Ethanol Reversal of Cellular Tolerance to Morphine in Rat Locus Coeruleus Neurons[S]

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ABSTRACT

Consumption of ethanol is a considerable risk factor for death in heroin overdose. We sought to determine whether a mildly intoxicating concentration of ethanol could alter morphine tolerance at the cellular level. In rat locus coeruleus (LC) neurons, tolerance to morphine was reversed by acute exposure of the brain slice to ethanol (20 mM). Tolerance to the opioid peptide [D-Ala^2,N-MePhe^4,Gly-ol]-enkephalin was not reversed by ethanol. Previous studies in LC neurons have revealed a role for protein kinase C (PKC)α in μ-opioid receptor (MOPr) desensitization by morphine and in the induction and maintenance of morphine tolerance, but we have been unable to demonstrate that 20 mM ethanol produces significant inhibition of PKCα. The ability of ethanol to reverse cellular tolerance to morphine in LC neurons was absent in the presence of the phosphatase inhibitor okadaic acid, indicating that dephosphorylation is involved. In human embryonic kidney 293 cells expressing the MOPr, ethanol reduced the level of MOPr phosphorylation induced by morphine. Ethanol reversal of tolerance did not appear to result from a direct effect on MOPr since acute exposure to ethanol (20 mM) did not modify the affinity of binding of morphine to the MOPr or the efficacy of morphine for G-protein activation as measured by guanosine 5′-O-(3-[^{35}S]thio)triphosphate binding. Similarly, ethanol did not affect MOPr trafficking. We conclude that acute exposure to ethanol enhances the effects of morphine by reversing the processes underlying morphine cellular tolerance.

Introduction

Opioid addicts are notorious polydrug users, often taking one or more of ethanol, benzodiazepines, cannabis, cocaine or crack, and ketamine in addition to illicit heroin or a prescription opioid. Ethanol is the most commonly detected substance other than heroin in opioid-related deaths, being detected in approximately 50% of heroin-related deaths and 30% of methadone-related deaths (Darke and Hall, 2003; Hickman et al., 2007; see also the National Treatment Agency Research briefing no. 27 by Oliver et al., 2007, available at http://www.nta.nhs.uk/uploads/nta_rb27_combined_opiate_overdose.pdf). A number of studies of heroin overdose deaths have reported a significant inverse relationship between blood ethanol and blood morphine concentrations at postmortem examination (Ruttenber et al., 1990; Darke and Ross 1999; see above-referenced

Web site of the National Treatment Agency briefing no. 27). Furthermore, in a significant proportion of deaths, the levels of opioids detected at autopsy were reported to be below the predicted fatal range for highly tolerant individuals, suggesting that opioid consumption was equal to or lower than that in living active heroin users (Tagliaro et al., 1998; Darke et al., 2002; Hickman et al., 2007). This finding suggests that some overdose deaths may be due to multiple drug toxicity and that the combination of ethanol and heroin or another opioid may pose a significant risk (Darke and Zador, 1996; Darke and Hall, 2003).

There are several ways in which ethanol consumption could enhance the effects of opioids (Hickman et al., 2008). At the simplest level of interaction, opioids and ethanol are depressants, and their effects may simply be additive or synergistic. However, another possibility is that ethanol could act to reduce tolerance to opioids and thus increase overdose risk. In this study, we sought to determine whether acute ethanol exposure can reduce morphine tolerance.

We previously reported that cellular tolerance to opioids can be induced in rat brainstem locus coeruleus (LC) neurons

ABBREVIATIONS: CI, confidence interval; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$; DAG, diacylglycerol; DAMGO, [D-Ala^2,N-MePhe^4, Gly-ol]-enkephalin; D-AP5, D-(−)-2-amino-5-phosphonopentanoic acid; DMEM, Dulbecco's modified Eagle's medium; GIRK, G protein–activated inwardly rectifying K channel; GRK, G protein–coupled receptor kinase; [^{35}S]GTPγS, guanosine 5′-O-(3-[^{35}S]thio)triphosphate; Gö6976, 5,6,7,13-tetrahydro-13-methyl-5-oxo–12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile; HEK, human embryonic kidney; LC, locus coeruleus; MOPr, μ-opioid receptor; NA, noradrenaline; NMDA, N-methyl-D-aspartic acid; oxo-M, oxotremorine-M; PKC, protein kinase C.

by either in vivo or in vitro exposure to morphine and that this cellular tolerance results from desensitization of the μ-opioid receptors (MOPrs) through a protein kinase C (PKC)-dependent mechanism (Bailey et al., 2009a). Here we demonstrate that acute exposure to a low concentration of ethanol that would be mildly intoxicating in humans reverses morphine tolerance in LC neurons.

Materials and Methods

Electrophysiological Recordings

Male Wistar rats (130–170 g) were killed by cervical dislocation, and horizontal brain slices (200–250 μm thick) containing the LC were prepared as described (Bailey et al., 2003). Slices were submerged in a slice chamber (0.5 ml) mounted on the microscope stage and superfused (2.5–3 ml/min) with a bathing solution composed of (mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 11.1 D-glucose, 21.4 $NaHCO_3$, 0.1 ascorbic acid, and saturated with 95% O_2/5% CO_2 at 33 to 34°C. For patch-clamp recording, LC neurons were visualized by Nomarski optics using infrared light, and individual cell somata were cleaned by gentle flow of bathing solution from a pipette. Whole-cell voltage clamp recordings ($V_h = -60$ mV) were made using electrodes (3–6 MΩ) filled with (mM) 115 K-gluconate, 10 HEPES, 11 EGTA, 2 $MgCl_2$, 10 NaCl, 2 MgATP, 0.25 Na_2GTP (pH 7.3, osmolarity 275 mOsm). Recordings of whole-cell current were filtered at 2 kHz using an Axopatch 200B amplifier and analyzed offline using pClamp (both from Molecular Devices, Sunnyvale, CA).

Activation of MOPrs evoked a transmembrane K^+ current, and by performing whole-cell patch-clamp recordings, a real-time index of MOPr activation could be continuously recorded (Bailey et al., 2009a,b). The opioid-evoked current was recorded at a holding potential of -60 mV. MOPrs and α_2 adrenoceptors couple to the same set of K^+ channels in LC neurons (North and Williams, 1985). To reduce variation between cells, the amplitudes of opioid-evoked currents were normalized to the maximum current evoked by 100 μM noradrenaline (NA) in the same cell. Maximum responses to NA in LC neurons were unchanged by any of the drug treatments: morphine in vivo, morphine or [D-Ala^2,N-MePhe^4,Gly-ol]-enkephalin (DAMGO) in vitro, ethanol in control or morphine-treated slice, okadaic acid (Supplemental Fig. 1). Therefore, any desensitization of MOPrs observed and any effects of ethanol must be at the level of the receptor and G protein rather than on the K^+ channels. To ensure that responses to NA were mediated through α_2 adrenoceptors and not attenuated by uptake, NA was always applied in the presence of 1 μM prazosin and 3 μM cocaine.

Induction of Morphine Tolerance

Rats In Vivo. Rats were injected s.c. with 200 mg/kg morphine base contained in a slow-release formulation that contained 200 mg/ml morphine base suspended in an emulsion containing 0.9% NaCl, liquid paraffin oil, and mannide monooleate (Sigma-Aldrich, Poole, Dorset, UK) in a ratio of 0.5:0.42:0.08 (v:v:v). Three days after injection of the slow-release morphine, the animals were killed and brain slices prepared as described above. This method of morphine treatment has previously been shown to induce significant tolerance to the cellular and antinociceptive effects of MOPr agonists (Santamarta et al., 2005; Bailey et al., 2009a).

Brain Slices In Vitro. Slices from rat brain were placed on a nylon mesh platform in a preincubation chamber containing approximately 250 ml of bathing solution at 33–34°C. Given adequate oxygenation, slices incubated in this manner remained viable for up to 12 hours. To induce opioid tolerance, 1 μM morphine or 100 nM DAMGO was added to the fluid, bathing the slices for up to 9 hours. The fluid bathing the slices in the recording chamber also contained morphine or DAMGO at the same concentration as used in the preincubation treatment.

All protocols and procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive 1986 (86/609/EEC), and the University of Bristol ethical review document.

Cell Culture

Human embryonic kidney (HEK)293 cell lines stably expressing N-terminally hemagglutinin or T7-tagged rat MOPrs were maintained at 37°C in 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 10 U/ml penicillin, 10 mg/ml streptomycin, and 250 μg/ml of the selective antibiotic G418 (PAA, Pasching, Austria).

Radioligand Binding Studies

Competition binding assays were performed on membrane fragments prepared from T7-tagged MOPr expressing HEK293 cells. Briefly, 10 μg of membrane protein was incubated with 4 nM [^{3}H]naloxone (1 mCi/ml) in Hanks' buffered saline solution containing 20 mM HEPES at pH 7.4 and increasing concentrations of unlabeled morphine. Total and nonspecific binding were measured in the absence and presence of D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$ (CTAP; 4.32 μM). Reaction tubes were incubated with agitation for 2 hours at 24°C to allow ligand binding to reach equilibrium. After this, the binding reactions were stopped by rapid filtration of samples on glass fiber filter paper using a Brandel cell harvester (Brandell, Gaithersburg, MD), followed by 3 × 3 ml washes with ice-cold 20 mM HEPES buffer. After scintillation counting, specific binding was calculated by subtracting nonspecific binding from total binding. Binding displacement curves were then fitted by nonlinear regression to a single-site competition binding model to obtain IC$_{50}$ values and converted to K_i values using the Cheng-Prusoff equation. The K_i value of [^{3}H]naloxone was taken as 1.5 nM.

[^{35}S]GTPγS Binding Assay. The binding of guanosine 5′-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) to membranes prepared from HA-tagged MOPr-expressing HEK293 cells was based on a previously described protocol (Harrison and Traynor, 2003; Johnson et al., 2006). Cells were grown to approximately 90% confluence and removed from the culture flask using ice-cold phosphate buffered saline and a cell scraper. Pelleted cells (87 g; 3 minutes) were suspended in ice-cold homogenization buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4) and homogenized by 10 strokes in a hand-held glass Teflon homogenizer. The resultant homogenate was centrifuged at 600g for 10 minutes at 4°C, the pellet discarded, and the supernatant centrifuged at 48,000g in a SORVALL DiscoveryTM SE ultracentrifuge (Thermo Fisher Scientific, Waltham, MA). The final pellet (P2) containing the plasma membrane fraction was suspended in homogenization buffer, and protein concentration was determined by Bradford assay (Bradford, 1976). Aliquots of plasma membrane suspension were centrifuged at 17,500g in a bench-top centrifuge, and the resultant pellets were maintained at -80°C until required.

For the assay itself, 10 μg of plasma membrane protein/tube was incubated in the presence or absence of 20 mM ethanol in a total volume of 500 μl of incubation buffer (20 mM HEPES, 100 mM NaCl, 4 mM $MgCl_2$, pH 7.4) containing 10 μM GDP, 200 μM tricine, 200 μM dithiothreitol, various concentrations of agonist (DAMGO and morphine), and 100 pM [^{35}S]GTPγS (1 mCi/ml; PerkinElmer, Waltham, MA). Nonspecific binding was determined by the addition of 10 μM GTPγS to the assay. Reactions were allowed to proceed for 30 minutes before being stopped by rapid filtration on glass fiber filters using a Brandel cell harvester followed by 3 × 3 ml washes with ice-cold incubation buffer. The amount of [^{35}S]GTPγS bound to membranes on individual filters was then determined by liquid scintillation counting.

MOPr Phosphorylation

T7-tagged MOPr-expressing HEK293 cells were incubated with [^{32}P]orthophosphate (0.15 mCi/ml) in phosphate-free DMEM for 2

hours. After exposure to opioid agonists, cells were scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Triton X-100, 40 mM β-glycerophosphate, 1 mM EDTA, 0.1 μM microcystin, 0.5 mM sodium orthovanadate, and protease inhibitor cocktail); nuclei and cell debris were removed by centrifugation, and MOPr was immunoprecipitated with 1 μg of anti T7-Tag monoclonal antibody (Novagen Merck Chemicals, Nottingham, UK) and 20 μl of a 50% slurry of protein A-Sepharose. Immunoprecipitates were washed three times in lysis buffer and subjected to SDS-PAGE. Autoradiography films exposed to the gels were scanned, and the amount of incorporated [^{32}P] material in each lane was quantified using Scion Image software.

Receptor Trafficking

Cell surface expression of T7-tagged MOPrs in HEK293 cells was measured by enzyme-linked immunosorbent assay using a colorimetric alkaline phosphatase assay, as described previously (Bailey et al., 2003). To measure surface receptor expression, cells were exposed to the primary antibody (T7-Tag monoclonal antibody, 1:5000; Novagen Merck Chemicals) after exposure to ethanol. To measure receptor internalization, cells were first incubated with primary anti T7 antibody and then exposed to opioid or ethanol. Cells were incubated with drugs in DMEM at 37°C, fixed in 3.7% formaldehyde, and incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase; 1:1000; Sigma-Aldrich). A colorimetric alkaline phosphatase substrate (Bio-Rad Laboratories, Hemel Hempstead, Herts, UK) was then added, and samples were assayed at 405 nm with a microplate reader. The background was subtracted by simultaneous assay of HEK293 cells not expressing MOPr. Cell surface receptor expression or receptor loss was calculated by normalizing data from each treatment group to corresponding control surface receptor levels determined from cells not exposed to drugs. All experiments were performed in triplicate.

Data Analysis

All data are expressed as means ± S.E.M. or means and 95% confidence intervals (CIs). Where appropriate, unpaired two-tailed Student's t test, one-sample t test, or analysis of variance followed by Newman-Keus test or Bonferroni tests was used to assess statistical significance.

Results

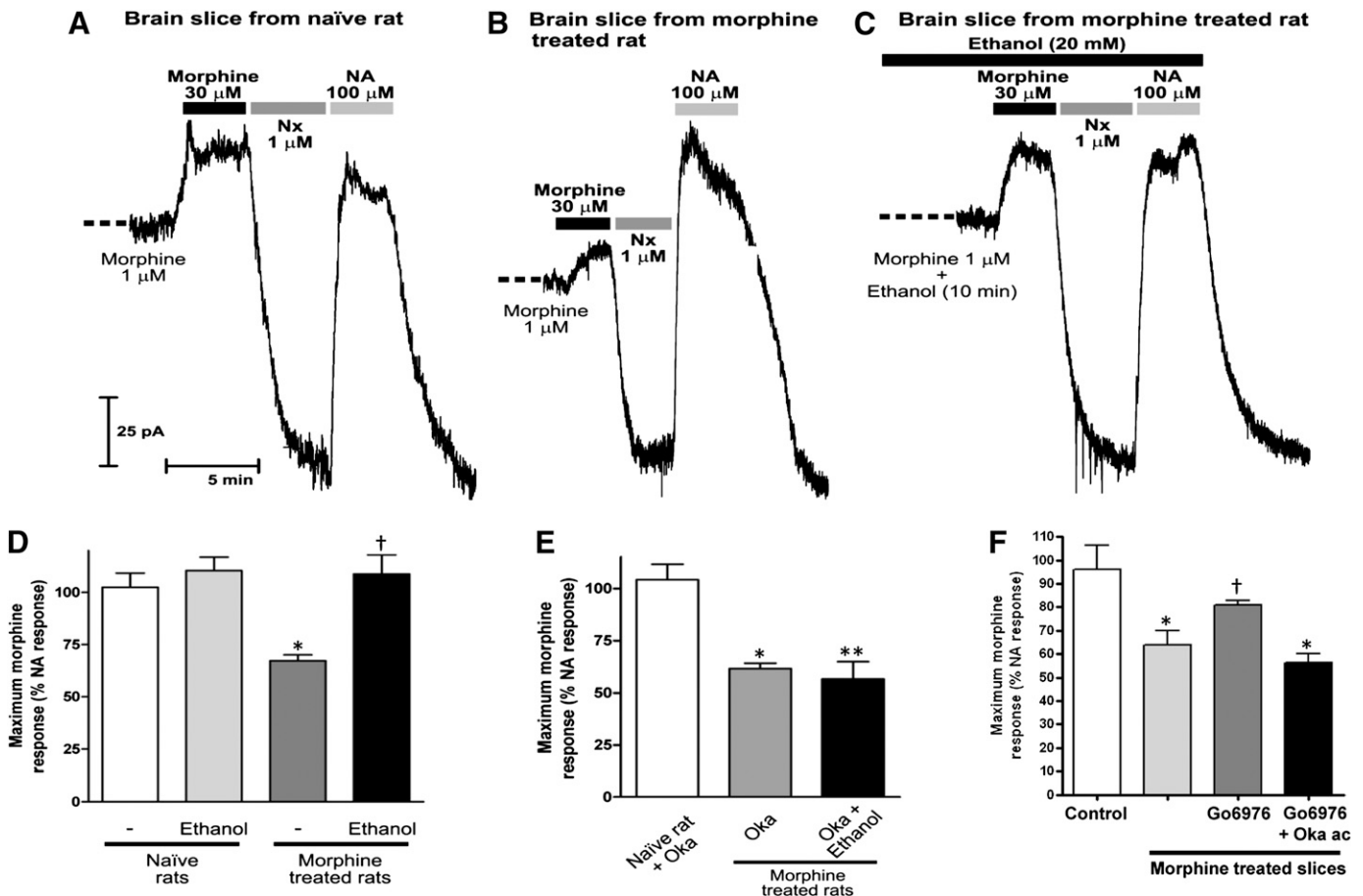**Ethanol Reversal of Cellular Tolerance to Morphine.** In LC neurons, MOPr activation results in the generation of an outward potassium current through G-protein–activated inwardly rectifying K channels (GIRKs), the amplitude of which can be used as a measure of receptor activation. We previously reported that prolonged exposure of LC neurons to morphine either in vivo or in vitro desensitizes the MOPr and that this receptor desensitization underlies acute cellular tolerance (Bailey et al., 2009a). Morphine is a partial agonist at MOPrs in LC neurons, evoking a lower maximum outward current than other opioids such as Met-enkephalin and DAMGO (Alvarez et al., 2002; Bailey et al., 2003). For any partial agonist, the maximum response is produced only when all the available receptors are occupied and any loss of MOPr function, as would occur if receptors were desensitized, results in a decrease in the maximum response evoked by morphine. Therefore, the MOPr desensitization underlying cellular tolerance to morphine can be measured as a decrease in the GIRK current evoked by a maximally effective, receptor-saturating concentration of morphine (Bailey et al., 2009a; Levitt and Williams 2012). In the present study, brain slices

were prepared either from rats pretreated in vivo with morphine for 3 days (to induce tolerance in vivo) or from naïve rats and then incubated in morphine (1 μM) for 5–9 hours (to induce tolerance in vitro) (Bailey et al., 2009a). Induction of tolerance to a submaximal concentration of morphine in vitro requires elevation of PKC activity, and so we included 10 μM oxotremorine-M (oxo-M) along with morphine in the bathing solution. In all experiments, during slice setting up and electrophysiological recording, the fluid bathing the brain contained 1 μM morphine to sustain tolerance and to prevent the neurons from going into withdrawal.

As reported previously (Bailey et al., 2009a), in LC neurons chronically exposed to morphine in vivo or in vitro, the response to a maximally effective concentration of morphine (30 μM) was significantly lower than that observed in parallel experiments on LC slices from untreated animals (Fig. 1, A, B, D, and F); that is, cellular tolerance had developed. By comparison, the maximum response evoked by NA (100 μM) was not affected by the morphine treatment (Supplemental Fig. 1; Bailey et al., 2009a). When morphine-tolerant neurons were exposed to ethanol (20 mM) for just 10 minutes before and then during the challenge with morphine (30 μM), the cellular tolerance was reversed (Fig. 1, C and D). Ethanol (20 mM) did not alter the maximum response evoked by NA in LC neurons in control slices or in slices prepared from morphine-treated animals (Supplemental Fig. 1). To determine how quickly ethanol reversed morphine tolerance, we first induced morphine tolerance in vitro and then, with the brain slice exposed to morphine (1 μM), applied ethanol (20 mM) and monitored the rate at which the opioid-activated current increased as tolerance was reversed. The effect of ethanol commenced as soon as the cell was exposed to the drug and was complete in 5 to 6 minutes (Fig. 2A).

The reversal of morphine-induced cellular tolerance by ethanol does not result from a direct effect of ethanol to potentiate current through the GIRK channels in LC neurons. At the low concentration of ethanol used (i.e., 20 mM), there was no change in the amplitude of the current evoked by morphine in neurons taken from nonmorphine-pretreated animals (control 166 ± 16 pA, $n = 5$; in presence of ethanol 173 ± 8 pA, $n = 4$, mean ± S.E.M.). We observed only a direct effect of ethanol to potentiate GIRK channel current in LC neurons from nonmorphine-pretreated animals at a concentration of ethanol of 100 mM (Fig. 2B).

To determine whether the reversal of morphine cellular tolerance by ethanol requires protein dephosphorylation, we applied the phosphatase inhibitor okadaic acid (1 μM) to the inside of LC neurons by including the drug in the recording pipette and allowing it to diffuse into the cell for at least 15–20 minutes before the application of a maximally effective concentration of morphine (30 μM). Okadaic acid did not affect the acute response to morphine in LC neurons from control, nonmorphine-pretreated animals and did not alter the degree of tolerance observed in slices taken from morphine-treated animals (Fig. 1E). However, okadaic acid did prevent the reversal of tolerance that was produced by a 10-minute exposure to ethanol 20 mM (Fig. 1E). We previously reported that cellular tolerance to morphine in LC neurons involves PKCα (Bailey et al., 2009a). In the present study, we observed that okadaic acid also prevented the reversal of cellular tolerance to morphine by the PKC inhibitor 5,6,7,13-tetrahydro-13-methyl-5-oxo–12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile

Fig. 1. Ethanol reversal of cellular tolerance to morphine in LC neurons taken from morphine-treated rats. Opioid-activated GIRK currents were recorded from individual LC neurons in rat brain slices. Recordings of GIRK current evoked by morphine (30 μM) and NA (100 μM) in slices from naïve and morphine-treated animals are shown. All slices were maintained in 1 μM morphine in vitro. (A) In a slice prepared from an untreated animal, the amplitude of the response to morphine (30 μM) is similar to the response to NA (100 μM). (B) In a slice from a morphine-treated animal, the response to morphine was reduced compared with that of noradrenaline, indicating the development of cellular tolerance. (C) In a slice from a morphine-treated animal subsequently exposed to ethanol for 10 minutes before and then during the challenge with morphine (30 μM), no tolerance was observed. All morphine-activated currents were reversed by naloxone (Nx). (D) Pooled data of the amplitude of the GIRK current evoked by morphine (30 μM) normalized to the response to noradrenaline (100 μM) in the same neuron (n = 4–6). Ethanol (20 mM) reversed the cellular tolerance to morphine in slices taken from morphine-treated rats. (E) Pooled data from at least three neurons in each treatment group showing that inclusion of the phosphatase inhibitor, okadaic acid (Oka, 1 μM) in the recording pipette solution prevented the reversal by ethanol of cellular tolerance to morphine in slices prepared from morphine-treated rats. (F) Pooled data from at least three neurons showing that inclusion of the phosphatase inhibitor Oka (1 μM) in the recording pipette solution prevented the reversal by the PKC inhibitor Gö6976 (1 μM) of cellular tolerance to morphine in slices prepared from morphine-treated rats. (E and F) Oka was allowed to dialyze into the cell from the recording pipette for 15–20 minutes before measuring the response to morphine. (D) *Indicates a significant difference ($P < 0.001$) from naïve control group; † indicates a significant difference ($P < 0.001$) from morphine-treated control group; (E) * and ** indicate a significant difference ($P < 0.05$ and $P < 0.01$, respectively) compared with naïve control group (analysis of variance followed by Bonferroni test). (F) *Indicates a significant difference ($P < 0.05$) from control (for morphine-treated) and from Gö6976 alone (for the morphine-treated + Gö6976) groups, and † indicates a significant difference ($P < 0.05$) from the morphine-treated control group.

(Gö6976) (Fig. 1F). These results indicate that the reversal of morphine tolerance by either ethanol or a PKC inhibitor involves protein dephosphorylation.

Effect of Ethanol on Tolerance Induced by the Opioid Peptide DAMGO. We previously reported that the mechanism of MOPr desensitization and cellular tolerance in LC neurons is agonist-dependent (Bailey et al., 2009a,b). Unlike desensitization induced by morphine, desensitization by the high-efficacy peptide agonist DAMGO is not PKC-dependent. DAMGO-induced desensitization may involve G protein–coupled receptor kinase (GRK) (Bailey et al., 2009b; but see Quillinan et al., 2011). We therefore sought to compare the effect of ethanol on cellular tolerance to DAMGO and morphine. As DAMGO is a peptide, we induced cellular tolerance in LC neurons using prolonged exposure to the opioid agonists in

vitro. We incubated brain slices prepared from opioid-naïve rats in bathing fluid containing either DAMGO (100 nM for 3–5 hours) or morphine (1 μM for 3–9 hours) at 33–34°C.

After prolonged DAMGO exposure, the maximum response to morphine was significantly lower than that observed in LC slices incubated for similar periods in bathing solution alone, indicating that DAMGO had induced MOPr desensitization and cellular tolerance (Fig. 2C). When slices preincubated with DAMGO were then exposed to ethanol (20 mM) for 10 minutes before and during the assessment of tolerance, there was no reversal of the DAMGO-induced tolerance (Fig. 2C). In contrast, cellular tolerance induced by prolonged in vitro exposure to morphine (1 μM plus oxo-M 10 μM)) was reversed by ethanol, 20 mM (Fig. 2D), but not by ethanol, 5 mM (unpublished data).
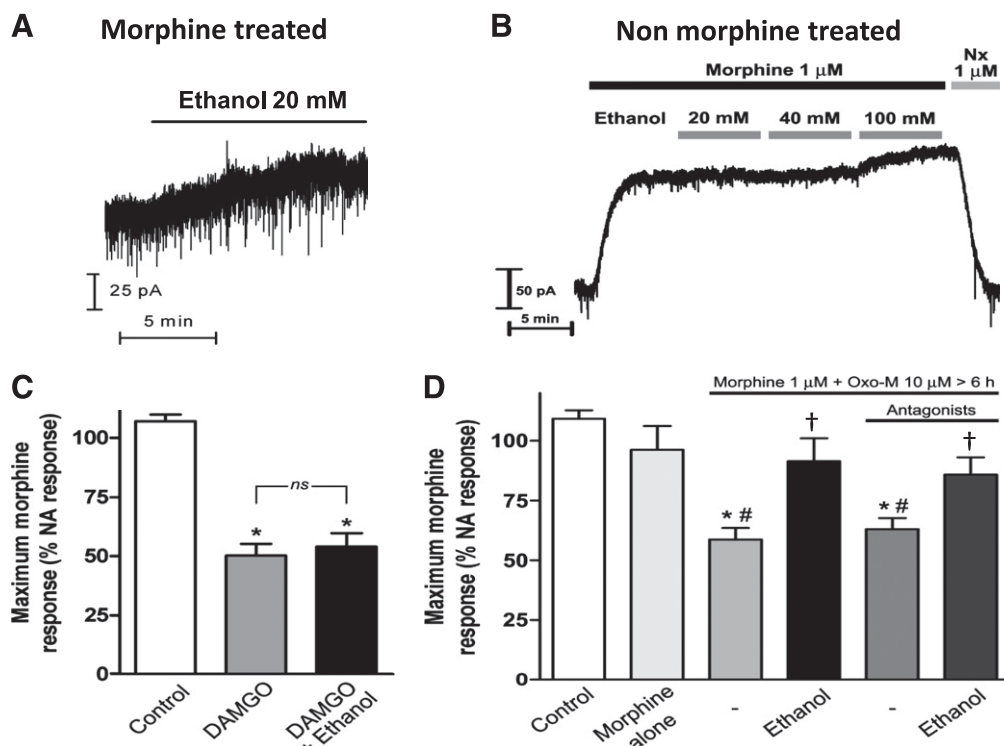
Fig. 2. Ethanol reversal of morphine-induced but not DAMGO-induced tolerance in LC neurons in vitro. (A) Membrane current recorded from an LC neuron in a slice prepared from a morphine-treated rat and maintained in morphine (1 μM). Exposure to ethanol evoked an outward current that developed over 5 to 6 minutes. (B) Recording from a single LC neuron from an untreated animal showing that the outward GIRK channel current evoked by morphine was potentiated significantly only when the ethanol concentration applied was 100 mM. All outward current was reversed by naloxone (Nx). (C) Brain slices were prepared from untreated, naïve animals and incubated with DAMGO (100 nM) or control bathing solution (control) for 3–5 hours in vitro. The histogram shows pooled data of the amplitude of the GIRK current evoked by a subsequent exposure of the slice to morphine (30 μM) to determine the level of cellular tolerance. The amplitude of the morphine response is normalized to the response to NA (100 μM) in the same neuron. Prolonged exposure of the slices to DAMGO reduced the subsequent response to morphine, indicating that cellular tolerance had been induced. Exposure of slices to ethanol (20 mM) for 10 minutes before and then during the challenge with morphine (30 μM) did not alter the level of cellular tolerance induced by DAMGO (n = 4 to 5). (D) Brain slices were prepared from untreated, naïve animals and incubated with morphine (1 μM) or morphine (1 μM) plus oxo-M (10 μM) for 6–9 hours in vitro. Neurons incubated with morphine plus oxo-M showed a reduced response to morphine (30 μM), indicating the development of cellular tolerance. Exposure of neurons to ethanol (20 mM) for 10 minutes before and then during the challenge with morphine (30 μM) reduced the level of cellular tolerance observed. No effect was observed on either the level of cellular tolerance induced by morphine plus oxo-M or the reversal of tolerance by acute exposure to ethanol when slices were perfused with a combination of the GABA$_A$ receptor antagonist bicuculline (10 μM), the NMDA receptor antagonist D-AP5 (25 μM), the glycine receptor antagonist strychnine (10 μM), and the L-type Ca^{2+} channel inhibitor nimodipine (3 μM) (antagonists) for at least 20 minutes before and during the application of morphine (30 μM) to determine the level of cellular tolerance. *Indicates a significant difference (P < 0.001) from control group; # indicates a significant difference (P < 0.01) from the respective morphine alone group; † indicates a significant difference (P < 0.05) from the respective morphine + oxo-M group; ns, not significant. (C) Analysis of variance (ANOVA) followed by Bonferroni test and in (D) ANOVA followed by Newman-Keus test.

Effect of GABA$_A$, Glycine, and *N*-Methyl-D-Aspartic Acid Receptor Antagonism and Calcium Channel Blockade on Ethanol Reversal of Morphine Tolerance. Ethanol is a drug with multiple actions (Harris et al., 2008). These include potentiation of agonist activation of GABA$_A$ and glycine receptors, inhibition of *N*-methyl-D-aspartic acid (NMDA) receptor-mediated responses, and inhibition of voltage-activated calcium channel currents. We sought to determine first whether any of these receptors and channels was involved in the maintenance of morphine tolerance in LC neurons and second whether they were involved in the reversal of tolerance by ethanol. To do this, we exposed slices to a cocktail of inhibitors comprising the GABA$_A$ receptor antagonist bicuculline (10 μM), the NMDA receptor antagonist D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5; 25 μM), the glycine receptor antagonist strychnine (10 μM), and the L-type Ca^{2+} channel blocker nimodipine (3 μM). In slices rendered tolerant to morphine in vitro (slices exposed to morphine 1 μM plus oxo-M 10 μM for 6–9 hours) exposure to

the cocktail of inhibitors for at least 20 minutes before and during the assessment of tolerance failed to modify the level of tolerance observed (Fig. 2D). Similarly, in the presence of the cocktail of inhibitors, ethanol still reversed the level of morphine tolerance (Fig. 2D). We conclude, therefore, that the effect of ethanol to reverse morphine tolerance in LC neurons is independent of the known effects of ethanol on GABA$_A$, glycine, and NMDA receptors as well as L-type calcium channels.

Effect of Ethanol on PKC and Phosphatase Activity. Previous studies have revealed a role for PKCα in morphine-induced MOPr desensitization in LC neurons (Bailey et al., 2009b) and several PKC isoforms, including PKCα in anti-nociception tolerance in vivo (Smith et al., 2007). Different groups have reported different results when examining the effects of ethanol on PKCα activity. Slater et al. (1997) and Reneau et al. (2011) reported a modest inhibition of PKCα by ethanol, whereas Rex et al. (2008) found no effect of ethanol on PKCα. We therefore sought to determine ourselves whether

ethanol, at the relatively low concentration that reversed morphine cellular tolerance (20 mM), could significantly inhibit PKCα. To do this, we used several assays of PKC activity: recombinant PKCα in vitro and recombinant PKCα in liposomes in vitro in the absence and presence of increasing concentrations of diacylglycerol (DAG). We did not observe a significant inhibition of PKC activity in the presence of ethanol 20 mM (see Supplemental Fig. 2, A–D). We did observe a small ~20%, statistically significant inhibition of PKCα activity with 100 mM ethanol when activity was measured in liposomes containing 4 and 8% DAG but no inhibition with 20 mM ethanol (Supplemental Fig. 2, B and C). We also measured the effect of ethanol on total endogenous PKC activity in supernatants from mouse cortex and striatum (Supplemental Fig. 2D). There appeared to be a small inhibition of endogenous PKC activity that increased as the ethanol concentration was increased, but even at 100 mM ethanol, this did not achieve statistical significance. We are therefore unable to conclude that ethanol 20 mM produces significant inhibition of PKC activity. We next examined whether the effect of ethanol could be to potentiate phosphatase activity rather than by inhibiting kinase activity. However, in supernatants from mouse cortex and striatum, the addition of ethanol (20 mM) did not alter phosphatase activity (Supplemental Fig. 2E).

Effect of Ethanol on the Interaction between Morphine and MOPr in HEK293 Cells. The reversal of MOPr tolerance observed in LC neurons already described herein could result not from a reversal of MOPr desensitization but rather from an enhancement of the response through the remaining functional MOPrs or an increase in the number of MOPrs on the plasma membrane. To investigate these potential mechanisms, we examined the effects of ethanol on agonist binding, agonist efficacy, receptor phosphorylation, and receptor trafficking to and from the plasma membrane in HEK293 cells stably expressing MOPrs.

To determine the effect of ethanol on morphine binding to MOPrs, we performed radioligand displacement studies with [^{3}H]naloxone. Na$^+$ ions were included in the assay buffer to mimic the situation in our brain slice experiments in which the extracellular buffer contains Na$^+$. The presence of Na$^+$ promotes a low-affinity state of the receptor, presumed to be uncoupled from G protein, thus reducing the affinity of agonist binding (Strange, 2008). The presence of ethanol (20 mM) did not alter the affinity of morphine binding to MOPr (Fig. 3A). The K_i for morphine binding in the absence and presence of ethanol was 544 nM (95% CI: 282 nM to 1 μM) and 458 nM (95% CI: 224–934 nM), respectively.

We next examined whether ethanol might enhance the agonist efficacy of morphine. We measured the concentration-dependence of morphine stimulation of [^{35}S]GTPγS binding in HEK293 cells stably expressing MOPrs. The presence of ethanol (20 mM) did not alter the ability of morphine to stimulate GTPγS binding (Fig. 3B).

To determine whether ethanol affects agonist-induced MOPr phosphorylation, cells were exposed to a receptor saturating concentration of morphine, 30 μM, for 10 minutes. At this concentration, morphine induced a modest increase in MOPr phosphorylation that was less than that induced by a receptor saturating concentration of DAMGO (10 μM, Fig. 3C) (Johnson et al., 2006). In experiments where cells were preincubated with ethanol (20 mM) for 15 minutes before and then during a 10-minute exposure to morphine, we observed

a consistent decrease in MOPr phosphorylation (Fig. 3, C and D). Phosphorylation induced by DAMGO was unaffected by the presence of ethanol.

To examine whether ethanol altered the trafficking of MOPrs from the plasma membrane, we used an enzyme-linked immunosorbent assay assay to determine the level of expression of T7-tagged MOPrs stably expressed in HEK293 cells. A 10- or 30-minute exposure to ethanol (20 mM) did not alter the level of MOPr expression in nonopioid-treated cells (Fig. 3E). Similarly, a brief, 10-minute exposure to ethanol 20 mM did not alter the internalization of MOPr in cells exposed to morphine (1 or 30 μM) or DAMGO (10 μM) for 30 minutes (Fig. 3F). As previously reported (McPherson et al., 2010), DAMGO induced greater receptor internalization than did morphine.

Discussion

We have observed that 20 mM ethanol reverses morphine tolerance in LC neurons. In the UK and in most states in the United States, the legal driving limit is 80 mg of ethanol per 100 ml of blood (17.4 mM). Therefore, the reversal of morphine tolerance occurs at an ethanol concentration that would be only mildly intoxicating in humans. The effect of ethanol was rapid in onset and was seen when LC neurons had already been exposed to morphine (i.e., ethanol reverses morphine tolerance after it has developed).

With the opioid treatments we have used in this study, tolerance results from a loss of MOPr function, that is, desensitization (Bailey et al., 2009a; Llorente et al., 2012). Longer morphine treatments may recruit a second component to tolerance (Levitt and Williams, 2012). We used activation of GIRK current in LC neurons as our readout of MOPr function (Bailey et al., 2009a). In control experiments, we demonstrated that ethanol (20 mM) did not affect the opioid-activated GIRK current. Similarly, Osmanović and Shefner (1990) observed no effect of ethanol 40–60 mM on the GABA$_B$ receptor-activated GIRK current in LC neurons. Recombinant GIRK currents in oocytes are potentiated by ethanol but at concentrations greater than 30 mM (Kobayashi et al., 1999; Lewohl et al., 1999). Furthermore, in LC neurons, ethanol (20 mM) did not reverse tolerance to DAMGO, also measured using GIRK activation. Therefore, the reversal of morphine tolerance is not simply an artifact of ethanol potentiating GIRK channel current.

Acute ethanol reversed the tolerance induced by prolonged exposure to morphine but not by prolonged exposure to DAMGO. This is most likely due to the underlying mechanism of desensitization being agonist specific (Kelly et al., 2008) and ethanol affecting only the desensitization mechanisms underlying morphine tolerance. In rat LC neurons, DAMGO-induced desensitization of MOPr is blocked by overexpression of a GRK-dominant negative mutant (Bailey et al., 2009b), although others using a genetic approach suggested that GRK was not involved in Met-enkephalin-induced desensitization (Quillinan et al., 2011). Ethanol does not inhibit GRK (Rex et al., 2008), and that would fit with ethanol not reversing DAMGO-induced tolerance. In contrast, morphine induces MOPr desensitization in LC neurons primarily through a PKC-dependent mechanism.

Exposure to morphine results in an enhancement of MOPr phosphorylation that is reduced in the presence of a PKC
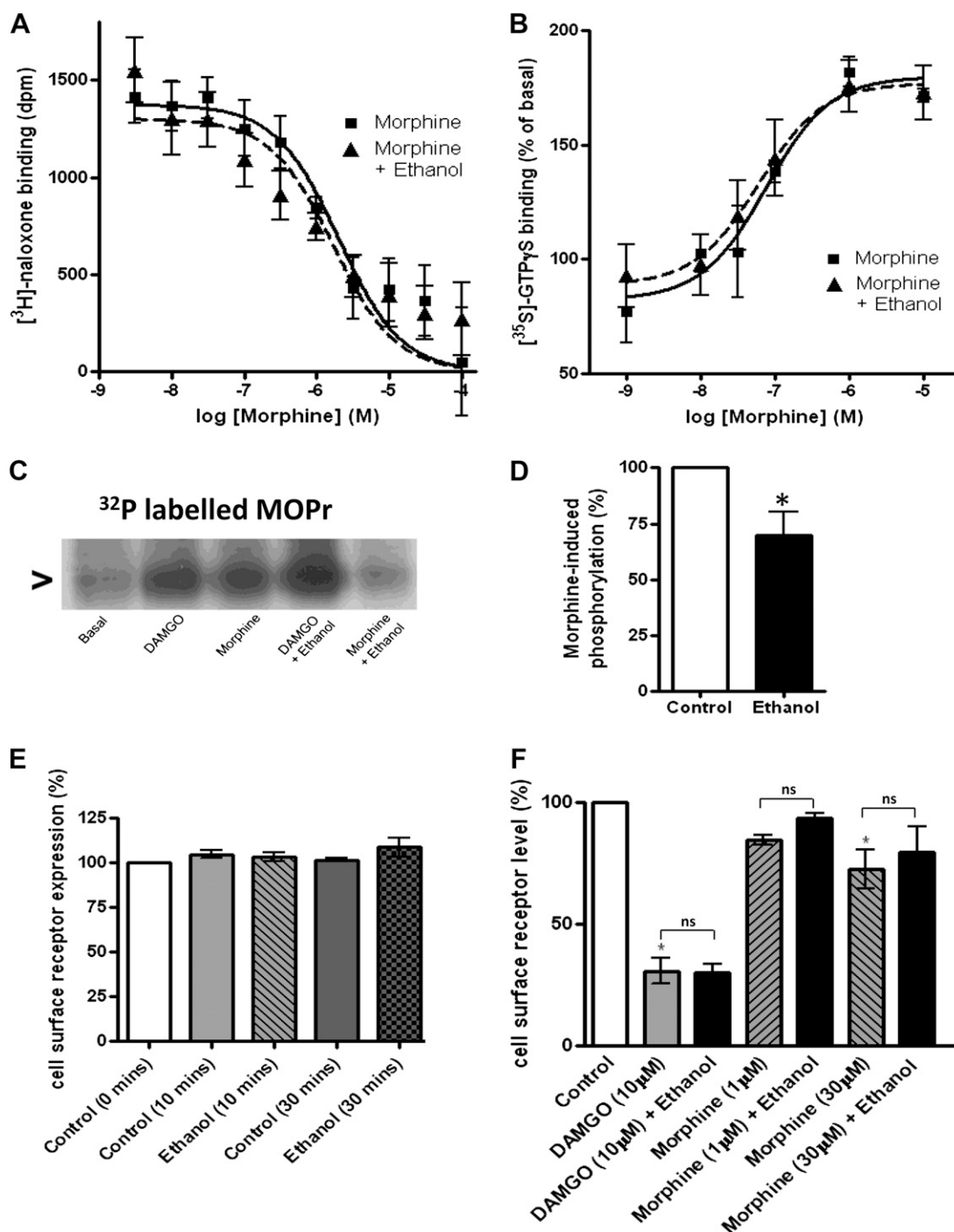
Fig. 3. Effect of ethanol on MOPr function in HEK293 cells. (A) Displacement of specific [³H]naloxone binding by morphine in the absence (squares) and presence of 20 mM ethanol (triangles) from membranes prepared from HEK293 cells stably expressing MOPrs ($n = 5$ for each). (B) Stimulation of [³⁵S]GTPγS binding to membranes prepared from HEK293 cells stably expressing MOPrs by morphine in the absence (squares) and presence (triangles) of 20 mM ethanol ($n = 4$ for each). (C) Phosphorimage of immunoprecipitated MOPr from [³²P]-labeled HEK293 cells stably expressing MOPrs and subjected to SDS-PAGE. Arrowhead indicates the position of MOPr on the gel at ~80 kDa. Cells were exposed to DAMGO (10 μM) or morphine (30 μM) for 10 minutes in the absence or presence of ethanol (20 mM). Cells were pre-exposed to ethanol for 15 minutes before the addition of the opioid agonists. (D) Quantification of seven experiments of the type shown in (C) to determine the level of MOPr phosphorylation by morphine (30 μM) in the absence (control) and presence of ethanol (20 mM). To reduce interexperimental variability, the density of the phosphorylated MOPr band from cells treated with morphine plus ethanol was calculated as a percentage of the density of the band from cells treated with morphine alone obtained from a sample loaded on the same gel. *Indicates $P < 0.05$ compared with morphine alone. (E and F) Measurement of cell surface T7-tagged MOPrs in HEK293 cells. (E) Cell surface receptors were labeled with primary antibody after exposure to ethanol, and the level of receptor expression on the plasma membrane measured by subsequent enzyme-linked immunosorbent assay. (F) Cells were first labeled with primary antibody and then exposed to DAMGO or morphine for 30 minutes. This gives a measure of drug-induced cell surface receptor loss. Ethanol was added for 10 minutes at the end of the exposure to opioid drug; $n = 3$–5 for each treatment. *Indicates a significant difference from control ($P < 0.05$). Ethanol had no effect on the level of cell surface receptors after treatment with either DAMGO or morphine. ns, not significant.

inhibitor, whereas phosphorylation of MOPr induced by DAMGO is not reduced (Johnson et al., 2006). PKC phosphorylates Ser363 on the C-tail of MOPr (Feng et al., 2011; Chen et al., 2013). Whilst this residue is constitutively phosphorylated on MOPrs expressed in HEK293 cells, it is not known whether morphine increases the phosphorylation of this residue. DAMGO, and to a much lesser extent morphine, phosphorylates Ser375 on MOPr (Doll et al., 2011, 2012; Just et al., 2013). The smaller effect of morphine is consistent with its reduced ability to induce arrestin binding to MOPr and MOPr internalization (McPherson et al., 2010). In the present study, we have shown that morphine-induced MOPr phosphorylation is reduced by exposure to ethanol. Given that ethanol does not reduce DAMGO-induced phosphorylation, then the most likely interpretation of these finding is that ethanol does not reduce Ser375 phosphorylation, but it does reduce phosphorylation of other residues, such as Ser363.

We previously demonstrated that PKCα is the isoform involved in morphine-induced MOPr desensitization in LC neurons (Bailey et al., 2009b). Thus, a potential mechanism for ethanol reversal of morphine tolerance could be a reduction in PKCα activity. Ethanol reverses the acute agonist-induced desensitization of dopamine D1 and D2 receptors (Rex et al., 2008; Nimitvilai et al., 2012). In these studies, the mechanism of the ethanol effect was not fully elucidated, but inhibition of PKC activity was suggested.

The reported effects of ethanol on PKC activity are confusing and contradictory, both activation and inhibition of PKC having been reported. This is in part because the effects of ethanol are PKC isoform specific and also vary with the location of the enzyme, how it is activated, the substrate used, and the cofactors present (Stubbs and Slater, 1999; Newton and Ron 2007). Slater et al. (1997), using lipid vesicles, observed that ethanol inhibited PKCα by 20% at 50 mM and that the effect was dependent upon the presence of DAG; and Reneau et al. (2011), using purified PKCα alone (i.e., not in lipid vesicles), observed a 20% inhibition of PKCα by 10 mM ethanol. In contrast, Rex et al. (2008) reported that at 100 mM ethanol did not inhibit lipid-activated PKCα but did inhibit PKCγ and PKCδ. We did not observe a significant effect of ethanol (20 mM) on PKCα activity in the absence or presence of lipid activators. In addition, we have performed differential centrifugation and live-cell confocal microscopy experiments in HEK 293 cells with GFP-tagged PKC isoforms and a membrane-localized PKC activity FRET reporter, MyrPalm-CKAR (Gallegos et al., 2006), to measure membrane translocation of PKC as well as PKC activity at the plasma membrane. However, we have been unable to measure translocation of PKC to the plasma membrane in response to morphine or membrane PKC activity using these methods (Cunningham, Withey, and Oldfield, unpublished observations) and were thus unable to determine whether ethanol has a specific effect on membrane-localized PKC. It is therefore still debatable whether the effect of ethanol to reverse morphine tolerance in LC neurons results from inhibition of PKCα as a specific effect on membrane localized PKC activity in the vicinity of the MOPrs cannot be ruled out. Recently, it has been suggested that c-Jun N-terminal kinase may be involved in morphine tolerance (Melief et al., 2010), but c-Jun N-terminal kinase inhibitors do not inhibit morphine cellular tolerance in LC neurons (Lowe and Henderson, unpublished observations; Levitt and Williams, 2012). We cannot

exclude the possibility that ethanol might reverse morphine tolerance by inhibiting another, as yet unidentified kinase involved in MOPr desensitization and tolerance.

Ethanol reversal of morphine tolerance was prevented by okadaic acid, a protein phosphatase inhibitor. This would indicate that protein dephosphorylation is important for tolerance reversal. If ethanol acts by inhibiting PKC (or another unidentified kinase), then the requirement for phosphatase activity could be to dephosphorylate the relevant protein(s) phosphorylated by PKC. However, another interpretation of the ability of okadaic acid to inhibit the effect of ethanol would be that ethanol works by enhancing phosphatase activity. This seems unlikely, as we did not observe any effect of ethanol on phosphatase activity, and others have reported that ethanol decreases rather than increases protein phosphatase 2A activity (Hong-Brown et al., 2007). In addition, the decrease in D_1 receptor phosphorylation induced by ethanol persists in the presence of phosphatase inhibitors (Rex et al., 2008).

Ethanol can alter the arrangement of water-filled cavities around the transmembrane domains of plasma membrane proteins, thus modifying their function (Jung et al., 2005). However, we could find no evidence for a direct effect of ethanol on MOPr. Ethanol did not alter morphine's ability to bind to or activate the MOPr. In addition, we found no evidence for ethanol altering MOPr expression at the plasma membrane, which is in contrast to results obtained from PC12 cells expressing MOPr using fluorescence correlation spectroscopy by Vukojević et al. (2008). They reported that ethanol increased both the lateral mobility of MOPrs and the density of MOPrs on the plasma membrane.

Potentially, ethanol could have altered the level of morphine tolerance indirectly by potentiating agonist activation of $GABA_A$ and glycine receptors, by inhibition of NMDA receptor-mediated responses, or by inhibition of voltage-activated calcium channel currents, well documented effects of ethanol (Harris et al., 2008). We recently reported that ethanol reversal of morphine antinociception tolerance in mice can be prevented by blocking $GABA_A$ receptors (Hull et al., 2013). A difficulty with such in vivo behavioral experiments, however, is in determining whether the effect of ethanol is directly on the $GABA_A$ receptor or results in subsequent $GABA_A$ receptor activation. In LC neurons, antagonists of $GABA_A$, glycine, NMDA, or calcium channels did not reverse the level of tolerance (i.e., mimic ethanol) or prevent the action of ethanol. Therefore, the effect of ethanol to reverse cellular tolerance to morphine in LC neurons does not involve an effect on these channels.

Chronic ethanol exposure reduces MOPr coupling to G proteins (Chen and Lawrence, 2000; Sim-Selley et al., 2002; Saland et al., 2004). In the whole animal, acute exposure to ethanol reverses morphine tolerance (Hull et al., 2013), whereas chronic exposure to ethanol reduced the antinociceptive effect of morphine and DAMGO (He and Whistler, 2011). Thus acute and chronic ethanol exposure produces opposite effects on MOPr function and morphine tolerance.

In conclusion, acute ethanol exposure reverses morphine tolerance at a cellular level by reversing MOPr desensitization. This finding may have important implications for opioid addiction in humans. If in opioid addicts acute ethanol consumption rapidly reverses tolerance to the respiratory depressant effects of opioids, this would increase the risk of death from heroin overdose.

Author Contributions

Participated in research design: Llorente, Withey, Cunningham, Cooke, Saxena, Dewey, Bailey, Kelly, Henderson.

Conducted experiments: Llorente, Withey, Cunningham, Cooke, Rivero, Saxena, McPherson, Oldfield, Kelly.

Performed data analysis: Llorente, Withey, Cunningham, Cooke, Rivero, McPherson, Kelly.

Wrote or contributed to the writing of the manuscript: Dewey, Bailey, Kelly, Henderson.

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