Generation of an inducible expression system to perform molecular pharmacology studies with mu opioid receptors

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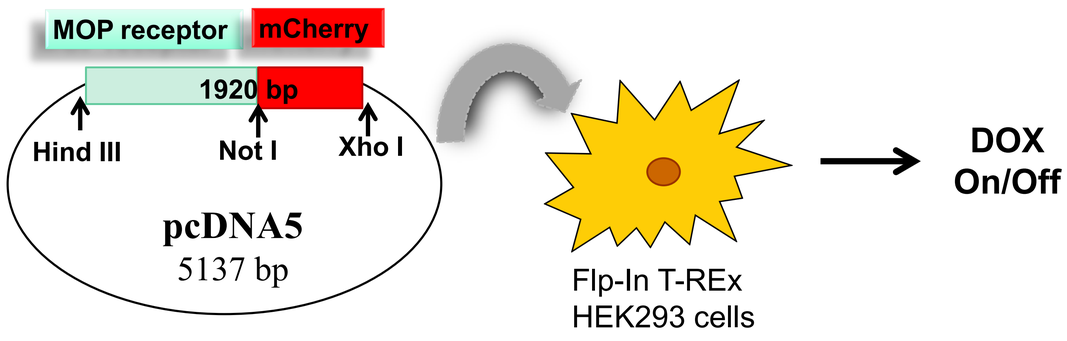
Summary

Opioid receptors are included in the superfamily of G-protein coupled receptors (GPCRs) and μ opioid (MOP) receptor is the subtype that essentially mediates the physiological actions of some alkaloid drugs clinically used such as morphine. Although morphine has been considered one of the most potent analgesic drugs, especially for the treatment of chronic and refractory pain, its use in clinical practice is limited by the occurrence of tolerance and dependence following prolonged treatments. Taking into account the central role played by MOP receptor in different cell signaling pathways as well as the physiological effects after its activation by morphine, we have generated an inducible heterologous expression system to conduct further investigations on molecular pharmacology of MOP receptor. By amplification and ligation of a cDNA encoding for human MOP receptor to the sequence corresponding to mCherry fluorescent protein, we obtained a unique open reading frame resulting in mCherry fused to the carboxyl terminus of MOP receptor. This construct was subcloned into pcDNA5/FRT/TO vector to be subsequently transfected into Flp-In T-REx HEK293 cells to get its insertion in the inducible locus by homologous recombination. Next, we assessed the expression and cellular distribution of MOP-Cherry by means fluorescence microscopy in living cells. Time course experiments of doxycycline (DOX) treatment revealed, firstly, a MOP Cherry expression depending on DOX induction, and secondly a time-dependent increase of MOP-Cherry receptor expression that was accompanied by the augmentation of intracellular vesicles. To further investigate on the nature of these trafficking vesicles, cells were treated for different periods with Concanavalin A, resulting in a potent inhibition of vesicle formation and demonstrating therefore that vesicles resulted from a constitutive endocytosis process. In order to test whether MOP-Cherry receptor constitutive internalization depends on G-protein coupling we treated cells with pertussis toxin resulting in a significant decrease of both intracellular vesicle formation and receptor expression levels. Finally, we explored some pharmacological characteristics of MOP-Cherry receptors by means radioligand binding assays using [^{3}H]diprenorphine and [^{35}S]GTPγS. Saturation of [^{3}H]diprenorphine binding sites showed affinity values (Kd) in agreement with the expected one for this antagonist. In addition, we observed a significant increase in receptor expression levels (Bmax) in samples from those cells treated with DOX for longer periods, corroborating then by a quantitative approach that receptor expression level increased across the induction time. Finally, [^{35}S]GTPγS binding experiments revealed that MOP-Cherry receptor is functional since the opioid agonist DAMGO stimulated the specific incorporation of [^{35}S]GTPγS in cell membranes in a concentration-dependent manner.

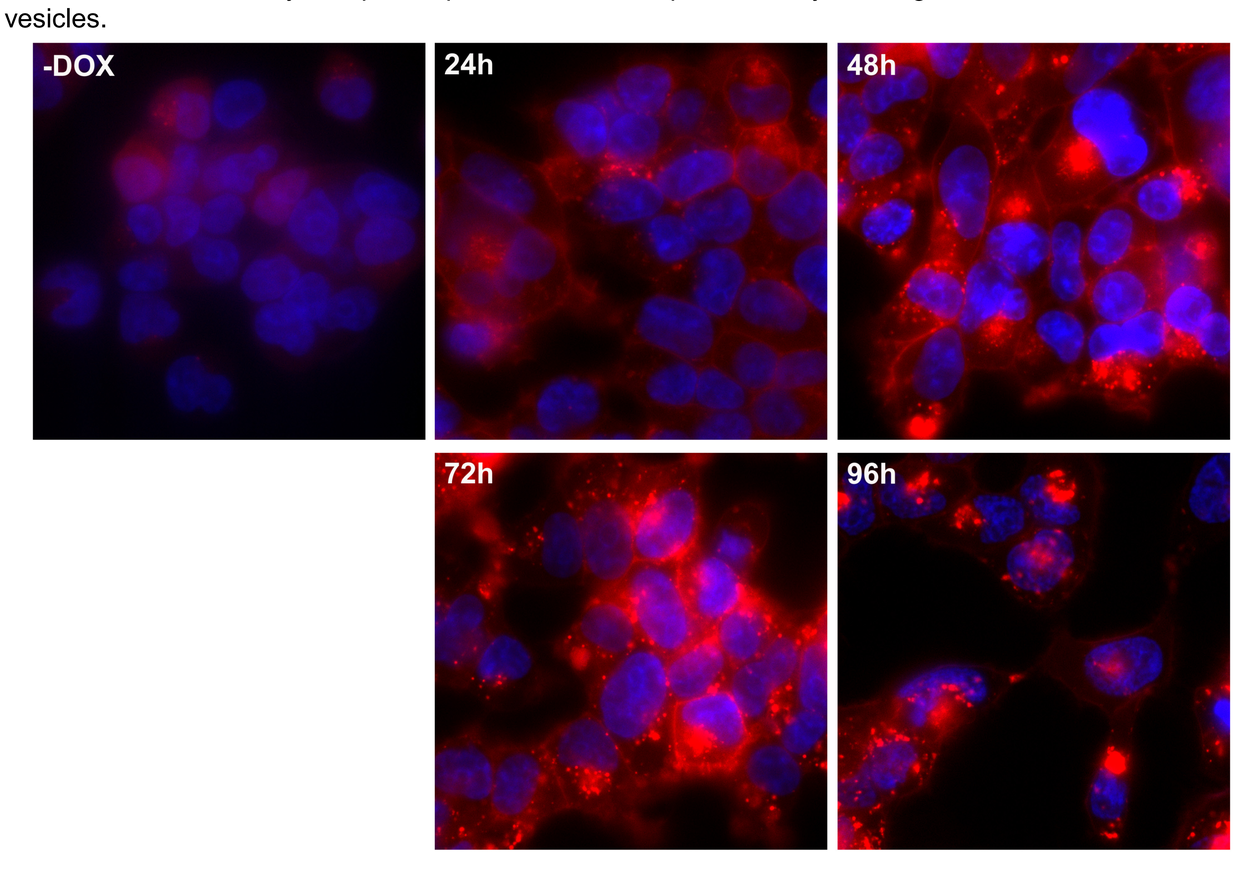
Generation of a stable Flp-In T-REx HEK293 cell line expressing MOP-Cherry in an inducible manner



Schematic representation of the stable cell line generation. cDNA encoding for human MOP receptor was amplified and ligated to the sequence corresponding to mCherry fluorescent protein in order to obtain an unique fusion protein from a single open reading frame. This construct was subcloned into pcDNA5/FRT/TO vector to be transfected into Flp-In T-REx HEK293 cells to get its insertion in the inducible locus by homologous recombination. After selection of positive clones by antibiotic resistance, we obtained a stable cell line expressing MOP-Cherry in an inducible manner.

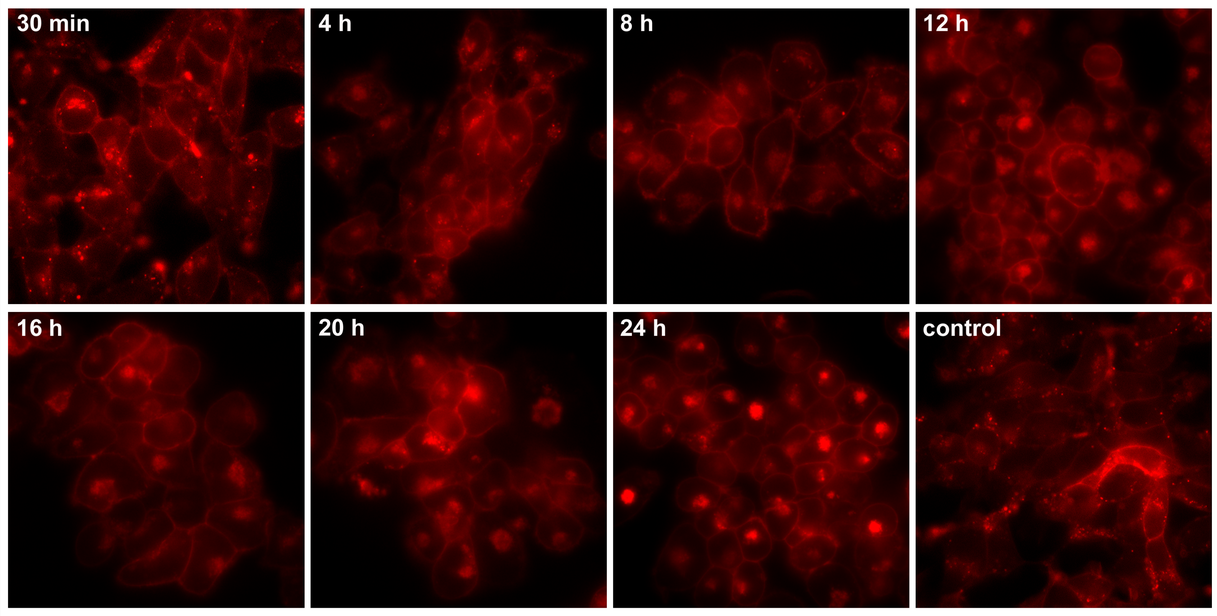
Visualization of the expression and cellular distribution of MOP-Cherry receptors in Flp-In HEK293 living cells by epifluorescent microscopy

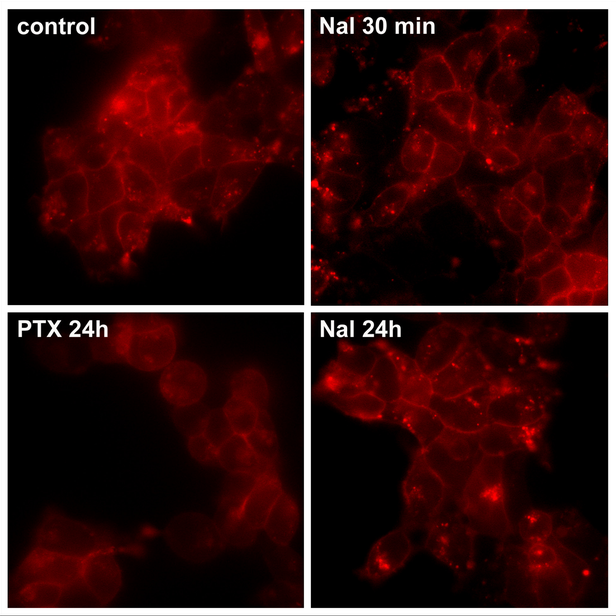
Time course of MOP-Cherry receptor expression by doxycycline (DOX) treatment in Flp-In HEK293 cells. Images were obtained after illuminating living cells with settings to detect mCherry (red) and Hoecht 33342 (blue) fluorescence. Cells were treated with DOX (0.01 μg/ml) for different times displayed at the upper left corner of each picture. Addition of DOX resulted in a time-dependent increase of MOP-Cherry receptor expression that was paralleled by the augmentation of intracellular vesicles.



Vesicle trafficking studies

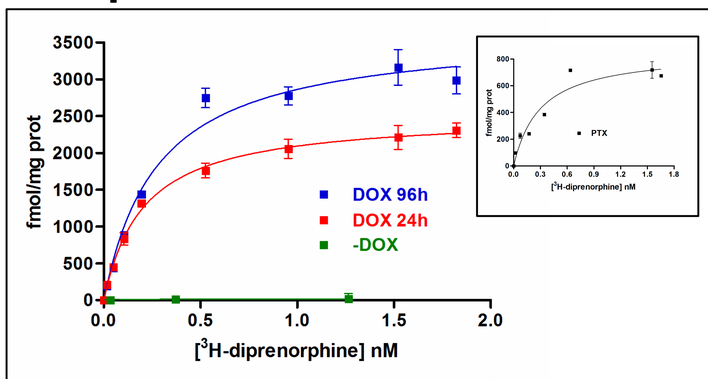
Effect of Concanavalin A on vesicle trafficking. Fluorescent microscopy images were obtained from living cells treated with DOX (0.01 μg/ml) for 24h. Concanavalin A (500 μg/ml) was added in parallel at the time point displayed in every picture. Inhibition of vesicle formation appeared 4 hours after the addition of the endocytosis inhibitor.





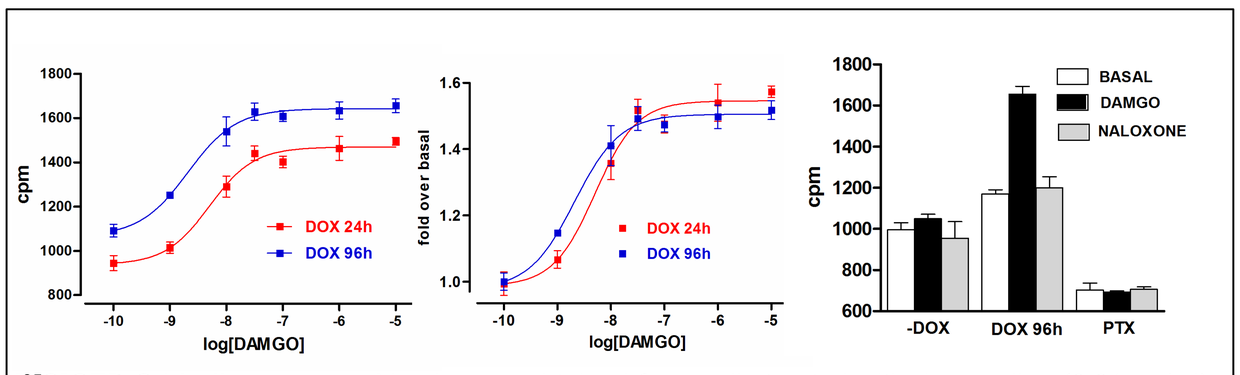
Effect of Pertussis toxin and Naloxone on constitutive receptor endocytosis.
Fluorescent microscopy images were obtained from living cells treated with DOX (0.01 μg/ml) for 24h to induce MOP Cherry receptor expression. Cells treated with Naloxone (Nal 10^{-5}M) for different periods are shown in the right panel. Left-bottom panel shows cells treated with pertussis toxin (PTX 100ng/ml) for 24h. A significant decrease of both intracellular vesicle formation and receptor expression level was observed in PTX treated cells.

Pharmacologycal characterization of MOP-Cherry receptors



	Bmax (fmol/mg)	Kd
DOX 96h	3656	0,17
DOX 24h	2521	0,20
DOX+PTX 24h	854.9	0,29

Saturation binding assays. Specific [^{3}H]diprenorphine saturation binding assays performed with membranes from cells non treated (green), treated with DOX for 24 hours (red), with DOX for 96 hours (blue) or with DOX plus pertussis toxin for 24 hours (black, inset) are displayed on the left. The pharmacological parameters obtained in these experiments are shown on the right. A significant increase in receptor expression level (Bmax) is observed in samples from cells treated with DOX for longer periods (96h). [^{3}H]diprenorphine saturation binding in membranes from cells treated with pertussis toxin shows a significant decrease in receptor expression level.



[^{35}S]GTPγS binding assays. Membranes obtained from cells treated with doxycycline (0.01 μg/ml) at different times to induce MOP-Cherry receptor expression were incubated with an agonist (DAMGO 10^{-5}M) or an antagonist/inverse agonist (naloxone 10^{-4}M). DAMGO stimulated the specific incorporation of [^{35}S]GTPγS in a dose-dependent manner (left panels) whereas naloxone did not show any effect (right panel). Membranes from cells treated with pertussis toxin plus DOX for 24h (right panel) were devoid of interaction between receptors and $G\alpha_{i/o}$ subunits.

Concluding remarks

This experimental system provides MOP-Cherry receptor expression by using doxycycline as an ON/OFF agent allowing the use of the same host cells as negative control when MOP-Cherry receptor is not induced to express. Additionally, the expression level of our gene of interest might be regulated depending on the doxycycline period of treatment. In this particular expression model, MOP-Cherry receptor distributes in trafficking vesicles as a result of constitutive endocytosis that is a G-protein coupling dependent process. Further investigations to determine whether MOP-Cherry constitutive endocytosis concurs with receptor functional constitutive activity will be conducted. If this is the case, this experimental system would represent a model of choice to pharmacologically characterize potential inverse agonist compounds.

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