*g* for 10 min at 4°C. The pellet was resuspended in assay buffer immediately before use. Receptor binding in crude membrane fractions was determined using [^{3}H]MPEP (46.85 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) over a concentration range of 0.075 to 10 nM, and nonspecific binding was determined using 10 μM MPEP. Binding studies were performed using 300 μg of protein in a 1-ml reaction volume. After incubation for 1 h at room temperature, tissues were rapidly filtered over Whatman GF/C filters, and radioactivity remaining on the filters was detected by liquid scintillation counting. Each binding experiment (n = 4 animals) was performed with three to five replicate experiments for each genotype and brain region.

Ethanol Consumption. For these studies, C57BL/6J × 129/SvJae PKCε heterozygous null mice (F2 generation; Hodge et al., 1999; Khasar et al., 1999) were backcrossed onto a C57BL/6J background for

10 generations, and mice heterozygous for the PKCε-null mutation were subsequently mated with female 129/SvJae PKCε heterozygous mice to generate F1 hybrid wild-type and PKCε-null mice for study. Mice were housed individually in cages equipped with two bottle grommets at one end and allowed simultaneous access for 16 h to one 50-ml bottle containing 10% (v/v) ethanol and another containing tap water, beginning at 5:00 PM. The next morning at 9:00 AM, the two bottles were removed from the cages, and the amount of each fluid consumed was recorded. Animals did not have access to either fluid from 9:00 AM to 5:00 PM. These 16-h two-bottle choice sessions were conducted four sequential days per week. For the remaining 3 days, animals were allowed 24-h access to both solutions. At least 2 weeks of baseline consumption was established before administration of any pharmacological agents. Mice were administered saline or MPEP (3, 10, or 20 mg/kg i.p.) in a volume of 10 ml/kg immediately before 16 h access sessions. All animals received saline or doses of MPEP in a balanced and randomized order, with a maximum of two injections per week. Ethanol consumption was defined as grams per kilogram per 16 h, ethanol preference was defined as (milliliters of ethanol consumed per 16 h/total milliliters consumed per 16 h) × 100, and water consumption was defined as milliliters per 16 h.

Statistical Analyses. Data from immunoblot and radioligand binding experiments were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls or Dunn's multiple comparisons tests, or by a Kruskal-Wallis one-way ANOVA on ranks when appropriate. Data from ethanol consumption studies were analyzed by two-way ANOVA with one-factor repetition (treatment) followed by Holm-Sidak multiple comparisons tests. $p < 0.05$ was considered statistically significant for all tests.

Results

Phospho-PKC Experiments. We used phospho-specific antibodies to detect phosphorylation of PKCε at T566 and S729, because phosphorylation of this kinase at these two sites is considered to reflect an increase in the activatable pool of PKCε (Parekh et al., 1999, 2000; Akita, 2002; Cenni et al., 2002). The activation loop of PKCε is phosphorylated at T566 by phospholipid-dependent kinase 1 (PDK1), which in turn is activated by lipid products of phosphatidylinositol-3 kinase (PI3K) (Parekh et al., 2000; Cenni et al., 2002). Once PKCε is phosphorylated at T566, it can be then be phosphorylated in the C-terminal at S729, either by autophosphorylation or by heterologous kinases (see *Discussion*).

Administration of the mGluR5 agonist CHPG to wild-type mice (Fig. 1a) increased the abundance of striatal phospho-PKCε (S729) immunoreactivity relative to total PKCε immunoreactivity [$F_{(2,14)} = 4.087$; $p < 0.05$]. This was blocked by systemic pretreatment with MPEP. It is surprising that CHPG also increased phosphorylation of PKCε at T566 [$H_{(2)} = 8.681$; $p < 0.05$], and this also was blocked by systemic MPEP pretreatment (Fig. 1b). The increase in phospho-PKCε (T566) induced by CHPG [$F_{(2,21)} = 3.901$; $p < 0.05$] also was inhibited by pretreatment with the PI3K inhibitor LY294002 (Fig. 1c). In contrast, CHPG did not modify the ratio of phospho-PKCα/βII (T638/641) to total PKCβII immunoreactivity [Fig. 1d; $F_{(2,9)} = 0.081$; $p > 0.05$]. Additional experiments revealed that a single peripheral administration of MPEP (20 mg/kg i.p.), but not saline, reduced basal levels of phospho-PKCε (S729) relative to total PKCε immunoreactivity [Fig. 1e; $F_{(1,12)} = 5.063$; $p < 0.05$].

Ethanol Consumption. As we reported previously (Hodge et al., 1999; Olive et al., 2000), baseline ethanol consumption (Fig. 2a) was significantly higher in wild-type mice compared with PKCε-null mice [$H_{(1)} = 9.694$; $p < 0.005$]. Likewise, baseline ethanol preference (Fig. 2b) was significantly higher in wild-type compared with PKCε-null mice [$H_{(1)} = 7.467$; $p < 0.01$]. Administration of MPEP dose dependently reduced ethanol consumption in wild-type but not in PKCε-null mice. Analysis of ethanol consumption in both genotypes revealed a significant main effect of genotype [$F_{(1,63)} = 4.630$; $p < 0.05$], MPEP treatment [$F_{(3,63)} = 3.726$; $p < 0.05$] and a genotype × treatment interaction [$F_{(3,63)} = 3.854$; $p < 0.05$]. Likewise, with regard to ethanol preference, there was a main effect of MPEP treatment [$F_{(3,63)} = 3.002$; $p < 0.05$] and a genotype × treatment interaction [$F_{(3,63)} = 4.841$; $p < 0.005$], with a trend toward a significant main effect of genotype on ethanol preference [$F_{(1,63)} = 3.208$; $p = 0.08$]. No effect of genotype [$F_{(1,58)} = 1.510$; $p > 0.05$] or MPEP treatment [$F_{(3,58)} = 0.903$; $p > 0.05$] on water intake was observed, nor was there a genotype × treatment interaction [$F_{(3,58)} = 0.368$; $p > 0.05$] (Fig. 2c).

Radioligand Binding. To control for the possibility that differential effects of MPEP on ethanol consumption in wild-type and PKCε-null mice might result from genotypic differences in mGluR5 density or affinity, we performed radioligand binding studies using [^{3}H]MPEP in crude cortical and striatal membrane preparations from wild-type and PKCε-null mice. Saturation binding curves are shown in Fig. 3. No genotypic differences in ligand binding density (B_{max}) were observed in the cortex [wild-type $B_{max} = 56.16 \pm 2.65$, PKCε null $B_{max} = 66.96 \pm 11.84$, $F_{(1,5)} = 0.579$; $p > 0.05$] or striatum [wild-type $B_{max} = 44.29 \pm 11.34$, PKCε null $B_{max} = 78.66 \pm 16.28$, $F_{(1,6)} = 2.190$; $p > 0.05$]. Likewise, no genotypic differences in ligand affinity (K_D) were observed in the cortex [wild-type $K_D = 6.45 \pm 0.48$, PKCε null $K_D = 6.32 \pm 1.42$, $F_{(1,5)} = 0.006$; $p > 0.05$] or striatum [wild-type $K_D = 4.60 \pm 0.35$, PKCε null $K_D = 5.04 \pm 0.50$, $F_{(1,6)} = 0.385$; $p > 0.05$].

Discussion

Our findings identify for the first time a novel mGluR5-PKCε signaling pathway in the brain that regulates ethanol consumption. Using PKCε-null mice, we found that the ability of the mGluR5 antagonist MPEP to reduce ethanol consumption was dependent on the presence of PKCε. These alterations in ethanol consumption were not a result of altered mGluR5 number or binding in PKCε-null mutant mice, because no differences in radiolabeled MPEP binding to the cortex and striatum between wild-type and PKCε-null mice were observed. Furthermore, using a phospho-specific antibody against phosphorylated T566 of PKCε and LY294002 (an inhibitor of PI3K), we found that activation of mGluR5 increases the activatable pool of PKCε via PI3K and PDK1. Until now, regulated phosphorylation of PKCε at T566 has only been demonstrated in vitro in NIH 3T3 cells stimulated with platelet-derived growth factor (Cenni et al., 2002) or in human embryonic kidney 293 cells stimulated with serum after serum starvation (Parekh et al., 1999). Our results provide the first in vivo evidence for regulated PDK1 mediated phosphorylation of PKCε through a G protein-coupled neurotransmitter receptor (mGluR5). Moreover, our results also indicate that mGluR5 is coupled to PI3K in the striatum. These findings provide in vivo functional significance to the recent in vitro demonstration that mGluR5 can associate

with PI3K via a PI3K enhancer/Homer protein complex (Rong et al., 2003).

We did not, however, examine the effects of LY294002 on basal levels of PKCε phosphorylation, because it has previously been shown that inhibition of PDK1 function (through the use of kinase inactive mutants of PDK1 rather than pharmacological inhibition of PI3K by LY294002) does not alter basal phosphorylation of PKCε (Cenni et al., 2002). Thus, we predict that administration of LY294002 alone would not alter basal levels of PKCε phosphorylation in vivo. Yet, given recent evidence that PI3K contributes to behavioral plasticity associated with repeated exposure to cocaine (Izzo et al., 2002), the role of this lipid kinase in drug and ethanol self-administration clearly merits further investigation.

Direct measurements of PKCε activity were not performed in the present study. Rather, we quantified levels of phosphorylation of PKCε using specific antisera against two phosphorylation sites on this enzyme, because phosphorylation of PKC isozymes is strongly correlated with increases in activity of these kinases and seems to be essential for expression of maximal kinase activity (Mitchell et al., 1989; Parekh et al., 1999; Newton, 2001). Most PKC isoforms contain phosphorylation sites in the activation loop and in the C-terminal hydrophobic region, and it is thought that a sequential phosphorylation of these sites leads to activation of the enzyme (Newton, 1997, 2001). For example, it has been demonstrated that *trans*-phosphorylation of PKCβII in its activation loop (at T500) by PDK1 renders it competent to autophosphorylate residues T641 and S660 (Dutil et al., 1994; Keranen et al., 1995). Once autophosphorylated, PKCβII remains catalytically competent but inactive until it translocates to the membrane, where it binds lipid activators such as DAG. Because PKCε contains similar phosphorylation sites in its activation loop (T566) and C terminus (S710 and S729), it is likely that this PKC isoform undergoes a similar sequential activation sequence (Parekh et al., 2000). The activation loop of PKCε is known to be phosphorylated by PDK1, which in

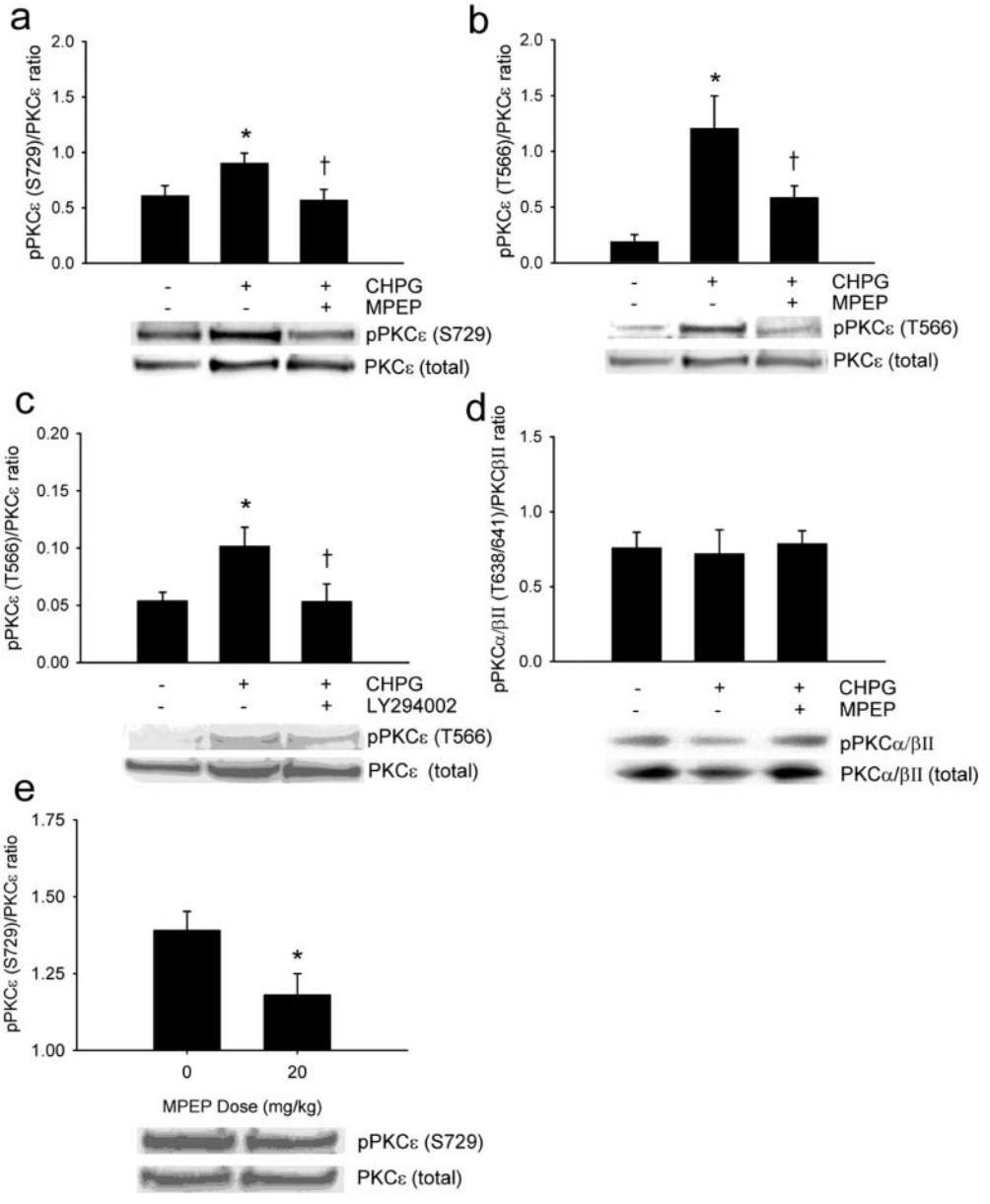

Fig. 1. The mGluR5 receptor is positively coupled to PKCε via a pathway dependent on PI3K. Administration of the mGluR5 agonist CHPG significantly increased levels of active PKCε as measured by the ratio of phospho-PKCε (S729) (a) or phospho-PKCε (T566) (b) immunoreactivities to total PKCε immunoreactivity (*, $p < 0.05$ versus saline i.p. followed by vehicle i.c.v.). These effects were significantly attenuated when animals were pretreated with MPEP (†, $p < 0.05$ versus saline i.p. followed by CHPG i.c.v.). c, PI3K kinase inhibitor LY294002 significantly attenuated the ability of CHPG to increase levels of phospho-PKCε (T566) relative to total PKCε (†, $p < 0.05$ versus vehicle before CHPG). d, no effect of CHPG treatment was observed on levels of phospho-PKCα/βII (T638/641) relative to total PKCβII. e, systemic administration of MPEP (20 mg/kg) but not saline (0 mg/kg) resulted in a reduction in levels of phospho-PKCε (S729) immunoreactivity relative to total PKCε immunoreactivity (*, $p < 0.05$ versus saline). Data shown represent mean ± S.E.M. of $n = 3$ animals per condition and four to eight immunoblot analyses.

turn is activated by lipid products of PI3K (Parekh et al., 2000; Cenni et al., 2002). PDK1-mediated phosphorylation of the activation loop is a constitutive event in the maturation of conventional PKC isozymes (α, β, and γ), but for other PKC isozymes it is regulated by the activity of PI3K (Newton, 2001). PDK1-mediated phosphorylation of PKCε at T566 then triggers sequential phosphorylation of S710 and S729. However, whether phosphorylation of S729 reflects autophosphorylation is a subject of debate, because some investigators have demonstrated a complete abolition of phosphorylation of PKCε at S729 in the presence of a PKC inhibitor (Parekh et al., 1999), whereas others found only partial reductions in phosphorylation at this site (Cenni et al., 2002). These latter findings suggest that under certain conditions, other kinases may participate in phosphorylation of PKCε at S729. However, these same authors also showed evidence for autophosphorylation processes in PKCε activation, because kinase inactive mutants of PKCε failed to show phosphorylation of PKCε at S729 (Cenni et al., 2002). Regardless of the mechanism, PKCε phosphorylation at S729 has been used in several studies as a marker for changes in PKCε activation (Saitoh et al., 2001; Bayer et al., 2003; Zhou et al., 2003).

In the present study, we showed that activation of mGluR5 by CHPG stimulated phosphorylation of PKCε at both T566 and S729, indicating that stimulation of mGluR5 increases the available pool of PKCε that can become active. Our finding that MPEP alone reduced phosphorylation of PKCε at S729 in the striatum indicates a role for mGluR5 in setting the basal level of activatable PKCε in this brain region. Consistent with this result, it has been reported that mGluR5 exhibits constitutive activity, because these receptors undergo endocytosis in the absence of agonists in cultured hippocampal neurons (Fourgeaud et al., 2003).

Both mGluR5 and PKCε are highly coexpressed in numerous regions of the adult rodent brain such as the cerebral cortex, olfactory bulb, striatum, nucleus accumbens, and hippocampus (Saito et al., 1993; Shigemoto et al., 1993; Romano et al., 1995; Minami et al., 2001). For the current study, we chose to examine the dorsal striatum, because this region contains very high levels of both proteins; is adjacent to the lateral ventricle, making it easily accessible to i.c.v.-administered drugs; and plays an important role in the control of drug self-administration and persistent drug-related habits (Winder et al., 2002; Gerdeman et al., 2003; Wang et al., 2003). However, the striatum also contains significant levels of PKCβ (Tanaka and Saito, 1992; Minami et al., 2001), and thus the possibility exists that mGluR5 in this region also may be coupled to this PKC isozyme. Yet, we found that CHPG did not modify the ratio of phospho-PKCα/βII (T638/

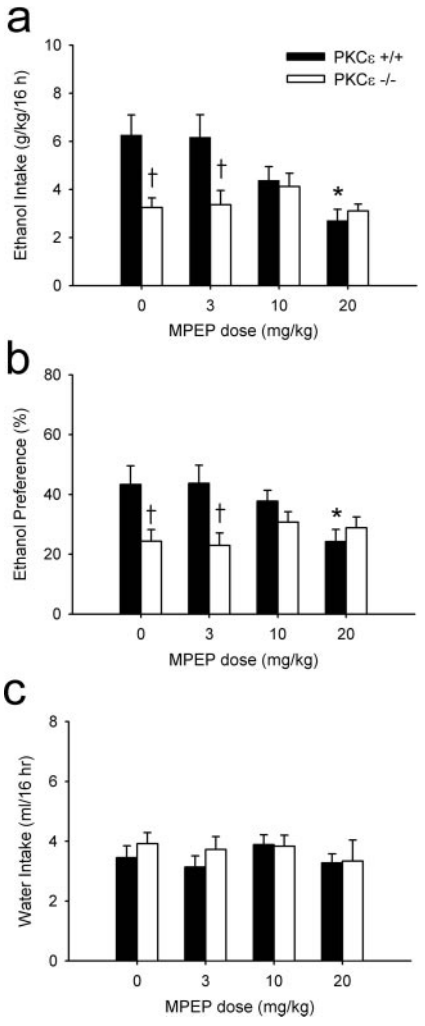

Fig. 2. PKCε is required for the ability of the mGluR5 antagonist MPEP to reduce ethanol consumption (a) and preference (b). Saline (0 mg/kg) or MPEP (3, 10, or 20 mg/kg) were administered immediately before 16-h two-bottle access sessions. c, lack of effect of MPEP administration on water intake. ∗, $p < 0.05$ versus saline-treated mice of the same genotype. †, $p < 0.05$ versus wild type at corresponding treatment. Values represent mean ± S.E.M. of $n = 8$ to 14 animals per dose.

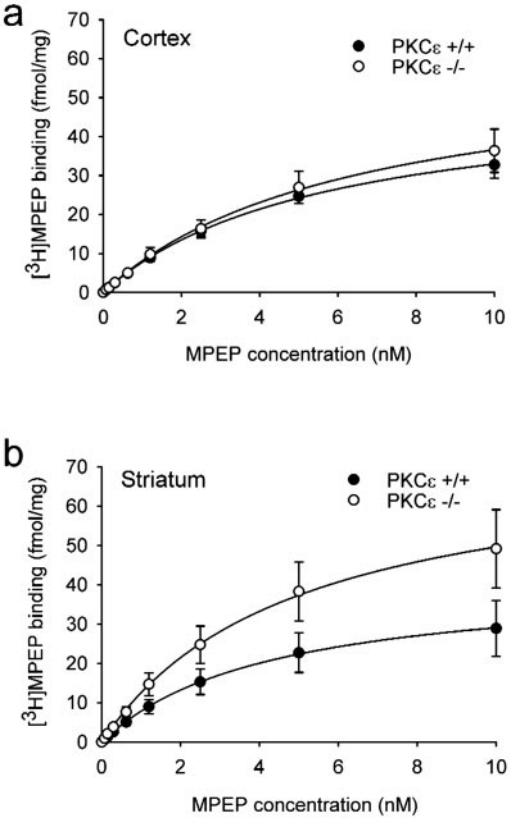

Fig. 3. Saturation curves for [^{3}H]MPEP binding to membranes from cerebral cortex (a) or striatum (b) of wild-type (●) and PKCε-null (○) mice. Values represent mean ± S.E.M. of $n = 3$ to 5 replicate experiments per group ($n = 4$ mice/experiment).

641) to total PKCβII immunoreactivity, indicating that the effect of mGluR5 stimulation on PKCϵ phosphorylation in the striatum is specific. Consistent with this, nonselective group I mGluR ligands have been reported to induce translocation of PKCϵ but not PKCβ in cortical synaptosomes (Pastorino et al., 2000), suggesting that mGluR5 coupling to PKCϵ may be specific in other forebrain regions as well.

The current study underlines the importance of PKCϵ, a downstream signaling target of mGluR5, in the ability of the mGluR5 antagonist MPEP to reduce ethanol self-administration. Further studies are needed to determine whether dysregulation of this signaling pathway contributes to disorders of excessive ethanol consumption such as alcohol abuse and alcoholism. Although it has been demonstrated that mGluR5 also plays an important role in the reinforcing and behavioral effects of other drugs of abuse such as cocaine and nicotine (Kenny and Markou, 2004; Olive, 2005), no studies to date have examined the role of PKCϵ activity in modulation of cocaine and nicotine self-administration as well as behavioral and neural plasticity induced by these drugs. Such studies should provide a more detailed understanding of the cellular substrates underlying drug addiction. It is interesting that although we have shown that rewarding effects of morphine are not altered by coadministration of MPEP at doses up to 20 mg/kg in mice (Mcgeehan and Olive, 2003) (although higher doses may be required in other rodent species; Popik and Wrobel, 2002), preliminary evidence suggests that morphine reward is enhanced in mice lacking PKCϵ (Newton et al., 2004). Thus, more research in needed aimed at identifying G protein-coupled receptor other than mGluR5 that are coupled to PKCϵ in brain regions known to be critically involved in the rewarding effects of morphine, such as the ventral tegmental area (Wise, 1989; McBride et al., 1999). In addition to drugs of abuse, this mGluR5-PKCϵ signaling cascade may have relevance for other central nervous system pathologies, because both antagonism of mGluR5 as well as deletion of PKCϵ produces parallel reductions in anxiety and hyperalgesia (Khasar et al., 1999; Spooren et al., 2001, 2003; Hodge et al., 2002; Kuhn et al., 2002; Conn, 2003).

Acknowledgments

We thank Dr. Peter Parker (Imperial Cancer Research Fund, London, UK) for the generous donation of the antisera to phospho-PKCϵ (T566).

References

Akita Y (2002) Protein kinase C-ϵ (PKC-ϵ): its unique structure and function. *J Biochem* **132:**847–852.
Backstrom P, Bachteler D, Koch S, Hyytia P, and Spanagel R (2004) mGluR5 antagonist MPEP reduces ethanol-seeking and relapse behavior. *Neuropsychopharmacology* **29:**921–928.
Bayer AL, Heidkamp MC, Patel N, Porter M, Engman S, and Samarel AM (2003) Alterations in protein kinase C isoenzyme expression and autophosphorylation during the progression of pressure overload-induced left ventricular hypertrophy. *Mol Cell Biochem* **242:**145–152.
Cenni V, Doppler H, Sonnenburg ED, Maraldi N, Newton AC, and Toker A (2002) Regulation of novel protein kinase C epsilon by phosphorylation. *Biochem J* **363:**537–545.
Chiamulera C, Epping-Jordan MP, Zocchi A, Marcon C, Cottiny C, Tacconi S, Corsi M, Orzi F, and Conquet F (2001) Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice. *Nat Neurosci* **4:**873–874.
Choi DS, Wang D, Dadgar J, Chang WS, and Messing RO (2002) Conditional rescue of protein kinase C ϵ regulates ethanol preference and hypnotic sensitivity in adult mice. *J Neurosci* **22:**9905–9911.
Conn PJ (2003) Physiological roles and therapeutic potential of metabotropic glutamate receptors. *Ann NY Acad Sci* **1003:**12–21.
Dutil EM, Keranen LM, DePaoli-Roach AA, and Newton AC (1994) In vivo regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. *J Biol Chem* **269:**29359–29362.
Fourgeaud L, Bessis AS, Rossignol F, Pin JP, Olivo-Marin JC, and Hemar A (2003) The metabotropic glutamate receptor mGluR5 is endocytosed by a clathrin-independent pathway. *J Biol Chem* **278:**12222–122230.
Franklin KBJ and Paxinos G (2001) *The Mouse Brain in Stereotaxic Coordinates.* Academic Press, San Diego.
Gerdeman GL, Partridge JG, Lupica CR, and Lovinger DM (2003) It could be habit forming: drugs of abuse and striatal synaptic plasticity. *Trends Neurosci* **26:**184–192.
Harrison AA, Gasparini F, and Markou A (2002) Nicotine potentiation of brain stimulation reward reversed by DHβE and SCH 23390, but not by eticlopride, LY 314582 or MPEP in rats. *Psychopharmacology* **160:**56–66.
Hermans E and Challiss RA (2001) Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. *Biochem J* **359:**465–484.
Hodge CW, Mehmert KK, Kelley SP, McMahon T, Haywood A, Olive MF, Wang D, Sanchez-Perez AM, and Messing RO (1999) Supersensitivity to allosteric $GABA_A$ receptor modulators and alcohol in mice lacking PKCϵ. *Nat Neurosci* **2:**997–1002.
Hodge CW, Raber J, McMahon T, Walter H, Sanchez-Perez AM, Olive MF, Mehmert K, Morrow AL, and Messing RO (2002) Decreased anxiety-like behavior, reduced stress hormones and neurosteroid supersensitivity in mice lacking protein kinase Cepsilon. *J Clin Investig* **110:**1003–1010.
Izzo E, Martin-Fardon R, Koob GF, Weiss F, and Sanna PP (2002) Neural plasticity and addiction: PI3 kinase and cocaine behavioral sensitization. *Nat Neurosci* **5:**1263–1264.
Kalivas PW (2004) Glutamate systems in cocaine addiction. *Curr Opin Pharmacol* **4:**23–29.
Kenny PJ, Gasparini F, and Markou A (2003a) Group II metabotropic and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate glutamate receptors regulate the deficit in brain reward function associated with nicotine withdrawal in rats. *J Pharmacol Exp Ther* **306:**1068–1076.
Kenny PJ and Markou A (2004) The ups and downs of addiction: role of metabotropic glutamate receptors. *Trends Pharmacol Sci* **25:**265–272.
Kenny PJ, Paterson NE, Boutrel B, Semenova S, Harrison AA, Gasparini F, Koob GF, Skoubis PD, and Markou A (2003b) Metabotropic glutamate 5 receptor antagonist MPEP decreased nicotine and cocaine self-administration but not nicotine and cocaine-induced facilitation of brain reward function in rats. *Ann NY Acad Sci* **1003:**415–418.
Keranen LM, Dutil EM, and Newton AC (1995) Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr Biol* **5:**1394–1403.
Khasar SG, Lin Y-H, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, et al. (1999) A novel nociceptor signaling pathway demonstrated in protein kinase Cϵ mutant mice. *Neuron* **24:**253–260.
Kuhn R, Pagano A, Stoehr N, Vranesic I, Flor PJ, Lingenhöhl K, Spooren W, Gentsch C, Vassout A, Pilc A, et al. (2002) In vitro and in vivo characterization of MPEP, an allosteric modulator of the metabotropic glutamate receptor subtype 5: review article. *Amino Acids* **23:**207–211.
McBride WJ, Murphy JM, and Ikemoto S (1999) Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. *Behav Brain Res* **101:**129–152.
Mcgeehan AJ, Janak PH, and Olive MF (2004) Effect of the mGluR5 antagonist 6-methyl-2-(phenylethynyl)pyridine (MPEP) on the acute locomotor stimulant properties of cocaine, d-amphetamine and the dopamine reuptake inhibitor GBR12909 in mice. *Psychopharmacology* **174:**266–273.
Mcgeehan AJ and Olive MF (2003) The mGluR5 antagonist MPEP reduces the conditioned rewarding effects of cocaine but not other drugs of abuse. *Synapse* **47:**240–242.
Minami H, Owada Y, Handa Y, and Kondo H (2001) Localization of mRNAs for novel, atypical as well as conventional protein kinase C (PKC) isoforms in the brain of developing and mature rats. *J Mol Neurosci* **15:**121–135.
Mitchell FE, Marais RM, and Parker PJ (1989) The phosphorylation of protein kinase C as a potential measure of activation. *Biochem J* **261:**131–136.
Newton AC (1997) Regulation of protein kinase C. *Curr Opin Cell Biol* **9:**161–167.
Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors and macromolecular interactions. *Chem Rev* **101:**2353–2364.
Newton PM, Kim JA, Camarini R, Chu K, Roberts AJ, and Messing RO (2004) Enhanced sensitivity to morphine reward and analgesia in mice lacking PKCϵ. *Soc Neurosci Abstr* **30:**594.5.
Olive MF (2005) mGluR5 receptors: neuroanatomy, pharmacology and role in drug addiction. *Curr Psychiatry Rev*, in press.
Olive MF, Mehmert KK, Messing RO, and Hodge CW (2000) Reduced operant ethanol self-administration and in vivo mesolimbic dopamine responses to ethanol in PKCϵ deficient mice. *Eur J Neurosci* **12:**4131–4140.
Parekh D, Ziegler W, Yonezawa K, Hara K, and Parker PJ (1999) Mammalian TOR controls one of two kinase pathways acting upon nPKCδ and nPKCϵ. *J Biol Chem* **274:**34758–34764.
Parekh DB, Ziegler W, and Parker PJ (2000) Multiple pathways control protein kinase C phosphorylation. *EMBO (Eur Mol Biol Organ) J* **19:**496–503.
Pastorino L, Colciaghi F, Gardoni F, Albani-Torregrossa S, Pellegrini-Giampietro DE, Moroni F, De Graan PN, Cattabeni F, and Di Luca M (2000) (+)-MCPG induces PKCepsilon translocation in cortical synaptosomes through a PLD-coupled mGluR. *Eur J Neurosci* **12:**1310–1318.
Paterson NE, Semenova S, Gasparini F, and Markou A (2003) The mGluR5 antagonist MPEP decreased nicotine self-administration in rats and mice. *Psychopharmacology* **167:**257–264.
Popik P and Wrobel M (2002) Morphine conditioned reward is inhibited by MPEP, the mGluR5 antagonist. *Neuropharmacology* **43:**1210–1217.
Romano C, Sesma MA, McDonald CT, O'Malley K, Van den Pol AN, and Olney JW

(1995) Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. *J Comp Neurol* **355:**455–469.
Rong R, Ahn JY, Huang H, Nagata E, Kalman D, Kapp JA, Tu J, Worley PF, Snyder SH, and Ye K (2003) PI3 kinase enhancer-Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis. *Nat Neurosci* **6:**1153–1161.
Saito N, Itouji A, Totani Y, Osawa I, Koide H, Fujisawa N, Ogita K, and Tanaka C (1993) Cellular and intracellular localization of epsilon-subspecies of protein kinase C in the rat brain; presynaptic localization of the epsilon-subspecies. *Brain Res* **607:**241–248.
Saitoh N, Hori T, and Takahashi T (2001) Activation of the epsilon isoform of protein kinase C in the mammalian nerve terminal. *Proc Natl Acad Sci USA* **98:**14017–14021.
Sharko AC, Iller K, Koenig H, Lau K, Ou CJ, Olive MF, Camarini R, and Hodge CW (2002) Involvement of metabotropic glutamate receptor subtype 5 (mglu5) in alcohol self-administration. *Alcohol Clin Exp Res* **26 (5 Suppl):**112A.
Shigemoto R, Nomura S, Ohishi H, Sugihara H, Nakanishi S, and Mizuno N (1993) Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. *Neurosci Lett* **163:**53–57.
Spooren W, Ballard T, Gasparini F, Amalric M, Mutel V, and Schrieber R (2003) Insight into the function of group I and group II metabotropic glutamate (mGlu) receptors: behavioural characterization and implications for the treatment of CNS disorders. *Behav Pharmacol* **14:**257–277.
Spooren WP, Gasparini F, Salt TE, and Kuhn R (2001) Novel allosteric antagonists shed light on mglu5 receptors and CNS disorders. *Trends Pharmacol Sci* **22:**331–337.
Tanaka C and Saito N (1992) Localization of subspecies of protein kinase C in the mammalian central nervous system. *Neurochem Int* **21:**499–512.
Tessari M, Pilla M, Andreoli M, Hutcheson DM, and Heidbreder CA (2004) Antagonism at metabotropic glutamate 5 receptors inhibits nicotine- and cocaine-taking behaviours and prevents nicotine-triggered relapse to nicotine-seeking. *Eur J Pharmacol* **499:**121–133.
Tzschentke TM and Schmidt WJ (2003) Glutamatergic mechanisms in addiction. *Mol Psychiatry* **8:**373–382.
Wang JQ, Mao L, Parelkar NK, Tang Q, Liu Z, Sarwar S, and Choe ES (2003) Glutamate-regulated behavior, transmitter release, gene expression and addictive plasticity in the striatum: roles of metabotropic glutamate receptors. *Curr Neuropharmacol* **1:**1–20.
Winder DG, Egli RE, Schramm NL, and Matthews RT (2002) Synaptic plasticity in drug reward circuitry. *Curr Mol Med* **2:**667–676.
Wise RA (1989) Opiate reward: sites and substrates. *Neurosci Biobehav Rev* **13:**129–133.
Zhou Y, Li GD, and Zhao ZQ (2003) State-dependent phosphorylation of ε-isozyme of protein kinase C in adult rat dorsal root ganglia after inflammation and nerve injury. *J Neurochem* **85:**571–580.

Address correspondence to: Dr. M. Foster Olive, Ernest Gallo Clinic and Research Center, Department of Neurology, University of California at San Francisco, 5858 Horton St., Suite 200, Emeryville, CA 94608. E-mail: folive@itsa.ucsf.edu