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## Activity of opioid ligands in cells expressing cloned $\mu$ opioid receptors

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### Abstract

**Background:** The aim of the present study was to describe the activity of a set of opioid drugs, including partial agonists, in a cell system expressing only  $\mu$  opioid receptors. Receptor activation was assessed by measuring the inhibition of forskolin-stimulated cyclic adenosine mono phosphate (cAMP) production. Efficacies and potencies of these ligands were determined relative to the endogenous ligand  $\beta$ -endorphin and the common  $\mu$  agonist, morphine.

**Results:** Among the ligands studied naltrexone, WIN 44,441 and SKF 10047, were classified as antagonists, while the remaining ligands were agonists. Agonist efficacy was assessed by determining the extent of inhibition of forskolin-stimulated cAMP production. The rank order of efficacy of the agonists was fentanyl = hydromorphone =  $\beta$ -endorphin > etorphine = lofentanil = butorphanol = morphine = nalbuphine = nalorphine > cyclazocine = dezocine = metazocine  $\geq$  xorphanol. The rank order of potency of these ligands was different from that of their efficacies; etorphine > hydromorphone > dezocine > xorphanol = nalorphine = butorphanol = lofentanil > metazocine > nalbuphine > cyclazocine > fentanyl > morphine >>>>  $\beta$ -endorphin.

**Conclusion:** These results elucidate the relative activities of a set of opioid ligands at  $\mu$  opioid receptor and can serve as the initial step in a systematic study leading to understanding of the mode of action of opioid ligands at this receptor. Furthermore, these results can assist in understanding the physiological effect of many opioid ligands acting through  $\mu$  opioid receptors.

### Background

Opioid ligands exhibit a variety of physiological activities and have been utilized extensively in medicine, most prominently in the treatment of pain. However, at analgesic doses, opioid receptor agonists or partial agonists can induce unwanted side effects such as ventilatory depression [1,2] and the development of physical tolerance and dependence [3,4]. Thus, the search for opioid ligands which possess analgesic effect and lack untoward effects has been a sought after goal of the medical community.

The overall hypothesis that drives the present work is that the ideal opioid analgesics that exhibit minimal side effects might be drugs that bind to more than one opioid receptor, but differentially activate each of the opioid receptor types ( $\mu$ ,  $\delta$ ,  $\kappa$ ). Such drugs would potentially act as a full agonist at a specific opioid receptor type, while acting as partial agonists or antagonists at the other receptor types. For example some investigators have suggested that opioid ligands with agonism at  $\mu$  opioid receptors and antagonism at  $\delta$  opioid receptors are potentially use-

ful analgesics [5–7]. In cases where the medicinal effect of a drug is mediated through the same opioid receptor type that also elicits the side effects, the use of drugs with mixed activity could be most beneficial [7]. In such a case, interaction with one receptor could reverse the unwanted side effects associated with activation of the other receptor. In order to test this hypothesis, the activation profiles of a set of non-selective opioid ligands need to be assessed *in vitro*, followed by *in vivo* evaluation of analgesic and unwanted effects. The completed data set can be used to determine the characteristics of ligands possessing analgesia in the absence of unwanted effects. One of the steps in such an approach is presented here.

In order to clearly understand the activity of any ligand for mechanistic characterization or rational drug design, it is essential that the ligands be tested in a well-defined environment under identical experimental conditions. Moreover, the use of a transfected cell system in which a single receptor type is expressed is critical for these types of modelling. Such tools were not available until recently when the three opioid receptor types were cloned. We have previously characterized these ligands in cells expressing only  $\delta$  opioid receptor [8]. The present study was devised to characterize the activity of a set of opioid ligands in a cell line expressing only  $\mu$  opioid receptors. The ligands selected were chosen based on our previous data suggesting that they bind to all three opioid receptor types [9]. Previous model tissue data [10] and *in vivo* data [11] had suggested that some of these drugs displayed differential activation profiles at each of the opioid receptor types. Thus, the present study was designed to achieve the following goals; (1) to describe the activation profiles of a set of opioid ligands not previously defined in an isolated cell system expressing only  $\mu$  opioid receptor, and (2) to compare the efficacies of these drugs to the known, highly efficacious  $\mu$  receptor agonist, fentanyl, the common opioid analgesic morphine and to the endogenous opioid ligand  $\beta$ -endorphin.

In the present study, we employed an HEK cell line stably expressing  $\mu$  opioid receptors (HEK- $\mu$ ) to characterize the activation and binding profiles of fifteen opioid ligands. These cells do not express endogenous  $\mu$  opioid receptors and are easily transfected. Furthermore, they have previously been used to study opioid receptor activation [12–16] by measuring inhibition of forskolin-stimulated cAMP production. The results obtained from this study can serve to clarify the categorization of each of the ligands studied as an agonist, weak/partial agonist or antagonist at  $\mu$  opioid receptors. Moreover, these results demonstrate that it is crucial to understand the interaction of each drug with a single receptor type at the molecular level in order to fully appreciate the mode of action of the drug in the body.

## Results

### Activation assays

Our results clearly characterize the ligands studied as agonists or antagonists at  $\mu$  opioid receptors with respect to inhibition of adenylyl cyclase. Naltrexone, SKF 10047 and WIN 44,441 were identified as antagonists. These ligands exhibited little or no measurable inhibitory effect on forskolin-stimulated cAMP production when used alone and were repeatedly able to block the inhibitory effect of 1 nM etorphine. All other ligands studied showed agonism at  $\mu$  opioid receptor with varying maximal effects or efficacies ranging from 29% – 71% (Table 1). The rank order of efficacy of the ligands tested were fentanyl = hydromorphone =  $\beta$ -endorphin > etorphine = lofentanil = butorphanol = morphine = nalbuphine = nalorphine > cyclazocine = dezocine = metazocine  $\geq$  xorphanol. The IC<sub>50</sub> of all ligands studied were between 1–10 nM, with the exception of etorphine, which was the most potent drug, with an IC<sub>50</sub> of 0.3 nM, morphine, which was the least potent drug with an IC<sub>50</sub> of 12 nM and the endogenous ligand  $\beta$ -endorphin with IC<sub>50</sub> of 500 nM. A low affinity is acceptable for an endogenous ligand that is released at very high concentrations at the site of action at the synaptic cleft. The rank order of potency of the drugs studied were; etorphine > hydromorphone > dezocine > xorphanol = nalorphine = butorphanol = lofentanil > metazocine > nalbuphine > cyclazocine > fentanyl > morphine >>>>  $\beta$ -endorphin. As can be seen, the rank order of efficacy was not related to the rank order of potency. In fact one of the most potent ligands, xorphanol, was the least efficacious.

Statistical analysis of the differences between the efficacies of different ligands was carried out to distinguish the full agonists from the partial agonists. Based on these analyses, there was no difference in the efficacy of butorphanol, etorphine, lofentanil or nalorphine compared to fentanyl or hydromorphone ( $p > 0.05$ ). However, morphine and nalbuphine both had efficacies that were less than fentanyl ( $p < 0.05$ ). Both fentanyl and hydromorphone showed maximal efficacies that were not significantly different compared to that of the endogenous ligand  $\beta$ -endorphin ( $P > 0.05$ ), thus categorizing them as full agonists compared to  $\beta$ -endorphin. Finally the four weakest agonists, cyclazocine, dezocine, metazocine and xorphanol all exhibited efficacies that showed statistically significant difference when compared to fentanyl ( $p < 0.01$ ), and thus were categorized as partial agonists.

Figure 1 represents the dose response curves of three agonists and one antagonist. The least efficacious (xorphanol, Fig 1a) and most efficacious (fentanyl, Fig 1b) ligands are shown for comparison. The dose response curve of nalbuphine that was previously described as an antagonist at  $\mu$  opioid receptor is also shown (Fig 1c) in order to demonstrate the robustness of its agonistic effect at  $\mu$  opioid re-

**Table 1: Activation Profiles of Opioid ligands in HEK- $\mu$  Cells**

Agonist	IC50 $\pm$ SEM (nM)	Max Inhibition (%)
$\beta$ -Endorphin	526 $\pm$ 105	71 $\pm$ 5
Butorphanol	3 $\pm$ 2	58 $\pm$ 5
Cyclazocine	21 $\pm$ 8	37 $\pm$ 2**###
DAMGO	35 $\pm$ 9	58 $\pm$ 5
Dezocine	1.3 $\pm$ 0.6	43 $\pm$ 2**###
Etorphine	0.6 $\pm$ 0.3	60 $\pm$ 6
Fentanyl	8.4 $\pm$ 1.6	69 $\pm$ 4 $\oplus$
Hydromorphone	0.8 $\pm$ 0.1	67 $\pm$ 4 $\oplus$
Lofentanil	3.8 $\pm$ 2.0	56 $\pm$ 6
Metazocine	2.0 $\pm$ 0.1	41 $\pm$ 4**###
Morphine	17 $\pm$ 8	48 $\pm$ 4**###
Nalbuphine	11 $\pm$ 2	49 $\pm$ 4**###
Nalorphine	2.7 $\pm$ 1.4	50 $\pm$ 2 $\#$
Xorphanol	3.4 $\pm$ 1.7	29 $\pm$ 2** $\oplus\oplus$ ###
Naltrexone	Antagonist	
SKF 10047	Antagonist	
WIN 44,441	Antagonist	

Inhibitory effect of opioid ligands on Forskolin-stimulated adenylyl cyclase activity was measured as described under "Materials and Methods". Data for IC50's represent the mean  $\pm$  SEM obtained from two or more experiments carried out in duplicate. Maximum inhibition data represent the mean  $\pm$  SEM obtained from the best fit curve for data from three to four experiments carried out in duplicate. (\*\* =  $P < 0.001$ , \* =  $P < 0.05$ ; compared to fentanyl), ( $\oplus\oplus$  =  $P < 0.001$ ,  $\oplus$  =  $P < 0.05$  compared to morphine), (### =  $P < 0.001$ , # =  $P < 0.05$ ; compared to  $\beta$ -endorphin).

**Table 2: Binding Profiles of Opioid ligands in HEK- $\mu$  cells**

LIGAND	AFFINITY Ki (nM)
Cyclazocine	0.22 $\pm$ 0.02
DAMGO	2.65 $\pm$ 0.75
Morphine	2.2 $\pm$ 0.5
Naltrexone	0.74 $\pm$ 0.06
SKF 10047	1.15 $\pm$ 0.05
WIN 44,441	0.99 $\pm$ 0.31
Xorphanol	0.25 $\pm$ 0.01

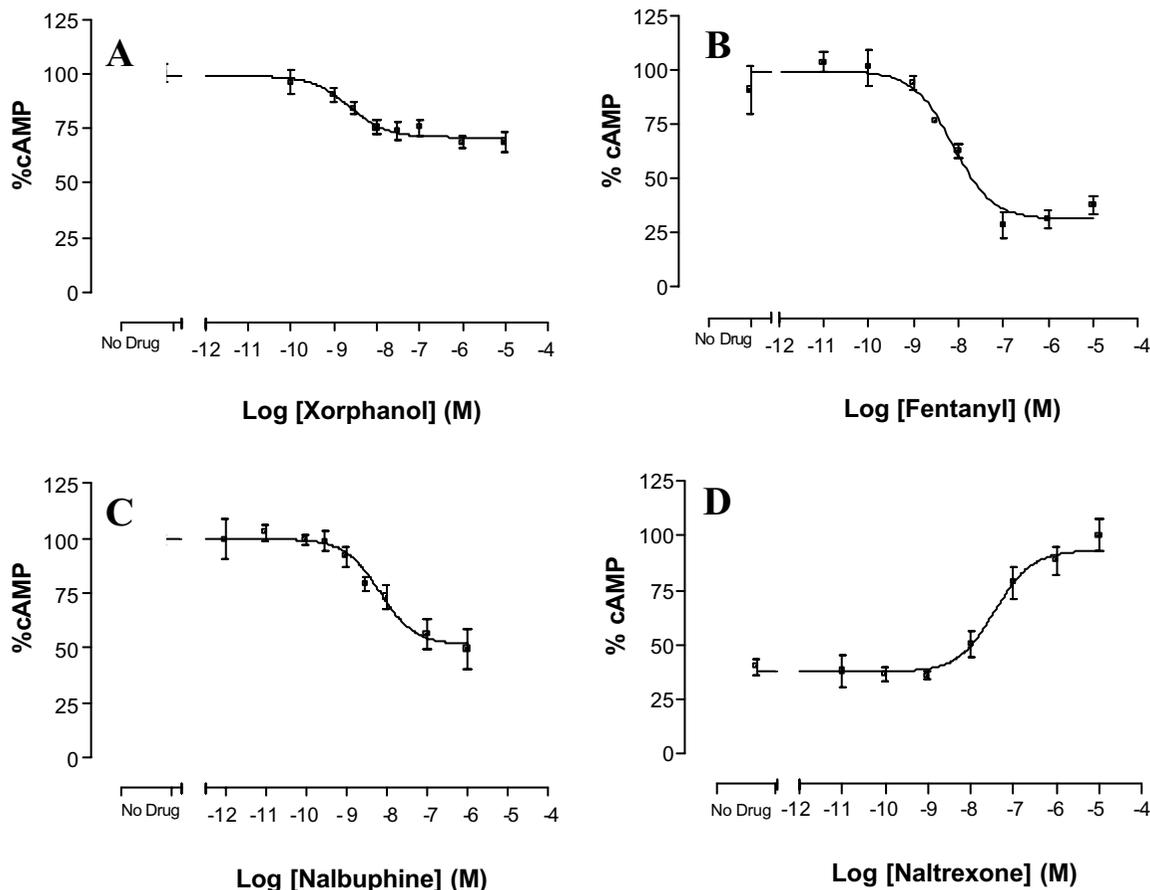
Competition binding assays were carried out in cell homogenates of HEK- $\mu$  cells as described under "Materials and Methods". Data represent mean  $\pm$  SEM of Ki values from two or more independent experiments carried out in triplicate.

ceptor. Finally, a dose response curve of naltrexone as it fully blocked the effect of 1 nM etorphine is shown (Fig 1d).

#### Binding assays

A single clone of transfected HEK cells stably expressing approximately 3 pmoles of  $\mu$  opioid receptors per mg of protein, as measured by [ $^3$ H]-DAMGO binding, was propagated and used as a model for characterization of the binding profiles of a set of opioid ligands at  $\mu$  opioid receptors. This level of expression is not uncommon for transfected cell systems [17,18], although such receptor expression levels may appear higher than reported values from brain tissues. The measured values from specific brain regions are average estimates over an entire tissue or

region and not that of a single cell and the actual expression on a single cell in the brain is most likely much higher. Furthermore, these high levels of receptor expression have been measured in cell lines that were established from various neuronal tissues. For example, similar levels of expression have been measured for neuronal cells in culture (SH-SY5Y cells) [19] or primary cultures of dorsal root ganglion sensory neurons [20]. Thus, the level of  $\mu$  receptor expression in the cell line used in this study is comparable to that of cells expressing the  $\mu$  opioid receptors in various regions of the nervous system. Competition binding studies were carried out for the ligands that were shown to act as antagonists or weak/partial agonists at  $\mu$  opioid receptors to confirm that these agents were in fact capable of binding to  $\mu$  opioid receptors with high affini-



**Figure 1**  
**Dose response curves of inhibition of adenylyl cyclase activity by representative ligands in HEK- $\mu$  cells.** Varying concentration of opioid ligands were used to determine the potency and efficacy of each ligand in inhibiting the effect of 5  $\mu$ M forskolin in producing cAMP, as described under methods. Maximal cAMP levels were in the range of 400–1000 pmole/well. The 100% on the x-axis corresponds to the cAMP levels in the absence of any drug, i.e.: forskolin alone for all figures including Fig. 1D. Data presented are the average data from 2 or more experiments carried out in duplicate. Data have been normalized as described under methods. Error bars represent standard error of the mean of the normalized data. (A) Xorphanol, (B) Fentanyl, (C) Nalbuphine and (D) Naltrexone.

ty. Competition binding studies indicated that the antagonists naltrexone, SKF 10047 and WIN 44,441 had binding affinities ( $K_i$ ) in nanomolar range and the two weakest agonists, xorphanol and cyclazocine bound the receptor with the highest affinities. The rank order of affinities was determined to be; cyclazocine = xorphanol > WIN 44,441 > naltrexone > SKF 10047 > morphine. This order was in agreement with the relative potencies of these drugs, showing that cyclazocine and xorphanol had higher affinities and potencies compared to morphine, although they were both less efficacious than morphine.

**Discussion**  
 A well-defined receptor expression system was used to characterize the activation profiles of a set of opioid ligands and to compare the efficacies and potencies of these ligands at  $\mu$  opioid receptors. The main advantage of using transfected HEK cells for this study is that these cells do not contain endogenous  $\mu$  opioid receptors, but express the G proteins necessary for the proper coupling of the transfected genes to the respective second messenger systems. A well established method for assessing G-protein activation by opioid receptors and characterizing activity of opioid ligands is measuring the extent of inhibition of

forskolin-stimulated adenylyl cyclase (AC) activity [12–14,21–23]. Adenylyl cyclase has been implicated in playing a role in mediating the analgesic effect of opioid ligands through  $\mu$  opioid receptors [24–28]. Thus, characterizing the ability of opioid ligands to inhibit cAMP production, such as described in this report, could be used as an index for assessing their activity as a correlate of their analgesic effects. Moreover, a simple well-defined system, such as the one used in this study, can be very beneficial in describing the mode of action of each ligand at a specific receptor. However, it is understood that once applied to the whole animal *in vivo*, the overall effect of the drug will be a composite of the effects of the drug on all receptor types interacting with it. Furthermore, the pharmacokinetic parameters, such as metabolism, tissue absorption and distribution of the drug will play a major role in the overall drug effect *in vivo*.

The present set of ligands exhibited a range of efficacies and potencies. The most efficacious drug was fentanyl, supporting previous *in vivo* studies describing fentanyl as a highly potent and efficacious analgesic drug [11]. On the other hand xorphanol was identified as a highly potent ligand with the lowest efficacy in inhibiting cAMP production through  $\mu$  opioid receptors. Clinically, xorphanol is a well tolerated, orally active analgesic that provides effective pain relief but shows low physical dependence liability [29,30]. It is possible that the low physical dependence liability of this drug is due to its partial agonism at  $\mu$  opioid receptors.

The present study can offer some explanation for the existing *in vivo* data by providing information on the relative coupling efficacies of the tested ligands from a controlled system. For example, one study characterizing butorphanol, [31] described this drug as a mild analgesic compared to morphine. However, in another study, butorphanol was reported to be equipotent in its antinociceptive effect to that of morphine [32]. In our assays, we have observed that butorphanol and morphine are equally efficacious in inhibiting cAMP production, although butorphanol is more potent than morphine in this assay. Based on these two sets of results, if the analgesic effect of butorphanol is mediated via  $\mu$  opioid receptors through the inhibitory effect of these receptors on adenylyl cyclase, the equal efficacy of morphine and butorphanol would support an equal antinociceptive effect of these two drugs. Thus, such results demonstrate that it is essential to describe the effect of ligands on specific receptor types in order to fully define the mode of action of each drug.

Our data clearly show that nalbuphine is a potent agonist at the  $\mu$  opioid receptor with an efficacy similar to that of morphine, thus agreeing with *in vivo* data showing that morphine and nalbuphine are equally potent as analge-

sics [33]. However, other reports have described nalbuphine as a "mixed agonist/antagonist" with agonism at  $\kappa$  opioid receptors and antagonism at  $\mu$  opioid receptors based on *in vivo* data [11,33]. This categorization is likely due to the fact that nalbuphine has been shown to reduce the ventilatory depressant effect of other opioids, while adding to their analgesic effect [33]. Nalbuphine is also used clinically to reduce the opioid-mediated side effects such as itching, without completely reversing the analgesic effects of the full agonists [34–36]. According to our data, although nalbuphine is clearly a potent agonist at  $\mu$  receptors, its levels of agonism is significantly lower than the very efficacious drug fentanyl and the endogenous ligand  $\beta$ -endorphin. Therefore, if combined with a drug such as fentanyl, or in the presence of an internal pool of  $\beta$ -endorphin, nalbuphine can act as an antagonist in inhibiting adenylyl cyclase by  $\mu$  opioid receptors. Thus, the net effect of the mixture of the two ligands at the level of the whole animal will be the combined effect. This can explain the usefulness of drugs such as nalbuphine in reducing the side effects associated with the more potent opioid analgesics while not totally reversing the analgesic action of those ligands.

Comparing the efficacies of the ligands in this study to the endogenous ligand  $\beta$ -endorphin indicates that fentanyl, etorphine, lofentanil, butorphanol and hydromorphone are all full agonists. Thus in physiological pathways where inhibition of adenylyl cyclase mediates the activity of  $\mu$  opioid receptors, these drugs will act as agonists at  $\mu$  opioid receptors. Other ligands with efficacies significantly different from that of  $\beta$ -endorphin, can act as agonists with different efficacies at  $\mu$  opioid receptors when administered alone. However, in the presence of the endogenous ligand  $\beta$ -endorphin (when released at the synapse) or another full agonist, a weaker agonist can potentially act as a competitive antagonist. Conversely, it is possible for ligands categorized and accepted as antagonists to act as agonists at  $\mu$  opioid receptor, if applied alone at high enough concentrations, albeit with a very low efficacy. This does not mean that when applied to the whole organism, an analgesic effect of such a drug will be observed. The reason is that the affinity, efficacy, dose and pharmacokinetic properties of the drug used will influence the ultimate effect seen in the organism, as will the presence of other ligands in the system. When drugs are used in combination, the net effect will depend on the intrinsic characteristics (efficacy and affinity) of the two drugs relative to each other and their relative concentrations at the site of action. Consequently, it is important to have a clear understanding of the characteristics of each drug and the molecular changes that occur at the receptor following receptor-drug interaction [37]. Such knowledge will allow one to target a specific receptor population with the aim of attaining a certain physiological effect; possibly by mix-

ing two or more drugs or using a single drug that possesses different activation profiles at various receptors.

## Conclusion

In summary, this is the first detailed comparative report of the inhibitory effect of a set of opioid ligands on the accumulation of cAMP in intact cells expressing  $\mu$  opioid receptors. The activation profiles of these ligands have been compared to that of the endogenous opioid ligand,  $\beta$ -endorphin, and the commonly used opioid analgesics, morphine and fentanyl. Moreover, this report serves to clarify the activation profiles of many previously uncharacterised ligands in cells expressing only  $\mu$  opioid receptors, thus leading to a better understanding of the mechanism of action of these drugs.

## Method

### Cell culture

Human Embryonic Kidney (HEK) 293 cells were maintained in D-MEM/F-12 (Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 1:1 mixture), supplemented with 10% (v/v) fetal calf serum (FCS), 200  $\mu$ g/ml G-418 (Geneticin<sup>®</sup>) in a humidified incubator with 5% CO<sub>2</sub> and 95% air, at 37°C. The incubation medium was changed every 3–4 days. Once a week, cells were re-plated at 20% density into 75 cm<sup>2</sup> tissue culture flasks.

### Establishing stable cells expressing $\mu$ opioid receptors

Stably transfected HEK cells were developed as described previously [12]. HEK 293 cells were transfected with mouse  $\mu$  opioid receptor cDNA in the pcDNA3 vector (a generous gift from Drs. Chris Evans and Duane Keith, UCLA) using the lipofectin<sup>®</sup> reagent (Life Technologies, Rockville, MD). One clone with a B<sub>max</sub> of 3326  $\pm$  674 fmoles/mg of protein as assessed by [<sup>3</sup>H]-DAMGO binding was propagated for use in this study.

### Binding assays

Saturation binding assays were carried out for [<sup>3</sup>H]-DAMGO in HEK cells as described previously [38]. Each assay was carried out in triplicates in a 250  $\mu$ l total reaction volume containing 20–25  $\mu$ g of crude cell homogenate per assay tube. Incubation was in 50 mM Tris HCl buffer, pH 7.4 at room temperature for 2 hours. The assay was terminated by rapid filtration through Whatman GF/B filters followed by three washes, with ice-cold buffer. Radioactivity retained on the filters was measured using liquid scintillation counting.

Competition binding assays were carried out in crude homogenate of HEK- $\mu$  cells. Binding was carried out in 250  $\mu$ l volume of 50 mM Tris HCl buffer, pH 7.4 in the presence of about 0.5–1 nM [<sup>3</sup>H]-DAMGO and increasing concentrations (24–32) of unlabeled ligand. Incubation and washing were as described above. Binding data were

analyzed using the Affinity Analysis Software as described before [38]. For preparation of crude cell homogenate, confluent cultures of HEK- $\mu$  cells were harvested using phosphate buffered saline. Following centrifugation, the cell pellet was resuspended in ice-cold 50 mM Tris HCl buffer pH 7.4 at about 10<sup>7</sup>cell/ml, and homogenized using a polytron at setting 6 for 10 seconds. The cell homogenate was stored in aliquots at -86°C until use. Protein content of the cell homogenate was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

### Whole cell adenylyl cyclase assays

Exponentially growing HEK- $\mu$  cells were harvested and resuspended in serum free DMEM/F12 medium. Cells were plated in 96 well micro titer plates at 5  $\times$  10<sup>4</sup> cells/well. To each well, phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added to a final concentration of 100  $\mu$ M, followed by addition of agonists at different concentrations and incubation at 37°C. Following incubation for 15 minutes, forskolin was added to each well to a final concentration of 5  $\mu$ M followed by another incubation for 15 minutes at 37°C. The reaction was terminated by aspiration of the medium and addition of lysis buffer from the Biotrak<sup>™</sup> cAMP Enzyme Immunoassay kit from Amersham Pharmacia Biotech (Buckinghamshire, England). The rest of the assay followed the protocol provided with the kit. Actual amount of cAMP was determined for each sample in comparison to a standard curve of known amounts of cAMP provided in the cAMP kit, as described in the kit protocol.

### Agonism

Agonistic activity of opioid ligands was assessed by measuring the inhibitory effect of the drugs on forskolin-stimulated cAMP accumulation. Data were normalized to the top of the curve (no drug, 100%), expressed as percent inhibition of forskolin-stimulated cAMP accumulation and were fitted to a sigmoidal function by using one site competition function as described below. The efficacy of each ligand was defined as percent inhibition of forskolin-stimulated cAMP production compared to no drug levels (0% inhibition, 100% cAMP production).

### Antagonism

Compounds with no or very small *in vitro* agonistic activity (<20% inhibition of cAMP production) for which the dose response curves could not be fitted due to the small effect, were tested for antagonism. Antagonists were defined as ligands that were able to repeatedly block the inhibitory effect of 1 nM etorphine on forskolin-stimulated cAMP production. Antagonist was added to the cells along with IBMX. After 15 minutes of incubation, the agonist was added and the cells were incubated with both drugs

for an additional 10 minutes. The rest of the assay was as described above.

### Curve fitting

The analysis of drug activity was performed using PRISM software (GraphPad Software, Inc. San Diego, CA). A computer-generated "best fit" of non-linear regression data was used to provide an estimate of the inhibitory concentration at 50% (IC<sub>50</sub>). Dose response data generated by cAMP enzyme immunoassay (EIA) system were fitted to the one site competition function.

### Data processing

Data from each dose response curve were normalized to the top of the respective curve. The normalized data from multiple dose response curves were combined and a new dose response curve was fitted to the combined data and the IC<sub>50</sub> and maximal inhibition were determined for the combined data.

### Drugs

Forskolin, fentanyl, IBMX, hydromorphone, and naltrexone were obtained from Sigma-Aldrich (St. Louis, MO), nalbuphine and  $\beta$ -endorphin were obtained from RBI (Natick, MA), cyclazocine, etorphine, metazocine, morphine, nalorphine, and SKF 10047 were obtained from National Institute of Drug Abuse (Bethesda, MD), lofentanil was from Janssen Pharmaceutical Inc. (Titusville, NJ), Dezocine was from Wyeth Laboratories (Philadelphia, PA), Win 444,441 was from Sterling Winthrop Pharmaceutical and Xorphanol was from Miles Inc. Pharmaceutical Division (West Haven, CT). All tissue culture reagents were purchased from Life Technologies (Rockville, MD). DAMGO was obtained from Peninsula Laboratories (San Carlos, CA), and [<sup>3</sup>H]-DAMGO was from Multiple Peptide Systems (San Diego, CA). All other reagents were of analytical grade from standard commercial sources. All ligands used were prepared as 10 mM stock solutions in water except WIN 44,441, which was 5 mM. All ligands were dissolved in distilled water except cyclazocine, dezocine, etorphine, which were dissolved in 100% ethanol. For the drugs dissolved in ethanol, the final concentration of ethanol was <0.01% which had no effect on the assays performed.

### Statistical Analysis

Maximal inhibitory effect of each ligand was compared to the levels of maximal inhibition by  $\beta$ -endorphin, fentanyl, morphine and xorphanol using ANOVA analysis with Dunnett's multiple comparison as post-test using PRISM software (GraphPad Software, Inc. San Diego, CA). Significant difference between the inhibitory effects of two ligands was determined whenever  $p < 0.05$ .

### Authors' contributions

PG carried out some of the cAMP assays, performed data and statistical analysis and drafted the manuscript. HD carried out a large portion of the cAMP assays and data analysis work. The contributions of the first two authors were equal to the overall goals of the study. JDC provided intellectual input and critical interpretation of the data. JL conceived of the study, participated in its design and coordination, carried out the binding assays and finalized the manuscript for publication.

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