

# Morphine Desensitization, Internalization, and Down-Regulation of the $\mu$ Opioid Receptor Is Facilitated by Serotonin 5-Hydroxytryptamine<sub>2A</sub> Receptor Coactivation<sup>[S]</sup>

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## ABSTRACT

Analysis of the distribution of mRNA encoding the serotonin (5-hydroxytryptamine) 5-HT<sub>2A</sub> receptor and the  $\mu$  opioid peptide receptor in rat brain demonstrated their coexpression in neurons in several distinct regions. These regions included the periaqueductal gray, an area that plays an important role in morphine-induced analgesia but also in the development of tolerance to morphine. To explore potential cross-regulation between these G protein-coupled receptors, the human  $\mu$  opioid peptide receptor was expressed stably and constitutively in Flp-In T-REx human embryonic kidney 293 cells that harbored the human 5-HT<sub>2A</sub> receptor at the inducible Flp-In locus. In the absence of the 5-HT<sub>2A</sub> receptor, pretreatment with the enkephalin agonist [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin but not with the alkaloid agonist morphine produced desensitiza-

tion, internalization, and down-regulation of the  $\mu$  opioid peptide receptor. Induction of 5-HT<sub>2A</sub> receptor expression in these cells resulted in up-regulation of  $\mu$  opioid peptide receptor levels that was blocked by both a 5-HT<sub>2A</sub> receptor inverse agonist and selective inhibition of signaling via G $\alpha_q$ /G $\alpha_{11}$  G proteins. After induction of the 5-HT<sub>2A</sub> receptor, coaddition of 5-HT with morphine now also resulted in desensitization, receptor internalization, and down-regulation of the  $\mu$  opioid peptide receptor. It has been argued that enhancement of  $\mu$  opioid peptide receptor internalization in response to morphine would limit the development of tolerance without limiting analgesia. These data suggest that selective activation of the 5-HT<sub>2A</sub> receptor in concert with treatment with morphine might achieve this aim.

Morphine is used widely as an analgesic in the treatment of chronic pain (Martini and Whistler, 2007). However, tolerance to morphine develops rapidly, restricting its clinical utility (Martini and Whistler, 2007). The analgesic effects of morphine are clearly mediated via the  $\mu$  opioid peptide

(MOP) receptor because they are absent in animals lacking this G protein-coupled receptor (GPCR) (Matthes et al., 1996). However, despite a vast range of studies that have attempted to understand the molecular basis of tolerance to morphine and that have explored why other agonists that also activate the MOP receptor have different functional profiles, this remains a contentious area generating many, apparently conflicting, views (Gintzler and Chakrabarti, 2008). It was assumed initially that the development of tolerance to morphine would reflect MOP receptor desensitization, which would be anticipated to preclude sustained function (Gainetdinov et al., 2004). Indeed, recent studies have suggested that repeated morphine administration can en-

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**ABBREVIATIONS:** MOP,  $\mu$  opioid peptide; GPCR, G protein-coupled receptor; CA3, field CA3 of hippocampus; DAMGO, [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin; 5-HT, serotonin, 5-hydroxytryptamine; PKC, protein kinase C; GTP $\gamma$ S, guanosine 5'-O-(3-thio)triphosphate; PCR, polymerase chain reaction; eCFP, enhanced cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; HEK, human embryonic kidney; FRT, Flp recombination target; MOPS, 3-(N-morpholino)propanesulfonic acid; ANOVA, analysis of variance; Ro318220, 3-[1-(3-(amidinothio)propyl-1H-indol-3-yl)]-3-(1-methyl-1H-indol-3-yl) maleimide (bisindolylmaleimide IX); YM254890, (1R)-1-((3S,6S,9S,12S,18R,21S,22R)-21-acetamido-18-benzyl-3-[(1R)-1-methoxyethyl]-4,9,10,12,16,22-hexamethyl-15-methylene-2,5,8,11,14,17,20-hepta-oxo-1,19-dioxo-4,7,10,13,16-pentaazacyclodocosan-6-yl)-2-methylpropyl rel-(2S,3R)-2-acetamido-3-hydroxy-4-methylpentanoate.

hance agonist potency and hence increase receptor desensitization and promote tolerance (Ingram et al., 2008). In contrast, a series of studies have suggested that tolerance to morphine may stem from a lack of rapid desensitization, resulting in other adaptive, and potentially slowly reversible, changes becoming dominant (for review, see Martini and Whistler, 2007).

A widely noted feature is that in many settings and circumstances, and in both transfected model systems and native tissues, morphine produces little internalization of the MOP receptor from the surface of cells. By contrast, other agonists, including both other alkaloids such as etorphine, and peptide enkephalin analogs such as [D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin (DAMGO), promote extensive receptor internalization and produce less tolerance (Keith et al., 1996; Sternini et al., 1996). It has been suggested, therefore, that strategies that result in enhanced internalization of the MOP receptor in response to morphine might be useful in limiting the development of tolerance (Finn and Whistler, 2001) without compromising analgesia (Koch et al., 2005). This concept has recently received substantial support following production of a knockin line of mice in which the MOP receptor was replaced by a variant in which a substantial region of the C-terminal tail of the MOP receptor was exchanged for the equivalent sequence from the  $\delta$  opioid peptide receptor (Kim et al., 2008). Such C-terminally modified forms of the MOP receptor had been shown previously to internalize in response to morphine when expressed heterologously and to limit the appearance of biochemical markers associated with the development of tolerance to morphine (Finn and Whistler, 2001). In these knockin mice, morphine caused internalization of the modified receptor, and, importantly, although still producing analgesia, it induced substantially reduced tolerance and physical dependence (Kim et al., 2008).

Although an excellent proof of concept demonstrating potential validity of the underlying hypothesis, introduction of modified receptors is limited to heterologous cell lines and mouse models. However, a range of strategies have been suggested to promote endocytosis of the wild-type MOP receptor in response to morphine that might have the potential for translation to a clinical setting. These strategies include modulation of the cellular content of regulatory polypeptides known to be involved in the fine control of signal transduction, including  $\beta$ -arrestins (Bohn et al., 2000), and, as a more immediately practical avenue, regulation of the activity of various protein kinases and phosphatases (Bailey et al., 2006; Kelly et al., 2008). Alternatively, the use of combinations of morphine in conjunction with other opioid ligands (He and Whistler, 2005; Roy et al., 2005) has also been suggested as a strategy to promote MOP receptor internalization and to limit the development of tolerance, dependence, or both.

Many studies on aspects of the molecular details of MOP receptor regulation are performed in heterologous cell systems that allow easy manipulation of signaling and regulatory polypeptides. However, in many of these studies the MOP receptor has been expressed in the absence of other GPCRs with which it is coexpressed in native systems. The serotonin (5-hydroxytryptamine) 5-HT<sub>2A</sub> receptor is widely expressed in the central nervous system of humans and rodents, and it is coupled strongly to activation of protein

kinase C (PKC) via G proteins of the G<sub>q</sub>/G<sub>11</sub> family (Sanders-Bush et al., 2003). PKC activation, either directly by addition of phorbol esters or via activation of the muscarinic M<sub>3</sub> acetylcholine receptor, has been reported to enhance morphine-induced rapid desensitization of the MOP receptor in rat locus ceruleus neurons (Bailey et al., 2004). We have, therefore, explored coexpression of the 5-HT<sub>2A</sub> receptor and MOP receptor in various regions of rat brain and whether coexpression of the 5-HT<sub>2A</sub> receptor with the MOP receptor in heterologous cells can imbue morphine with the ability to cause internalization and desensitization of the MOP receptor.

## Materials and Methods

### Materials

All materials for tissue culture were from Invitrogen (Paisley, UK). [<sup>3</sup>H]Ketanserin, [<sup>3</sup>H]diprenorphine, and [<sup>35</sup>S]GTP $\gamma$ S were from PerkinElmer Life and Analytical Sciences (Boston, MA). Doxycycline, 5-HT, mianserin, DAMGO, naloxone, and morphine were from Sigma-Aldrich. YM254890 was a kind gift from Astrellas Pharma Inc. (Osaka, Japan).

### Receptor Fusions with Fluorescent Proteins

Generation and subcloning of the human 5-HT<sub>2A</sub> receptor construct was essentially as described previously (Carrillo et al., 2004). Briefly, the c-Myc epitope tag was added at the amino terminus of the receptor by PCR techniques using forward primers containing the sequence of the c-Myc epitope (amino acid sequence EQKLI-SEEDL). A c-Myc-5-HT<sub>2A</sub> receptor C-terminally tagged with enhanced cyan fluorescent protein (eCFP) was constructed by amplifying the sequence corresponding to the receptor by PCR and by removing the stop codon. This PCR product was ligated to the fluorescent protein sequence amplified by PCR and containing the same endonuclease restriction site (NotI). The final product of this ligation corresponds to a single open reading frame encoding the receptor-fluorescent protein fusion. c-Myc-5-HT<sub>2A</sub>-eCFP was subcloned into the vector pcDNA5/FRT/TO (Invitrogen) for the subsequent generation of Flp-In T-REx HEK293 cell lines. MOP-eYFP receptor (Canals and Milligan, 2008) was obtained after amplification of human MOP using PCR primers containing a SacI endonuclease site at the 5' end and an ApaI endonuclease site at the 3' end and in the process removing the stop codon. This PCR product was subcloned into pYFP-N1 vector, resulting in a single open reading frame consisting of the MOP receptor with eYFP fused to the receptor carboxyl terminus. All the constructs were verified by DNA sequencing.

### Generation of Stable Flp-In T-REx HEK293 Cell Lines

To generate Flp-In T-REx HEK293 cell lines able to express c-myc-5-HT<sub>2A</sub>-eCFP in an inducible manner, cells were transfected with a mixture containing the c-myc-5-HT<sub>2A</sub>-eCFP receptor cDNA in the pcDNA5/FRT/TO vector and pOG44 vector (1:9) using Effectene transfection reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. When cotransfected with the pcDNA5/FRT plasmid into the Flp-In mammalian host cell line, the Flp recombinase expressed from pOG44 mediates integration of the pcDNA5/FRT vector containing the gene of interest into the genome via Flp recombination target (FRT) sites. Cell maintenance and selection were as described previously (Canals and Milligan 2008). Clones resistant to blasticidin were screened for c-myc-5-HT<sub>2A</sub>-eCFP expression by both fluorescence and Western blotting. To induce expression of c-myc-5-HT<sub>2A</sub>-eCFP cells were treated with varying concentrations of doxycycline for different periods. The optimal expression of c-myc-5-HT<sub>2A</sub>-eCFP was achieved after 24-h treatment with 0.01  $\mu$ g of doxycycline/ml growth medium (see *Results*). A double stable cell line expressing MOP-eYFP constitutively and c-Myc-

5-HT<sub>2A</sub>-eCFP in an inducible manner was generated from the Flp-In T-REx HEK293 cells described above. Cells were transfected using Effectene (QIAGEN) with the vector containing MOP-eYFP. Following transfection, cells were selected for resistance to Geneticin (G418; 1 mg/ml; Invitrogen), and the resistant clones were screened for receptor expression by fluorescence microscopy. Dialyzed fetal calf serum was used for cell growth to avoid activation of c-Myc-5-HT<sub>2A</sub>-eCFP by 5-HT that is present routinely in serum.

### [Ca<sup>2+</sup>]<sub>i</sub> Measurements

Cells were introduced into 96-well plates, loaded with the Ca<sup>2+</sup>-sensitive dye Fura-2 acetoxymethyl ester (1.5 μM), by incubation (30 min; 37°C) under reduced light in Dulbecco's modified Eagle's medium. Calcium imaging and analysis were then performed using a FLEXstation (Molecular Devices, Sunnyvale, CA).

### Cell Membrane Preparation

Harvested pellets from Flp-In T-REx HEK293 cells kept at -80°C were thawed and resuspended in 10 mM Tris and 0.1 mM EDTA, pH 7.4 (Tris/EDTA buffer). The cells were homogenized by 25 passes of a glass-on-Teflon homogenizer. The resulting suspension was centrifuged at 1200g for 10 min to remove unbroken cells and nuclei. The supernatant was subsequently centrifuged at 218,000g for 30 min in an Optima TLX ultracentrifuge (Beckman Coulter, Fullerton, CA). Resulting pellets were resuspended in Tris/EDTA buffer and passed 10 times through a 25-gauge needle. Protein concentration was determined, and the membranes were stored at -80°C until use.

### Radioligand Binding Assays

**[<sup>3</sup>H]Ketanserin Binding.** Binding assays were initiated by the addition of 15 to 20 μg of cell membrane to an assay buffer (50 mM Tris-HCl, 100 mM NaCl, and 3 mM MgCl<sub>2</sub>, pH 7.4) containing [<sup>3</sup>H]ketanserin (0.2–20 nM). Nonspecific binding was determined in the presence of 10 μM mianserin. Reactions were incubated for 60 min at 25°C, and bound ligand was separated from free ligand by vacuum filtration through GF/B filters (Semat, St. Albans, Hertfordshire, UK) using a cell harvester (Brandel Inc., Gaithersburg, MD). The filters were washed twice with ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 10 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 8 mM Na<sub>2</sub>HPO<sub>4</sub>), and bound ligand was estimated by liquid scintillation spectrometry.

**[<sup>3</sup>H]Diprenorphine Binding.** Cell membranes (15–20 μg of protein) were incubated with [<sup>3</sup>H]diprenorphine (0.02–2 nM in saturation assays; 1–2 nM in single point assays) in a total volume of 1 ml of buffer (50 mM Tris-HCl, 1 mM EDTA, and 10 mM MgCl<sub>2</sub>, pH 7.4). Nonspecific binding was determined by the inclusion of 100 μM naloxone. Binding was initiated by the addition of membranes, and the tubes were incubated at 25°C for 60 min. The assay was terminated by rapid filtration using a cell harvester (Brandel Inc.) with three 5-ml washes of ice-cold phosphate-buffered saline. The filters were soaked in 3 ml of scintillation fluid, and radioactivity was determined by liquid scintillation spectrometry.

**[<sup>35</sup>S]GTPγS Binding.** Cell membranes (10 μg) were incubated in buffer (20 mM HEPES, 100 mM NaCl, and 4 mM MgCl<sub>2</sub>, pH 7.4) containing 10 μM GDP and various concentrations of agonist ligands. All experiments were performed in triplicate. The reaction was initiated by the addition of cell membranes, and then membranes were incubated at 30°C for 60 min in the presence of 0.1 nM [<sup>35</sup>S]GTPγS. The reaction was terminated by rapid filtration with a cell harvester (Brandel Inc.) and three 4-ml washes with ice-cold phosphate-buffered saline. Radioactivity was determined as described above.

**[<sup>35</sup>S]GTPγS Binding by Immunocapture of Gα<sub>q</sub>/Gα<sub>11</sub>.** Experiments were initiated by the addition of membranes to assay buffer (20 mM HEPES, pH 7.4, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, and 100 nCi of [<sup>35</sup>S]GTPγS). Nonspecific binding was determined under the same conditions but in the presence of 100

μM GTPγS. Reactions were incubated for 30 min at 30°C and terminated by the addition of 0.5 ml of ice-cold buffer containing 20 mM HEPES, pH 7.4, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, and 0.2 mM ascorbic acid. The samples were centrifuged at 16,000g for 10 min at 4°C, and the resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1.25% Nonidet P-40) plus 0.2% SDS. Samples were precleared with Pansorbin (Calbiochem, Nottingham, UK), followed by immunoprecipitation with antiserum CQ (Mitchell et al., 1993) that selectively identifies the C-terminal decapeptide common to Gα<sub>q</sub> and Gα<sub>11</sub>. Finally, the immunocomplexes were washed twice with solubilization buffer, and bound [<sup>35</sup>S]GTPγS was measured by liquid scintillation spectrometry.

### Living Cell Epifluorescence Microscopy

Cells expressing receptors tagged with eCFP or eYFP were grown on poly-D-lysine-treated coverslips. The coverslips were placed into a microscope chamber containing physiological saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 20 mM HEPES, and 10 mM D-glucose, pH 7.4). Fluorescent images of the cells were acquired using a TE2000-E inverted microscope (Nikon, Melville, NY) equipped with a 40× (numerical aperture, 1.3) oil immersion Plan Fluor lens and a cooled digital CoolSNAP<sub>HQ</sub> charge-coupled device camera (Photometrics, Tucson, AZ). For internalization experiments in real time, drugs diluted in physiological saline solution were perfused into the microscope chamber, and pictures were taken every 3 min during a period of 30 to 35 min.

### Image Quantitation

The number of spots for each image time frame was quantitatively measured using the manual segmentation tool within the object counting module of AutoVisualize software version 9.3.6 (Autoquant Imaging, Watervliet, NY), which allowed us to manually segment and classify different sizes of spots to be counted in each time series image. On completion of the manual segmentation process, new images were created in which all pixels of the raw image that were not classified as part of the segmentation mask were effectively removed from the analysis.

### Western Blotting

Samples were heated at 65°C for 15 min and subjected to SDS-polyacrylamide gel electrophoresis analysis using 4 to 12% bis-Tris gels (NuPAGE; Invitrogen) and MOPS buffer. After electrophoresis, proteins were transferred onto nitrocellulose membranes that were incubated in a solution of 5% nonfat milk and 0.1% Tween 20 in Tris-buffered saline at room temperature on a rotating shaker for 2 h to block nonspecific binding sites. The membranes were incubated overnight with a rabbit anti-c-Myc polyclonal antibody (Cell Signaling, Hertfordshire, UK) or a sheep anti-MOP antiserum generated in-house (Canals and Milligan, 2008). Protein-antibody interactions were detected using horseradish peroxidase-linked anti-rabbit IgG or anti-goat IgG secondary antisera (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Immunoblots were developed by application of enhanced chemiluminescence solution (Pierce Chemical Rockford, IL).

### Distribution of mRNA Encoding Receptors

**Tissue Preparation.** Adult male Wistar rats were purchased from Iffa Credo (Lyon, France). Animal care followed the Spanish legislation on "Protection of animals used in experimental and other scientific purposes" in agreement with the European regulations [European Communities Council directive 86/609/EEC of November 24 1986 (O.J. of E.C. L358, 18/12/1986)]. Experimental procedures were approved by the required ethical committees and local authorities. Animals were killed by decapitation. The brains were quickly removed, frozen on dry ice, and kept at -20°C. Tissue sections (20 μm in thickness) were cut on a microtome-cryostat (HM500 OM; Microm, Walldorf, Germany), thaw-mounted onto 3-aminopropyltri-



ethoxysilane (Sigma-Aldrich)-coated slides, and kept at  $-20^{\circ}\text{C}$  until use.

**Hybridization Probes.** For the detection of 5-HT<sub>2A</sub> receptor mRNA, six different oligomers were used simultaneously. They were complementary to the following bases of the rat 5-HT<sub>2A</sub> receptor mRNA (GenBank accession no. X13971): 669 to 716, 723 to 767, 1379 to 1423, 1482 to 1530, 1844 to 1888, and 1923 to 1970. MOP receptor mRNA was detected with four oligomers complementary to the following bases of the rat MOP receptor mRNA (GenBank accession no. NM 013071): bases 187 to 231, 803 to 850, 1279 to 1323, and 1431 to 1475. Oligonucleotides were synthesized and high-performance liquid chromatography purified by Isogen Bioscience BV (Maarsden, The Netherlands). Comparison of the oligonucleotide sequences with European Molecular Biology Laboratory and GenBank databases with basic local alignment search tool indicated that the probes do not show any significant similarity with mRNAs in the rat other than their corresponding targets. Oligonucleotides were labeled at their 3' end either radioactively with [ $\alpha$ -<sup>33</sup>P]dATP (3000 Ci/mmol; PerkinElmer Life and Analytical Sciences) and terminal deoxynucleotidyltransferase (Calbiochem, San Diego, CA) or nonradioactively with digoxigenin-labeled nucleotides (digoxigenin-11-dUTP; Roche Diagnostics, Mannheim, Germany) and terminal deoxynucleotidyltransferase (recombinant; Roche Applied Science, Penzberg, Germany). Labeled probes were purified from nonincorporated nucleotides with ProbeQuant G-50 micro columns (GE Healthcare).

**In Situ Hybridization Histochemistry Procedure.** The protocols for single- and double-label in situ hybridization histochemistry were based on previously described procedures (Vilaró et al., 1996; Landry et al., 2000). Briefly, tissue sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (1 $\times$ ; 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 136 mM NaCl, and 2.6 mM KCl); washed in 1 $\times$  PBS; incubated in predigested pronase (Calbiochem) at a final concentration of 24 U/ml in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA; immersed in 2 mg/ml glycine in 1 $\times$  PBS to stop proteolytic activity; rinsed in 1 $\times$  PBS; and dehydrated through a graded series of ethanol. For hybridization, radioactively and nonradioactively labeled probes were diluted in a solution containing 50% formamide, 4 $\times$  standard saline citrate (1 $\times$ ; 150 mM NaCl and 15 mM sodium citrate), 1 $\times$  Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl (sodium lauryl sarcosinate), 20 mM phosphate buffer, pH 7.0, 250  $\mu\text{g/ml}$  yeast tRNA, and 500  $\mu\text{g/ml}$  salmon sperm DNA. The final concentrations of radioactive and digoxigenin-labeled probes in the hybridization buffer were in the same range (approximately 1.5–2 nM for each probe).

Tissue sections were covered with 60 to 70  $\mu\text{l}$  of hybridization solution, overlaid with Nescofilm coverslips (Bando Chemical Industries, Kobe, Japan), and incubated overnight at  $42^{\circ}\text{C}$  in humid boxes. Sections were washed extensively in 0.6 M NaCl and 10 mM Tris-HCl, pH 7.5, at  $60^{\circ}\text{C}$ . Sections hybridized with <sup>33</sup>P- and digoxigenin-labeled probes simultaneously were washed in the same buffer at room temperature for a further 10 min, whereas sections hybridized with radioactive probes only were dehydrated through an ethanol series and allowed to air dry before exposure to film or dipping into liquid emulsion.

**Development of Radioactive and Nonradioactive Hybridization Signal.** Sections hybridized with <sup>33</sup>P- and digoxigenin-labeled probes simultaneously were treated essentially as described by Landry et al. (2000). Briefly, slides were immersed for 30 min in a buffer containing 0.1 M Tris-HCl, pH 7.5, 1 M NaCl, 2 mM MgCl<sub>2</sub>, and 0.5% bovine serum albumin (fraction V; Sigma-Aldrich) and incubated overnight at  $4^{\circ}\text{C}$  in the same solution containing an alkaline-phosphatase-conjugated anti-digoxigenin antibody (Fab fragments, 1:5000 dilution; Roche Applied Science). They were washed in the same buffer and then in alkaline buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 5 mM MgCl<sub>2</sub>). Alkaline phosphatase activity was developed with 3.3 mg of nitroblue tetrazolium and 1.65 mg of bromochloroindolyl phosphate (Roche Applied Science) diluted in 10

ml of alkaline buffer overnight at room temperature in the dark. After washing in alkaline buffer containing 1 mM EDTA, the sections were briefly dipped in water, 70 and 100% ethanol, and air-dried.

Sections incubated with digoxigenin-labeled and <sup>33</sup>P-labeled probes simultaneously were dipped in a solution of 2% collodion in amyl acetate (Electron Microscopy Sciences, Hatfield, PA) and allowed to dry. All sections were then dipped into Ilford K5 nuclear emulsion (Harman Technology Ltd., Mobberley, Cheshire, UK) diluted 1:1 with distilled water. They were exposed in the dark at  $4^{\circ}\text{C}$  for 6 weeks and finally developed in D19 developer (Eastman Kodak, Rochester, NY).

## Analysis of Results

Tissue sections were examined under bright- and dark-field illumination in a Wild 420 macroscope (Leica, Heerbrugg, Germany) or in an Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with bright- and dark-field condensers for transmitted light and with an illuminated slide holder (Darklite; Micro Video Instruments, Avon, MA). Cells containing the mRNA detected with digoxigenin-labeled probes were identified as cellular profiles exhibiting a dark precipitate (alkaline phosphatase reaction product). Hybridization signal obtained with <sup>33</sup>P-labeled probes was considered positive when accumulation of silver grains over the stained cellular profiles, or unstained equivalent areas, was at least 2- to 3-fold higher than the background levels of signal in areas known to be devoid of the corresponding receptor mRNA. In all the experiments, control sections were included that were incubated with the labeled probes plus a large excess of the same unlabeled oligonucleotides. These sections served as control for specificity of positive signals and also as an estimate of background levels of signal.

## Data Analysis

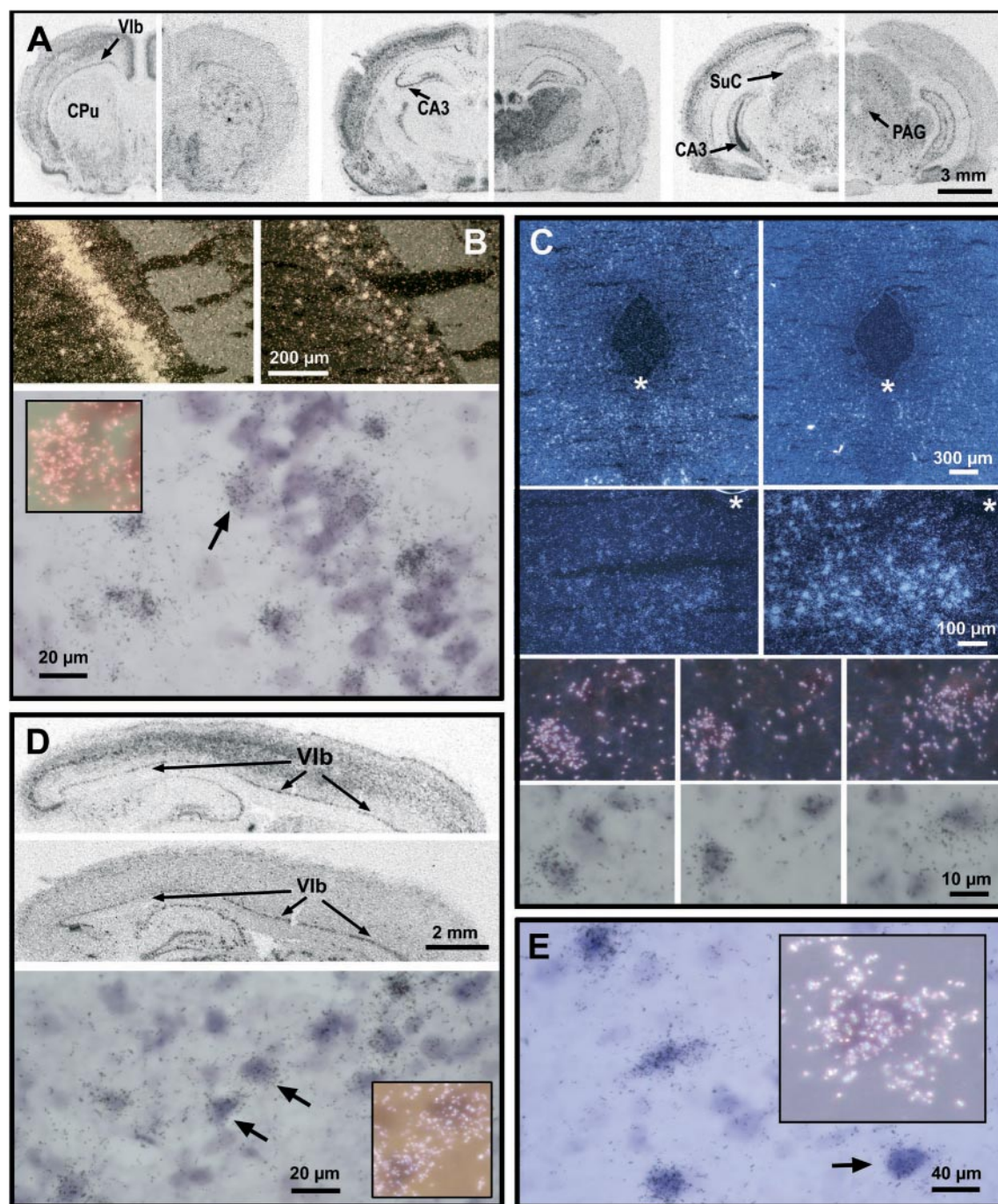
Data were analyzed using Prism (GraphPad Software Inc., San Diego, CA), and statistical significance was determined using either Student's *t* test or ANOVA followed by the post hoc analysis as appropriate. *P* < 0.05 determined statistical significance.

## Preparation of Figures

Microphotography was performed with a digital camera (DXM1200 F; Nikon) and ACT-1 Nikon software for in situ hybridization experiments. Figures were prepared for publication using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

## Results

A series of <sup>33</sup>P- and digoxigenin-labeled oligonucleotide probes were used individually or in combination to detect the presence of mRNAs encoding the 5-HT<sub>2A</sub> receptor and the MOP receptor in sections of rat brain. mRNA encoding each receptor was expressed widely (Fig. 1A). Detailed analysis indicated these to be coexpressed in individual cells in several areas, including neurons in field CA3 of hippocampus (CA3) (Fig. 1B), the periaqueductal gray (Fig. 1C), layer VIb of the neocortex (Fig. 1D), and the dorsal endopiriform nucleus (Fig. 1E). Specificity of the signals was confirmed in several routine control experiments performed with each of the oligonucleotide probes used for in situ hybridization. Controls for the 5-HT<sub>2A</sub> receptor probes have been illustrated previously (Mengod et al., 1990). Controls for the MOP receptor probes are shown in Supplemental Fig. 1. In brief, 1) when the various probes targeted at the same mRNA species were used independently as hybridization probes in consecutive sections, identical patterns of hybridization signal were observed. 2) For any given labeled probe, inclusion during hybridization of an excess of the same unlabeled oligonucle-



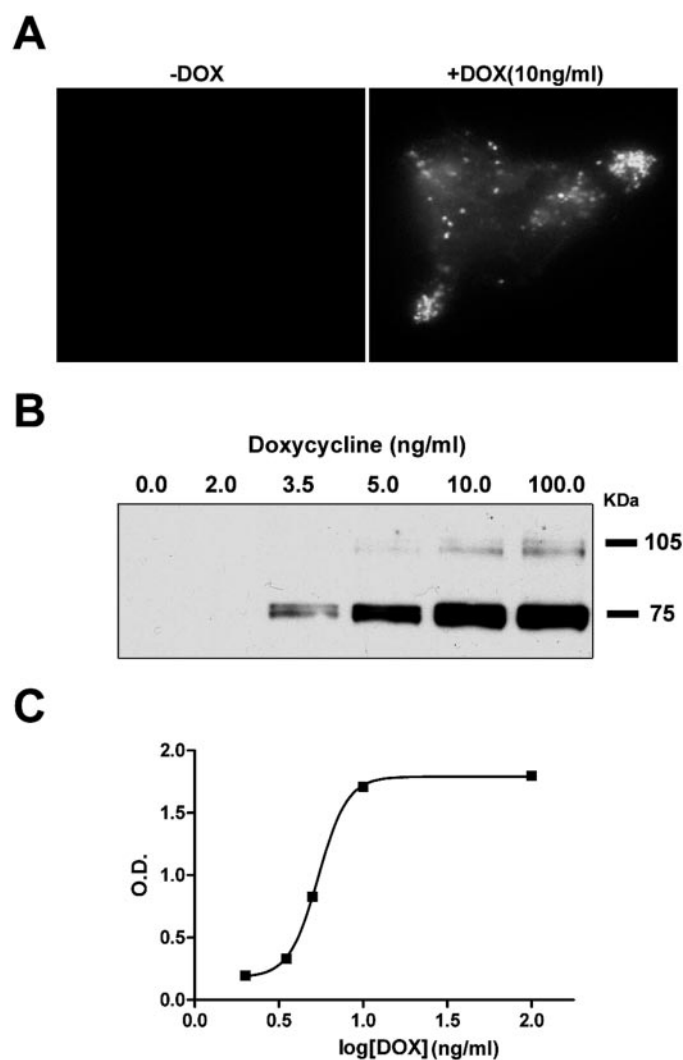
**Fig. 1.** mRNAs encoding the 5-HT<sub>2A</sub> receptor and the MOP receptor are coexpressed in many regions of rat brain. A, low-power images of film autoradiographs obtained after hybridizing consecutive coronal sections with <sup>33</sup>P-labeled probes for 5-HT<sub>2A</sub> receptor mRNA (left) or MOP receptor mRNA (right). B, colocalization of 5-HT<sub>2A</sub> and MOP receptor mRNAs in field CA3 of hippocampus. Top, dark-field photomicrographs of 5-HT<sub>2A</sub> (left) and MOP (right) receptor mRNAs visualized with <sup>33</sup>P-labeled probes and liquid photographic emulsion. Bottom, bright-field photomicrograph of a section where both mRNAs are visualized simultaneously with digoxigenin-labeled probes for 5-HT<sub>2A</sub> mRNA (dark precipitate) and <sup>33</sup>P-labeled probes for MOP mRNA (dark silver grains). The arrow points to a cell shown at higher magnification in the inset, where silver grains are seen as bright points with a Darklite device. C, colocalization of 5-HT<sub>2A</sub> and MOP receptor mRNAs in the periaqueductal gray. The first and second rows are dark-field images of 5-HT<sub>2A</sub> (left) and MOP (right) receptor mRNAs visualized with <sup>33</sup>P-labeled probes in consecutive sections. The asterisks are placed immediately underneath the fourth ventricle. Examples of cells coexpressing both mRNAs (dark precipitate, MOP mRNA; silver grains, 5-HT<sub>2A</sub> mRNA) are shown in the third (dark-field) and fourth (bright-field) rows. D, colocalization of 5-HT<sub>2A</sub> and MOP receptor mRNAs in layer VIb of neocortex. Low-power images of film autoradiographs obtained after hybridizing consecutive sagittal sections with <sup>33</sup>P-labeled probes for 5-HT<sub>2A</sub> (top) or MOP receptor mRNA (central). Bottom, a bright-field photomicrograph of a section where 5-HT<sub>2A</sub> mRNA is visualized with digoxigenin-labeled probes (dark precipitate) and MOP mRNA with <sup>33</sup>P-labeled probes (dark silver grains). The arrows point to cells shown at higher magnification in the inset. E, colocalization of 5-HT<sub>2A</sub> and MOP receptor mRNAs in the dorsal endopiriform nucleus. Bright-field photomicrograph showing 5-HT<sub>2A</sub> mRNA (dark precipitate) and MOP mRNA (dark silver grains). The arrow points to a cell shown in the inset. The size of the scale bars are indicated in the corresponding panels.



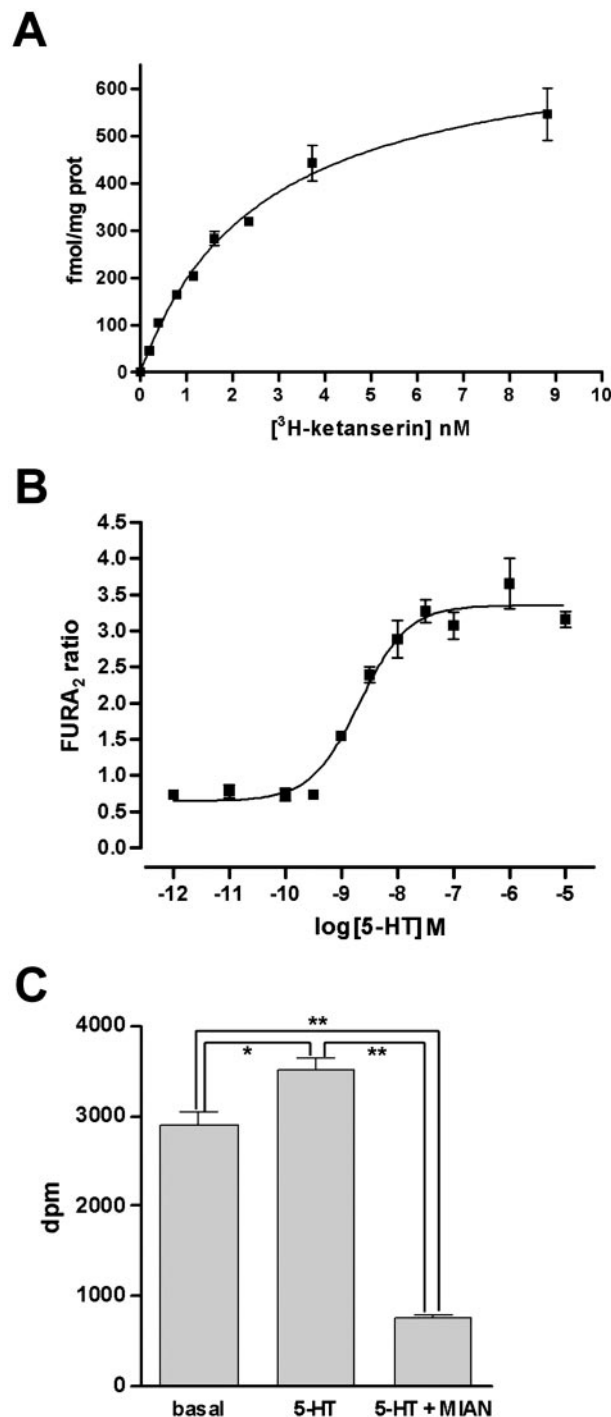
otide resulted in the absence of specific hybridization signals. The autoradiographic signal remaining under these conditions was taken as background or nonspecific tissue signal. 3) Inclusion during hybridization of an excess of an unrelated, unlabeled oligonucleotide left specific hybridization signals unaffected. 4) The thermal stability of the hybrids was studied by washing a series of consecutive sections at increasing temperatures. The intensity of specific signals suffered a very sharp decrease as washing temperature increased, whereas no such sharp decrease was observed in background levels of signal.

Because coexpressed GPCRs are often able to produce cross-regulation and alteration of function (Hur and Kim, 2002), the significant extent of colocalization of mRNA encoding these two receptors encouraged us to explore this possibility for the 5-HT<sub>2A</sub> and MOP receptors. cDNA encoding the human 5-HT<sub>2A</sub> receptor was modified to introduce the

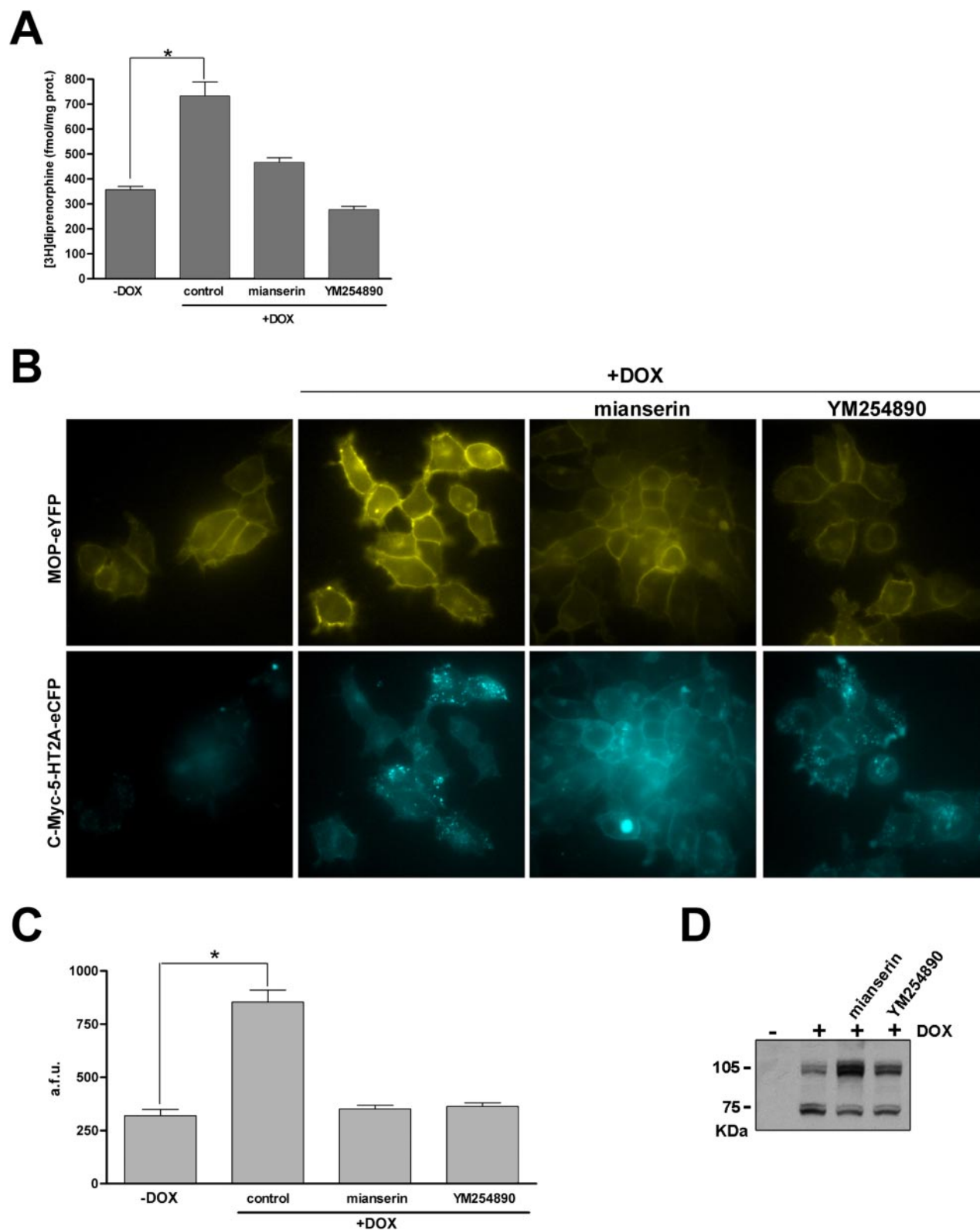
c-Myc epitope tag at the N terminus of the protein and at the C terminus by in-frame fusion of eCFP. This construct (c-Myc-5-HT<sub>2A</sub>-eCFP) was cloned into the tetracycline/doxycycline-inducible Flp-In locus of Flp-In T-REx HEK293 cells,



**Fig. 2.** Generation of Flp-In T-REx HEK293 cells that express c-Myc-5-HT<sub>2A</sub>-eCFP in an inducible manner. cDNA encoding c-Myc-5-HT<sub>2A</sub>-eCFP was cloned into the Flp-In locus of Flp-In T-REx HEK293 cells, and a population of positive cells was isolated. A, these cells were maintained in the absence (-DOX) or in the presence (+DOX) of 10 ng/ml doxycycline for 24 h and then imaged to detect eCFP. Membranes were prepared from such cells treated with varying concentrations of doxycycline for 24 h, and expression of c-Myc-5-HT<sub>2A</sub>-eCFP was detected by immunoblotting samples with an anti-c-Myc antibody (B). Such blots were quantitated by densitometry (C).



**Fig. 3.** Expressed c-Myc-5-HT<sub>2A</sub>-eCFP is functional. A, membranes from Flp-In T-REx HEK293 cells treated with doxycycline (10 ng/ml; 24 h) were used to measure the specific binding of the 5-HT<sub>2A</sub> receptor blocker [<sup>3</sup>H]ketanserin. B, these cells were also used to measure functionality of the 5-HT<sub>2A</sub> receptor via the ability of varying concentrations of 5-HT to elevate [Ca<sup>2+</sup>]<sub>i</sub>. C, membranes from these cells were used to measure binding of [<sup>35</sup>S]GTPγS in Gα<sub>q</sub>/Gα<sub>11</sub> immunoprecipitates in the absence of ligands (basal) or in the presence of 5-HT (10<sup>-5</sup> M) or 5-HT + mianserin (10<sup>-5</sup> M). One-way ANOVA significantly different ( $P < 0.0001$ ). Post hoc test; \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .



**Fig. 4.** Up-regulation of MOP-eYFP levels by 5-HT<sub>2A</sub> receptor expression reflects signaling via G $\alpha_q$ /G $\alpha_{11}$ . Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT<sub>2A</sub>-eCFP at the Flp-In locus were untreated (-DOX) or treated with doxycycline (+DOX). +DOX cells were also treated with either the 5-HT<sub>2A</sub> receptor antagonist/inverse agonist mianserin (10<sup>-5</sup> M) or the G $\alpha_q$ /G $\alpha_{11}$  inhibitor YM254890 (10<sup>-7</sup> M). **A**, specific binding of [<sup>3</sup>H]diprenorphine (2 nM) is displayed. Data represent means  $\pm$  S.E.M.,  $n = 3$ . \*,  $P < 0.001$ , significantly different. **B**, images of MOP-eYFP (top) or c-Myc-5-HT<sub>2A</sub>-eCFP (bottom) fluorescence of cells grown on coverslips. **C**, eYFP fluorescence from cells treated as described in **B** was measured directly. Data represent means  $\pm$  S.E.M.,  $n = 3$ . a.f.u., arbitrary fluorescence units. \*,  $P < 0.001$ , significantly different. **D**, although without major effect of total levels of c-Myc-5-HT<sub>2A</sub>-eCFP, treatment with mianserin and to a lesser extent YM254890, increased levels of cell surface located c-Myc-5-HT<sub>2A</sub>-eCFP (**B**, bottom), and this was accompanied by greater levels of the mature, 105-kDa terminally *N*-glycosylated form of this receptor.

and a pool of stably transfected cells was isolated. In the absence of doxycycline, expression of c-Myc-5-HT<sub>2A</sub>-eCFP was undetectable based either by the autofluorescence of eCFP (Fig. 2A) or immunoblot detection of c-Myc-reactive polypeptides (Fig. 2B). Addition of doxycycline resulted in concentration-dependent expression of c-Myc-5-HT<sub>2A</sub>-eCFP (Fig. 2, B and C), whereas visualization of individual living cells suggested that, at steady state, the bulk of c-Myc-5-HT<sub>2A</sub>-eCFP was present in punctate, intracellular vesicles

(Fig. 2A; Supplemental Fig. 2). Following induction of c-Myc-5-HT<sub>2A</sub>-eCFP by treatment with doxycycline for 24 h, specific [<sup>3</sup>H]ketanserin binding to membranes from these cells was monophasic, with  $K_d = 2.6 \pm 0.2$  nM and  $B_{max} = 665 \pm 89$  fmol/mg membrane protein (Fig. 3A). The expressed c-Myc-5-HT<sub>2A</sub>-eCFP was functional because addition of 5-HT resulted in a concentration-dependent increase in  $[Ca^{2+}]_i$ , with  $pEC_{50} = 8.6 \pm 0.2$  (Fig. 3B) (means  $\pm$  S.E.M.;  $n = 3$ ). In [<sup>35</sup>S]GTP $\gamma$ S binding studies performed on membranes from

TABLE 1

Expression of c-Myc-5-HT<sub>2A</sub>-eCFP and addition of 5-HT enhances G protein activation by DAMGO and morphine

[<sup>35</sup>S]GTP $\gamma$ S binding studies were performed on membranes of Flp-In T-REx HEK293 cells constitutively expressing MOP-eYFP and in which c-Myc-5-HT<sub>2A</sub>-eCFP expression could be induced by addition of doxycycline. The  $pEC_{50}$  for DAMGO and morphine and the extent of stimulation over maximal agonist-induced binding of [<sup>35</sup>S]GTP $\gamma$ S produced without induction of the 5-HT<sub>2A</sub> receptor construct are shown. Parentheses indicate number of independent experiments performed. Values are presented as mean  $\pm$  S.E.M. The values in parentheses represent  $n$ .

	$pEC_{50}$	$E_{max} > \text{Control}$ %
DAMGO		
Control	7.58 $\pm$ 0.09 (8)	
Doxycycline (10 ng/ml)	7.88 $\pm$ 0.16 (8)	10.59 $\pm$ 5.52 (8)
Doxycycline (10 ng/ml) 5-HT (1 $\mu$ M)	7.91 $\pm$ 0.08 (8)	33.33 $\pm$ 3.69* (8)
Morphine		
Control	7.63 $\pm$ 0.07 (4)	
Doxycycline (10 ng/ml)	7.65 $\pm$ 0.13 (4)	7.92 $\pm$ 1.68 (4)
Doxycycline (10 ng/ml) 5-HT (1 $\mu$ M)	7.92 $\pm$ 0.09 (4)	23.75 $\pm$ 4.65* (4)

\*  $P < 0.001$  by Dunnett's post hoc test analysis of variance.

TABLE 2

Coactivation of the 5-HT<sub>2A</sub> receptor with 5-HT and the MOP receptor with morphine results in desensitization to morphine

Flp-In T-REx HEK293 cells not treated with doxycycline lack expression of 5-HT<sub>2A</sub>-eCFP. Cells were pretreated with either DAMGO or morphine for the indicated times, and membranes prepared from the cells were then used to assess the potency (top) and relative efficacy (bottom) of DAMGO or morphine to stimulate binding of [<sup>35</sup>S]GTP $\gamma$ S. Data are presented as  $pEC_{50}$  or percentage of maximal agonist effect ( $E_{max}$ ). Desensitization was assessed by reduction in potency of the ligand following pretreatment. Numbers in parentheses indicate number of independent experiments. Values are presented as mean  $\pm$  S.E.M.

	Not Pretreated	30 min	60 min	120 min	240 min	Pretreatment
$pEC_{50}$						
DAMGO	7.70 $\pm$ 0.14 (4)	7.13 $\pm$ 0.10 (4)*	7.16 $\pm$ 0.09 (4)*	7.16 $\pm$ 0.04 (4)*	7.24 $\pm$ 0.04 (4)*	DAMGO 10 $\mu$ M
Morphine	7.73 $\pm$ 0.05 (3)	7.56 $\pm$ 0.09 (3)	7.50 $\pm$ 0.17 (3)	7.62 $\pm$ 0.13 (3)	7.53 $\pm$ 0.04 (3)	Morphine 10 $\mu$ M
% Not pretreated $E_{max}$						
DAMGO	100	93.21 $\pm$ 3.07 (4)	90.10 $\pm$ 4.28 (4)	91.72 $\pm$ 4.98 (4)	88.47 $\pm$ 3.10 (4)	DAMGO 10 $\mu$ M
Morphine	100	99.97 $\pm$ 1.90 (3)	102.48 $\pm$ 7.36 (3)	103.28 $\pm$ 5.70 (3)	92.43 $\pm$ 4.24 (3)	Morphine 10 $\mu$ M

\* Different from not treated.  $P < 0.05$  by Dunnett's post hoc test analysis of variance.

TABLE 3

Coactivation of the 5-HT<sub>2A</sub> receptor with 5-HT and the MOP receptor with morphine results in desensitization to morphine

Cells treated with doxycycline (0.01  $\mu$ g/ml) 24 h express 5-HT<sub>2A</sub>-eCFP. Cells were pretreated with either DAMGO or morphine for the indicated times, and membranes prepared from the cells were then used to assess the potency (top) and relative efficacy (bottom) of DAMGO or morphine to stimulate binding of [<sup>35</sup>S]GTP $\gamma$ S. Data are presented as  $pEC_{50}$  or percentage of maximal agonist effect ( $E_{max}$ ). Desensitization was assessed by reduction in potency of the ligand following pretreatment. Numbers in parentheses indicate number of independent experiments. Values are presented as mean  $\pm$  S.E.M.

	Not Pretreated	30 min	60 min	120 min	240 min	Pretreatment
$pEC_{50}$						
DAMGO	8.03 $\pm$ 0.16 (5)	7.23 $\pm$ 0.10 (5)*	7.32 $\pm$ 0.06 (5)*	7.07 $\pm$ 0.11 (5)*	7.28 $\pm$ 0.10 (5)*	DAMGO 1 $\mu$ M
Morphine	7.64 $\pm$ 0.11 (5)	7.80 $\pm$ 0.08 (5)	7.71 $\pm$ 0.11 (5)	7.78 $\pm$ 0.14 (5)	7.81 $\pm$ 0.18 (5)	Morphine 1 $\mu$ M
DAMGO	8.00 $\pm$ 0.15 (5)	8.13 $\pm$ 0.24 (5)	7.92 $\pm$ 0.22 (5)	7.96 $\pm$ 0.17 (5)	8.03 $\pm$ 0.20 (5)	5-HT 1 $\mu$ M
DAMGO	7.64 $\pm$ 0.11 (5)	8.07 $\pm$ 0.13 (5)	8.20 $\pm$ 0.08 (5)*	8.15 $\pm$ 0.07 (5)*	8.14 $\pm$ 0.11 (5)*	5-HT 1 $\mu$ M
DAMGO	8.00 $\pm$ 0.02 (3)	6.98 $\pm$ 0.14 (3)*	7.15 $\pm$ 0.08 (3)*	6.92 $\pm$ 0.31 (3)*	6.99 $\pm$ 0.20 (3)*	DAMGO 1 $\mu$ M
						5-HT 1 $\mu$ M
Morphine	7.86 $\pm$ 0.14 (3)	7.25 $\pm$ 0.10 (3)*	7.24 $\pm$ 0.10 (3)*	7.20 $\pm$ 0.18 (3)*	7.22 $\pm$ 0.16 (3)*	Morphine 1 $\mu$ M
						5-HT 1 $\mu$ M
% Not pretreated $E_{max}$						
DAMGO	100 (5)	87.30 $\pm$ 5.53 (5)	89.99 $\pm$ 4.00 (5)	86.98 $\pm$ 3.90 (5)	85.57 $\pm$ 3.05 (5)	DAMGO 1 $\mu$ M
Morphine	100 (5)	102.26 $\pm$ 2.06 (5)	100.73 $\pm$ 1.84 (5)	96.03 $\pm$ 5.66 (5)	98.15 $\pm$ 3.80 (5)	Morphine 1 $\mu$ M
DAMGO	100 (5)	94.77 $\pm$ 3.42 (5)	102.89 $\pm$ 3.15 (5)	93.44 $\pm$ 3.37 (5)	92.31 $\pm$ 4.71 (5)	5-HT 1 $\mu$ M
Morphine	100 (5)	101.05 $\pm$ 2.43 (5)	100.09 $\pm$ 6.72 (5)	102.20 $\pm$ 7.69 (5)	103.99 $\pm$ 5.71 (5)	5-HT 1 $\mu$ M
DAMGO	100 (3)	96.27 $\pm$ 5.44 (3)	90.88 $\pm$ 5.30 (3)	74.73 $\pm$ 4.41 (3)*	78.51 $\pm$ 1.74 (3)*	DAMGO 1 $\mu$ M
						5-HT 1 $\mu$ M
Morphine	100 (3)*	79.37 $\pm$ 1.26 (3)*	70.59 $\pm$ 3.01 (3)*	64.10 $\pm$ 3.08 (3)*	72.40 $\pm$ 3.15 (3)*	Morphine 1 $\mu$ M
						5-HT 1 $\mu$ M

\* Different from not treated.  $P < 0.05$  by Dunnett's post hoc test analysis of variance.



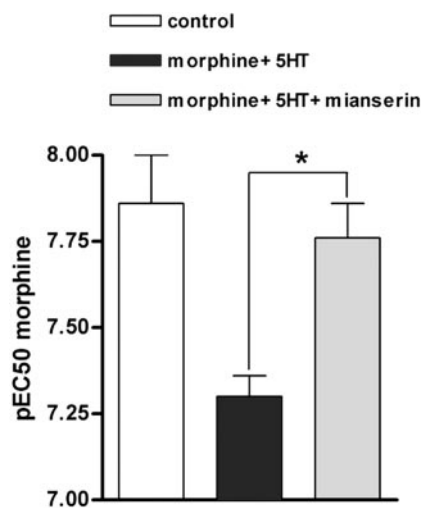
these cells, end of assay immunocapture of  $G\alpha_q/G\alpha_{11}$  indicated significant basal activity of the 5-HT<sub>2A</sub> receptor with a relatively limited capacity of 5-HT ( $10^{-5}$  M) to further elevate binding of [<sup>35</sup>S]GTP $\gamma$ S (Fig. 3C). By contrast, coaddition of the 5-HT<sub>2A</sub> receptor blocker mianserin ( $10^{-5}$  M) not only reversed the effect of 5-HT but also reduced the amount of bound [<sup>35</sup>S]GTP $\gamma$ S considerably below basal levels, consistent with constitutive activity of the c-Myc-5-HT<sub>2A</sub>-eCFP construct and with mianserin functioning as an inverse agonist (Fig. 3C).

The Flp-In T-REx HEK293 cells harboring c-Myc-5-HT<sub>2A</sub>-eCFP at the Flp-In locus were then transfected with the human MOP receptor modified at the C terminus by in-frame fusion of enhanced yellow fluorescent protein (MOP-eYFP), and individual clones constitutively expressing MOP-eYFP were isolated. Specific [<sup>3</sup>H]diprenorphine binding studies confirmed expression of MOP-eYFP (Fig. 4A), and induction of c-Myc-5-HT<sub>2A</sub>-eCFP increased levels of MOP-eYFP (Fig. 4A) by  $43 \pm 8\%$  (mean  $\pm$  S.E.M.;  $n = 10$ ;  $P < 0.01$ , one-way ANOVA), an effect that was prevented by coaddition of either the 5-HT<sub>2A</sub> receptor antagonist/inverse agonist mianserin ( $10^{-5}$  M) or the  $G\alpha_q/G\alpha_{11}$  inhibitor YM254890 ( $10^{-7}$  M) (Fig. 4A). Following doxycycline-induced expression, levels of c-Myc-5-HT<sub>2A</sub>-eCFP, assessed via saturation [<sup>3</sup>H]ketanserin binding studies, were not different ( $660 \pm 28$  fmol/mg membrane protein; mean  $\pm$  S.E.M.;  $n = 3$ ) from the cells lacking MOP-eYFP (Fig. 3A). Visual inspection and quantitation of eYFP fluorescence confirmed the binding data and that MOP-eYFP was predominantly plasma membrane-localized both in the absence and presence of c-Myc-5-HT<sub>2A</sub>-eCFP (Fig. 4, B and C). In these cells, the expressed c-Myc-5-HT<sub>2A</sub>-eCFP again displayed a predominantly punctate, intracellular vesicle pattern of distribution (Fig. 4B). Although without major effect on total levels of c-Myc-5-HT<sub>2A</sub>-eCFP, treatment with mianserin, and to a lesser extent YM254890, increased levels of cell surface-located c-Myc-5-HT<sub>2A</sub>-eCFP (Fig. 4B, bottom). This was associated with the presence of a higher proportion of the mature, 105-kDa terminally *N*-glycosylated form of this receptor construct (Fig. 4D). Visual inspection of cells to which 5-HT ( $10^{-5}$  M) was added during the period of c-Myc-5-HT<sub>2A</sub>-eCFP induction suggested that the level of MOP-eYFP was further increased. However, saturation [<sup>3</sup>H]diprenorphine binding studies indicated this not to be statistically significant (data not shown).

The expressed MOP-eYFP construct was also functional. The highly MOP-selective enkephalin analog DAMGO stimulated binding of [<sup>35</sup>S]GTP $\gamma$ S in membranes of these cells in a concentration-dependent manner (Table 1; Supplemental Fig. 3). As suggested by the higher levels of MOP-eYFP in the doxycycline- and 5-HT treated cells, the extent of [<sup>35</sup>S]GTP $\gamma$ S binding in response to DAMGO was greater in the treated than in the untreated cells (Table 1; Supplemental Fig. 3), an effect achieved without a significant alteration in the potency of DAMGO (Table 1; Supplemental Fig. 3). These effects of DAMGO were lacking in pertussis toxin-treated cells (data not shown), indicating that [<sup>35</sup>S]GTP $\gamma$ S binding via MOP-eYFP was to pertussis toxin-sensitive members of the  $G_i$  subgroup. Although often described as a MOP receptor partial agonist (Clark et al., 2006; Johnson et al., 2006), morphine stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in membranes of untreated MOP-eYFP-expressing Flp-In T-REx HEK293 cells to a similar extent as DAMGO (Supplemental Fig. 3), and

this was also further increased in the doxycycline- and 5-HT-treated cells (Table 1; Supplemental Fig. 3).

Many studies on the MOP receptor have noted profound desensitization of responses following pretreatment with ligands such as DAMGO but not with morphine (for review, see Connor et al., 2004). When Flp-In T-REx HEK293 cells expressing MOP-eYFP and harboring c-Myc-5-HT<sub>2A</sub>-eCFP at the Flp-In locus were pretreated with DAMGO ( $10^{-5}$  M) for times between 30 and 240 min, subsequent responses to DAMGO were desensitized within 30 min as measured by the reduction of potency of DAMGO to stimulate [<sup>35</sup>S]GTP $\gamma$ S binding in cell membranes (Table 2). However, this was not accompanied by a reduction in the extent of stimulation that could be achieved by a maximally effective concentration of DAMGO (Table 2). By contrast, pretreatment with morphine ( $10^{-5}$  M) over the same time period did not result in desensitization to subsequent morphine challenge (Table 2). Following induction of c-Myc-5-HT<sub>2A</sub>-eCFP expression, pretreatment with DAMGO ( $10^{-5}$  M) also invoked profound desensitization to subsequent exposure to DAMGO (Table 3). When the cells were pretreated with a combination of DAMGO and 5-HT, desensitization was now also noted as a reduction in  $E_{max}$  of DAMGO to enhance [<sup>35</sup>S]GTP $\gamma$ S binding as well as a decrease in potency (Table 3). In the presence of c-Myc-5-HT<sub>2A</sub>-eCFP, morphine ( $10^{-5}$  M) pretreatment again failed to desensitize the subsequent capacity of morphine to stimulate [<sup>35</sup>S]GTP $\gamma$ S binding (Table 3). However, following induction of expression of c-Myc-5-HT<sub>2A</sub>-eCFP, pretreatment with a combination of 5-HT ( $10^{-5}$  M) and morphine ( $10^{-5}$  M) resulted in rapid and profound desensitization of subsequent responses to morphine, measured as a reduction in both ligand potency and  $E_{max}$  (Table 3). This did not reflect a direct heterologous desensitization of MOP-eYFP via the 5-HT<sub>2A</sub> receptor. Treatment of cells expressing MOP-eYFP constitutively and induced to express c-Myc-5-HT<sub>2A</sub>-eCFP with only 5-HT ( $10^{-5}$  M) for between 30 and 240 min did not result in a desensitization of subsequent responses to mor-



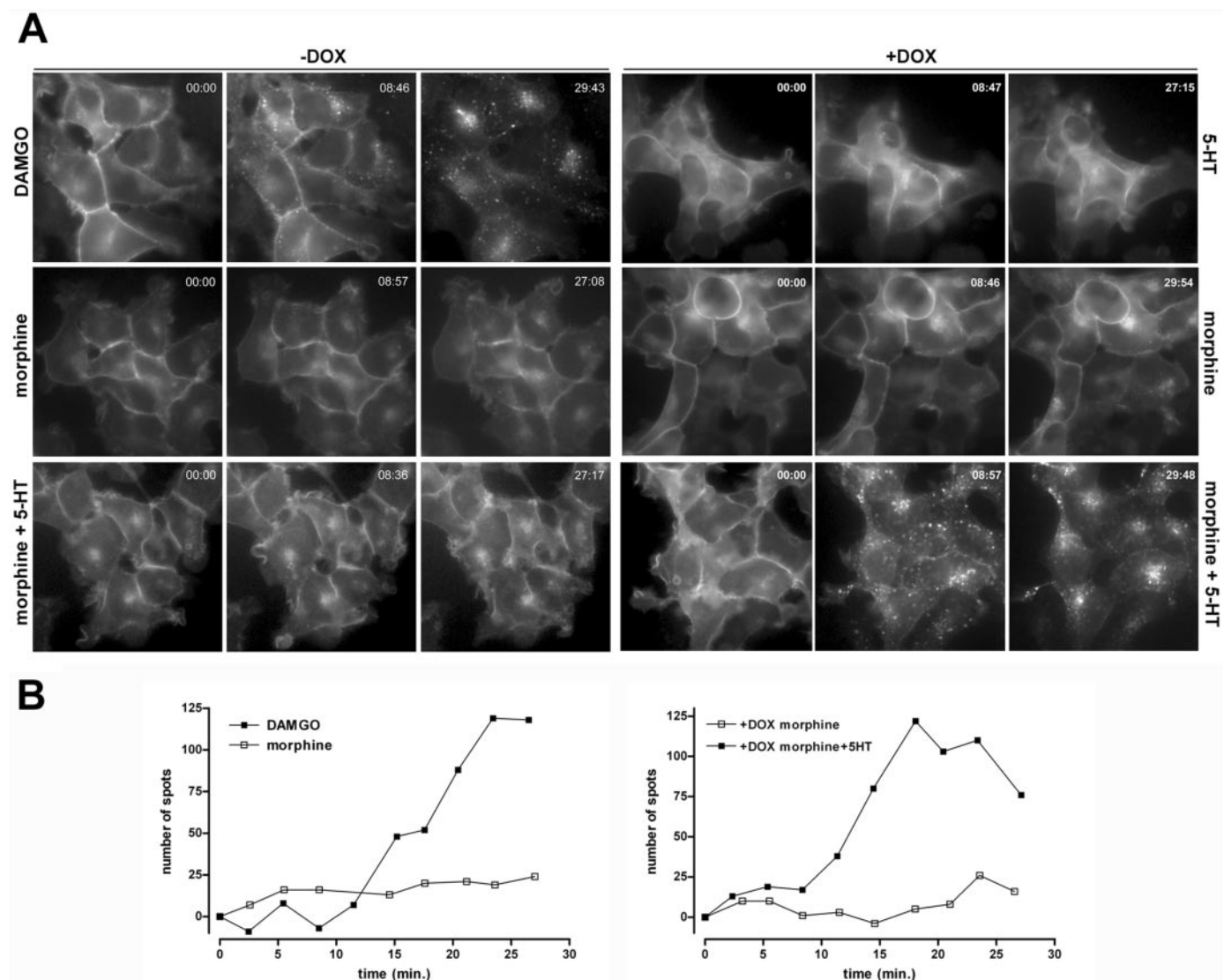
**Fig. 5.** Mianserin prevents desensitization of the MOP receptor produced in the presence of the 5-HT<sub>2A</sub> receptor by a combination of morphine and 5-HT. Membranes prepared from cells treated with doxycycline (10 ng/ml; 24 h) to express c-Myc-5-HT<sub>2A</sub>-eCFP and then treated or not for 1 h with morphine ( $10^{-5}$  M) + 5-HT ( $10^{-5}$  M) or a combination of morphine ( $10^{-5}$  M) + 5-HT ( $10^{-5}$  M) + mianserin ( $10^{-5}$  M). The potency of morphine to stimulate binding of [<sup>35</sup>S]GTP $\gamma$ S was then assessed. Data represent means  $\pm$  S.E.M.,  $n = 3$ . \*,  $P < 0.001$ , significantly different.

phine (Table 3). Indeed, a leftward shift in the EC<sub>50</sub> value for morphine was consistent with a supersensitization of the morphine response in these conditions (Table 3). The desensitization of subsequent responses to morphine produced by the combination of 5-HT and morphine did require the presence and activity of the 5-HT<sub>2A</sub> receptor. Addition of mianserin (10<sup>-5</sup> M) prevented 5-HT plus morphine-mediated MOP receptor desensitization measured in subsequent [<sup>35</sup>S]GTP $\gamma$ S binding studies (Fig. 5).

As noted in a range of other studies on the MOP receptor (Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996), in the Flp-In T-REx HEK293 cells expressing MOP-eYFP and in the absence of induction of c-Myc-5-HT<sub>2A</sub>-eCFP, DAMGO (10<sup>-5</sup> M) was able to cause extensive internalization of MOP-eYFP (Fig. 6; Supplemental Fig. 4). However, neither morphine (10<sup>-5</sup> M) nor a combination of morphine (10<sup>-5</sup> M) plus 5-HT (10<sup>-5</sup> M) was able to produce this effect (Fig. 6; Supplemental Fig. 5). Although induction of c-Myc-5-HT<sub>2A</sub>-

eCFP and addition of 5-HT (10<sup>-5</sup> M) did not alter DAMGO-induced internalization of MOP-eYFP (data not shown), after induction of c-Myc-5-HT<sub>2A</sub>-eCFP, addition of a combination of 5-HT (10<sup>-5</sup> M) and morphine (10<sup>-5</sup> M) resulted in substantial internalization of MOP-eYFP in the time-dependent manner (Fig. 6; Supplemental Fig. 6). Once more this was not an effect mediated by 5-HT/5-HT<sub>2A</sub> receptor in isolation because induction of c-Myc-5-HT<sub>2A</sub>-eCFP expression and addition of only 5-HT (10<sup>-5</sup> M) did not cause internalization of MOP-eYFP (Fig. 6).

Although incomplete in causing blockade, the coaddition of mianserin slowed the kinetics of MOP-eYFP internalization in response to the combination of 5-HT and morphine when the 5-HT<sub>2A</sub> receptor was present (Fig. 7). However, this ligand was without effect on DAMGO-mediated internalization of MOP-eYFP (Fig. 7). Coincubation with YM254890 (10<sup>-7</sup> M) also prevented internalization of MOP-eYFP induced by the combination of 5-HT plus morphine (Fig. 7) but not internalization



**Fig. 6.** In the presence of the 5-HT<sub>2A</sub> receptor, a combination of morphine and 5-HT causes internalization of MOP-eYFP. A, Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT<sub>2A</sub>-eCFP at the Flp-In locus were untreated (-Dox) or treated with doxycycline (10 ng/ml; 24 h) (+DOX). Cells were treated with the ligands indicated at the side of the panels and with the concentrations described in the text. The cells were then imaged over time to monitor the cellular location of MOP-eYFP. The insets record the time when the images were collected. B, quantitation of the time course of internalization in experiments akin to A was performed as described under *Materials and Methods*. Cells either uninduced (left) or induced to express c-Myc-5-HT<sub>2A</sub>-eCFP from the Flp-In locus (right) were challenged with the indicated ligands for varying periods.



stimulated by DAMGO (Fig. 7). A key role for PKC in this process was evident because the selective PKC inhibitor Ro318220 ( $10^{-7}$  M) also blocked internalization of MOP-eYFP induced by the combination of 5-HT plus morphine (Fig. 7) but not internalization induced by DAMGO (Fig. 7).

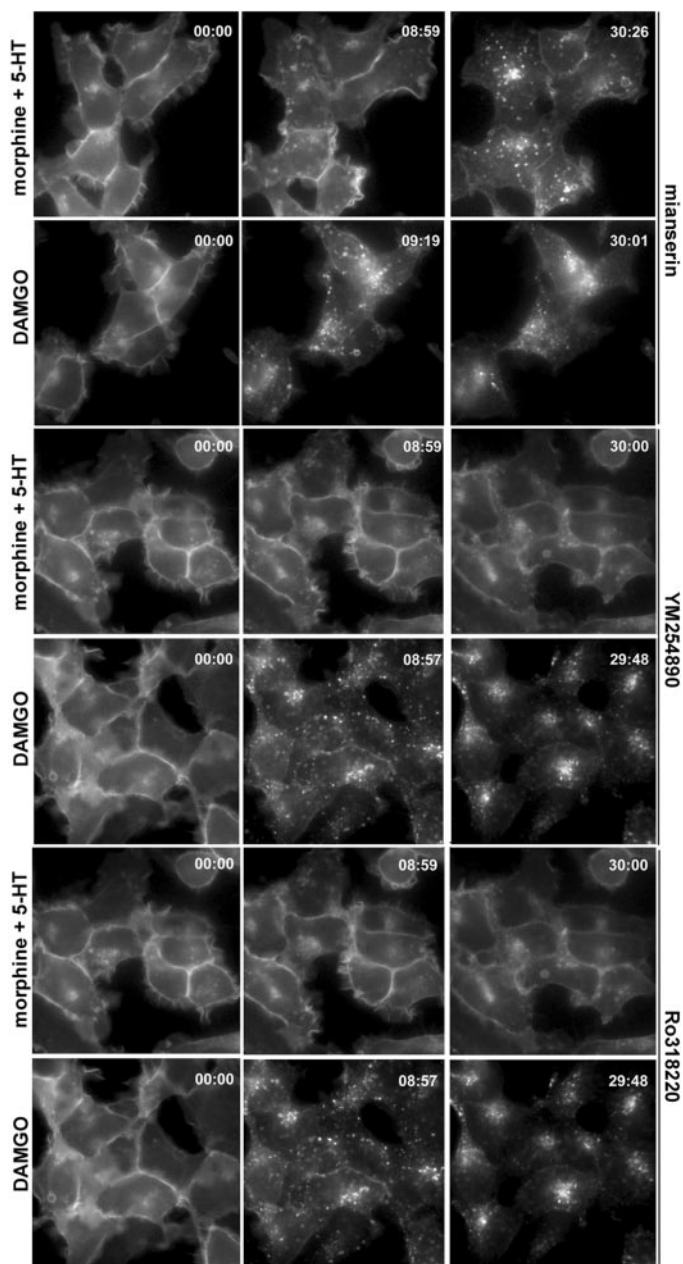
The capacity of DAMGO but not morphine treatment to down-regulate MOP receptor levels has also been described previously (Yabaluri and Medzihradsky, 1997). In the Flp-In T-REx HEK293 cells expressing MOP-eYFP, DAMGO ( $10^{-5}$  M) produced a rapid and extensive down-regulation of the number of [ $^3$ H]diprenorphine binding sites (Fig. 8A). Equiv-

alent treatment with morphine ( $10^{-5}$  M) did not cause significant down-regulation (Fig. 8A). Following doxycycline induction of c-Myc-5-HT<sub>2A</sub>-eCFP expression, combined treatment with DAMGO ( $10^{-5}$  M) and 5-HT ( $10^{-5}$  M) also resulted in extensive down-regulation of MOP-eYFP and now, treatment with morphine ( $10^{-5}$  M) plus 5-HT ( $10^{-5}$  M) also resulted in profound down-regulation of MOP-eYFP, albeit with a slower time course than produced by DAMGO plus 5-HT (Fig. 8B). Once more, this was not an effect mediated by 5-HT/5-HT<sub>2A</sub> receptor in isolation because receptor induction, addition, or both of 5-HT did not directly cause down-regulation of [ $^3$ H]diprenorphine binding sites (Fig. 8C). The conclusions from the above-mentioned experiments were supported by immunoblot studies performed on cell lysates to detect the presence of MOP-eYFP following the various treatments (Fig. 8D).

## Discussion

GPCRs are the largest group of trans-plasma membrane signal-transducing polypeptides, and it has been estimated that greater than 90% of the nonchemosensory GPCRs are expressed at some level in the central nervous system. Although not yet examined in any level of detail, it is clear that multiple GPCRs are coexpressed by individual cells. It is likely, therefore, that cross-regulation of signals may occur and that concurrent exposure to agonist ligands for two distinct GPCRs may alter signal output and receptor regulation (Hur and Kim, 2002). Based on observations that pharmacological activation of PKC, either directly by phorbol esters or by stimulation of the G<sub>q/11</sub>-coupled muscarinic acetylcholine M<sub>3</sub> receptor, is able to produce rapid morphine-induced desensitization of MOP receptors in locus ceruleus neurons from rat (Bailey et al., 2004), we explored potential colocalization of mRNAs encoding the MOP receptor and the serotonin 5-HT<sub>2A</sub> receptor in various regions of rat brain. The regional patterns of hybridization signal obtained for both receptor mRNAs were in agreement with previous studies (Mansour et al., 1994). Importantly, comparison of autoradiograms obtained from consecutive sections of rat brain hybridized with  $^{33}$ P-labeled probes for 5-HT<sub>2A</sub> or MOP receptor mRNA showed regions of codistribution of both mRNAs and other regions in which only one of the receptors was expressed. Regions of codistribution included some cortical layers, the nucleus accumbens, the hippocampal formation, some nuclei of the amygdala, and the periaqueductal gray. Because neurons of the periaqueductal gray contribute to both the clinically beneficial antinociceptive effects of morphine and the clinically restricting development of tolerance (Bagley et al., 2005; Ingram et al., 2008), we explored whether heterologous coexpression of the 5-HT<sub>2A</sub> and MOP receptors would result in their cross-regulation and alteration in the ability of morphine to desensitize or cause internalization of the MOP receptor.

Recently, we have made considerable use of Flp-In T-REx HEK293 cells to explore interactions between pairs of GPCRs (Ellis et al., 2006; Canals et al., 2008). These cells allow one GPCR to be harbored at the Flp-In T-REx locus and to be expressed only upon addition of tetracycline or doxycycline, whereas the second GPCR can be expressed constitutively. With such an arrangement the function, pharmacology and regulation of the constitutively expressed GPCR can be as-



**Fig. 7.** In the presence of the 5-HT<sub>2A</sub> receptor, a combination of morphine and 5-HT causes internalization of MOP-eYFP. The role of G<sub>q</sub>/G<sub>11</sub> and protein kinase C. Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT<sub>2A</sub>-eCFP at the Flp-In locus were treated with doxycycline (10 ng/ml; 24 h) (+DOX). The ability of DAMGO or morphine + 5-HT to cause internalization of MOP-eYFP was then assessed in the presence of mianserin ( $10^{-5}$  M), YM254890 ( $10^{-7}$  M), or Ro318220 ( $10^{-7}$  M).



essed, and then the same characteristics of the receptor can be reassessed, in the same cells, following induction of expression of the second GPCR. We therefore generated a double-stable cell line harboring c-Myc-5-HT<sub>2A</sub>-eCFP at the Flp-In T-REx locus and constitutively expressing MOP-eYFP. Induction of c-Myc-5-HT<sub>2A</sub>-eCFP expression resulted in up-regulation of MOP-eYFP, an effect that was blocked by the 5-HT<sub>2A</sub> receptor antagonist/inverse agonist mianserin and also by the highly selective G<sub>αq/11</sub> inhibitor YM254890 (Canals et al., 2006). The up-regulation of MOP-eYFP by induction of c-Myc-5-HT<sub>2A</sub>-eCFP is likely to be a selective effect because induction of expression of a human CB1 cannabinoid receptor from the equivalent Flp-In T-REx locus does not result in up-regulation of constitutively expressed MOP-eYFP (Canals et al., 2006). Interestingly, an increase of MOP receptor level in several regions of rat brain has been observed after chronic treatment with fluoxetine, a selective serotonin reuptake inhibitor (de Gandarias et al., 1999) that should produce elevated brain levels of 5-HT. Similar studies in animals that use selective 5-HT<sub>2A</sub> receptor antagonists might clarify the involvement of this or other related receptors for serotonin in MOP receptor up-regulation in native neural tissue.

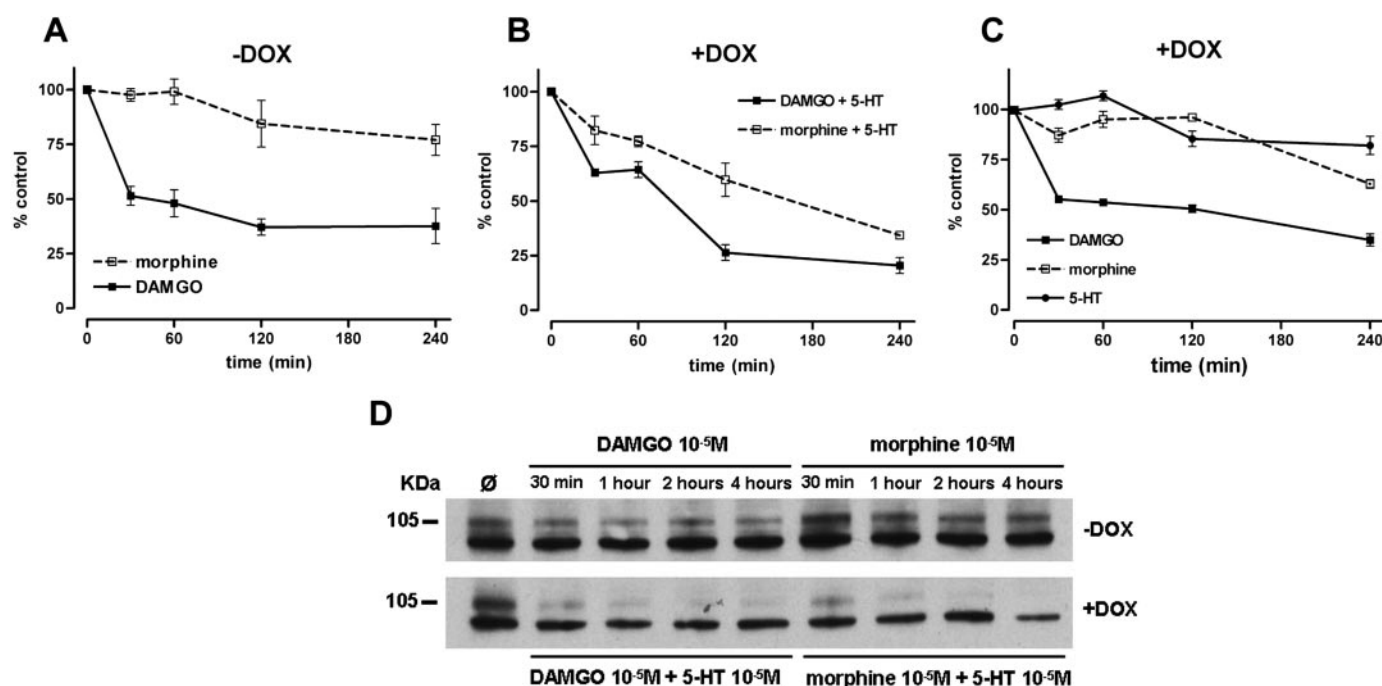
As reported in a wide range of studies (for review, see Connor et al., 2004; Martini and Whistler, 2007) with stable expression of the MOP receptor in isolation pretreatment with morphine was unable to cause desensitization, internalization, or down-regulation of MOP-eYFP. In contrast the MOP receptor-selective synthetic enkephalin DAMGO produced robust and rapid desensitization that was maximal within 30 min. Similar differences between these opioid agonists have been described previously in various studies. How-

ever, there are numerous discrepancies depending on the cellular model studied, the pharmacological assay used to assess MOP receptor function, or a combination (for review, see Connor et al., 2004; Bailey and Connor, 2005). We concentrated on [<sup>35</sup>S]GTPγS binding studies because these measure the initial step in the generation of downstream signals after receptor activation produced by agonist binding.

By contrast with the cells that expressed only MOP-eYFP, following induction of expression of the 5-HT<sub>2A</sub> receptor morphine was able to recapitulate the pattern of regulation of MOP-eYFP produced by DAMGO. However, this was only observed when 5-HT was added in concert with morphine. These effects required expression of the 5-HT<sub>2A</sub> receptor because coaddition of 5-HT and morphine without 5-HT<sub>2A</sub> receptor induction was ineffective, and the effect of 5-HT/5-HT<sub>2A</sub> receptor was blocked by the 5-HT<sub>2A</sub> receptor antagonist/inverse agonist mianserin.

Other strategies have been described to induce MOP receptor internalization by morphine in heterologous expression systems. These strategies include overexpression of other accessory proteins involved in GPCR desensitization/endocytosis processes, such as arrestins or G protein-coupled receptor kinases (Zhang et al., 1998), and the combination of morphine with other opioid agonists at concentrations that are too low, when used in isolation, to cause internalization (He and Whistler, 2005). Some of the findings observed at the cellular level have been extended to experimental animal models of nociception to demonstrate that internalization of MOP receptors by morphine may avoid the development of tolerance when using this opioid agonist chronically (Kim et al., 2008).

Although the use of combinations of opioid ligands has the



**Fig. 8.** In the presence of the 5-HT<sub>2A</sub> receptor, a combination of morphine and 5-HT causes down-regulation of MOP-eYFP. Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT<sub>2A</sub>-eCFP at the Flp-In locus were untreated (A) or treated with doxycycline (10 ng/ml; 24 h) (B and C). The cells were then exposed to morphine (□), DAMGO (■), 5-HT (○), or combinations of 5-HT with either morphine or DAMGO for times up to 4 h. Membranes were prepared and used to measure the specific binding of 2 nM [<sup>3</sup>H]diprenorphine. Data are presented (means ± S.E.M.; n = 3) as percentage of [<sup>3</sup>H]diprenorphine binding sites in cells before exposure to the ligands. D, immunoblots performed on lysates of cells samples treated as described above. The 105-kDa band corresponds to MOP-eYFP receptor. Ø, untreated cells.

potential for direct translation to a clinical setting, we wanted to explore other avenues for potential combination therapy. Our approach was based on a requirement to identify other GPCRs coexpressed with the MOP receptor in individual neurons in regions of the brain known to be important in morphine-induced analgesia and the development of tolerance. One example proved to be the 5-HT<sub>2A</sub> receptor, and we demonstrate herein that coactivation of this receptor allows morphine to cause each of desensitization, internalization, and down-regulation of the MOP receptor. Given the literature that indicates that strategies that enhance morphine-induced desensitization and internalization of the MOP receptor are likely to maintain analgesic efficacy while reducing antinociceptive tolerance (Martini and Whistler, 2007; Kim et al., 2008), it will be interesting, in time, to explore whether coactivation of the 5-HT<sub>2A</sub> receptor or other GPCRs that regulate similar signaling cascades and that are coexpressed with the MOP receptor will produce such effects.

In broken cell membrane [<sup>35</sup>S]GTPγS binding studies, the 5-HT<sub>2A</sub> receptor displayed high levels of constitutive activity. However, in the intact cell situation the level of constitutive activity was insufficient to cause substantial regulation of the MOP receptor in response to morphine. It is possible that cell homogenization and membrane preparation results in loss of soluble factors or proteins only weakly associated with the 5-HT<sub>2A</sub> receptor that apply a brake to constitutive activity. Furthermore, although it has become commonplace to ascribe effects of receptor coexpression on function to the propensity of the receptors to heterodimerize (Milligan and Smith, 2007), this seems unlikely to contribute to the current effects because the coexpressed MOP and 5-HT<sub>2A</sub> receptors had very distinct cellular distributions. Drugs targeting the 5-HT<sub>2A</sub> receptor have been suggested to be useful in the treatment of a range of disorders, including psychosis and sleep disorders (de Angelis, 2002; Morairty et al., 2008) and the regulation of platelet aggregation (Uchiyama et al., 2007). These programs aim to block activity of the 5-HT<sub>2A</sub> receptor. However, selective serotonin reuptake inhibitors are effective antidepressants that function by maintaining elevated levels of 5-HT. It is interesting, therefore, that the selective serotonin reuptake inhibitor fluoxetine has been reported to suppress morphine tolerance and dependence (Singh et al., 2003).

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