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Morphine desensitization, internalization and down-regulation of the mu opioid receptor is facilitated by serotonin 5-HT_{2A} receptor co-activation

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Running title 5-HT_{2A} regulation of mu receptor function

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Abbreviations

CA3, field CA3 of hippocampus; CPu, caudate putamen; DOP, delta opioid peptide; GPCR, G protein-coupled receptor; GTPγS, guanosine 5'-O-(thiotriphosphate); MOP, mu opioid peptide; PAG, periaqueductal gray; PKC, protein kinase C; SuC, superior colliculus.

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ABSTRACT

Analysis of the distribution of mRNA encoding the 5-HT_{2A} receptor and the mu opioid peptide receptor in rat brain demonstrated their co-expression in neurones in a number of distinct regions. These included the periacqueductal grey, 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 HEK293 cells that harbored the human 5-HT_{2A} receptor at the inducible Flp-In locus. In the absence of the 5-HT_{2A} receptor pre-treatment with the enkephalin agonist DAMGO but not with the alkaloid agonist morphine produced desensitization, internalization and down-regulation of the mu opioid peptide receptor. Induction of 5-HT_{2A} receptor expression in these cells resulted in up-regulation of mu opioid peptide receptor levels that was blocked by both a 5-HT_{2A} receptor inverse agonist and selective inhibition of signalling via $G\alpha_q/G\alpha_{11}$ G proteins. Following induction of the 5-HT_{2A} receptor co-addition of 5-HT with morphine now also resulted in each of 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_{2A} receptor in concert with treatment with morphine might achieve this aim.

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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 as 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 explored why other agonists which 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 enhance 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 (see Martini and Whistler, 2007 for review).

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 DAMGO, promote extensive receptor internalization and produce less tolerance (Keith et al., 1996,

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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 knock-in 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 (DOP) receptor (Kim et al., 2008). Such C-terminally modified forms of the MOP receptor had previously been shown 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 knock-in mice, morphine caused internalization of the modified receptor and, importantly, although still producing analgesia, 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 has 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 include modulation of the cellular content of regulatory polypeptides known to be involved in the fine control of signal transduction including β-arrestins (Bohn et al., 2000) and, as a more immediately practical avenue, regulation of the activity of various protein kinases and phosphatases

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(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 limit the development of tolerance and/or dependence.

Many studies on aspects of the molecular details of MOP receptor regulation are performed in heterologous cell systems that allow easy manipulation of signalling 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 co-expressed in native systems. The serotonin 5-HT_{2A} receptor is widely expressed in the central nervous system of man and rodents and is coupled strongly to activation of protein kinase C (PKC) via G proteins of the G_q/G_{11} family (Sanders-Bush et al., 2003). PKC activation, either directly by addition of phorbol esters or via activation of the muscarinic M_3 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 co-expression of the 5-HT_{2A} receptor and MOP receptor in various regions of rat brain and if co-expression of the 5-HT_{2A} 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

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All materials for tissue culture were from Invitrogen (Paisley, U.K.). [^{3}H]ketanserin, [^{3}H]diprenorphine and [^{35}S]GTPγS were from Perkin Elmer Life and Analytical Sciences (Boston, MA). Doxycycline, serotonin (5-HT), mianserin, DAMGO (Tyr-D-Ala-Gly-N-methyl-Phe-Gly-ol), naloxone and morphine were from Sigma-Aldrich. YM254890 was the kind gift of Astrellas Pharma Inc. (Osaka, Japan).

Receptor fusions with fluorescent proteins

Generation and subcloning of the human 5-HT_{2A} receptor construct was essentially as described before (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 EQKLISEEDL). A c-Myc-5-HT_{2A} receptor c-terminally tagged with enhanced cyan fluorescent protein (eCFP) was constructed by amplifying the sequence corresponding to the receptor by PCR and 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_{2A}-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 peYFP-N1 vector resulting in a single

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open reading frame consisting of the MOP receptor with eYFP fused to the receptor carboxy 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_{2A}-eCFP in an inducible manner cells were transfected with a mixture containing the c-myc-5-HT_{2A}-eCFP receptor cDNA in the pcDNA5/FRT/TO vector and pOG44 vector (1:9) using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. When cotransfected with the pcDNA5/FRT plasmid into a 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 (Canals and Milligan 2008). Clones resistant to blasticidin were screened for c-myc-5-HT_{2A}-eCFP expression by both fluorescence and Western blotting. To induce expression of c-myc-5-HT_{2A}-eCFP cells were treated with varying concentrations of doxycycline for different periods of time. The optimal expression of c-myc-5-HT_{2A}-eCFP was achieved after 24 hours treatment with 0.01µg doxycycline/ml growth medium (see Results). A double stable cell line expressing MOP-eYFP constitutively and c-Myc-5-HT_{2A}-eCFP in an inducible fashion 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 G418 (1mg/ml) and the resistant clones screened for receptor expression by fluorescence microscopy.

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Dialyzed fetal calf serum was used for cell growth to avoid activation of c-Myc-5-HT_{2A}-eCFP by 5-HT that is present routinely in serum.

$[Ca^{2+}]_i$ measurements

Cells were introduced into 96 well plates, loaded with the Ca^{2+}-sensitive dye Fura-2 AM, (1.5 µM), by incubation (30 min; 37°C) under reduced light in DMEM growth medium. Calcium imaging and analysis were then performed using a FLEXstation (Molecular Devices.

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 1200*g* for 10 min to remove unbroken cells and nuclei. The supernatant was subsequently centrifuged at 218,000*g* for 30 min in a Beckman Optima TLX Ultracentrifuge (Palo Alto, 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

[^{3}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 $MgCl_2$,

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pH 7.4) containing [^{3}H]ketanserin (0.2–20 nM). Non-specific 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, U.K.) using a Brandel cell harvester. The filters were washed twice with ice-cold phosphate-buffered saline (140 mM NaCl, 10 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4), and bound ligand estimated by liquid scintillation spectrometry.

[^{3}H]diprenorphine binding. Cell membranes (15-20 µg of protein) were incubated with [^{3}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 $MgCl_2$, pH 7.4). Non-specific 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 Brandel cell harvester with three 5-ml washes of ice-cold phosphate-buffered saline. The filters were soaked in 3 ml of scintillation fluid and radioactivity determined by liquid scintillation spectrometry.

[^{35}S]GTPγS binding. Cell membranes (10 µg) were incubated in buffer (20 mM HEPES, 100 mM NaCl, 4 mM $MgCl_2$, 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 incubated at 30°C for 60 min in the presence of 0.1 nM [^{35}S]GTPγS. The reaction was terminated by rapid filtration with a

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Brandel cell harvester and three 4-ml washes with ice-cold phosphate-buffered saline. Radioactivity was determined as described above.

[^{35}S]GTPγS binding by immunocapture of $G\alpha_q$/$G\alpha_{11}$. Experiments were initiated by the addition of membranes to assay buffer (20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, and 100 nCi of [^{35}S]GTPγS). Non-specific 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 $MgCl_2$, 100 mM NaCl, and 0.2 mM ascorbic acid. The samples were centrifuged at 16,000 x *g* 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 pre-cleared with Pansorbin (Calbiochem, Nottingham, U.K.), followed by immunoprecipitation with antiserum CQ (Mitchell et al., 1993) that selectively identifies the C-terminal decapeptide common to $G\alpha_q$ and $G\alpha_{11}$. Finally, the immunocomplexes were washed twice with solubilization buffer, and bound [^{35}S]GTPγS 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 $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 10 mM D-glucose, pH 7.4). Fluorescent images of the cells were

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acquired using a Nikon TE2000-E inverted microscope (Nikon Instruments, Melville, NY) equipped with a x40 (numerical aperture = 1.3) oil immersion Plan Fluor lens and a cooled digital CoolSNAP$_{HQ}$ 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 minutes during a period of 30-35 minutes.

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 process.

Western blotting

Samples were heated at 65 °C for 15 min and subjected to SDS-PAGE analysis using 4–12% BisTris 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 non-specific binding sites. The membranes were incubated overnight with a rabbit anti-c-Myc polyclonal antibody (Cell Signaling, Hertfordshire,

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U.K.) or a sheep anti-MOP antiserum generated in house (Canals and Milligan 2008) and protein/antibody interactions detected using horseradish peroxidase-linked anti-rabbit IgG or anti-goat IgG secondary antisera (Amersham Biosciences, Buckinghamshire, U.K.). Immunoblots were developed by application of enhanced chemiluminescence solution (Pierce).

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 -20ºC. Tissue sections, 20 μm thick, were cut on a microtome-cryostat (Microm HM500 OM, Walldorf, Germany), thaw-mounted onto 3-aminopropyltriethoxysilane- (Sigma, St Louis, MO) coated slides, and kept at -20ºC until used.

Hybridization probes

For the detection of 5-HT_{2A} receptor mRNA, 6 different oligomers were used simultaneously. They were complementary to the following bases of the rat 5-HT_{2A} receptor mRNA (GenBank accession number X13971): 669-716, 723-767, 1379-1423,

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1482-1530, 1844-1888, and 1923-1970. MOP receptor mRNA was detected with 4 oligomers complementary to the following bases of the rat MOP receptor mRNA (GenBank accession number NM 013071): bases 187-231, 803-850, 1279-1323, and 1431-1475. Oligonucleotides were synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands). Comparison of the oligonucleotide sequences with EMBL and GenBank databases with basic local alignment search tool (BLAST) indicated that the probes do not show any significant similarity with mRNAs in the rat other than their corresponding targets. Oligonucleotides were labelled at their 3'-end either radioactively with [^{33}P] α-dATP (3000 Ci/mmol, New England Nuclear, Boston, MA, USA) and terminal deoxynucleotidyltransferase (TdT) (Oncogene Research Products, San Diego, CA, USA) or non-radioactively with digoxigenin-labelled nucleotides (Dig-11-dUTP, Roche Applied Science, Mannheim, Germany) and TdT (recombinant, Roche Applied Science, Penzberg, Germany). Labelled probes were purified from non-incorporated nucleotides with ProbeQuant G-50 micro columns (GE Healthcare, Little Chalfont, U.K.).

In situ hybridization histochemistry procedure

The protocols for single- and double-label *in situ* hybridization histochemistry were based on previously described procedures (Vilaro et al., 1996, Landry et al., 2000). Briefly, tissue sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (1xPBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed in 1xPBS, incubated in predigested pronase (Calbiochem, San Diego, CA, USA) at a final concentration of 24 U/ml in 50 mM Tris-HCl pH 7.5, 5 mM EDTA, immersed in 2

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mg/ml glycine in 1xPBS to stop proteolytic activity, rinsed in 1xPBS and dehydrated through a graded series of ethanol. For hybridization, radioactively- and non-radioactively labelled probes were diluted in a solution containing 50% formamide, 4x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate), 1x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 µg/ml yeast tRNA and 500 µg/ml salmon sperm DNA. The final concentrations of radioactive and Dig-labelled probes in the hybridization buffer were in the same range (approximately 1.5 - 2 nM each probe). Tissue sections were covered with 60-70 µl of hybridization solution, overlaid with Nescofilm coverslips (Bando Chemical Ind, Kobe, Japan) and incubated overnight at 42ºC in humid boxes. Sections were washed extensively in 0.6M NaCl, 10mM Tris-HCl pH 7.5 at 60ºC. Sections hybridized with ^{33}P- and digoxigenin-labelled 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 ethanols and allowed to air-dry before exposure to film or dipping into liquid emulsion.

Development of radioactive and non-radioactive hybridization signal

Sections hybridized with ^{33}P- and digoxigenin-labelled 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 $MgCl_2$ and 0.5% bovine serum albumin (Fraction V, Sigma-Aldrich) and incubated overnight at 4ºC in the same solution containing an alkaline-phosphatase-conjugated anti-digoxigenin antibody (Fab fragments, 1:5000 dilution; Roche Applied Science, Penzberg,

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Germany). They were washed in the same buffer, and then in alkaline buffer (0.1 M Tris-HCl pH 9.5; 0.1 M NaCl; 5 mM $MgCl_2$). Alkaline phosphatase activity was developed with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Roche Applied Science, Penzberg, Germany) 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-labelled and ^{33}P-labelled probes simultaneously were dipped in a solution of 2% collodion in amyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA) 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°C for 6 weeks, and finally developed in Kodak D19 developer (Kodak, Rochester, NY, USA).

Analysis of results

Tissue sections were examined under bright- and dark-field illumination in a Wild 420 macroscope (Leica, Heerbrugg, Germany) or in a Zeiss 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, USA). Cells containing the mRNA detected with digoxigenin-labelled probes were identified as cellular profiles exhibiting a dark precipitate (alkaline phosphatase reaction product). Hybridization signal obtained with ^{33}P-labelled 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

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areas known to be devoid of the corresponding receptor mRNA. In all the experiments, control sections were included that were incubated with the labelled probes plus a large excess of the same unlabelled 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 analysed using PRISM (GraphPAD Software Inc., San Diego, CA) and statistical significance 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 Software, San Jose, CA, USA).

RESULTS

A series of [^{33}P]- and digoxigenin-labelled oligonucleotide probes were used individually or in combination to detect the presence of mRNAs encoding the 5-HT_{2A} receptor and the MOP receptor in sections of rat brain. mRNA encoding each receptor was expressed widely (**Figure 1A**). Detailed analysis indicated these to be co-expressed in individual cells in a number of areas including neurones of the CA3 field of the hippocampus (**Figure 1B)**, the periaqueductal grey (**Figure 1C**), in layer VIb of the neocortex (**Figure 1D**) and the dorsal endopiriform nucleus (**Figure 1E**). Specificity of the signals was

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confirmed in several routine control experiments performed with each of the oligonucleotide probes used for *in situ* hybridization. Controls for the 5-HT_{2A} receptor probes have been illustrated previously (Mengod et al., 1990). Controls for the MOP receptor probes are shown in **Supplementary Figure** 1. In brief, a) 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. b) For any given labelled probe, inclusion during hybridization of an excess of the same unlabelled oligonucleotide resulted in the absence of specific hybridization signals. The auto-radiographic signal remaining under these conditions was taken as background or non-specific tissue signal. c) Inclusion during hybridization of an excess of an unrelated, unlabelled oligonucleotide left specific hybridization signals unaffected. d) 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 co-expressed GPCRs are often able to produce cross-regulation and alteration of function (Hur and Kim, 2002), the significant extent of co-localization of mRNA encoding these two receptors encouraged us to explore this possibility for the 5-HT_{2A} and MOP receptors. cDNA encoding the human 5-HT_{2A} 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 enhanced cyan fluorescent protein (eCFP). This construct (c-Myc-5-HT_{2A}-eCFP) was cloned into the tetracycline/doxycycline-inducible Flp-In locus of Flp-

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In T-REx HEK293 cells and a pool of stably transfected cells isolated. In the absence of doxycycline, expression of c-Myc-5-HT_{2A}-eCFP was undetectable based either on the autofluorescence of eCFP (**Figure 2A**) or immunoblot detection of c-Myc reactive polypeptides (**Figure 2B**). Addition of doxycycline resulted in concentration-dependent expression of c-Myc-5-HT_{2A}-eCFP (**Figures 2B, 2C**) whilst visualization of individual living cells suggested that, at steady-state, the bulk of c-Myc-5-HT_{2A}-eCFP was present in punctate, intracellular vesicles (**Figure 2A and Supplementary Figure 2**). Following induction of c-Myc-5-HT_{2A}-eCFP by treatment with doxycycline for 24h, specific [^{3}H]ketanserin binding to membranes from these cells was monophasic with K_d = 2.6 +/- 0.2 nM and B_{max} 665 +/- 89 fmol/mg membrane protein (**Figure 3A**). The expressed c-Myc-5-HT_{2A}-eCFP was functional because addition of 5-HT resulted in a concentration-dependent increase in $[Ca^{2+}]_i$ with pEC_{50} 8.6 +/- 0.2 (**Figure 3B**) (means +/- SEM, n = 3). In [^{35}S]GTPγS binding studies performed on membranes from these cells, end of assay immunocapture of $G\alpha_q/G\alpha_{11}$ indicated significant basal activity of the 5-HT_{2A}-receptor with a relatively limited capacity of 5-HT (10^{-5}M) to further elevate binding of [^{35}S]GTPγS (**Figure 3C)**. By contrast, co-addition of the 5-HT_{2A} receptor blocker mianserin (10^{-5}M) not only reversed the effect of 5-HT but reduced the amount of bound [^{35}S]GTPγS considerably below basal levels, consistent with constitutive activity of the c-Myc-5-HT_{2A}-eCFP construct and with mianserin functioning as an inverse agonist (**Figure 3C)**.

The Flp-In T-REx HEK293 cells harboring c-Myc-5-HT_{2A}-eCFP at the Flp-In locus were then transfected with the human MOP receptor modified at the C-terminus by

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in-frame fusion of enhanced yellow fluorescent protein (MOP-eYFP) and individual clones constitutively expressing MOP-eYFP isolated. Specific [^{3}H]diprenorphine binding studies confirmed expression of MOP-eYFP (**Figure 4A**) and induction of c-Myc-5-HT_{2A}-eCFP increased levels of MOP-eYFP (**Figure 4A**) by 43 +/- 8% (mean +/- SEM, n = 10, $p < 0.01$ one-way ANOVA), an effect that was prevented by co-addition of either the 5-HT_{2A} receptor antagonist/inverse agonist mianserin (10^{-5}M) or the $G\alpha_q/G\alpha_{11}$ inhibitor YM254890 (10^{-7}M) (**Figure 4A**). Following doxycycline-induced expression, levels of c-Myc-5-HT_{2A}-eCFP, assessed via saturation [^{3}H]ketanserin binding studies, were not different (660 +/- 28 fmol/mg membrane protein, mean +/- SEM, n = 3) from the cells lacking MOP-eYFP (**Figure 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_{2A}-eCFP (**Figures 4B, C**). In these cells the expressed c-Myc-5-HT_{2A}-eCFP again displayed a predominantly punctate, intracellular vesicle pattern of distribution (**Figure 4B**). Although without major effect on total levels of c-Myc-5-HT_{2A}-eCFP, treatment with mianserin and, to a lesser extent, YM254890 increased levels of cell surface located c-Myc-5-HT_{2A}-eCFP (**Figure 4B, lower panels).** This was associated with the presence of a higher proportion of the mature, 105 kDa terminally N-glycosylated form of this receptor construct **(Figure 4D).** Visual inspection of cells to which 5-HT (10^{-5}M) was added during the period of c-Myc-5-HT_{2A}-eCFP induction suggested that the level of MOP-eYFP was further increased. However, saturation [^{3}H]diprenorphine binding studies indicated this not to be statistically significant (**data not shown**).

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The expressed MOP-eYFP construct was also functional. The highly MOP selective enkephalin analog DAMGO stimulated binding of [^{35}S]GTPγS in membranes of these cells in a concentration-dependent manner (**Table 1 and Supplementary Figure 3**). As suggested by the higher levels of MOP-eYFP in the doxycycline and 5-HT treated cells, the extent of [^{35}S]GTPγS binding in response to DAMGO was greater in the treated than in the untreated cells (**Table 1 and Supplementary Figure 3**), an effect achieved without a significant alteration in the potency of DAMGO (**Table 1 and Supplementary Figure 3**). These effects of DAMGO were lacking in pertussis toxin-treated cells (**data not shown**), indicating that [^{35}S]GTPγS 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 [^{35}S]GTPγS binding in membranes of untreated MOP-eYFP expressing Flp-In T-REx HEK293 cells to a similar extent as DAMGO (**Supplementary Figure 3**) and this was also further increased in the doxycycline and 5-HT treated cells (**Table 1, Supplementary Figure 3**). Many studies on the MOP receptor have noted profound desensitization of responses following pre-treatment 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_{2A}-eCFP at the Flp-In locus were pre-treated 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 [^{35}S]GTPγS binding in cell membranes (**Table 2A**). However, this was not accompanied by a reduction in the extent of stimulation that could be achieved by a

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maximally effective concentration of DAMGO (**Table 2A**). By contrast, pre-treatment with morphine (10^{-5}M) over the same time period did not result in desensitization to subsequent morphine challenge (**Table 2A**). Following induction of c-Myc-5-HT_{2A}-eCFP expression, pre-treatment with DAMGO (10^{-5}M) also invoked profound desensitization to subsequent exposure to DAMGO (**Table 2B**). When the cells were pre-treated with a combination of DAMGO and 5-HT, desensitization was now also noted as a reduction in E_{max} of DAMGO to enhance [^{35}S]GTPγS binding as well as a decrease in potency (**Table 2B**). In the presence of c-Myc-5-HT_{2A}-eCFP, morphine (10^{-5}M) pre-treatment again failed to desensitize the subsequent capacity of morphine to stimulate [^{35}S]GTPγS binding (**Table 2B**). However, following induction of expression of c-Myc-5-HT_{2A}-eCFP, pre-treatment 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 2B**). This did not reflect a direct heterologous desensitization of MOP-eYFP via the 5-HT_{2A}-receptor. Treatment of cells expressing MOP-eYFP constitutively and induced to express c-Myc-5-HT_{2A}-eCFP with only 5-HT (10^{-5}M) for between 30 and 240 min did not result in a desensitization of subsequent responses to morphine (**Table 2B**). Indeed, a leftward shift in the EC_{50} for morphine was consistent with a super-sensitization of the morphine response in these conditions (**Table 2B**). 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_{2A}-receptor. Addition of mianserin (10^{-5}M) prevented 5-HT plus

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morphine-mediated MOP receptor desensitization measured in subsequent [^{35}S]GTPγS binding studies (**Figure 5**).

As noted in a range of other studies on the MOP receptor (Keith et al., 1996, Sternini et al., 1996, Arden et al., 1995), in the Flp-In T-REx HEK293 cells expressing MOP-eYFP and in the absence of induction of c-Myc-5-HT$_{2A}$-eCFP, DAMGO (10^{-5}M) was able to cause extensive internalization of MOP-eYFP (**Figure 6 and Supplementary Figure 4**). However, neither morphine (10^{-5}M), nor a combination of morphine (10^{-5}M) plus 5-HT (10^{-5}M), was able to produce this effect (**Figure 6 and Supplementary Figure 5**). Although induction of c-Myc-5-HT$_{2A}$-eCFP and addition of 5-HT (10^{-5}M) did not alter DAMGO-induced-internalization of MOP-eYFP (**not shown**), after induction of c-Myc-5-HT$_{2A}$-eCFP, addition of a combination of 5-HT (10^{-5}M) and morphine (10^{-5}M) resulted in substantial internalization of MOP-eYFP in the time dependent manner (**Figure 6 and Supplementary Figure 6**). Once more this was not an effect mediated by 5-HT/the 5-HT$_{2A}$ receptor in isolation because induction of c-Myc-5-HT$_{2A}$-eCFP expression and addition of only 5-HT (10^{-5}M) did not cause internalization of MOP-eYFP (**Figure 6**).

Although incomplete in causing blockade, the co-addition of mianserin slowed the kinetics of MOP-eYFP internalization in response to the combination of 5-HT and morphine when the 5-HT$_{2A}$-receptor was present (**Figure 7**). However, this ligand was without effect on DAMGO-mediated internalization of MOP-eYFP (**Figure 7**). Co-incubation with YM254890 (10^{-7}M) also prevented internalization of MOP-eYFP induced by the combination of 5-HT plus morphine (**Figure 7**) but not internalization

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stimulated by DAMGO (**Figure 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 (**Figure 7**) but not internalization induced by DAMGO (**Figure 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 (**Figure 8A**). Equivalent treatment with morphine (10^{-5}M) did not cause significant down-regulation (**Figure 8A**). Following doxycycline induction of c-Myc-5-HT$_{2A}$-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 (**Figure 8B).** Once more, this was not an effect mediated by 5-HT/5-HT$_{2A}$-receptor in isolation because receptor induction and/or addition of 5-HT did not directly cause down-regulation of [^{3}H]diprenorphine binding sites (**Figure 8C**). The conclusions from the above experiments were supported by immunoblot studies performed on cell lysates to detect the presence of MOP-eYFP following the various treatments (**Figure 8D**).

DISCUSSION

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GPCRs are the largest group of trans-plasma membrane signal transducing polypeptides and it has been estimated that greater than 90% of the non-chemosensory 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 co-expressed 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 $G_{q/11}$-coupled muscarinic acetylcholine M_3 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 co-localization of mRNA's encoding the MOP receptor and the serotonin 5-HT_{2A} 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-labelled probes for 5-HT_{2A} or MOP receptor mRNA showed regions of co-distribution of both mRNAs, and other regions where only one of the receptors was expressed. Regions of co-distribution included some cortical layers, the nucleus accumbens, the hippocampal formation, some nuclei of the amygdala, and the periaqueductal gray. As neurons of the periaqueductal gray contribute to both the clinically beneficial anti-nociceptive effects of morphine and the clinically restricting development of tolerance (Bagley et al., 2005, Ingram et al., 2008) we explored whether heterologous co-expression of the 5-HT_{2A} and MOP receptors would

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result in their cross-regulation and alteration in the ability of morphine to desensitize or cause internalization of the MOP receptor.

In recent times 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, whilst the second can be expressed constitutively. With such an arrangement the function, pharmacology and regulation of the constitutively expressed GPCR can be assessed and then the same characteristics of the receptor can be re-assessed, 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_{2A}-eCFP at the Flp-In T-REx locus and constitutively expressing MOP-eYFP. Induction of c-Myc-5-HT_{2A}-eCFP expression resulted in up-regulation of MOP-eYFP, an effect that was blocked by the 5-HT_{2A} receptor antagonist/inverse agonist mianserin and also by the highly selective $G_{\alpha q/11}$ inhibitor YM254890 (Canals et al., 2006). The up-regulation of MOP-eYFP by induction of c-Myc-5-HT_{2A}-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 employ selective 5-HT_{2A} receptor antagonists might clarify the involvement of this, or

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other related receptors for serotonin in MOP receptor up-regulation in native neural tissue.

As reported in a wide range of studies (see Connor et al., 2004, Martini and Whistler, 2007 for review) with stable expression of the MOP receptor in isolation pre-treatment 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 minutes. Similar differences between these opioid agonists have been described previously by various authors. However, there are numerous discrepancies depending on the cellular model studied and/or the pharmacological assay employed to assess MOP receptor function (for review see (Connor et al, 2004, Bailey and Connor, 2005). We concentrated on [^{35}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_{2A} receptor morphine was able to re-capitulate 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_{2A} receptor because co-addition of 5-HT and morphine without 5-HT_{2A} receptor induction was ineffective and the effect of 5-HT/5-HT_{2A} receptor was blocked by the 5-HT_{2A} receptor antagonist/inverse agonist mianserin.

Other strategies have been described to induce MOP receptor internalization by morphine in heterologous expression systems**.** These include over-expression of other **MOL 48272**

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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 potential for direct translation to a clinical setting, we wished to explore other avenues for potential combination therapy. Our approach was based on a requirement to identify other GPCRs co-expressed 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_{2A} receptor and we demonstrate herein that co-activation 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 which enhance morphine-induced desensitization and internalization of the MOP receptor are likely to maintain analgesic efficacy whilst reducing anti-nociceptive tolerance (Martini and Whistler, 2007, Kim et al., 2008), then it will be interesting, in time, to explore whether co-activation of the 5-HT_{2A} receptor or other GPCRs that regulate similar signaling cascades and which are co-expressed with the MOP receptor will produce such effects.

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In broken cell membrane [^{35}S]GTPγS binding studies the 5-HT_{2A} 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_{2A} receptor that apply a brake to constitutive activity. Furthermore, although it has become fashionable to ascribe effects of receptor co-expression on function to the propensity of the receptors to hetero-dimerize (Milligan and Smith, 2007), this appears unlikely to contribute to the current effects as the co-expressed MOP and 5-HT_{2A} receptors had very distinct cellular distributions. Drugs targeting the 5-HT_{2A} 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 programmes aim to block activity of the 5-HT_{2A} receptor. However, selective serotonin re-uptake inhibitors are effective anti-depressants that function by maintaining elevated levels of 5-HT. It is interesting, therefore, that the selective serotonin re-uptake inhibitor fluoxetine has been reported to suppress morphine tolerance and dependence (Singh et al., 2003).

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Figure Legends

Figure 1 mRNAs encoding the 5-HT_{2A} receptor and the MOP receptor are co-expressed in many regions of rat brain

A: Low power images of film autoradiograms obtained after hybridizing consecutive coronal sections with ^{33}P-labelled probes for 5-HT_{2A} receptor mRNA (left side) or MOP receptor mRNA (right side). **B:** Co-localization of 5-HT_{2A} and MOP receptor mRNAs in field CA3 of hippocampus. Upper panels are dark field photomicrographs of 5-HT_{2A} (left) and MOP (right) receptor mRNAs visualized with ^{33}P-labelled probes and liquid photographic emulsion. The lower panel is a bright field photomicrograph of a section where both mRNAs are visualized simultaneously with digoxigenin-labelled probes for 5-HT_{2A} mRNA (dark precipitate) and ^{33}P-labelled 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:** Co-localization of 5-HT_{2A} and MOP receptor mRNAs in the periaqueductal grey (PAG). The first and second rows are dark field images of 5-HT_{2A} (left) and MOP (right) receptor mRNAs visualized with ^{33}P-labelled probes in consecutive sections. The asterisks are placed immediately underneath the 4th ventricle. Examples of cells co-expressing both mRNAs (dark precipitate: MOP mRNA; silver grains: 5-HT_{2A} mRNA) are shown in the third (dark field) and fourth (bright field) rows. **D:** Co-localization of 5-HT_{2A} and MOP receptor mRNAs in layer VIb of neocortex. Low power images of film autoradiograms obtained after hybridizing consecutive sagittal sections with ^{33}P-labelled probes for 5-HT_{2A} (upper panel) or MOP receptor mRNA (central panel). The lower panel is a bright field

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photomicrograph of a section where 5-HT_{2A} mRNA is visualized with digoxigenin-labelled probes (dark precipitate) and MOP mRNA with ^{33}P-labelled probes (dark silver grains). The arrows point to cells shown at higher magnification in the inset. **E**: Co-localization of 5-HT_{2A} and MOP receptor mRNAs in the dorsal endopiriform nucleus. Bright field photomicrograph showing 5-HT_{2A} 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.

Figure 2 Generation of Flp-In T-REx HEK293 cells that express c-Myc-5-HT_{2A}-eCFP in an inducible fashion

cDNA encoding c-Myc-5-HT_{2A}-eCFP was cloned into the Flp-In locus of Flp-In T-REx HEK293 cells and a population of positive cells isolated.

A. These were maintained in the absence (- DOX) or in the presence (+ DOX) of 10 ng/ml doxycyline for 24 h and then imaged to detect eCFP.

Membranes were prepared from such cells treated with varying concentrations of doxycyline for 24 h and expression of c-Myc-5-HT_{2A}-eCFP detected by immunoblotting samples with an anti-c-Myc antibody (**B**). Such blots were quantitated by densitometry (**C**).

Figure 3 Expressed c-Myc-5-HT_{2A}-eCFP is functional

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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_{2A} receptor blocker [^{3}H]ketanserin.

B. These cells were also used to measure functionality of the 5-HT_{2A} receptor via the ability of varying concentrations of 5-HT to elevate $[Ca^{2+}]_i$.

C. Membranes from these cells were used to measure binding of [^{35}S]GTPγS in $G\alpha_q/G\alpha_{11}$ immunoprecipitates in the absence of ligands (basal) or in the presence of 5-HT (10^{-5}M) or 5-HT + mianserin (10^{-5}M). One-way ANOVA significantly different (P<0.0001). Post-hoc test *P<0.05, **P<0.001.

Figure 4 Up-regulation of MOP-eYFP levels by 5-HT_{2A} receptor expression reflects signalling via $G\alpha_q/G\alpha_{11}$.

Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT_{2A}-eCFP at the Flp-In locus were untreated (-DOX) or treated with doxycycline (+ DOX). + DOX cells were also treated with either the 5-HT_{2A} receptor antagonist/inverse agonist mianserin (10^{-5}M) or the $G\alpha_q/G\alpha_{11}$ inhibitor YM254890 (10^{-7}M).

A. The specific binding of [^{3}H]diprenorphine (2 nM) is displayed. Data represent means +/- SEM n = 3. * significantly different P< 0.001.

B. Images of MOP-eYFP (**upper panels**) or c-Myc-5-HT_{2A}-eCFP (**lower panels**) fluorescence of cells grown on coverslips.

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C. eYFP fluorescence from cells treated as in **B**. was measured directly. Data represent means +/- SEM n = 3. a.f.u.: arbritary fluorescence units * significantly different P< 0.001.

D. Although without major effect of total levels of c-Myc-5-HT_{2A}-eCFP, treatment with mianserin and to a lesser extent YM254890 increased levels of cell surface located c-Myc-5-HT_{2A}-eCFP (**B, lower panels)** and this was accompanied by greater levels of the mature, 105 kDa terminally N-glycosylated form of this receptor**.**

Figure 5 Mianserin prevents desensitization of the MOP receptor produced in the presence of the 5-HT_{2A} 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_{2A}-eCFP and then treated or not for 1 hour 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 [^{35}S]GTPγS was then assessed. Data represent means +/- SEM n = 3. * significantly different P< 0.001.

Figure 6 In the presence of the 5-HT_{2A} receptor a combination of morphine and 5-HT causes internalization of MOP-eYFP

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A. Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT_{2A}-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 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 in Materials and Methods. Cells either un-induced (**left hand panel**) or induced to express c-Myc-5-HT_{2A}-eCFP from the Flp-In locus (**right hand panel**) were challenged with the indicated ligands for varying periods of time.

Figure 7 In the presence of the 5-HT_{2A} receptor a combination of morphine and 5-HT causes internalization of MOP-eYFP. The role of G_q/G_{11} and protein kinase C

Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT_{2A}-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).

Figure 8 In the presence of the 5-HT_{2A} receptor a combination of morphine and 5-HT causes down-regulation of MOP-eYFP

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Flp-In T-REx HEK293 cells stably expressing MOP-eYFP and harboring c-Myc-5-HT_{2A}-eCFP at the Flp-In locus were untreated (**A**) or treated with doxycycline (10 ng/ml, 24 h (**B, C**). The cells were then exposed to morphine (open squares), DAMGO (filled squares), 5-HT (open circles) or combinations of 5-HT with either morphine or DAMGO for times up to 4h. Membranes were prepared and used to measure the specific binding of 2nM [^{3}H]diprenorphine. Data are presented (means +/- SEM, n = 3) as % of [^{3}H]diprenorphine binding sites in cells prior to exposure to the ligands. (**D**) Immunoblots performed on lysates of cells samples treated as above. The 105 kDa band corresponds to MOP-eYFP receptor. Ø: untreated cells.

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Tables

Table 1

Expression of c-Myc-5-HT_{2A}-eCFP and addition of 5-HT enhances G protein activation by DAMGO and morphine

DAMGO mean ± SEM (n)	**pEC_{50}**	**% E_{max} over control**
Control	7.58 ± 0.09 (8)	
Doxycycline (10 ng/ml)	7.88 ± 0.16 (8)	10.59 ± 5.52 (8)
Doxycycline (10 ng/ml) 5-HT (10^{-5}M)	7.91 ± 0.08 (8)	33.33 ± 3.69* (8)
morphine mean ± SEM (n)	**pEC_{50}**	**% E_{max} over control**
Control	7.63 ± 0.07 (4)	
Doxycycline (10 ng/ml)	7.65 ± 0.13 (4)	7.92 ± 1.68 (4)
Doxycycline (10 ng/ml) 5-HT (10^{-5}M)	7.92 ± 0.09 (4)	23.75 ± 4.65* (4)

[^{35}S]GTPγS binding studies were performed on membranes of Flp-In T-REx HEK293 cells constitutively expressing MOP-eYFP and in which c-Myc-5-HT_{2A}-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 [^{35}S]GTPγS produced without induction of the 5-HT_{2A} receptor construct are shown. Parantheses indicate number of independent experiments performed. Data are expressed as means +/- SEM. *P< 0.001. Dunnett's post hoc test analysis of variance.

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Table 2 Co-activation of the 5-HT_{2A} receptor with 5-HT and the MOP receptor with morphine results in desensitization to morphine. **A** Flp-In T-REx HEK293 cells not treated with doxycycline lack expression of 5-HT_{2A}-eCFP. Cells were pre-treated with either DAMGO or morphine for the indicated times and membranes prepared from the cells then employed to assess the potency (upper panels) and relative efficacy (lower panels) of DAMGO or morphine to stimulate binding of [^{35}S]GTPγS. Data are presented as pEC_{50} +/-SEM or % of maximal agonist effect (E_{max}). Numbers in parentheses indicate number of independent experiments. Desensitization was assessed by reduction in potency of the ligand following pre-treatment. * Different from not –treated. $p < 0.05$ Dunnett's post hoc test analysis of variance.

pEC_{50} ± SEM (n)	Not pre-treated	30 min	60 min	120 min	240 min	Pre-treatment
DAMGO	7.70 ± 0.14 (4)	7.13 ± 0.10 (4)*	7.16 ± 0.09 (4)*	7.16 ± 0.04 (4)*	7.24 ± 0.04 (4)*	DAMGO 10^{-5}M
morphine	7.73 ± 0.05 (3)	7.56 ± 0.09 (3)	7.50 ± 0.17 (3)	7.62 ± 0.13 (3)	7.53 ± 0.04 (3)	morphine 10^{-5}M

% Not pre-treated E_{max} ± SEM (n)	Not pre-treated	30 min	60 min	120 min	240 min	Pre-treatment
DAMGO	100	93.21 ± 3.07 (4)	90.10 ± 4.28 (4)	91.72 ± 4.98 (4)	88.47 ± 3.10 (4)	DAMGO 10^{-5}M
morphine	100	99.97 ± 1.90 (3)	102.48 ± 7.36 (3)	103.28 ± 5.70 (3)	92.43 ± 4.24 (3)	morphine 10^{-5}M

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Table 2 Co-activation of the 5-HT_{2A} receptor with 5-HT and the MOP receptor with morphine results in desensitization to morphine

B Cells treated with doxycycline (0.01µg/ml) 24 hours express 5-HT_{2A}-eCFP. The protocol was as in **A** with pre-treatments employing the ligand shown. * Different from not-treated. $p < 0.05$ Dunnett's post hoc test analysis of variance.

pEC_{50} ± SEM (n)	Not pre-treated	30 min	60 min	120 min	240 min	Pre-treatment
DAMGO	8.03 ± 0.16 (5)	7.23 ± 0.10 (5)*	7.32 ± 0.06 (5)*	7.07 ± 0.11 (5)*	7.28 ± 0.10 (5)*	DAMGO 10^{-5}M
morphine	7.64 ± 0.11 (5)	7.80 ± 0.08 (5)	7.71 ± 0.11 (5)	7.78 ± 0.14 (5)	7.81 ± 0.18 (5)	morphine 10^{-5}M
DAMGO	8.00 ± 0.15 (5)	8.13 ± 0.24 (5)	7.92 ± 0.22 (5)	7.96 ± 0.17 (5)	8.03 ± 0.20 (5)	5-HT 10^{-5}M
morphine	7.64 ± 0.11 (5)	8.07 ± 0.13 (5)	8.20 ± 0.08 (5)*	8.15 ± 0.07 (5)*	8.14 ± 0.11 (5)*	5-HT 10^{-5}M
DAMGO	8.00 ± 0.02 (3)	6.98 ± 0.14 (3)*	7.15 ± 0.08 (3)*	6.92 ± 0.31 (3)*	6.99 ± 0.20 (3)*	DAMGO 10^{-5}M 5-HT 10^{-5}M
morphine	7.86 ± 0.14 (3)	7.25 ± 0.10 (3)*	7.24 ± 0.10 (3)*	7.20 ± 0.18 (3)*	7.22 ± 0.16 (3)*	morphine 10^{-5}M 5-HT 10^{-5}M

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% Not pre-treated Emax ± SEM (n)	Not pre-treated	30 min	60 min	120 min	240 min	Pre-treatment
DAMGO	100 (5)	87.30 ± 5.53 (5)	89.99 ± 4.00 (5)	86.98 ± 3.90 (5)	85.57 ± 3.05 (5)	DAMGO 10^{-5}M
morphine	100 (5)	102.26 ± 2.06 (5)	100.73 ± 1.84 (5)	96.03 ± 5.66 (5)	98.15 ± 3.80 (5)	morphine 10^{-5}M
DAMGO	100 (5)	94.77 ± 3.42 (5)	102.89 ±3.15 (5)	93.44 ± 3.37 (5)	92.31 ± 4.71 (5)	5-HT 10^{-5}M
morphine	100 (5)	101.05 ± 2.43 (5)	100.09 ± 6.72 (5)	102.20 ± 7.69 (5)	103.99 ± 5.71 (5)	5-HT 10^{-5}M
DAMGO	100 (3)	96.27 ± 5.44 (3)	90.88 ± 5.30 (3)	74.73 ± 4.41 (3)*	78.51 ± 1.74 (3)*	DAMGO 10^{-5}M 5-HT 10^{-5}M
morphine	100 (3)*	79.37 ± 1.26 (3)*	70.59 ± 3.01 (3)*	64.10 ± 3.08 (3)*	72.40 ± 3.15 (3)*	morphine 10^{-5}M 5-HT 10^{-5}M

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Footnotes

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Figure 1

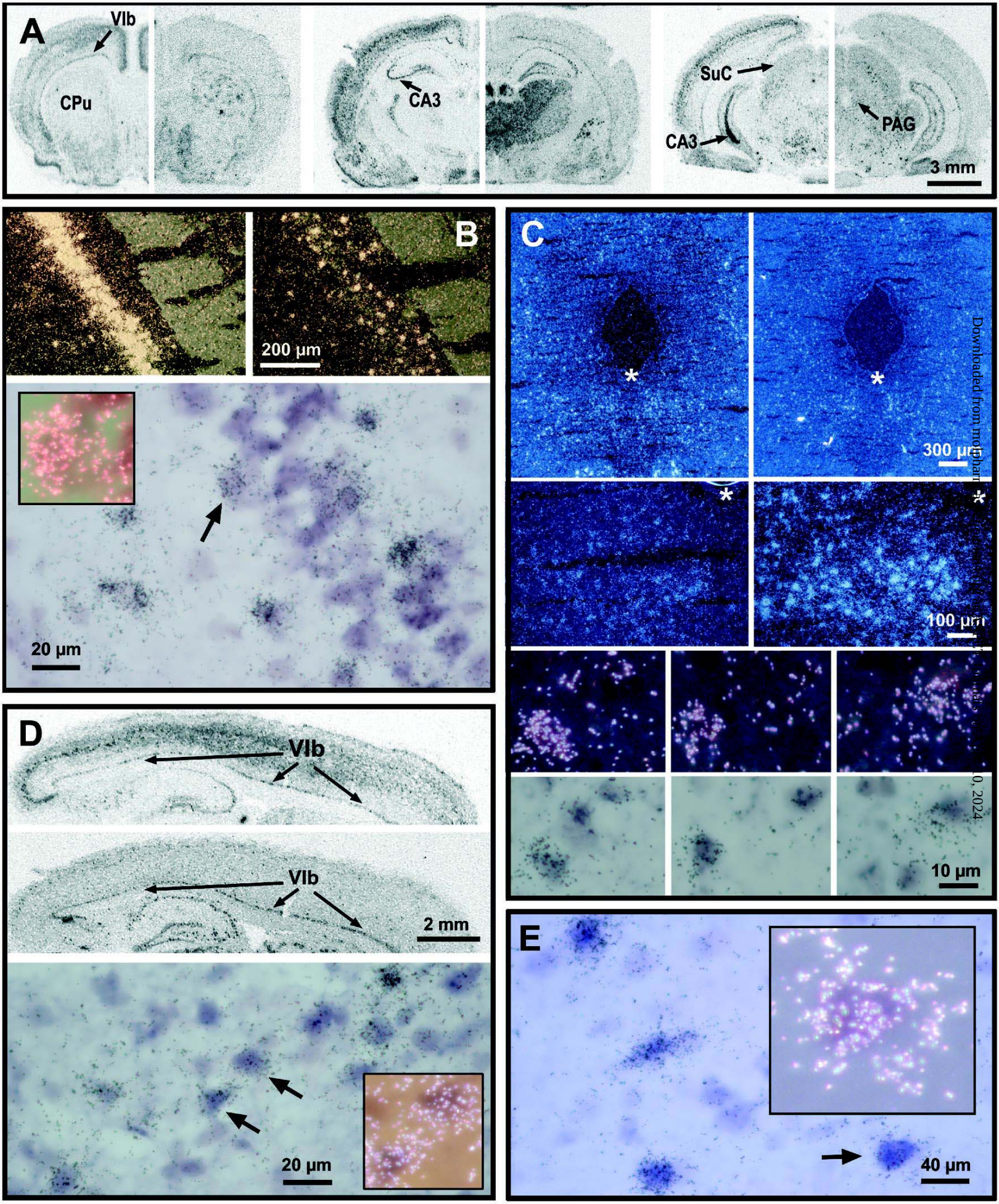

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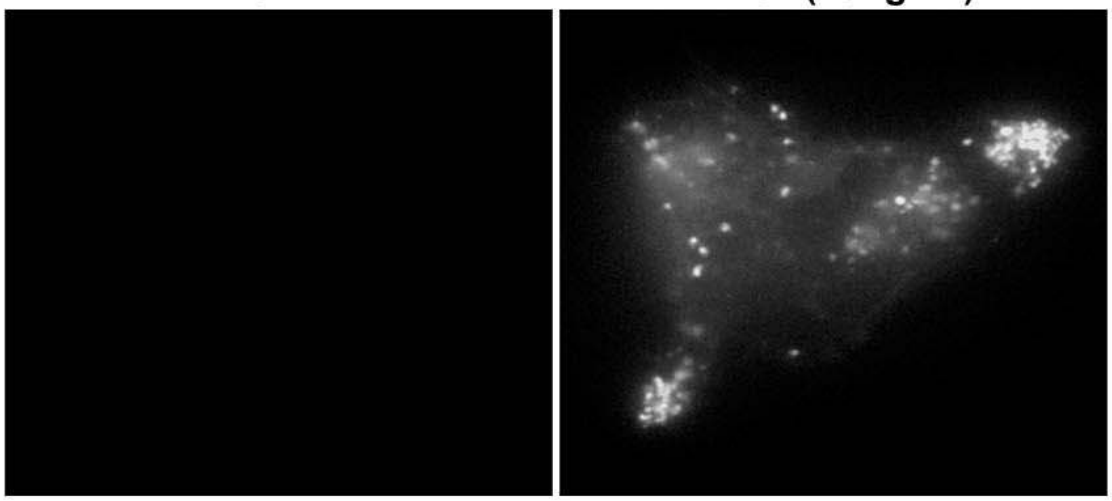
Figure 2

A



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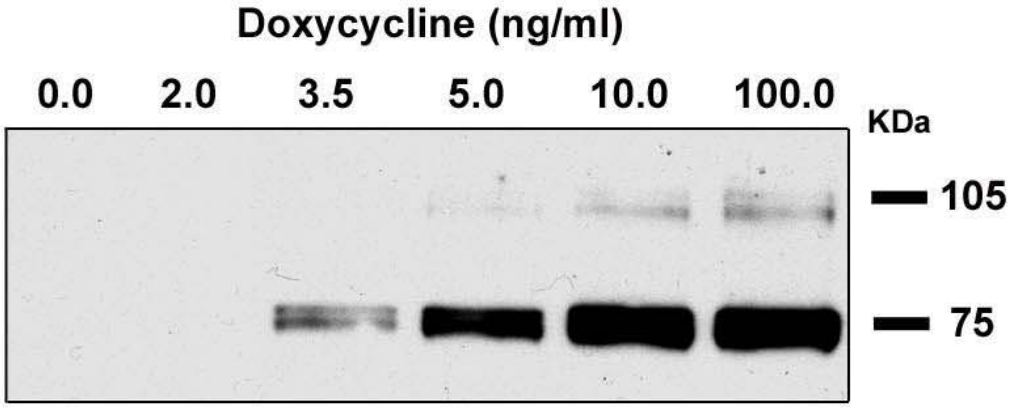

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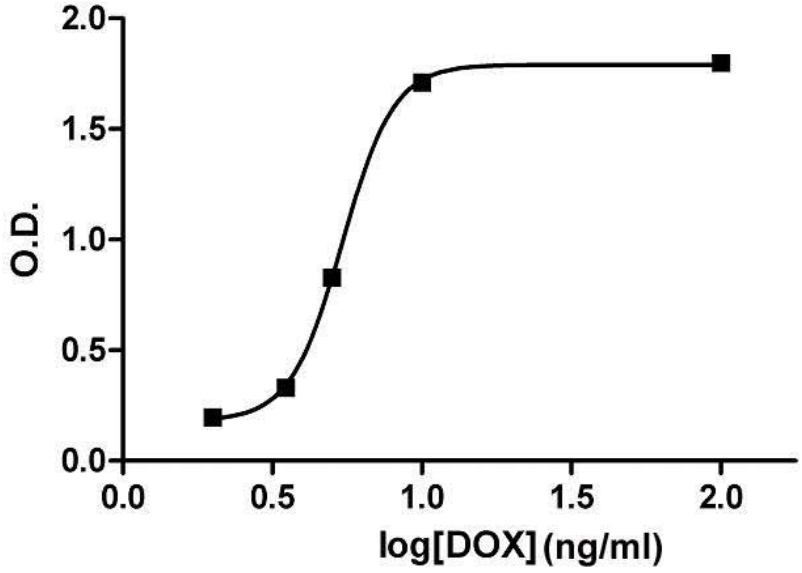

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Figure 3

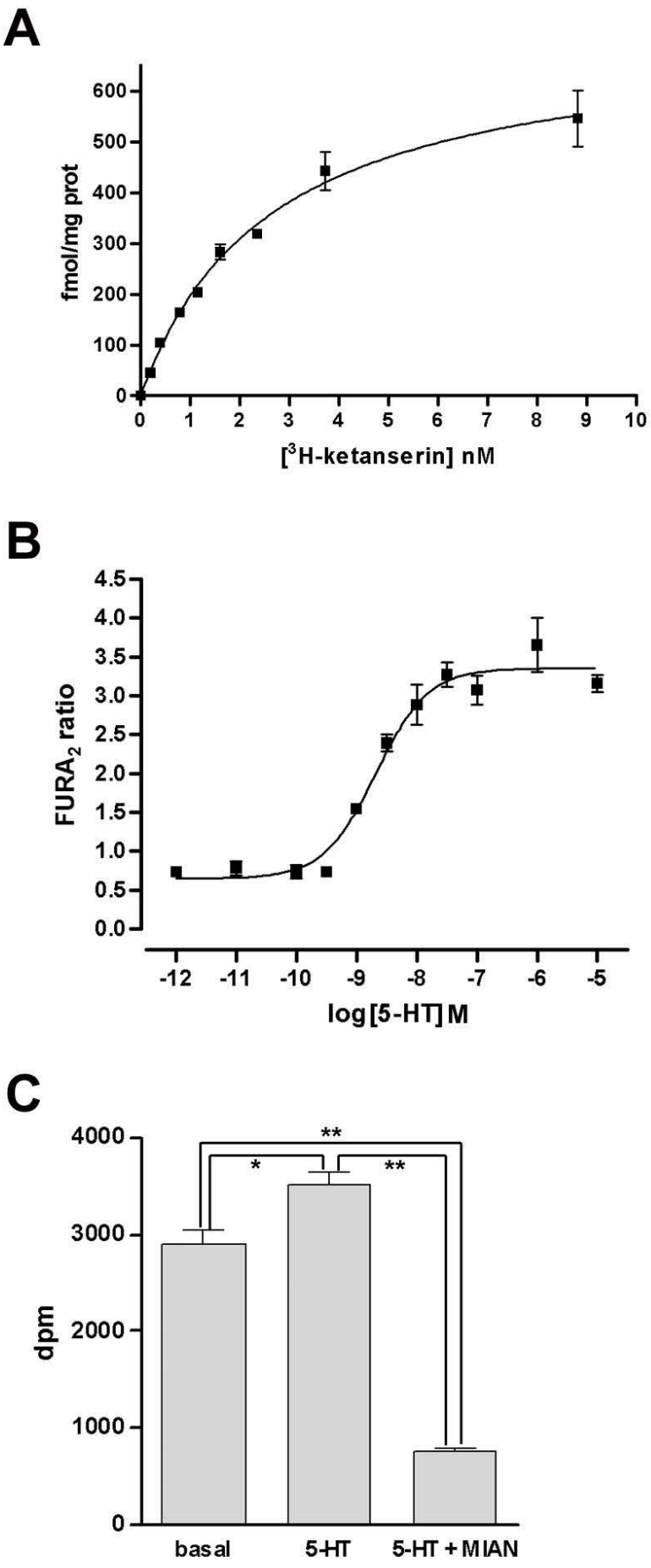

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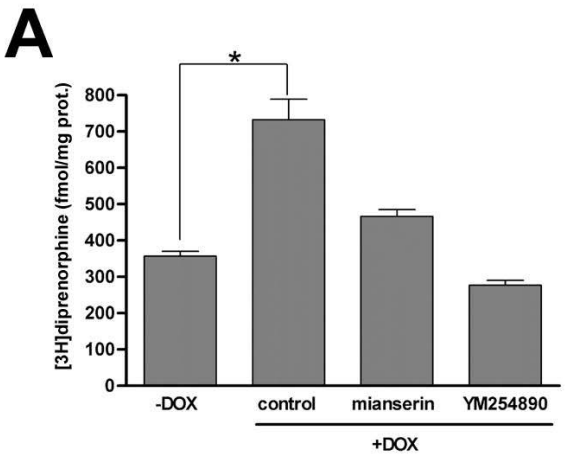
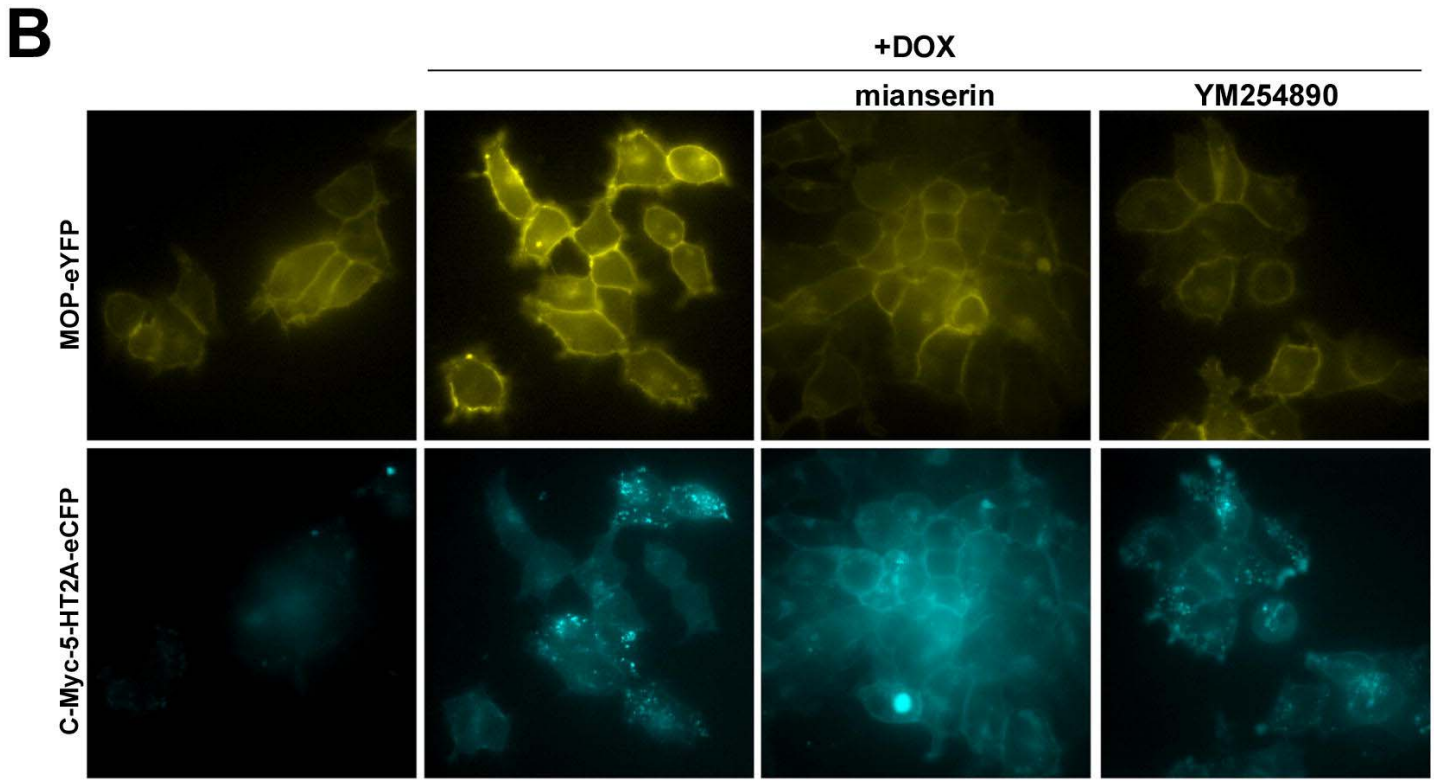
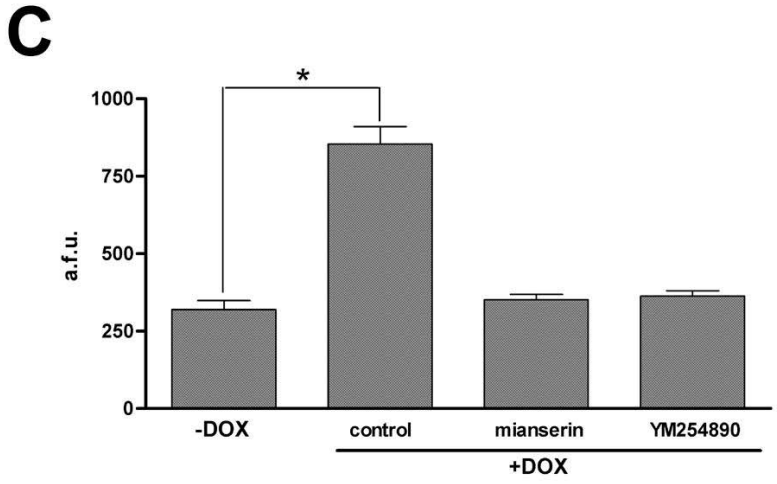
Figure 4







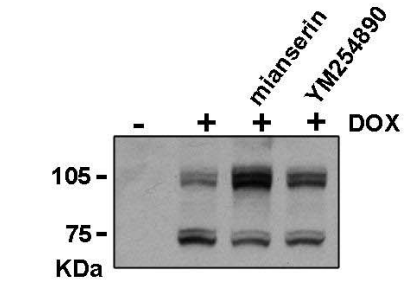


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Figure 5

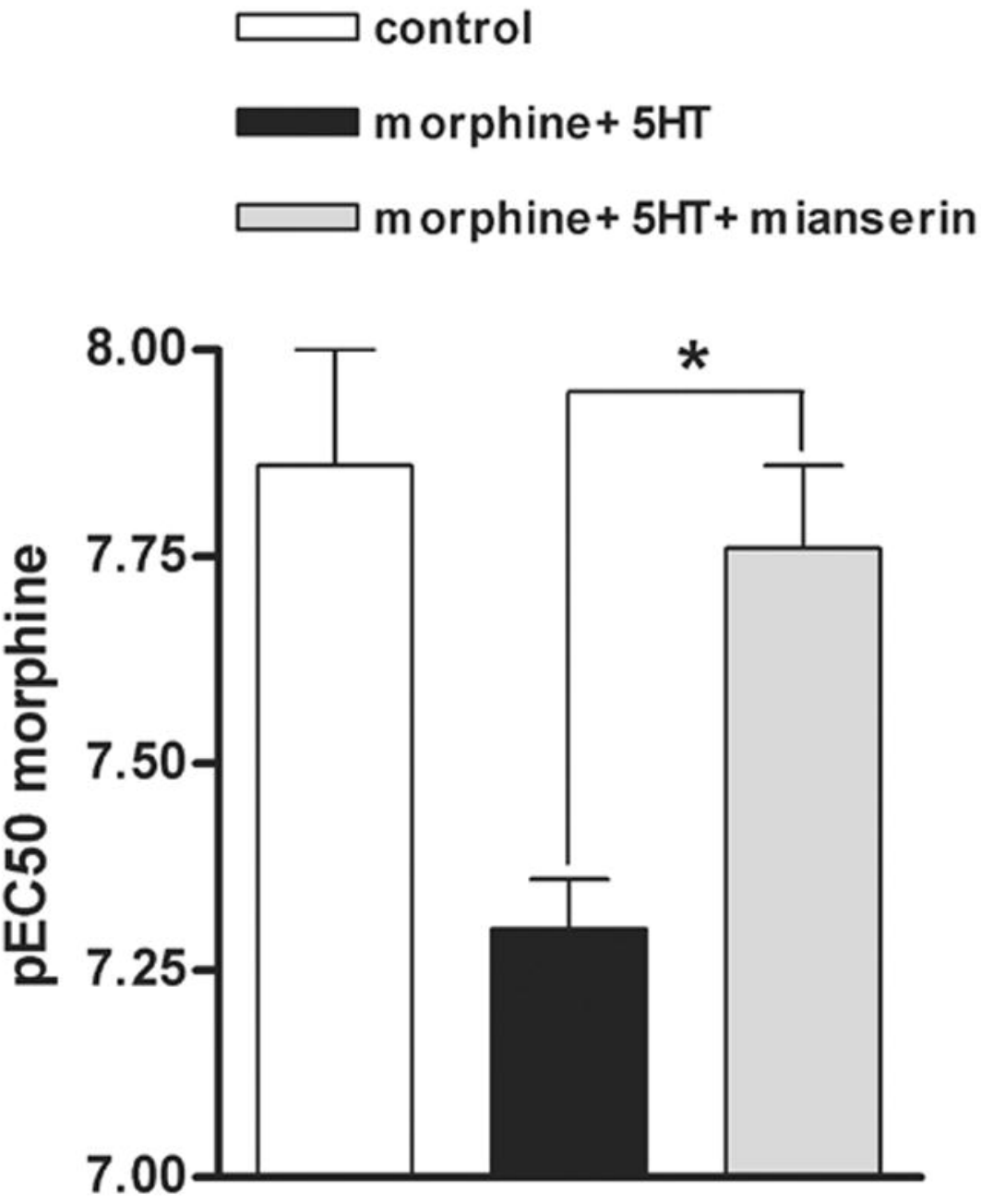
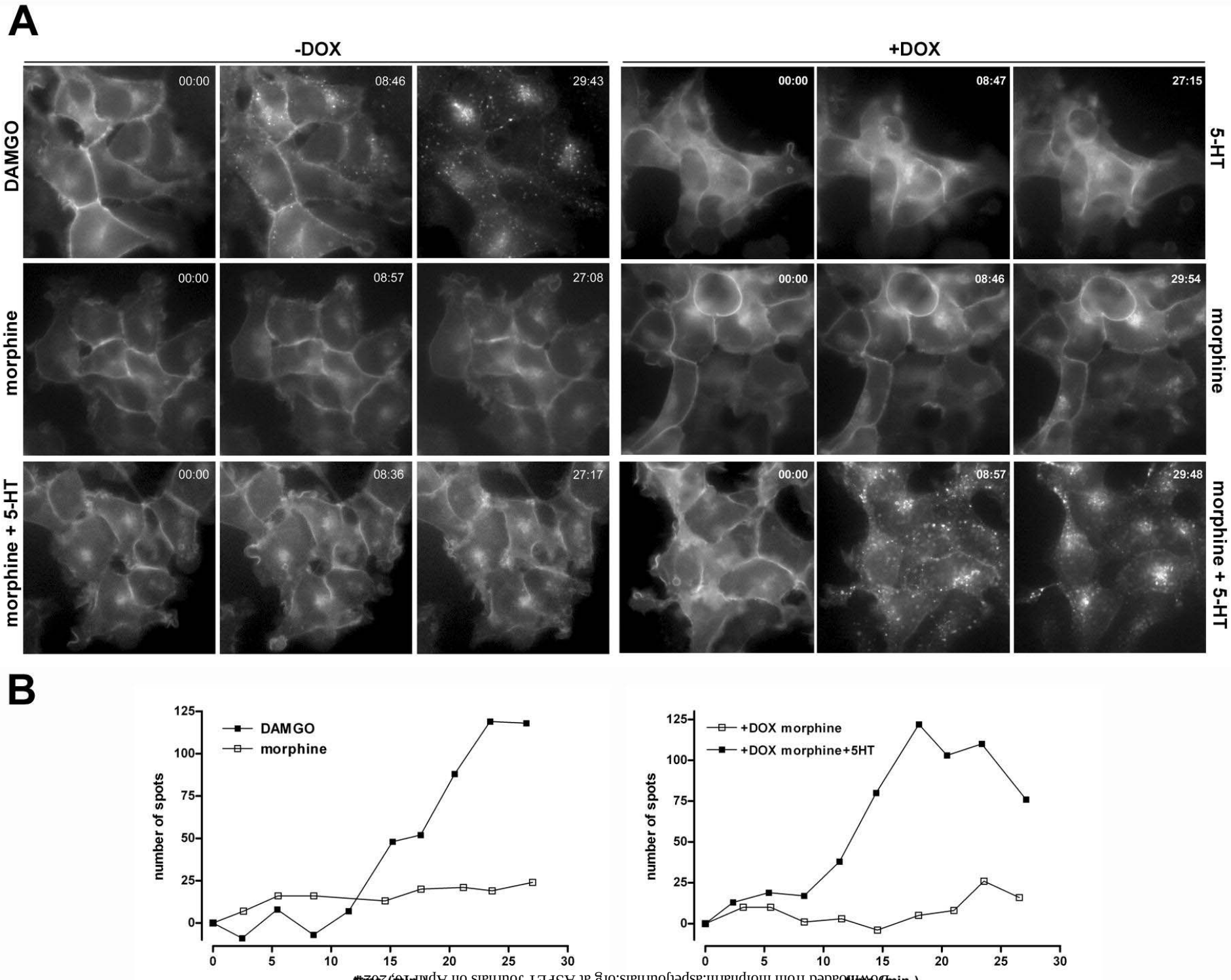


Figure 6



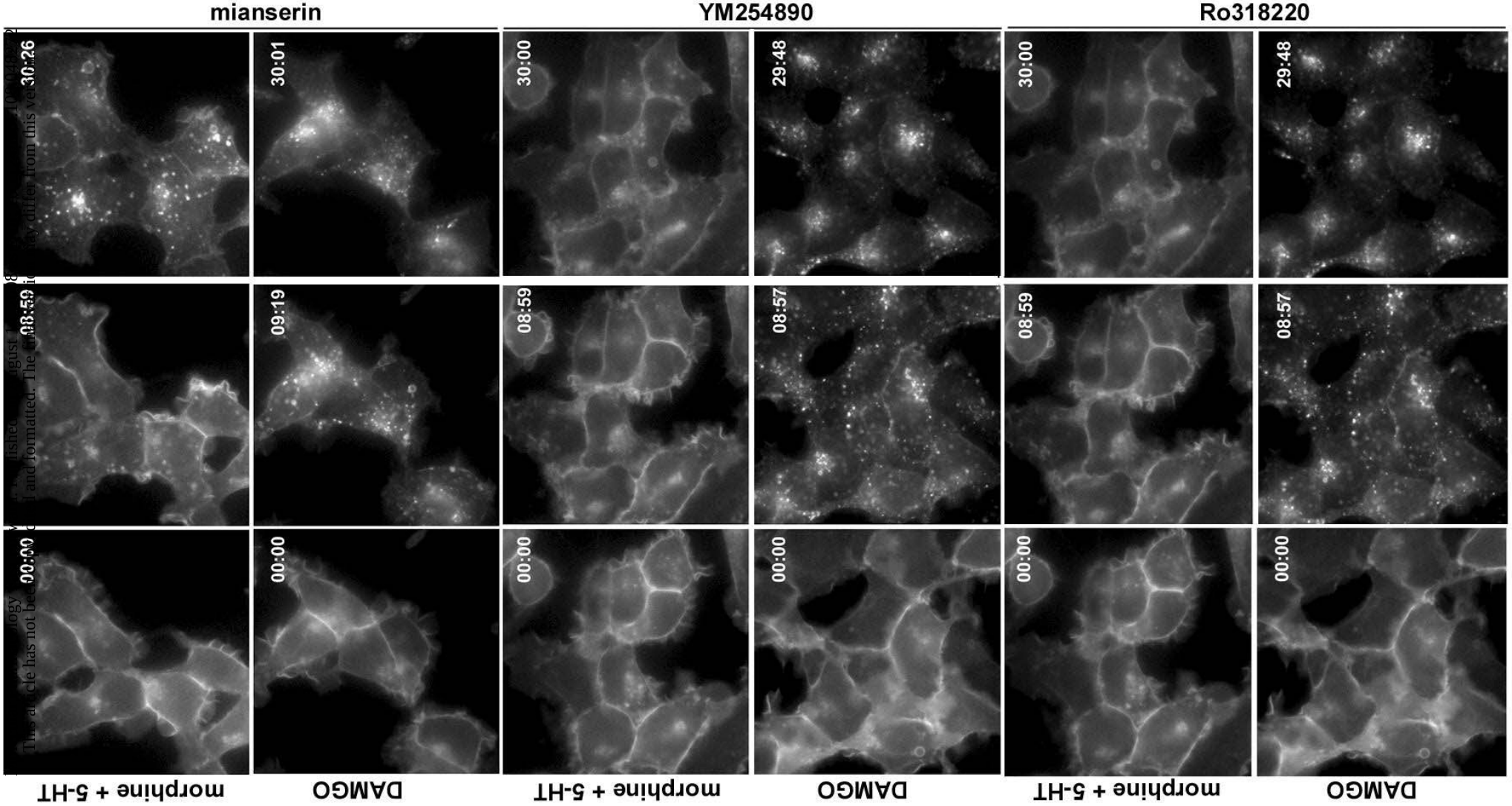


Figure 7

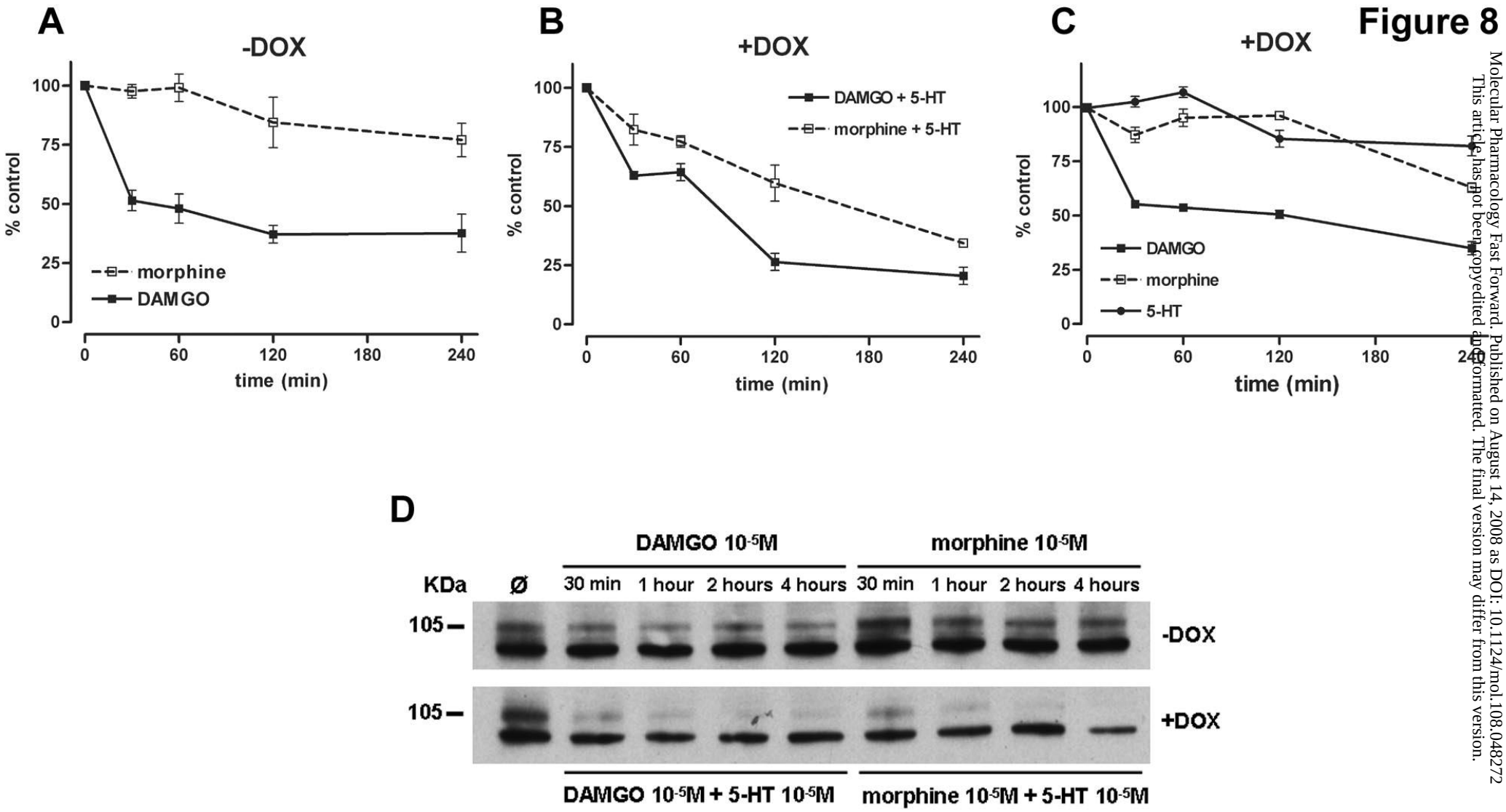


Figure 8