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A continuous, fluorescence based assay of μ -opioid receptor activation in AtT-20 cells

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Abstract:	Opioids are widely prescribed analgesics, however their use is limited due to development of tolerance and addiction, as well as high variability in individual response. The development of improved opioid analgesics requires high-throughput functional assays to assess large numbers of potential opioid ligands. In this study, we assessed the ability of a proprietary "no wash" fluorescent membrane potential dye to act as a reporter of μ -opioid receptor (MOR) activation and desensitization via activation of G protein-coupled inwardly rectifying potassium channels. AtT-20 cells stably expressing mouse MOR were assayed in 96-well plates using the Molecular Devices FLIPR membrane potential dye. Dye emission intensity decreases upon membrane hyperpolarization. Fluorescence decreased in a concentration-dependent manner upon application of range of opioid ligands to the cells, with high efficacy agonists producing a decrease of 35% to 40% in total fluorescence. The maximum effect of morphine faded in the continued presence of agonist, reflecting receptor desensitization. The effects of opioids were prevented by prior treatment with pertussis toxin and blocked by naloxone. We have demonstrated this assay to be an effective method for assessing ligand signalling at MOR which may potentially be scaled up as an additional HTS technique for characterizing novel opioid ligands.

A continuous, fluorescence based assay of μ -opioid receptor activation in AtT-20 cells

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Keywords: GIRK channels, opioids, fluorescence, high-throughput screening, cell-based assay

ABSTRACT

Opioids are widely prescribed analgesics, however their use is limited due to development of tolerance and addiction, as well as high variability in individual response. The development of improved opioid analgesics requires high-throughput functional assays to assess large numbers of potential opioid ligands. In this study, we assessed the ability of a proprietary “no wash” fluorescent membrane potential dye to act as a reporter of μ -opioid receptor (MOR) activation and desensitization via activation of G protein-coupled inwardly rectifying potassium channels. AtT-20 cells stably expressing mouse MOR were assayed in 96-well plates using the Molecular Devices FLIPR membrane potential dye. Dye emission intensity decreases upon membrane hyperpolarization. Fluorescence decreased in a concentration-dependent manner upon application of range of opioid ligands to the cells, with high efficacy agonists producing a decrease of 35% to 40% in total fluorescence. The maximum effect of morphine faded in the continued presence of agonist, reflecting receptor desensitization. The effects of opioids were prevented by prior treatment with pertussis toxin and blocked by naloxone. We have demonstrated this assay to be an effective method for assessing ligand signalling at MOR which may potentially be scaled up as an additional HTS technique for characterizing novel opioid ligands.

INTRODUCTION

Opioid analgesics are the most widely prescribed drugs in the treatment of moderate to severe pain. Despite their powerful analgesic effects, the use of opioids in the treatment of chronic pain can be problematic due to the development of tolerance and physical or psychological dependence¹. Over time, increasing doses of opioids become necessary to maintain analgesia. The increased toxic side effects such as respiratory depression, constipation and nausea associated with escalating doses of opioids can reach unacceptable levels, leading to inadequate pain relief or overdose. Opioids, however, continue to be the mainstay of chronic pain treatment due to a lack of suitable alternative drugs. As such, there is a substantial need for the development of new opioid analgesics, with reduced adverse effects and a decreased ability to produce tolerance. The μ opioid receptor (MOR) is the primary site of action for most clinically important opioid drugs, and as such is the major target for the development of improved opioid analgesic drugs². It is generally accepted that there are not functionally important subtypes of MOR³, and so aside from novel formulations or delivery strategies, the development of new pharmacotherapies targeting MOR is likely to focus on subtleties of receptor signalling and regulation.

MOR is a member of the G protein-coupled receptor (GPCR) superfamily, ubiquitous cell-surface receptors that act as cellular switches to regulate most cellular signalling processes⁴. GPCR agonists stabilise active conformations of their receptors, leading to signalling via both the α and $\beta\gamma$ subunits of the associated heterotrimeric G protein complex and sometimes also via non-G protein-dependent pathways⁵. GPCR signalling is complex, with different ligands preferentially activating (or inhibiting) distinct signalling pathways at the same receptor⁵. There is also increasing evidence

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3 that distinct ligands also differentially engage pathways which regulate receptor
4 activity during prolonged agonist exposure, a topic of intense investigation with
5 regard to opioid receptors^{6,7,8}. These ideas have lead to renewed interest in the
6 possibility of developing ligands targeting MOR which engage only subsets of
7 signalling systems or regulatory pathways, potentially leading to drugs with more
8 favourable clinical profiles.
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10 Drug development typically involves the screening of large libraries of lead
11 compounds to identify those capable of binding to and signalling via a receptor. The
12 vast array of lead compounds available requires high-throughput screening methods
13 for efficient detection of potential therapeutic compounds. Radioligand binding
14 studies are often used to identify candidates, but determination of ligand efficacy
15 requires some sort of signalling response. For opioid receptors, this can be
16 achieved using cell lines expressing engineered G proteins which couple to
17 processes such as intracellular calcium ($[Ca]_i$) mobilization⁹, cAMP-dependent gene
18 transcription or more traditional assays of adenylyl cyclase (AC) activity which
19 require harvesting or lysing of cells¹⁰. In this study we sought to develop a minimally
20 invasive assay that reflected a naturalistic coupling of MOR and which could
21 potentially be used to readily examine agonist regulation of receptor signalling. In
22 mouse pituitary AtT-20 cells, heterologously expressed MOR inhibit native calcium
23 channels (I_{Ca})¹¹ and activate endogenous G protein gated inwardly rectifying
24 potassium channels¹² (GIRKs), and in both cases this signalling is subject to rapid,
25 agonist-induced regulation. We assessed the suitability of a proprietary fluorescent
26 membrane potential assay to act as a robust reporter of MOR activation and
27 desensitization in AtT-20 cells, with the view to potentially providing an assay for
28 high throughput screening of both these important aspects of MOR function.
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MATERIALS AND METHODS

FLAG-MOR Transfection and Cell Culture

Mouse AtT-20 neuroblastoma cells were stably transfected with the cDNA encoding the FLAG epitope-tagged mouse μ -opioid receptor using the transfectant Lipofectamine (Gibco BRL) as previously described¹¹. The pcDNA3 FLAG-MOR construct was a kind gift from Dr. Lakshmi Devi (Mt Sinai School of Medicine, New York, USA). Geneticin (500 μ g/ml) was added to select for clones expressing FLAG-MOR protein. During *in situ* identification of positive clones using Alexa-488 coupled FLAG-MOR antibody (Sigma, F7425), 48 potentially suitable single cells were transferred to single wells using a micropipette and were grown to confluence for subsequent determination of cell surface MOR binding density. MOR binding density was determined on intact cells by incubation with increasing triplicate concentrations of [³H] DAMGO (0.125 - 32 nM; Perkin Elmer, USA) at 4°C in 50mM Tris-Cl, pH7.4 for 2h. Briefly, approximately 1×10^5 cells were plated in 24-well plate coated with poly-L-lysine overnight. Cells were then rinsed gently twice with 50mM Tris-Cl, pH7.4, placed on ice and incubations in the radioligand were commenced. Non-specific binding (less than 2% of total binding at [³H] DAMGO 5 nM) was determined in the presence of unlabelled DAMGO (10 μ M). At the end of the incubation plated cells were rinsed three times with 1 ml 50mM Tris-Cl, pH 7.4 at 4°C. Cells in each well were then digested for 1 h at room temperature with 100 μ l of 1N NaOH. 100 μ l 1N HCl was then added to each well and collected into scintillation vials and bound ligand determined using a liquid scintillation counter (Packard Tricarb, USA). Specific binding was plotted, and Kd and Bmax for each clone determined using GraphPad Prism. One clone expressing a moderate density of surface FLAG-MOR was selected for subsequent experiments. The Kd for [³H]-

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3 DAMGO binding was 1.2 nM and receptor density was 10.2 pmol/mg protein. After
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5 counting cell numbers used for protein determination it was estimated that 2.5×10^7
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7 cells yielded one mg protein. Therefore 10.2 pmol/mg protein represents
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9 approximately 2.5×10^5 receptors per cell. The selected clone of AtT-20 cells stably
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11 expressing mouse FLAG-MOR was then cultured in Dulbecco's Modified Eagle
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13 Medium (DMEM) containing 10% fetal bovine serum (FBS), 100U
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15 penicillin/streptomycin and 300µg/mL G418. Cells were passaged at 80%
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17 confluency as required. Assays were carried out on cells up to 25 passages. Cells
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19 for assays were grown in 75 cm² flasks and used at 90% confluence. The day
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21 before the assay cells were detached from the flask with trypsin/EDTA (Sigma) and
22
23 resuspended in 10 ml of Leibovitz's L-15 media supplemented with 1% FBS, 100U
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25 penicillin/streptomycin and 15 mM glucose. The cells were plated in volume of 90 µl
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27 in black walled, clear bottomed 96-well microplates (Corning) and incubated
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29 overnight in ambient CO₂.
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38 **Membrane Potential Assay**

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40 Membrane potential was measured using a FLIPR Membrane Potential Assay kit
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42 (blue) from Molecular Devices. The dye was reconstituted with assay buffer supplied
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44 with the kit or with a low-K modification. The standard assay buffer contained (mM),
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46 NaCl 145, HEPES 22, Na₂HPO₄ 0.338, NaHCO₃ 4.17, KCl 5.33, KH₂PO₄ 0.441,
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48 MgSO₄ 0.407, MgCl₂ 0.493, CaCl₂ 1.26, Glucose 5.56 (pH 7.4, osmolarity 315 ± 5).
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50 The modified buffer was formulated without the addition of 5.33 mM KCl. Taking into
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52 account the K concentration of L-15, the final in-well concentrations of K were 5.55
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54 mM (standard) and 2.88 mM (low K) respectively. Prior to the assay, cells were
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3 loaded with 90 μ l/well of the dye solution without removal of the L-15, giving an initial
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5 assay volume of 180 μ l/well. Plates were then incubated at 37°C at ambient CO₂ for
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7 45 minutes. Fluorescence was measured using a FlexStation 3 (Molecular Devices)
8
9 microplate reader with cells excited at a wavelength of 530 nm and emission
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11 measured at 565 nm. Baseline readings were taken every 2 seconds for at least 2
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13 minutes, at which time either drug or vehicle was added in a volume of 20 μ l.
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15 Further additions were made in volumes of 20 μ l, as indicated. The background
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17 fluorescence of cells without dye or dye without cells was negligible. Changes in
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19 fluorescence were expressed as a percentage of baseline fluorescence after
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21 subtraction of the changes produced by vehicle addition, which was less than 2 % for
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23 drugs dissolved in assay buffer or DMSO. The final concentration of DMSO was not
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25 more than 0.1%.
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32 **Data Analysis and Calculation of Z' values**

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35 Concentration response data was analysed using PRISM (GraphPad Software Inc.,
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37 San Diego, CA), using four-parameter non-linear regression to fit concentration-
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39 response curves. The time course of morphine-induced receptor desensitization
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41 was fit with a single phase exponential to obtain an estimated t/2 for the process. To
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43 calculate Z', a measure of the robustness of the assay and its suitability for HTS, the
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45 membrane potential assay was performed on 3 separate occasions using assay
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47 buffer as the negative control and 300 nM DAMGO as the positive control in 96-well
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49 plates. The Z' factor was calculated as outlined in Zhang *et al.*, (1999)¹³.
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53 **RESULTS**

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3 In AtT-20 cells loaded with the proprietary membrane potential dye, addition of the
4 peptidergic MOR agonist DAMGO or the prototypic alkaloid opioid morphine
5 produced a rapid decrease in fluorescence, consistent with hyperpolarization of the
6 cells (Figure 1). It took about 30s for high concentrations of morphine to maximally
7 hyperpolarise the cells (Table 1). The decrease in fluorescence produced by
8 morphine was concentration dependent and reversed by addition of the opioid
9 receptor antagonist naloxone (Figure 2A). Addition of increasing concentrations of
10 naloxone produced a parallel shift in the concentration response curve for morphine,
11 with a pA_2 of -8.5 ± 0.1 , (2.9 ± 0.5 nM, $n=3$), a value consistent with the reported
12 binding affinity of naloxone at mouse MOR (2 nM)¹⁴ (Figure 2B). Pretreatment of
13 cells overnight with pertussis toxin (200 ng/ml) prevented the decrease in
14 fluorescence by DAMGO and morphine (Figure 3). Addition of morphine or DAMGO
15 to AtT-20 cells not transfected with MOR produced no change in fluorescence.
16 Using the assay buffer supplied with the kit, the maximal decrease in fluorescence
17 produced by DAMGO was $31 \pm 1\%$ ($n=6$). We sought to optimise the assay by
18 decreasing extracellular $[K]_{Ex}$ from 5.6 mM to 2.9 mM in order to make the reversal
19 potential (K_e) for K more negative. The reduction in $[K]_{Ex}$ was achieved by dissolving
20 the dye in saline containing no added KCl. The reduction of $[K]_{Ex}$ resulted in
21 maximally effective concentrations of DAMGO producing a 7% greater decrease in
22 total fluorescence ($P < 0.01$, Students T-test). The E_{max} and pEC_{50} for DAMGO in
23 5.6 mM $[K]_{Ex}$ were $31 \pm 1\%$ and 8.3 ± 0.1 respectively, while in 2.9 mM $[K]_{Ex}$, E_{max}
24 and pEC_{50} for DAMGO were $38 \pm 2\%$ and 8.3 ± 0.1 respectively (Figure 4).
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54 The maximum response elicited by DAMGO was similar to that elicited by
55 somatostatin ($E_{max} = 39 \pm 3.2\%$, pEC_{50} 8.7), which acts at endogenous sst_2 and sst_5
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3 receptors to activate GIRK channels in AtT-20 cells¹⁵. We independently assessed
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5 the changes in fluorescence produced by altering the membrane K⁺ permeability by
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7 incubating AtT-20 cells with nigericin, a potassium selective antibiotic ionophore¹⁶. A
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9 maximally effective concentration of nigericin (1 μM) produced a decrease in
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11 fluorescence signal of 50 ± 3%, which was not decreased any further upon the
12
13 addition of 300 nM DAMGO. The fluorescent signal observed after nigericin
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15 incubation may reflect the signal when the membrane potential of the cells is driven
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17 to E_K¹⁶ (Figure 5).
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22 We assessed the capacity of the fluorescence assay to reliably detect opioid ligands
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24 of differing efficacy by treating AtT-20MOR cells with a range of structurally distinct
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26 opioid ligands. All agonists tested produced a concentration-dependent decrease in
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28 cellular fluorescence. High efficacy agonists such as fentanyl, DAMGO and β-
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30 endorphin produced a maximum decrease in fluorescence of 35-40%, while
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32 morphine, buprenorphine and pentazocine were shown to be partial agonists (Figure
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34 6). A rank order of ligand efficacy was established (Table 1).
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41 We assessed the suitability of this assay for HTS by calculating the Z' factor, a
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43 measure of the assay robustness. An assay with a Z' factor of between 0.5 - 1 is an
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45 excellent assay in terms of signal/noise ratio and data reproducibility¹³. The Z' factor
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47 for this assay was calculated in multiple experiments, with Z' values of 0.6, 0.7 and
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49 0.7, indicating that the assay is suitable for HTS.
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53 A hallmark of opioid signalling is agonist-dependent desensitization, where
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55 continuous application of agonist results in a relatively rapid decline in receptor
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3 activation. The decrease in fluorescence produced by application of high
4 concentrations of morphine faded in the continued presence of agonist, reaching a
5 plateau after about 30 minutes (Figure 7A). In order to assess whether the decline in
6 fluorescence reflected a change in MOR signalling, we incubated cells with a high
7 concentration of morphine (1 μ M) and then challenged them with subsequent
8 addition of 10 μ M morphine. The response to 10 μ M morphine declined significantly
9 over time. When fit with a one phase exponential association function, the peak
10 response to 10 μ M morphine declined with a τ of 490 s (95 % C.I. 413–603 s) to a
11 maximum inhibition of 72 % (95 % C.I. 67–76 %, , Figure 7B). In order to assess
12 whether the decline in morphine effectiveness reflected heterologous
13 desensitization, we repeated the experiments using 1 μ M somatostatin as the
14 challenge drug. The response to somatostatin also declined during continuous
15 morphine exposure (τ of 460 s, 95 % C.I. 331-748 s), the maximum inhibition of the
16 somatostatin response was 30 % (95 % C.I. 27-34 %). Preincubation with naloxone
17 (1 μ M) did not affect the hyperpolarization produced by somatostatin (10 nM; control
18 36 ± 1 %, in naloxone 35 ± 1 %).

43 DISCUSSION

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45 In this study, we have developed a real time, no wash, fluorescence based assay for
46 MOR-mediated hyperpolarisation of intact AtT-20 cells. We used a proprietary
47 membrane potential sensitive dye from Molecular Devices to measure membrane
48 hyperpolarisation following MOR mediated GIRK channel activation¹². The
49 fluorescent signal rapidly and reliably decreased after opioid application, and this
50 decrease in signal could be completely reversed by the opioid naloxone applied for
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3 up to at least 2 hours after agonist, indicating the stability of the dye signal and
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5 capacity of the systems to report continued activation of the μ -receptors for
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7 prolonged periods, albeit in the face of receptor desensitization. The ability to
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9 continuously measure the consequences of opioid receptor activation in cells for
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11 such prolonged periods of time has only previously been possible using high
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13 resistance electrode recordings from single neurons in brain slices^{7,17}. Activation of
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15 endogenous sst receptors in AtT-20 cells¹⁵ produced a reduction in fluorescence
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17 similar that seen following MOR activation, and in both cases this reduction was less
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19 than that produced by application of the K-selective ionophore nigericin¹⁶.
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25 The potency and efficacy of the opioid agonists correlate well with previous studies
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27 in AtT-20 cells, and with studies in native neurons where activation of GIRK channels
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29 or inhibition of I_{Ca} was used to determine agonist intrinsic activity^{11,18,19,20}. The data
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31 we obtained with the hyperpolarization assay is similar to that we obtained when we
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33 measured MOR inhibition of calcium currents in these cells – the rank order of
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35 potency in both studies is DAMGO > methadone = morphine > pentazocine¹¹, and in
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37 both studies morphine and pentazocine have reduced efficacy when compared with
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39 DAMGO. The only notable inconsistencies are the apparently greater efficacy of
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41 endomorphin 1 and 2 compared with methadone in the present study. Many studies
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43 have reported the endomorphins as being partial agonists^{18,21}, while methadone has
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45 been reported to be an agonist with a similar efficacy to DAMGO^{11,22,23}. The most
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47 likely explanation for the discrepancy in our study is that methadone appeared to
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49 have a reduced maximal effect because of its propensity to block K channels,
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51 including the GIRK channels likely to contribute to the hyperpolarisation measured in
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53 the present study^{22,24}. Conversely, the observation that the maximum effect of the
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3 endomorphins was similar to that of well recognized high efficacy agonists suggests
4 the presence of some spare receptors in our system, as noted previously with similar
5 cell lines¹¹. It is also possible that the efficacy discrepancies reflect subtle bias in
6 ligand signalling to one pathway over another in different tissues, and it is worth
7 noting that endomorphins have recently been reported to show such bias in assays
8 of β -arrestin recruitment^{25,26}.
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19 Continuous application of opioid agonists usually results in desensitization of
20 receptor signalling, a complex process that may involve receptor phosphorylation
21 and sequestration. The apparent stability of the membrane potential assay led us to
22 explore whether it could be used to investigate μ -opioid receptor desensitization.
23 The hyperpolarisation produced by high concentrations of morphine or DAMGO
24 appeared to wane over time, and when the cells were challenged with a
25 concentration of agonist that should saturate the cell surface receptors there was a
26 marked decrease in this response after only a few minutes exposure to agonist,
27 consistent with analogous studies performed in locus coeruleus neurons²⁷ or cell
28 lines transfected with μ -opioid receptors and GIRK channels²⁸. We were able to
29 make use of the endogenous sst receptors in the AtT-20 cells to determine whether
30 exposure to desensitizing concentrations of opioid agonist affected signalling through
31 other receptors. The time constant for the desensitization of signalling produced by
32 morphine was slower than that reported in our previous study of opioid signalling
33 which utilized inhibition of I_{Ca} in AtT-20 cells, this may reflect a distinct recruitment of
34 desensitization processes by morphine in intact cells compared with cells dialyzed by
35 patch-clamp recording¹¹. A major limitation of using the Flexstation for
36 desensitization assays is the inability to wash off drugs, which makes it impossible to
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3 do experiments where receptor function is repeatedly probed with concentrations of
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5 drugs that activate only a portion of receptors^{11,27}. Nevertheless, the potential of this
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7 assay to quickly test multiple agonists or putative modulators of receptor
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9 desensitization in an intact system makes it an attractive option.
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14 GIRK channel activation has most commonly been assessed using
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16 electrophysiological techniques, but assays potentially suitable for high throughput
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18 screening have been reported utilizing thallium flux²⁹ or commercially available
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20 membrane potential dyes^{30,31}. Thallium is toxic and apparently unsuitable for all cell
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22 lines³¹ while other membrane potential sensitive dyes (Di-Bac, HLB 021-152) give
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24 results qualitatively similar to those reported here^{30,31}. The EC₅₀ value for the
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26 somatostatin-induced hyperpolarisation of our AtT-20 cells was 2 nM, very similar to
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28 that previously reported using another dye (4 nM)³¹. However, it should be noted
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30 that under our standard conditions the proprietary dye gives a change in
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32 fluorescence of approximately 40% following GPCR activation, which compares with
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34 changes of approximately 10% using DiBAC₄ in HL-1 or HLB 021-152 in AtT-20
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36 cells^{30,31}. Removal of media and/or washing of the cells is also required during dye
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38 loading of commercial dyes used previously, this increases assay time and may
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40 promote cell detachment from the microplate wells^{31,32}.
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47 This assay has a number of advantages as a rapid screen for assessing ligand
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49 potency and efficacy at μ -opioid receptors. It is rapid, real-time, only requires the
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51 addition of a single reagent and is performed in intact cells. When compared with
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53 assays of G α subunit activation – either [³⁵S]GTP γ S binding assays or assays which
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55 directly measure agonist-stimulated GTPase activity³³, the GIRK assay is far simpler,
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3 requires no handling of radioactivity and provides a real time measure of receptor
4 activation rather than a single point determination. However, the $G\alpha$ subunit
5 activation assays will provide a more sensitive discriminator of efficacy in cells with a
6 low to moderate expression of receptors because the assay is constrained only by
7 the amount of accessible $G\alpha$ subunit in the cells, and this is usually not limiting³³. By
8 contrast, assays of AC regulation via measurement of cAMP accumulation have
9 been adapted for use in whole cells, however these assays usually require
10 incubation steps and either cell lysis followed by addition of several reagents or the
11 use of cells transfected with enzymes which catalyse the production of a fluorescent
12 substrate or with fluorescently labelled reporter proteins¹⁰. Assays of AC activity can
13 be very sensitive and also detect cAMP levels over a large range of concentrations
14 but in general when assaying the activity of G_i/G_o -coupled receptors such as the μ -
15 opioid receptor it is usually necessary to artificially elevate cAMP levels with forskolin
16 in order to obtain an appropriate signal window for determining AC inhibition by the
17 G_i/G_o -derived $G\alpha$ subunits. Nevertheless, assays of AC inhibition represent the most
18 flexible and straightforward way of studying $G\alpha$ (as opposed to $G\beta\gamma$) signalling in a
19 high throughput environment.
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43 While both $GTP\gamma S$ binding and cAMP accumulation assays have been widely used
44 for studying desensitization of opioid receptor signalling, the usual requirement for
45 control cells to be incubated with agonist for at least 10-15 minutes makes
46 interpretation of the data problematic, as receptor desensitization will be occurring
47 during this time (e.g. Figure 7)⁷.
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3 Other relatively straightforward strategies for real-time measurement of MOR-
4 mediated signalling involve measuring $[Ca]_i$ concentration using Ca^{2+} sensitive
5 fluorescent dyes. This approach provides relatively rapid real time response, and
6 assays of $[Ca]_i$ concentration can be amenable to studying receptor
7 desensitization^{34,35}. Interestingly, we were unable to activate intracellular Ca release
8 in AtT-20 cells via MOR or any of the $G\alpha_q$ receptors reportedly expressed in this cell
9 line³⁶ (data not shown).
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20 This assay offers an alternative approach for measuring MOR activation and
21 desensitisation by targeting a naturalistic $G\beta\gamma$ -mediated signalling pathway. Our
22 results show the membrane potential assay to be a rapid, reliable and inexpensive
23 method for identifying ligands that modulate GIRK activity, and may be scaled up to
24 enable high-throughput screening for novel opioid drugs.
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Table 1: Potencies and efficacies of the range of structurally distinct opioid ligands tested using the membrane potential assay in AtT-20 cells. Ligands are ranked in order of efficacy. Ligands with Emax significantly different to that of DAMGO are marked with * ($P < 0.05$, extra sum of squares F test). Latency is the time taken for the peak signal to be reached after a maximal concentration of drug was added.

OPIOID AGONIST	pEC50	Emax (%)	Hill Slope	Latency (s)
DAMGO	8.3 ± 0.1	38 ± 2	0.8 ± 0.1	29 ± 1
Endomorphin 2	8.4 ± 0.1	37 ± 2	1.0 ± 0.1	32 ± 2
Endomorphin 1	8.7 ± 0.1	36 ± 1	0.9 ± 0.1	27 ± 1
B-endorphin	7.0 ± 0.1	36 ± 3	1.2 ± 0.2	33 ± 4
Fentanyl	9.3 ± 0.1	35 ± 1	1.0 ± 0.1	27 ± 2
Met-Enkephalin *	8.5 ± 0.1	34 ± 1	0.8 ± 0.1	30 ± 3
Methadone *	7.6 ± 0.1	32 ± 1	1.2 ± 0.2	29 ± 3
Morphine *	7.7 ± 0.1	31 ± 1	1.0 ± 0.2	29 ± 2
Oxycodone *	6.7 ± 0.1	28 ± 1	1.2 ± 0.1	24 ± 3
Buprenorphine *	8.0 ± 0.1	21 ± 1	0.8 ± 0.3	12 ± 4
Pentazocine *	6.2 ± 0.2	5 ± 1	1.6 ± 0.3	18 ± 2

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3 **Figure 1:** Example traces of fluorescent signal in the membrane potential assay over
4 300 sec. Baseline readings were taken every 2 sec for 120 sec, at which point
5 increasing concentrations of DAMGO (3 nM, 30 nM, 300 nM) were added to AtT-
6 20MOR cells, resulting in concentration-dependent decreases in fluorescent signal
7 as shown.
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16 **Figure 2:** (A) Trace of fluorescent signal illustrating the reversal of DAMGO
17 stimulated decrease in signal by MOR antagonist naloxone. 1 μ M DAMGO/HBSS
18 was added to AtT-20MOR cells at 120 secs, followed by addition of 1 μ M naloxone
19 at 240 secs. The DAMGO stimulated decrease in fluorescent signal was completely
20 reversed by naloxone. Naloxone had no effect when applied without previous
21 addition of DAMGO. (B) Concentration response curve for morphine, both alone and
22 with the addition of 3 nM, 10 nM and 100 nM naloxone. The addition of naloxone
23 produced a parallel shift in the morphine concentration response curve with a pA_2 of
24 -8.5 ± 0.1 , (2.9 ± 0.5 nM, $n=3$).
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Figure 3: The decrease in fluorescent signal observed with 30 nM DAMGO or 100
nM morphine was abolished following overnight incubation of AtT-20-MOR cells with
200ng/mL PTX.

Figure 4: Decreasing extracellular potassium concentration ($[K]_{Ex}$) increases the
maximum change in fluorescent signal observed with DAMGO. Concentration-
response curves were generated for DAMGO activation of GIRK channels in both
low $[K]_{Ex}$ (2.9 mM) and high $[K]_{Ex}$ (5.5 mM) conditions. E_{max} in low $[K]_{Ex}$ was $38 \pm 2\%$,
21% higher than in high $[K]_{Ex}$ (E_{max} $31 \pm 1\%$; $P < 0.01$).

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5 **Figure 5:** Treatment of AtT-20-MOR cells with nigericin, a K selective antibiotic
6 ionophore, caused a greater decrease in fluorescent signal than the maximally
7 effective concentration of DAMGO ($P < 0.05$). 1 μM nigericin (heavy trace), or
8 vehicle (light trace) was added to cells at 120 secs. Nigericin caused a decrease of
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10 50 \pm 3% in fluorescent signal. Membrane potential was allowed to reach equilibrium
11 before the addition of 300 nM DAMGO at 1500 sec, which did not cause any further
12 decrease in signal.
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22 **Figure 6:** Concentration response curves for DAMGO, morphine, buprenorphine and
23 pentazocine activation of GIRK channels, illustrating the differences in agonist
24 potency and efficacy. Morphine, buprenorphine and pentazocine all showed partial
25 agonist activity with E_{max} values of 31 \pm 1%, 21 \pm 1% and 5 \pm 1% respectively.
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33 **Figure 7:** Desensitization of MOR signalling in AtT-20 cells. Continuous application
34 of morphine (1 μM) reduces the response to a subsequent addition of a high
35 concentration of morphine or SRIF. Panel A shows example traces from an
36 experiment where a high concentration of morphine (10 μM) or SRIF (1 μM) was
37 added 30 minutes after the desensitizing concentration of 1 μM morphine (heavy
38 trace) or 30 minutes after the addition of vehicle (light trace). Panel B shows the time
39 course of the decline in response to 10 μM morphine or 1 μM SRIF in the continued
40 presence of 1 μM morphine. Data are expressed as a percentage of the control
41 response to drug added at the same time after the run commenced, and represent
42 the mean \pm s.e.m. of 3 - 8 determinations, each in duplicate or triplicate. The data
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3 was fit with an single exponential function to derive a $t/2$ for desensitization (490s for
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5 morphine/morphine, 460s for morphine/somatostatin).
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For Peer Review

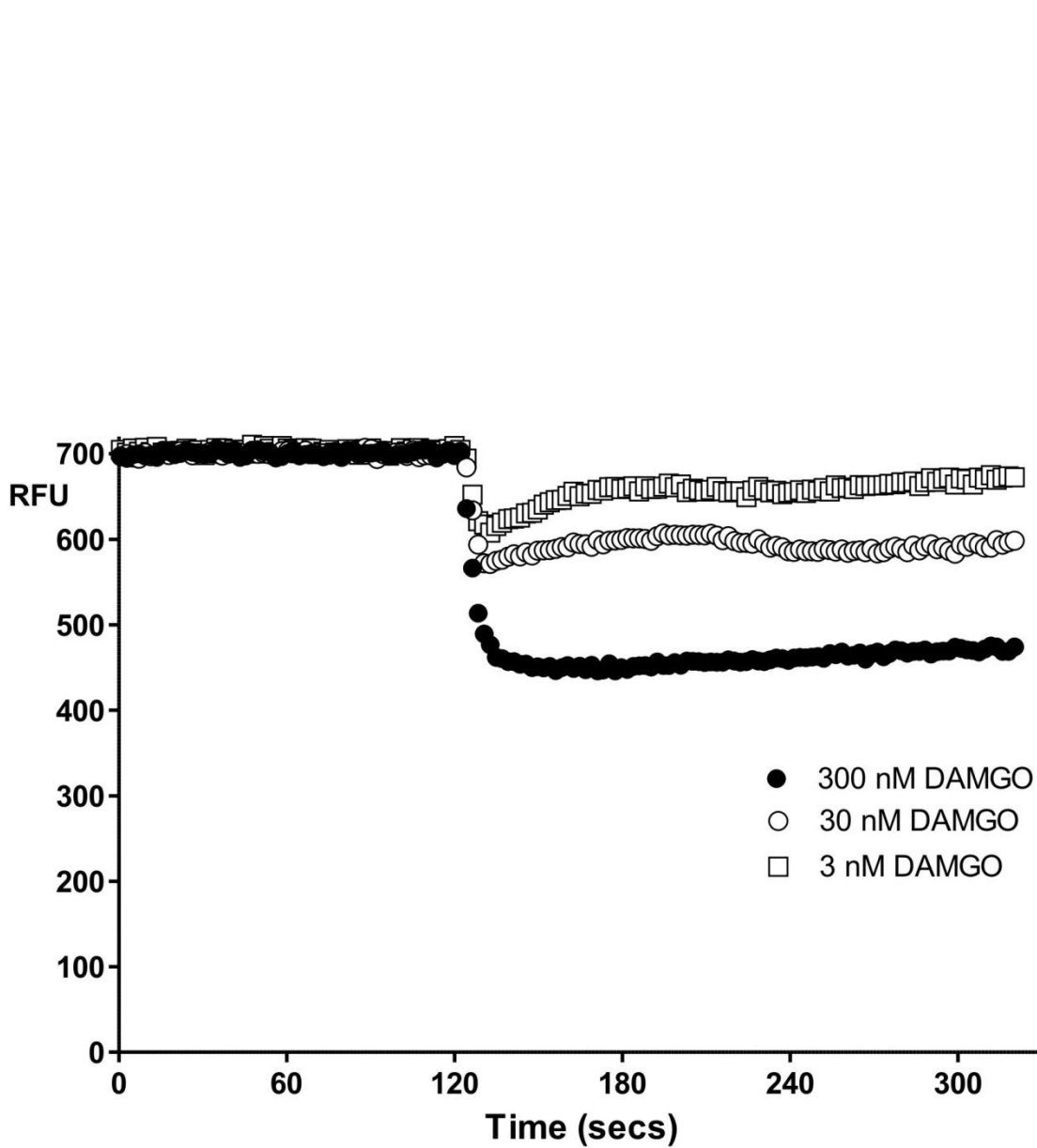


Figure 1

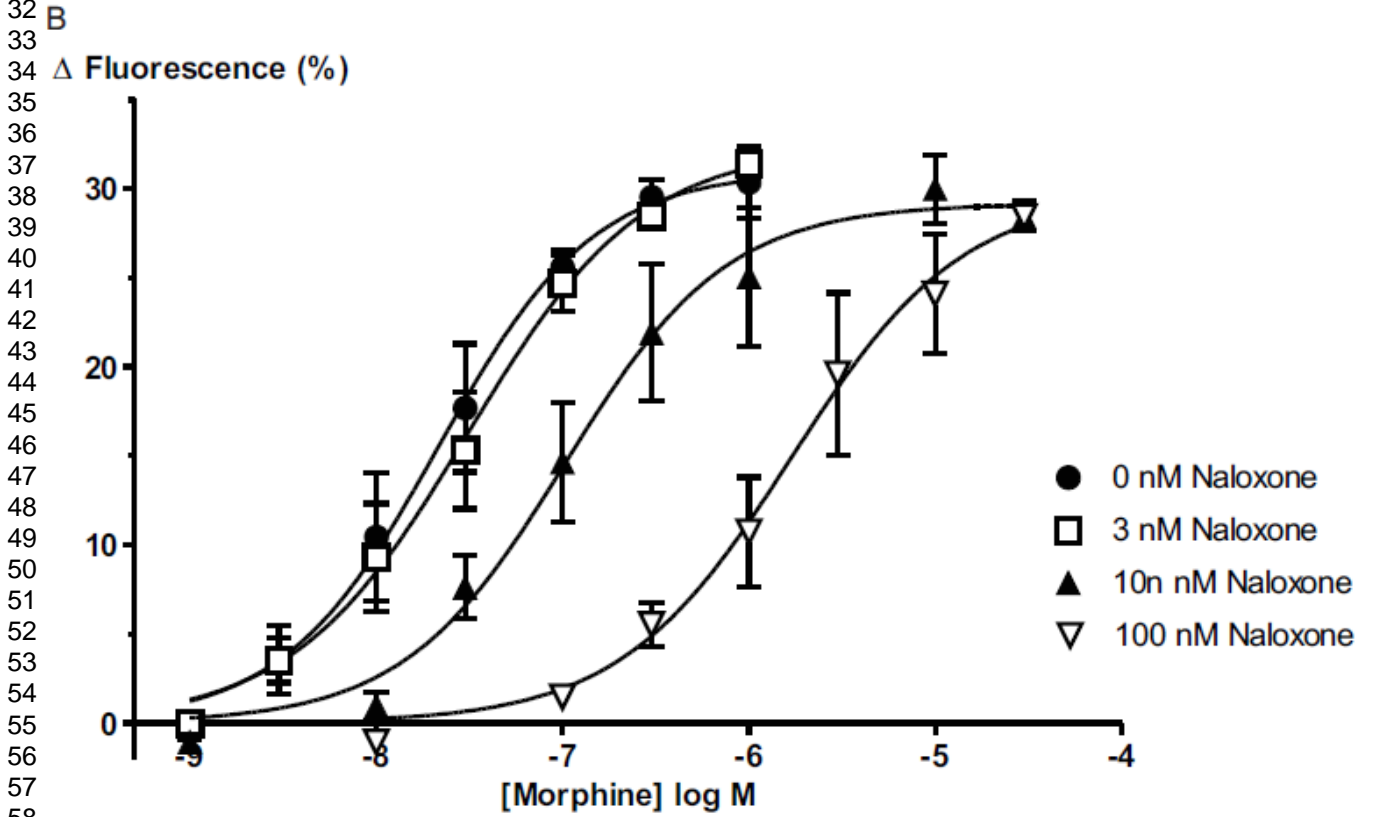
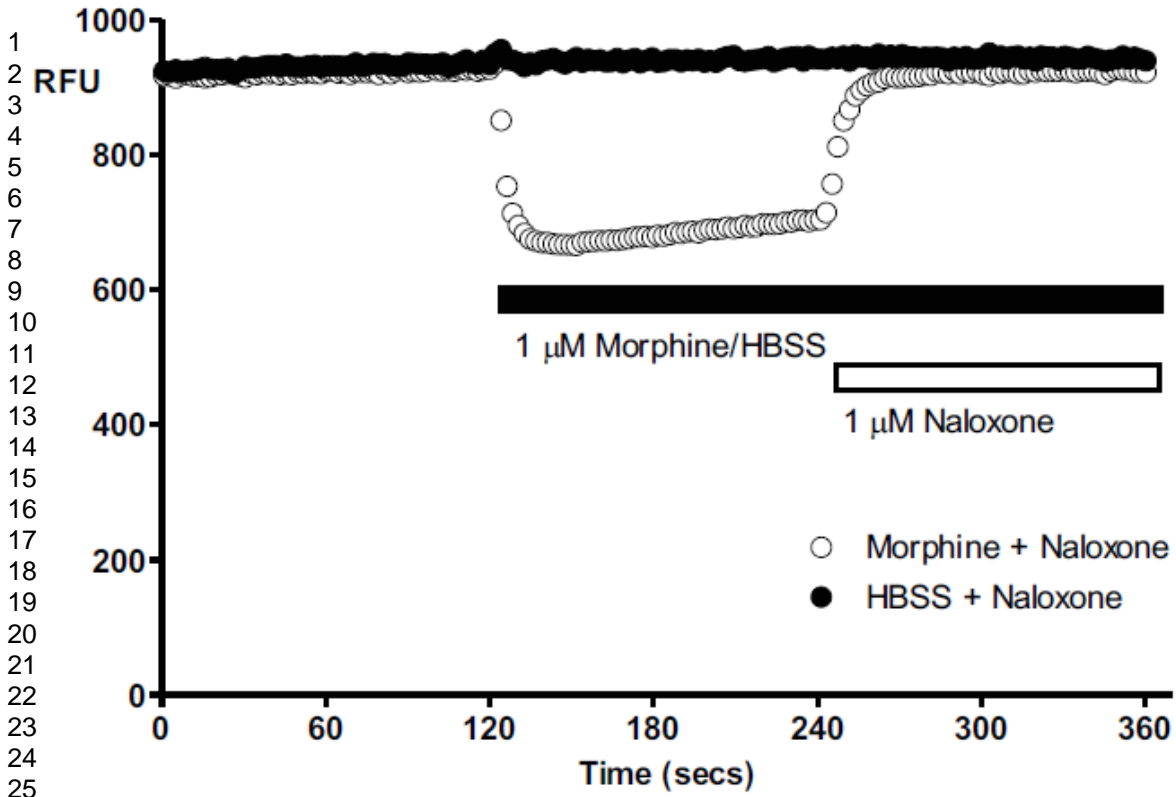


Figure 2

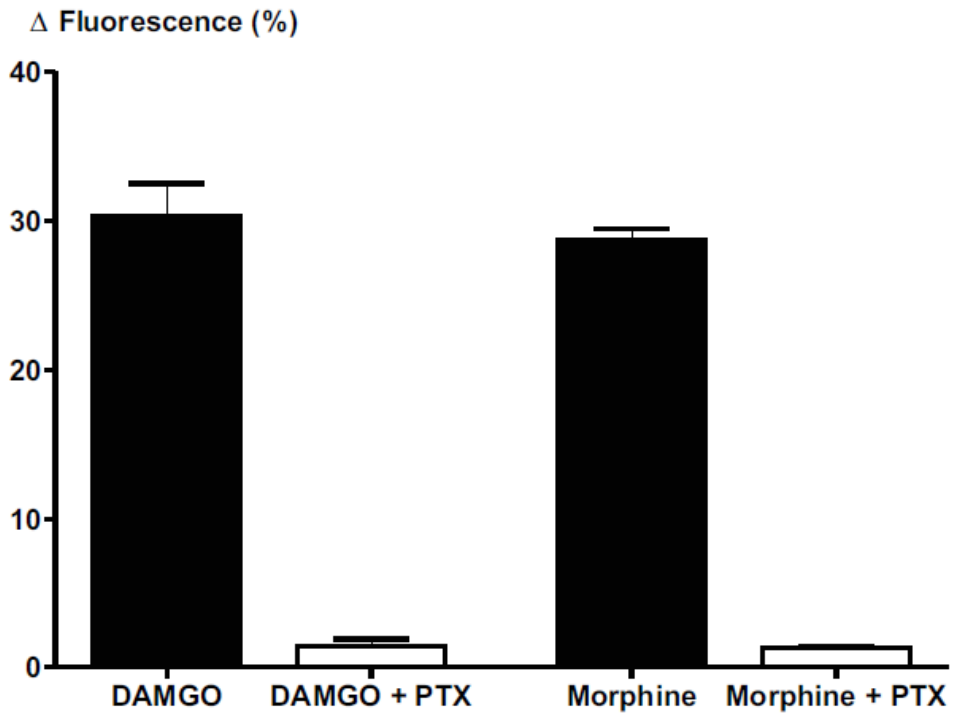


Figure 3

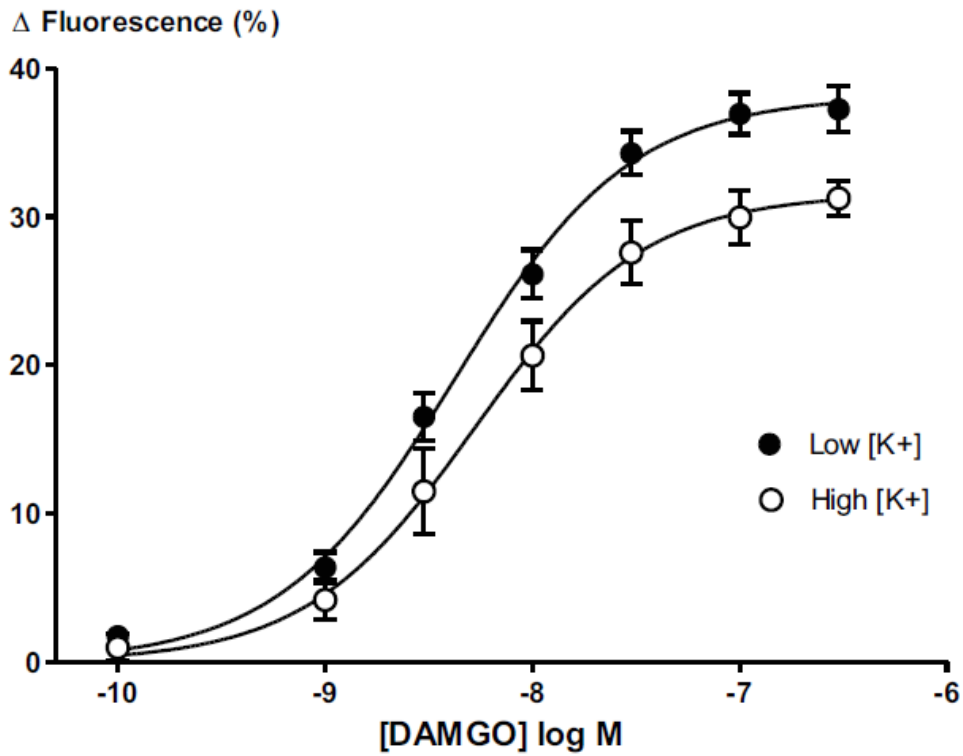


Figure 4

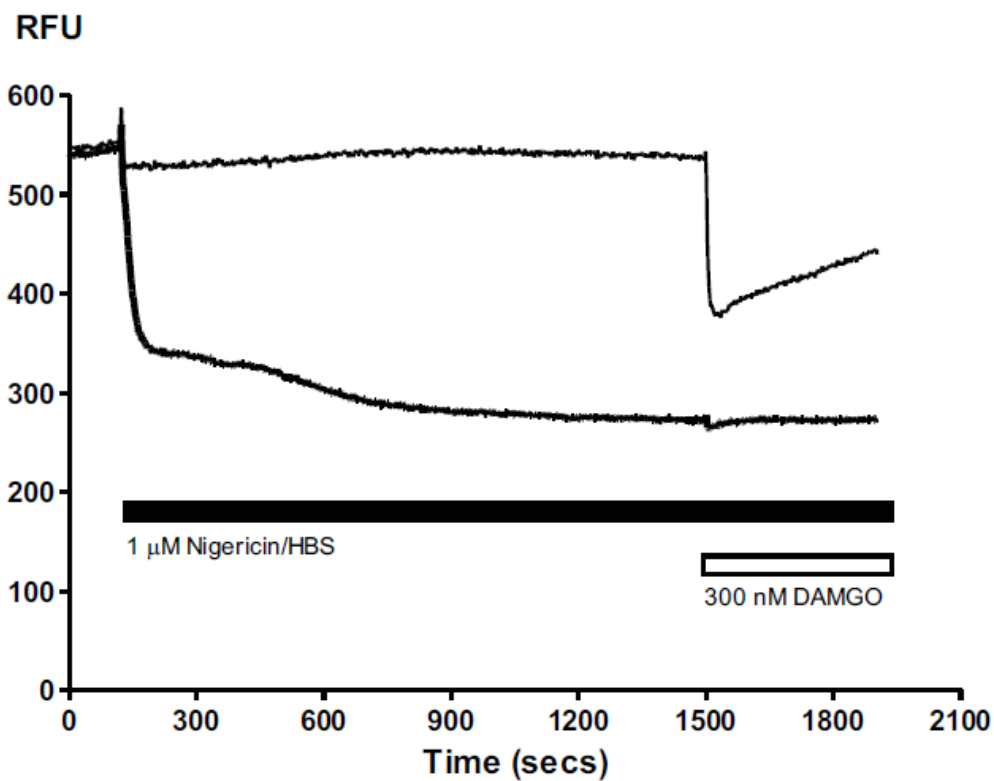


Figure 5

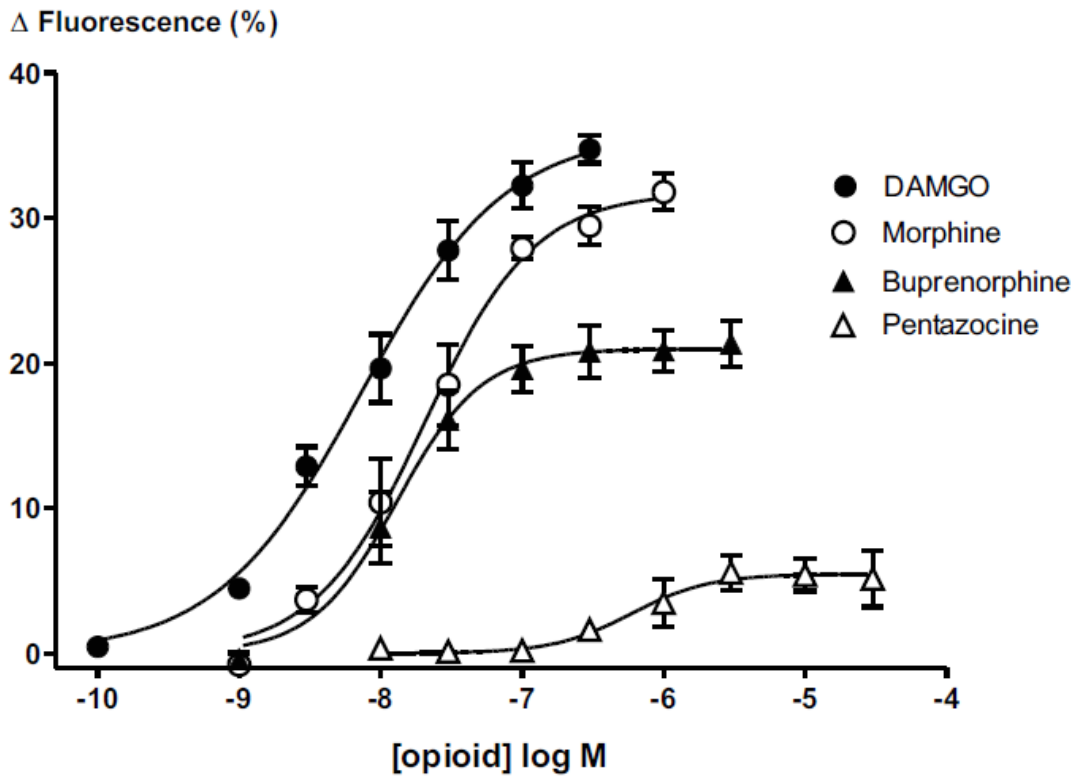
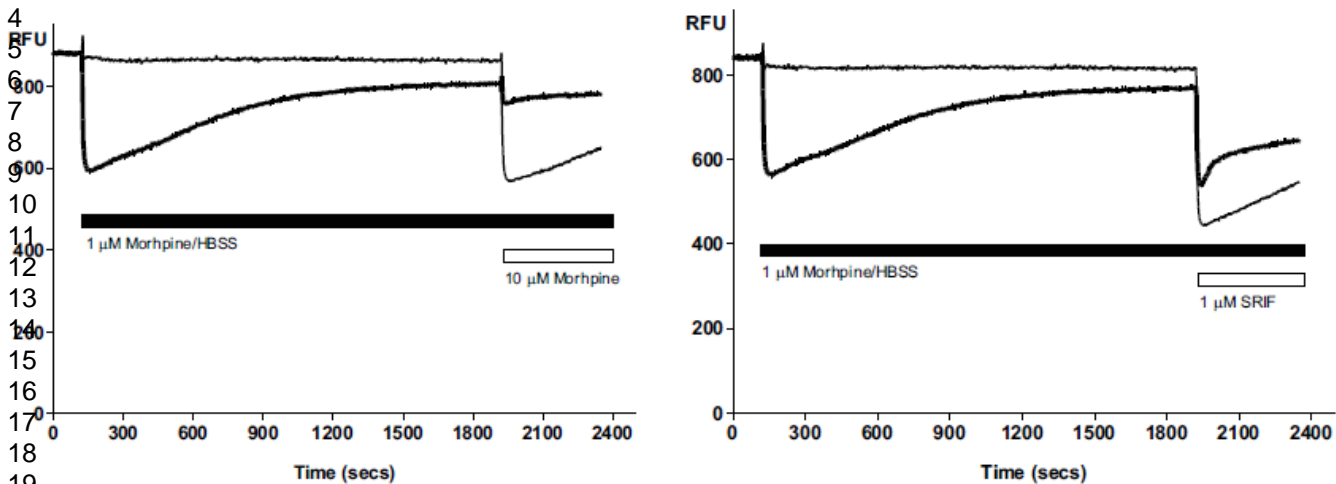


Figure 6

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Reduction of control response (%)

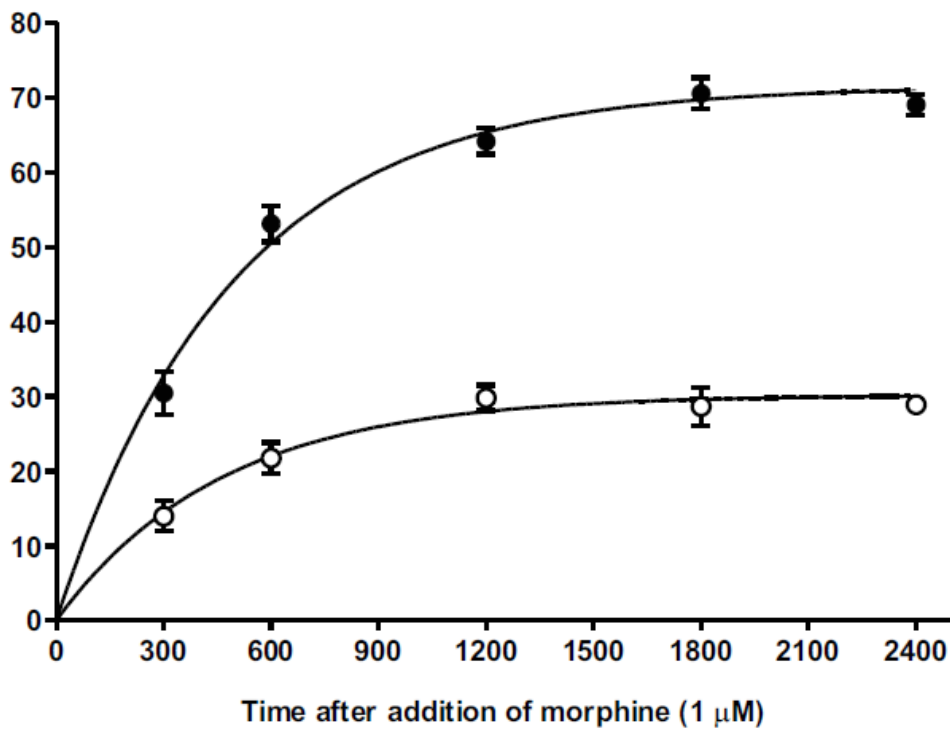


Figure 7