

Ethanol reversal of cellular tolerance to morphine in rat locus coeruleus neurons

Javier Llorente, Sarah Withey, Guadalupe Rivero, Margaret Cunningham, Alex Cooke, Kunal Saxena, Jamie McPherson, Sue Oldfield, William L Dewey, Chris P Bailey, Eamonn Kelly and Graeme Henderson

School of Physiology and Pharmacology, University of Bristol, UK (JL, SW, GR, MC, AC, KS, JM, SO, EK, GH)

Department of Pharmacology and Toxicology, Virginia Commonwealth University, US (WLD)

Department of Pharmacy and Pharmacology, University of Bath, UK (CPB)

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Author for correspondence:

Graeme Henderson
School of Physiology & Pharmacology
University of Bristol
Bristol, BS8 1TD
UK
Email: Graeme.henderson@bris.ac.uk

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Abbreviations

CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; DAG, diacylglycerol; DAMGO [D-Ala²,N-MePhe⁴,Gly-ol]-enkephalin; DMEM, Dulbecco's modified Eagle's medium; FCS, fluorescence correlation spectroscopy; GABA, γ -aminobutyric acid; GDP, guanosine diphosphate; GIRK, G protein-gated K channel; GRK, G protein-coupled receptor kinase; GTP γ S guanosine 5'-O-[gamma-thio]triphosphate; LC, locus coeruleus, LC; μ -opioid receptor, MOPr; HEK, human embryonic kidney; NA, noradrenaline; NMDA,N-methyl-D-aspartic acid; oxo-M, oxotremorine-M; PKC, protein kinase C

ABSTRACT

Consumption of ethanol is a considerable risk factor leading to deaths in heroin overdose. We have 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-Ala²,N-MePhe⁴,Gly-ol]-enkephalin (DAMGO) was not reversed by ethanol. Previous studies in LC neurons have revealed a role for 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 PKC α . 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 HEK293 cells expressing the μ -opioid receptor (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 [³⁵S]-GTP γ S 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; Oliver *et al.*, 2007). A number of studies of heroin overdose deaths have reported a significant inverse relationship between blood ethanol and blood morphine concentrations at post-mortem (Ruttenber *et al.*, 1990; Darke and Ross 1999; Oliver *et al.*, 2007). 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 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 depressant and their effects may simply be additive or synergistic. However, another possibility is that ethanol could act to reduce tolerance to opioids and thus increases overdose risk. In this paper we have sought to determine whether acute ethanol exposure can reduce morphine tolerance.

We have previously reported that cellular tolerance to opioids can be induced in rat brainstem locus coeruleus (LC) neurons 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; saturated with 95% O_2 /5% CO_2 at 33 – 34 $^\circ\text{C}$. 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 $\text{M}\Omega$) 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 analysed off-line using pClamp.

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 continually recorded (Bailey *et al.*, 2009a & b). The opioid-evoked current was continuously 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 noradrenaline (NA; 100 μM) 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-Ala²,N-MePhe⁴,Gly-ol]-enkephalin (DAMGO) *in vitro*, ethanol in control or morphine treated slice, okadaic acid (Supplementary data 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 prazosin (1 μ M) and cocaine (3 μ M).

Induction of morphine tolerance

Rats in vivo. Rats were injected subcutaneously 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 (Arlacel A) 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 pre-incubation 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 h. To induce opioid tolerance, morphine (1 μ M) or DAMGO (100 nM) was added to the fluid bathing the slices for periods of up to 9 h. The fluid bathing the slices in the recording chamber also contained morphine or DAMGO at the same concentration as used in the pre-incubation treatment.

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

Cell culture

HEK293 cell lines stably expressing either N-terminally HA or T7 tagged rat MOPr were maintained at 37 °C in 5% CO₂, 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 [³H]-naloxone (1 mCi/mL) in Hank's Buffered Saline Solution (HBSS) containing 20 mM HEPES at pH 7.4 and increasing concentrations of unlabelled morphine. Total and non-specific binding were measured in the absence and presence of D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP, 4.32 µM). Reaction tubes were incubated with agitation for 2 hours at 24 °C to allow ligand binding to reach equilibrium. Following this, the binding reactions were stopped by rapid filtration of samples on glass fibre filter paper using a Brandel cell harvester, followed by 3 x 3 ml washes with ice cold 20 mM HEPES buffer. Following scintillation counting, specific binding was calculated by subtracting non-specific binding from total binding. Binding displacement curves were then fitted by non-linear regression to a single site competition binding model in order to obtain IC₅₀ values and converted to Ki values using the Cheng-Prusoff equation. The Ki value of [³H]-naloxone was taken as 1.5 nM.

[³⁵S]-GTP γ S binding assay. The binding of [³⁵S]-GTP γ S 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 min) 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 600 g for 10 min at 4 °C, the pellet discarded and the supernatant centrifuged at 48,000 g in a SORVALL® Discovery™ SE ultracentrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The final pellet (P2) containing the plasma membrane fraction was suspended in homogenization buffer and protein concentration determined by Bradford assay (Bradford, 1976). Aliquots of plasma membrane suspension were centrifuged at 17,500 g in a bench-top centrifuge and the resultant pellets maintained at -80 °C until required.

For the assay itself, 10 μ g of plasma membrane protein/tube were incubated in the presence or absence of 20 mM ethanol in a total volume of 500 μ l of incubation buffer (20 mM HEPES, 100 mM NaCl, 4 mM MgCl₂, pH 7.4) containing 10 μ M GDP, 200 μ M Tricine, 200 μ M DTT, various concentrations of agonist (DAMGO and morphine) and 100 pM [³⁵S]GTP γ S (1 mCi/mL; Perkin Elmer, Waltham, MA). Nonspecific binding was determined by the addition of 10 μ M GTP γ S to the assay. Reactions were allowed to proceed for 30 min before being stopped by rapid filtration on glass fibre filters using a Brandel cell harvester followed by 3 x 3 ml washes with ice-cold incubation buffer. The amount of [³⁵S]GTP γ S bound to membranes on individual filters was then determined by liquid scintillation counting.

MOPr phosphorylation.

T7-tagged MOPr expressing HEK293 cells were incubated with [³²P]-orthophosphate (0.15 mCi/mL) in phosphate-free DMEM for 2 h. 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, 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 [³²P] 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 (ELISA) 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, Nottingham, UK) after exposure to ethanol. Whereas, to measure receptor internalization cells were first incubated with primary anti T7 antibody and then exposed to opioid and/or ethanol. Cells were incubated with drugs in DMEM at 37 °C, then fixed in 3.7% formaldehyde and incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase; 1:1000; Sigma-Aldrich, Poole, Dorset, UK). 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 were 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 \pm standard error of the mean or means and 95% confidence intervals. Where appropriate unpaired two-tailed Student's t test, one-sample t test or ANOVA followed by Newman-Keus test or Bonferroni tests were 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 potassium channels (GIRKs) the amplitude of which can be used as a measure of receptor activation. We have 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 of 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 pre-treated *in vivo* with morphine for 3 days (to induce tolerance *in vivo*) or from naïve rats and then incubated in morphine (1 μ M) for 5-9 h (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 oxotremorine-M (oxo-M; 10 μ M) along with morphine in the bathing solution. In all experiments during slice setting up and electrophysiological recording the fluid bathing the brain contained morphine (1 μ M) 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 μ M) was significantly lower than that observed in parallel experiments on LC slices from untreated animals (Fig. 1A, B, D and F) i.e. cellular tolerance had developed. By comparison the maximum response evoked by NA (100 μ M) was not affected by the morphine treatment (Supplementary data Fig. 1 and Bailey *et al.*, 2009a). When morphine tolerant neurons were exposed to ethanol (20 mM) for just 10 min prior to and then during the challenge with morphine (30 μ M) the cellular tolerance was reversed (Fig. 1C & D). Ethanol (20 mM) did not alter the maximum response evoked by NA in LC neurons in control slices or slices prepared from morphine-treated animals (Supplementary data 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 μ M) 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 – 6 min (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, 20 mM, there was no change in the amplitude of the current

evoked by morphine in neurons taken from non-morphine pretreated animals (control 166 ± 16 pA, $n=5$; in presence of ethanol 173 ± 8 pA, $n=4$, mean \pm SEM). We only observed a direct effect of ethanol to potentiate GIRK channel current in LC neurons from non-morphine 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 μ M), 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 min prior to the application of a maximally effective concentration of morphine (30 μ M). Okadaic acid did not affect the acute response to morphine in LC neurons from control, non morphine-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 min exposure to ethanol 20 mM (Fig. 1E). We have previously reported that cellular tolerance to morphine in LC neurons involves PKC α (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 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 have previously reported that the mechanism of MOPr desensitization and cellular tolerance in LC neurons is agonist-dependent (Bailey *et al.*, 2009a and 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 h) or morphine (1 μ M for 3-9 h) at 33 – 34 °C.

Following prolonged DAMGO exposure the maximum response to morphine was significantly lower than that observed in LC slices incubated for similar periods of time in bathing solution alone indicating that DAMGO had induced MOPr desensitisation and cellular tolerance (Fig. 2C). When slices preincubated with DAMGO were then exposed to ethanol (20 mM) for 10 min prior to 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 μ M plus oxo-M 10 μ M)) was reversed by ethanol, 20 mM, (Fig. 2D) but not by ethanol, 5mM (data not shown).

Effect of GABA_A, glycine and NMDA 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 NMDA receptor-mediated responses and inhibition of voltage-activated calcium channel currents. We sought to determine first whether any of these receptors and channels were 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 μ M), the NMDA receptor antagonist D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5; 25 μ M), the glycine receptor antagonist strychnine (10 μ M) and the L-type Ca²⁺ channel blocker nimodipine (3 μ M). In slices rendered tolerant to morphine

in vitro (slices exposed to morphine 1 μ M plus oxo-M 10 μ M for 6 – 9 h) exposure to the cocktail of inhibitors for at least 20 min 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 PKC α in morphine-induced MOPr desensitization in LC neurons (Bailey *et al.*, 2009b) and several PKC isoforms including PKC α in antinociception tolerance *in vivo* (Smith *et al.* 2007). Different groups have reported different results when examining the effects of ethanol on PKC α activity. Slater *et al.* (1997) and Reneau *et al.* (2011) reported a modest inhibition of PKC α by ethanol whereas Rex *et al.* (2008) found no effect of ethanol on PKC α . We therefore sought to determine ourselves whether ethanol, at the relatively low concentration that reversed morphine cellular tolerance (20 mM), could significantly inhibit PKC α . To do this we utilised several assays of PKC activity – recombinant PKC α *in vitro* and recombinant PKC α 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 Supplementary data Fig. 2A-D). We did observe a small ~20%, statistically-significant inhibition of PKC α activity with 100 mM ethanol when activity was measured in liposomes containing 4% and 8% DAG but no inhibition with 20 mM ethanol (Supplementary data Fig. 2B & C). We have also measured the effect of ethanol on total endogenous PKC activity in in supernatants from mouse cortex and striatum (Supplementary data 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 addition of ethanol (20 mM) did not alter phosphatase activity (Supplementary data Fig. 2E).

Effect of ethanol on the interaction between morphine and MOPr in HEK293 cells

The reversal of MOPr tolerance observed in LC neurons described above could result, not from a reversal of MOPr desensitization, but 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 [³H]-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 nM to 934 nM) respectively.

We next examined whether ethanol might enhance the agonist efficacy of morphine. We measured the concentration-dependence of morphine stimulation of [³⁵S]-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 min. 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 and see also Johnson *et al.*, 2006). In experiments where cells were pre-incubated with ethanol (20 mM) for 15 min prior to and then during a 10 min exposure to morphine we observed a consistent decrease in MOPr phosphorylation (Fig. 3C & 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 ELISA assay to determine the level of expression of T7-tagged MOPrs stably expressed in HEK293 cells. A 10 or 30 min exposure to ethanol (20 mM) did not alter the level of MOPr expression in non-opioid treated cells (Fig. 3E). Similarly a brief, 10 min 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 min (Fig. 3F). As previously reported (McPherson *et al.*, 2010) DAMGO induced greater receptor internalization than morphine.

DISCUSSION

We have observed that 20 mM ethanol reverses morphine tolerance in LC neurons. In the UK and in most states in the USA, 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 only be 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 i.e. 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 read-out 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 artefact of ethanol to 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 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 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). While 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 internalisation (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 reduces phosphorylation of other residues such as Ser363.

We have previously demonstrated that PKC α 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 PKC α 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 co-factors present (Stubbs and Slater, 1999; Newton and Ron 2007). Slater *et al.*, 1997 observed using lipid vesicles that ethanol inhibited PKC α by 20% at 50 mM and that the effect was dependent upon the presence of DAG and Reneau *et al.* (2011) using purified PKC α alone (i.e. not in lipid vesicles), observed a 20% inhibition of PKC α by 10 mM ethanol. In contrast, Rex *et al.* (2008) reported that at 100 mM ethanol did not inhibit lipid-activated PKC α but did inhibit PKC γ and PKC δ . We have failed to observe a significant effect of ethanol (20 mM) on PKC α 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-localised 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 localised PKC. It is therefore still debatable whether the effect of ethanol to reverse morphine tolerance in LC neurons results from inhibition of PKC α as a specific effect on membrane localised PKC activity in the vicinity of the MOPrs cannot be ruled out. Recently, it has been suggested that c-Jun N-terminal kinase (JNK) may be involved in morphine tolerance (Melief *et al.*, 2010 but JNK inhibitors do not inhibit

morphine cellular tolerance in LC neurons (Lowe & Henderson, unpublished observations, Levitt & 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 D₁ 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. This is in contrast to results obtained from PC12 cells expressing MOPr using fluorescence correlation spectroscopy (FCS) 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 have 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* behavioural 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 then this would increase the risk of death due to heroin overdose.

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Author Contributions

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

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Footnotes

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FIGURE LEGENDS

Figure 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 μ M) and noradrenaline (100 μ M; NA) in slices from naïve and morphine-treated animals are shown. All slices were maintained in morphine 1 μ M *in vitro*. **A.** In a slice prepared from an untreated animal the amplitude of the response to morphine (30 μ M) is similar to that to noradrenaline (100 μ M, NA). **B.** In a slice from a morphine-treated animal the response to morphine was reduced compared to that of noradrenaline, indicating the development of cellular tolerance. **C.** In a slice from a morphine treated animal subsequently exposed to ethanol for 10 min prior to and then during the challenge with morphine (30 μ M) 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 μ M) normalized to the response to noradrenaline (100 μ M) 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 3 neurons in each treatment group showing that inclusion of the phosphatase inhibitor, okadaic acid (1 μ M; Oka) 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 3 neurons showing that inclusion of the phosphatase inhibitor, okadaic acid (1 μ M; Oka) in the recording pipette solution prevented the reversal by the PKC inhibitor Gö6976 (1 μ M) of cellular tolerance to morphine in slices prepared from morphine-treated rats. In **E** and **F** Okadaic acid was allowed to dialyse into the cell from the recording pipette for 15 - 20 min prior to measuring the response to morphine. In **D** * indicates a significant difference (p<0.001) from naïve control group and † indicates a significant difference (p<0.001) from morphine-treated control group. In **E** * and ** indicate a

significant difference ($p < 0.05$ and $p < 0.01$ respectively) compared to naïve control group (ANOVA followed by Bonferroni test). In **F** * indicates a significant difference ($p < 0.05$) from control (for morphine-treated) and from G6976 alone (for morphine-treated +G6976) groups and † indicates a significant difference ($p < 0.05$) from morphine-treated control group

Figure 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 5-6 min. **B.** Recording from a single LC neuron from an untreated animal showing that the outward GIRK channel current evoked by morphine was only significantly potentiated 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 h *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 noradrenaline (100 μM ; NA) 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 min prior to and then during the challenge with morphine (30 μM) did not alter the level of cellular tolerance induced by DAMGO ($n = 4 - 5$). **D.** Brain slices were prepared from untreated, naïve animals and incubated with morphine (1 μM) or morphine (1 μM) plus oxotremorine-M (10 μM ; Oxo-M) for 6 - 9 h *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 min prior to

and then during the challenge with morphine (30 μ M) 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 μ M), the NMDA receptor antagonist D-AP5 (25 μ M), the glycine receptor antagonist strychnine (10 μ M) and the L-type Ca²⁺ channel inhibitor nimodipine (3 μ M) (Antagonists) for at least 20 min before and during the application of morphine (30 μ M) 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 and † indicates a significant difference (p<0.05) from the respective morphine + Oxo-M group. In **C** ANOVA followed by Bonferroni test and in **D** ANOVA followed by Newman-Keus test).

Figure 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 ethanol 20 mM (triangles) from membranes prepared from HEK293 cells stably expressing MOPrs (n = 5 for each). **B.** Stimulation of [³⁵S]-GTP γ S binding to membranes prepared from HEK293 cells stably expressing MOPrs by morphine in the absence (squares) and presence (triangles) of ethanol 20 mM (n = 4 for each). **C.** Phosphorimage of immunoprecipitated MOPr from [³²P]-labeled HEK293 cells stably expressing MOPrs and subjected to SDS-PAGE. The arrow head indicates the position of MOPr on the gel at ~80 kDa. Cells were exposed to DAMGO (10 μ M) or morphine (30 μ M) for 10 min in the absence or presence of ethanol (20 mM). Cells were pre-exposed to ethanol for 15 min 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 μ M) in the absence (Control) and presence of ethanol (20 mM). In order to reduce inter experimental 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 to morphine alone. **E and F.** Measurement of cell surface T7 tagged MOPr in HEK293 cells. In **E** cell surface receptors were labelled with primary antibody after exposure to ethanol and the level of receptor expression on the plasma membrane measured by subsequent ELISA. In **F** cells were first labelled with primary antibody and then exposed to DAMGO or morphine for 30 min. This gives a measure of drug-induced cell surface receptor loss. Ethanol was added for 10 min 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.

Figure 1

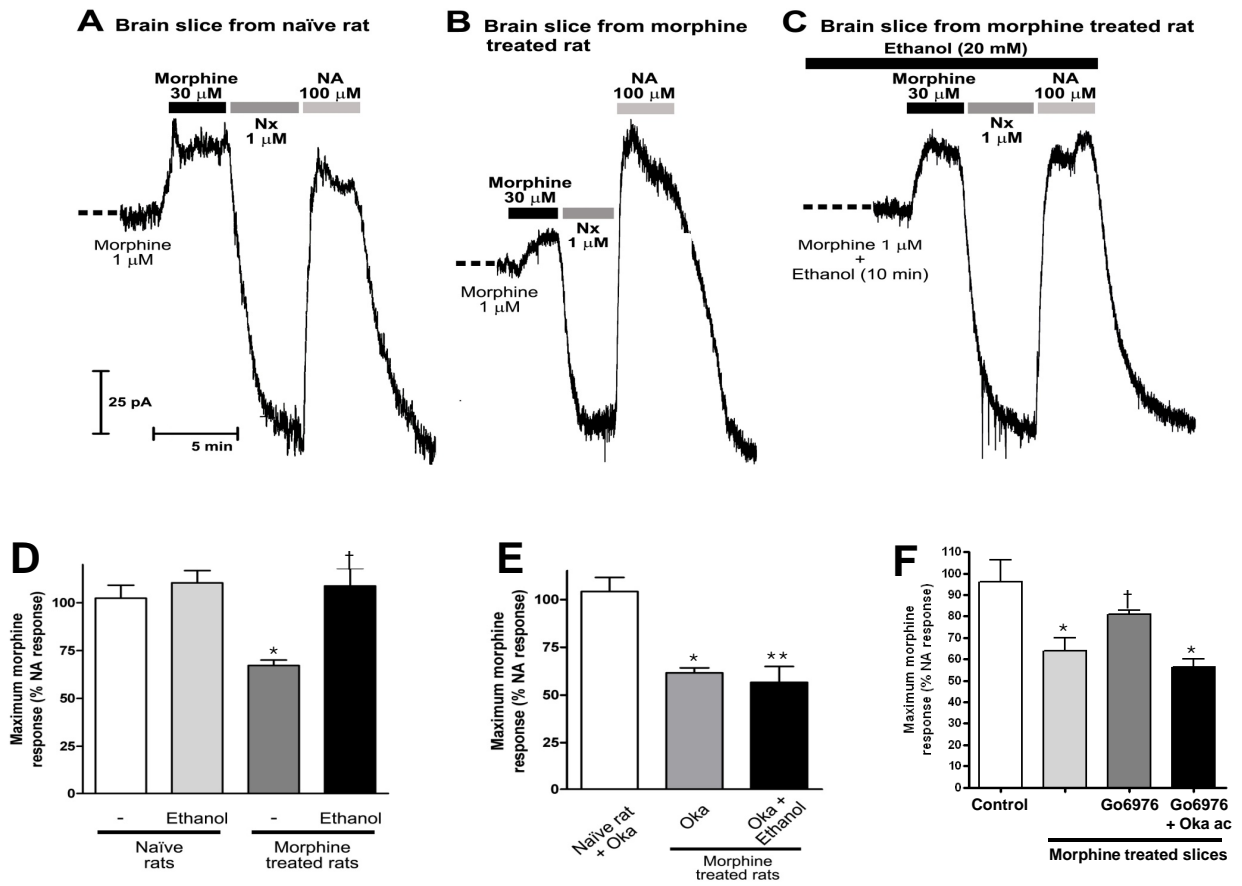
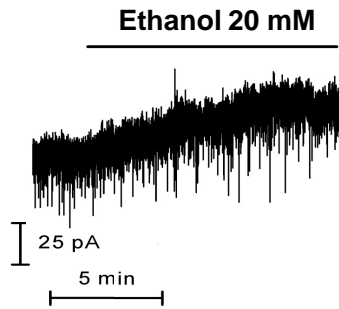
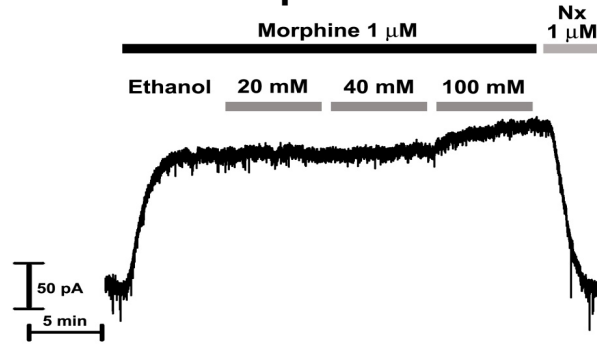


Figure 2

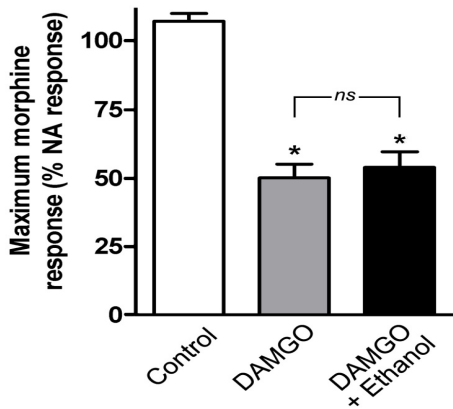
A Morphine treated



B Non morphine treated



C



D

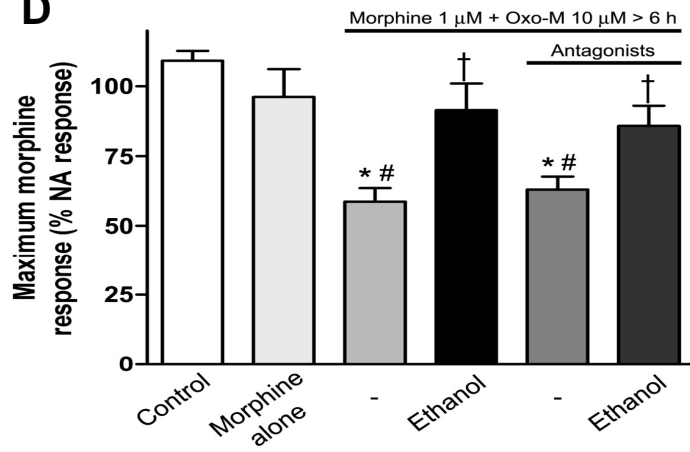
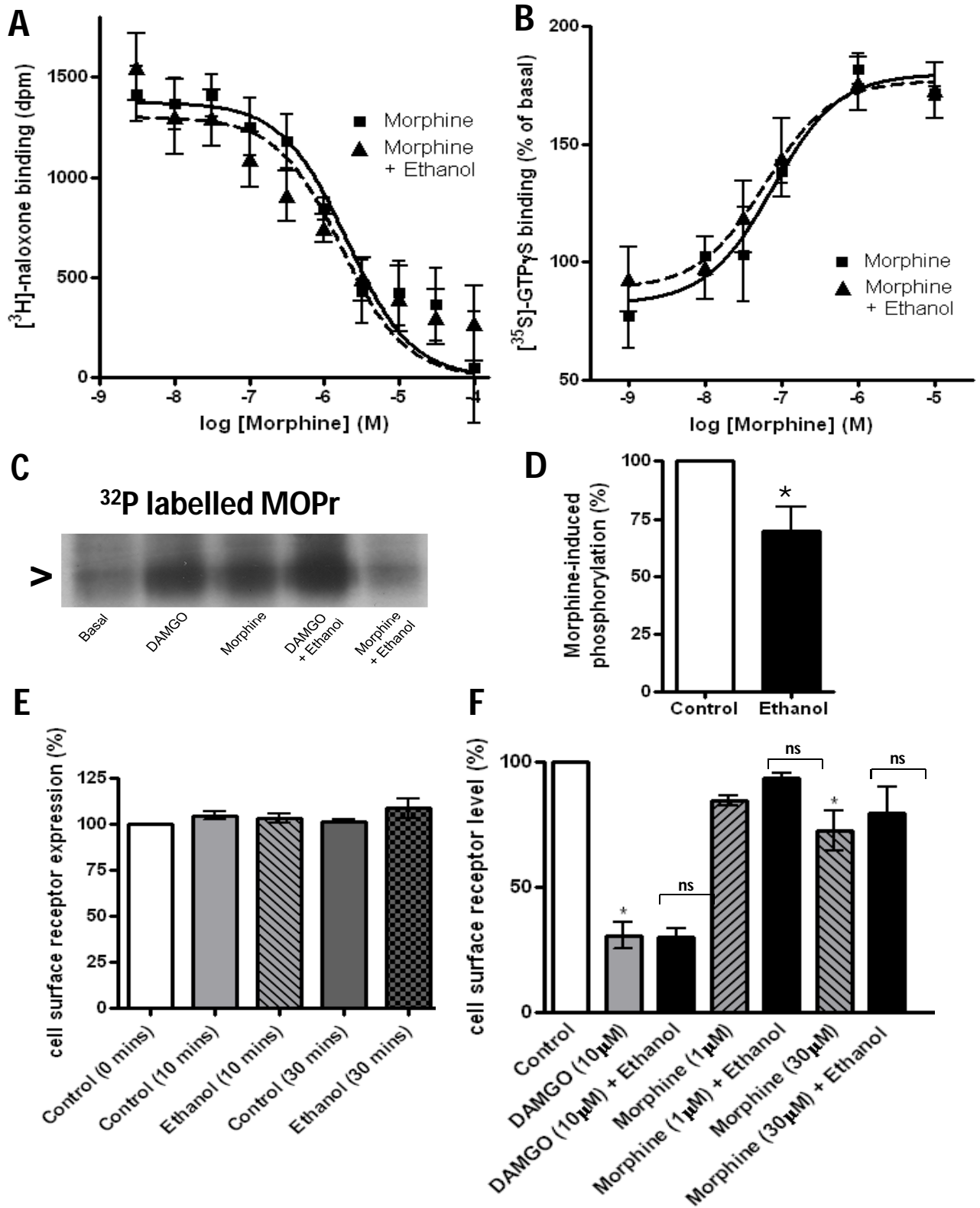


Figure 3

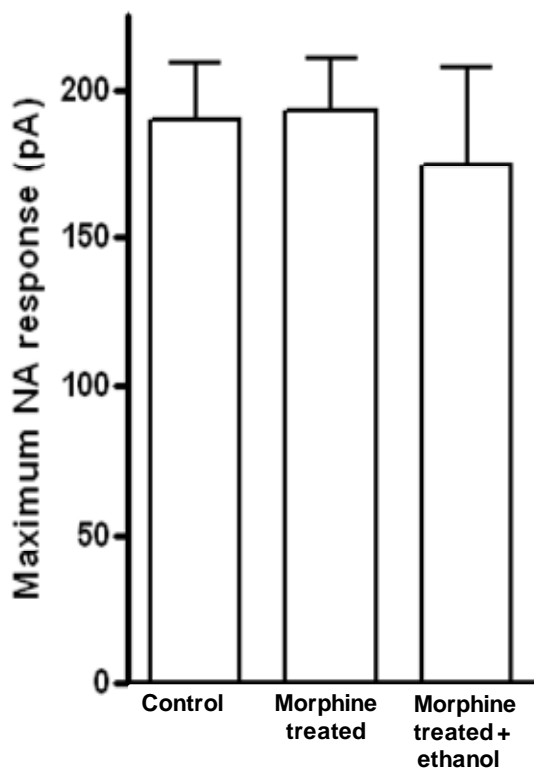


Supplemental Data Figure 1

Ethanol reversal of cellular tolerance to morphine in rat locus coeruleus neurons

Llorente, Withey, Rivero, Cunningham, Saxena, McPherson, Oldfield, Dewey, Bailey, Kelly and Henderson

Molecular Pharmacology



Morphine treatment and ethanol exposure do not change the amplitude of the maximum current evoked by noradrenaline in LC neurons.

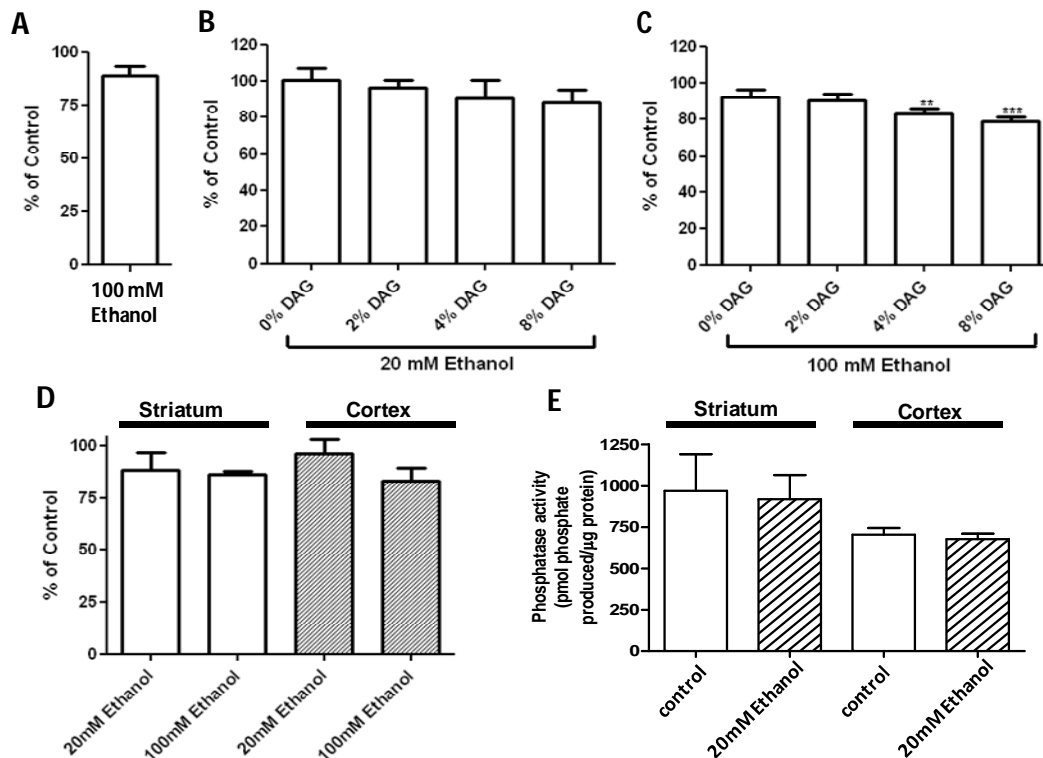
Graph shows the amplitude in pA of the response to noradrenaline (100 mM; NA) in LC neurons from control animals and morphine treated animals in the absence and presence of ethanol (20 mM). Methods are as described in main text

Supplemental Data Figure 2

Ethanol reversal of cellular tolerance to morphine in rat locus coeruleus neurons

Llorente, Withey, Rivero, Cunningham, Saxena, McPherson, Oldfield, Dewey, Bailey, Kelly and Henderson

Molecular Pharmacology



Lack of effect of ethanol on purified PKC α activity and mouse brain phosphatase activity

A-D PKC activity A nonradioactive PKC detection kit (PepTag assay kit; Promega) was used to measure kinase activity *in vitro* according to the manufacturer's instructions. Recombinant PKC α protein (10 ng) in lipid vesicles containing diacylglycerol (DAG; 0%, 2%, 4% or 8%), phosphatidylcholine (80%, 78%, 76% or 72%) and phosphatidylserine (20%) were prepared and enzyme activity determined in the presence and absence of ethanol by incubation with Peptag C1 substrate peptide for 30 min at 30°C. Phosphorylated peptide was resolved by SDS-PAGE and quantified by

densitometry after detection by UV illumination. **A** Ethanol (100 mM) did not alter PKC α activity in the absence of lipid vesicles. **B** Ethanol (20 mM) had no significant effect on PKC α activity in the presence of lipid vesicles. **C** Ethanol (100 mM) caused a decrease in PKC α activity which did reach significance however it cannot be concluded that ethanol reverses cellular tolerance by inhibiting PKC α activity. Due to the highly intoxicating effects of ethanol (100 mM), the effect on PKC α activity maybe unrelated to the reversal of tolerance. **D** Ethanol (20 or 100 mM) did not inhibit endogenous PKC activity in supernatants from mouse cortex and striatum. mouse brains were homogenized using a Polytron[®] homogenizer, in PKC extraction buffer (25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, 0.5 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin). The homogenate was centrifuged at 14,000 x g at 4°C for 5 min and the supernatant saved. Supernatant was passed over a 1 ml column of DEAE cellulose, pre-equilibrated in PKC extraction buffer and washed through with 2 ml PKC extraction buffer. The PKC containing fraction was eluted using PKC extraction buffer containing NaCl (200 mM). PKC activity was analysed as above. Ethanol (20 and 100 mM) had no significant effect on endogenous PKC activity in the cortex or the striatum. **E** Ethanol (20 mM) did not alter phosphatase activity in supernatants from mouse cortex and striatum. Phosphatase activity measured with a kit-based colorimetric assay (Promega Serine/Threonine Phosphatase Assay System), according to the supplier's instructions. Brain tissue from mouse striatum or cortex was dissected and frozen at -80°C. For the assay, tissue was rapidly thawed and homogenized using a Polytron[®] homogenizer, and the resulting homogenate centrifuged at 100,000 x g at 4°C for 1h. The supernatant was subsequently used for the phosphatase assay, which measures the production of free phosphate from a phosphopeptide substrate as the development of green colour with a molybdate dye, and is determined using a plate reader. Ethanol was added to the brain supernatants just before the plate assay was begun.