Pharmacology, Biochemistry and Behavior 91 (2008) 115–120

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

# The analgesic efficacy of fentanyl: Relationship to tolerance and μ-opioid receptor regulation

Sunil Sirohi [a], Shveta V. Dighe [a], Ellen A. Walker [b], Byron C. Yoburn [a,*]

[a] Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Queens, NY 11439, USA
[b] Department of Pharmaceutical Sciences, School of Pharmacy, Temple University, 3307 North Broad Street, Philadelphia, PA 19140, USA

ARTICLE INFO

*Article history:*
Received 7 April 2008
Received in revised form 18 June 2008
Accepted 24 June 2008
Available online 30 June 2008

*Keywords:*
Fentanyl
Opioid agonist
Analgesia
Efficacy
Tolerance
μ-Opioid receptor
Receptor downregulation

ABSTRACT

This study determined if fentanyl analgesic efficacy predicts the magnitude of tolerance and μ-opioid receptor regulation. To estimate efficacy, mice were injected i.p. with saline or clocinnamox (CCAM), an irreversible μ-opioid receptor antagonist, (0.32–25.6 mg/kg) and 24 h later fentanyl cumulative dose–response studies were conducted. CCAM dose dependently shifted the fentanyl dose–response function to the right. The apparent efficacy ($\tau$) of fentanyl, based on the operational model of agonism, was estimated as 58, indicating that fentanyl is a high analgesic efficacy agonist. Next, mice were infused with fentanyl (1, 2 or 4 mg/kg/day) for 7 days. Controls were implanted with placebo pellets. At the end of 7 days, morphine cumulative dose–response studies or μ-opioid receptor saturation binding studies were conducted. Fentanyl infusions dose dependently decreased morphine potency with the highest fentanyl dose reducing morphine potency by ≈6 fold. Chronic infusion with fentanyl (4 mg/kg/day) significantly reduced μ-opioid receptor density by 28% without altering affinity, whereas lower infusion doses had no effect. Taken together, the present results strengthen the proposal that opioid analgesic efficacy predicts μ-opioid receptor regulation and the magnitude of tolerance.



## 1. Introduction

Efficacy has been defined as the ability of a drug to impart a physiological response to a biological system at a given receptor occupancy (Kenakin, 2002). Recent formulations of this concept have suggested that there are multiple efficacies for ligands at the same receptor; a property which has been variously termed 'functional selectivity', 'biased agonism' and 'protean agonism' (e.g., Kenakin, 2007; Galandrin and Bouvier, 2006; Mailman, 2007). Consistent with this concept, opioid analgesics have been shown to vary in terms of efficacy for a variety of effects. For example, opioid agonists have differential effects on μ-opioid receptor regulation (e.g., internalization, downregulation), regulation of trafficking proteins (e.g., β-arrestin, Dynamin) and the magnitude of tolerance (Bohn et al., 2004; Duttaroy and Yoburn, 1995; Keith et al., 1998; Whistler and von Zastrow, 1998; Pawar et al., 2007).

Previously, opioid agonist efficacy has been considered as a characteristic of a drug irrespective of the outcome measure. Many experiments using this unitary measure of efficacy have suggested that chronic treatment (e.g., continuous s.c. infusion) with agonists proposed as having higher efficacy (e.g., etorphine, DAMGO) can induce internalization and downregulation of μ-opioid receptors in vitro and in vivo (Keith et al., 1998; Duttaroy and Yoburn, 1995; Pawar et al., 2007; Stafford et al., 2001; Whistler and von Zastrow, 1998). Conversely, agonists with lower efficacy (e.g., morphine, oxycodone) are less likely to produce either internalization or downregulation (Keith et al., 1998; Pawar et al., 2007; however; see Haberstock-Debic et al., 2005; Rodríguez-Muñoz et al., 2007). Furthermore, the ability of opioid analgesics to internalize and downregulate μ-opioid receptors appears to be inversely related to the magnitude of tolerance. Higher efficacy opioid agonists produce substantially less tolerance in vivo than lower efficacy opioid agonists at equi-effective (e.g., equi-multiples of the $ED_{50}$) doses (Duttaroy and Yoburn, 1995; Paronis and Holtzman, 1992; Pawar et al., 2007; Walker and Young, 2001).

In a previous report (Pawar et al., 2007), we have used the operational model of agonism (Black and Leff, 1983) to estimate the analgesic efficacy of several opioid analgesics. This model, which is based on the use of an irreversible antagonist to deplete receptors, calculates the parameter $\tau$. $\tau$ is used to compare analgesic efficacy; with larger values representing agonists with greater efficacy. Morphine and oxycodone were found to have relatively low $\tau$ values, whereas etorphine was identified as a higher efficacy opioid analgesic. This more quantitative estimate of analgesic efficacy confirmed previous results suggesting a relationship between opioid efficacy, μ-opioid receptor regulation and tolerance (e.g., Duttaroy and Yoburn, 1995; Paronis and Holtzman, 1992; Pawar et al., 2007).

In the present study, we have extended this approach to the clinically used opioid analgesic fentanyl. The analgesic efficacy of fentanyl was quantitatively estimated (i.e., $\tau$) and this was used to predict tolerance and μ-opioid receptor regulation. In addition, in a previous paper

* Corresponding author. Tel.: +1 718 990 1623; fax: +1 718 990 6036.
*E-mail address:* yoburnb@stjohns.edu (B.C. Yoburn).

0091-3057/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2008.06.019

(Duttaroy and Yoburn, 1995), we reported that opioid efficacy was not a major factor in predicting the magnitude of tolerance when opioid analgesics were administered intermittently. Therefore, in this study we also examined tolerance and μ-opioid receptor regulation following intermittent treatment with fentanyl.

## 2. Materials and methods

### 2.1. Subjects

Male Swiss Webster mice (23–35 g) obtained from Taconic Farms (Germantown, NY), were used throughout this study. Animals were housed 10 per cage for at least 24 h after arrival with food and water available *ad libitum*. All protocols were approved by the St John's University Institutional Animal Care and Use Committee.

### 2.2. General procedure

Initially, the analgesic $ED_{50}$ (tailflick; see below) was estimated using standard and cumulative dosing protocols (see below), at the time of peak effect of fentanyl. Next, mice were injected i.p. with saline (8–10/group) or CCAM (0.32–25.6 mg/kg) (5–10/group) and 24 h later fentanyl cumulative dose–response studies were conducted. In subsequent studies, tolerance to morphine following fentanyl treatment was determined. Morphine was used as the test drug, as we have done previously, so that the magnitude of potency shifts induced by fentanyl could be directly compared among opioid drugs (see Pawar et al., 2007). For chronic treatment studies, mice (8–10/group) were implanted s.c. with osmotic mini pumps (Alzet Model 2001; DURECT Corporation, CA) infusing fentanyl (1, 2 or 4 mg/kg/day) for 7 days. Controls were implanted with placebo pellets. At the end of 7 days, pumps and pellets were removed and 16 h later morphine cumulative dose–response studies were conducted or, 16–24 h later mice were sacrificed and spinal cords were collected for radioligand binding studies. For acute (single injection) and intermittent (one injection/day for 7 days) studies, mice (8–10/group) were injected with fentanyl (100 or 200 μg/kg) and 24 h later, morphine cumulative dose–response studies were conducted or mice were sacrificed and spinal cords were collected for radioligand binding studies. Pump and pellet implantation and removal were conducted while mice were lightly anesthetized with isoflurane: oxygen (4:96).

### 2.3. Analgesia assay

Antinociception was assayed using the tailflick assay (Model TF6, Emdie Instrument Co., Maidens, VA), in which a beam of light was focused on the dorsal surface of the tail of the mouse, approximately 2 cm from the tip of the tail. The intensity of the light was adjusted so that baseline tailflick latency was 1–3 s. If a mouse did not remove its tail from the heat source by 10 s, the test was terminated, a latency of 10 s was recorded and the mouse was defined analgesic. All testing was conducted by an experimenter who was unaware of the treatment of an individual mouse.

### 2.4. Dose–response protocol

Dose–response studies were conducted according to standard and cumulative dosing protocols (see Duttaroy et al., 1997). Briefly, in the standard dosing protocol, mice (5–6/dose) were injected with fentanyl (10–40 μg/kg) and tested once for analgesia at the time of peak effect of fentanyl (15 min; as determined previously).

In the cumulative dosing protocol (available on request), mice (8/group) were injected s.c. with a starting dose of morphine or fentanyl and tested for analgesia 30 and 15 min later, respectively. If the mouse displayed a tailflick latency of 10 s, it was defined as analgesic and not tested further. Otherwise, the mouse was immediately injected s.c. with a second dose (increment dose) of morphine or fentanyl and retested. This procedure was continued until all mice were analgesic. The cumulative dosing protocol is useful to reduce the number of mice required and the cost of supplies (e.g. osmotic pumps).

### 2.5. Radioligand binding studies

Mice (10/dose) were infused with fentanyl (1, 2 or 4 mg/kg/day) for 7 days. For all treatment groups, controls were implanted with placebo pellets for 7 days. At the end of treatment, the pumps and pellets were removed and 16 or 24 h later mice were sacrificed. Other mice (10/dose) were injected s.c. with fentanyl 200 μg/kg once a day for 7 days. Controls were injected s.c. with saline. Twenty four hours after the last injection, mice were sacrificed and spinal cords were rapidly removed. Spinal cord was used to examine changes in μ-opioid receptors since numerous studies have shown that opioids produce analgesic effects at the spinal level, and this tissue is readily removed and relatively homogenous compared to brain (e.g., Shah et al., 1994; Patel et al., 2003). Spinal cords were pooled in tubes containing 15 ml ice cold 50 mM TRIS buffer (pH 7.4). Samples were homogenized (Brinkmann Polytron Homogenizer, Westbury, NY) at 20,000 rpm on ice for 40 s. Homogenates were centrifuged at 15,000 rpm (≈26,000 ×g) for 15 min (3–9 °C). The supernatant was discarded and pellets were stored (−80 °C) until analysis. On the day of assay, the pellet was thawed on ice, re-suspended in 15 ml TRIS buffer and centrifuged at 15,000 rpm for 15 min (3–9 °C) and the supernatant was discarded. The pellet was re-suspended in 35 ml TRIS buffer and incubated for 30 min in a shaking water bath (25 °C). Samples were centrifuged again for 15 min, the supernatant was discarded and pellets were suspended in 18 ml of ice cold 50 mM potassium phosphate buffer (pH 7.2). An aliquot of homogenate was assayed in triplicate in saturation binding assays using [$^3$H] DAMGO (range: 0.02–10 nM) (μ-opioid receptor ligand; PerkinElmer Life Sciences, Boston, MA). Non-specific binding was determined in triplicate in the presence of levorphanol (1000 nM). Tubes were incubated for 90 min at 25 °C. Incubation was terminated by the addition of ice cold phosphate buffer and filtering the samples over GF/B filters (Brandel, Gaithersburg, MD). Filters were washed three times with phosphate buffer and than placed in vials containing scintillation cocktail and counted. Counts per minute (cpm) were converted into disintegration per minute (dpm) using the external standard method. Protein was assayed by the Bradford method (Bradford, 1976) using reagent from Bio-Rad (Richmond, CA). Binding studies were conducted 1–8 times for each treatment.

### 2.6. Drugs

Fentanyl hydrochloride was obtained from the Sigma Chemical Company (St. Louis, MO). Morphine sulphate and inert placebo pellets were obtained from the Research Triangle Institute (Research Triangle Park, NC). Clocinnamox mesylate was obtained from Tocris Bioscience (Ellisville, MO). Inert placebo pellets were wrapped in nylon mesh before implantation. Fentanyl and morphine were dissolved in 0.9% saline and doses are expressed as the free base. Clocinnamox (CCAM) was dissolved in $dH_2O$ with ≈4% DMSO (dimethyl sulfoxide) to enhance solubility and CCAM doses are expressed as the salt.

### 2.7. Data analysis

Dose–response studies were analyzed as quantal (percent analgesic) or graded (tailflick latency) data. The estimation of agonist $ED_{50}$ necessitates the use of quantal data. Quantal dose–response data were analyzed with the program BLISS-21 (Department of Statistics, University of Edinburgh). This program uses Probit Analysis (Finney, 1973) to calculate $ED_{50}$ values, standard errors, potency ratios and 95% confidence intervals. Potency changes are based on the ratio of the $ED_{50}$ value in the treated groups relative to control. This change is referred to as the shift in

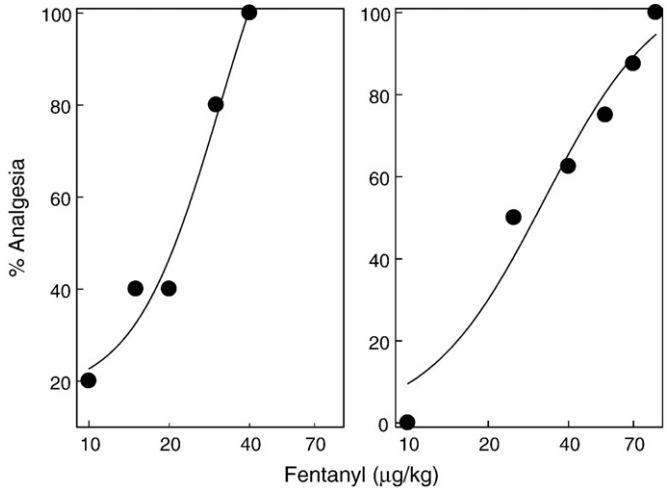


**Fig. 1.** Dose–response functions for fentanyl using the standard (left) and cumulative (right) dose–response protocols. For standard dosing, mice (5–6/dose) were injected s.c. with fentanyl as described in the methods. For cumulative dosing, mice ($N$=8) were injected cumulatively s.c. with fentanyl as described in the methods. The $ED_{50}$s for standard dosing for fentanyl from 2 independent studies were 18.1 and 22.0 μg/kg. The mean (±sem) $ED_{50}$ for cumulative dosing for fentanyl was estimated as 40.8±5.2 μg/kg based on 8 independent studies. Data presented are the results of a single representative experiment for each protocol. Dose–response data were fit using nonlinear regression (4 parameter logistic) using Sigmaplot (version 10; Systat Software, Inc., San Jose, CA).

the $ED_{50}$. The calculation of agonist efficacy following receptor depletion using the operational model requires graded data (see details below). Dose–response data were fit using nonlinear regression (4 parameter logistic) using Sigmaplot (version 10; Systat Software, Inc., San Jose, CA).

The operational model of agonism (Black and Leff, 1983; Black et al., 1985; Leff et al., 1990), allows quantitative comparison of various agonists in terms of efficacy. In this model, apparent efficacy is represented by $\tau$, a dimensionless proportionality factor which is generally considered to be analogous to traditional estimates of efficacy (Motulsky and Christopoulos, 2004). In this model, as in other models that estimate efficacy (Furchgott, 1966; Furchgott and Bursztyn, 1967), the experimental protocol requires partial receptor inactivation or depletion. Clocinnamox (CCAM), an irreversible μ-opioid receptor antagonist is a reasonable choice for estimating $\tau$ since it has been shown to dose dependently decrease the μ-opioid receptor density (Burke et al., 1994; Chan et al., 1995).

Calculation methods for the operational model of agonism described by Zernig et al. (1996) were employed to estimate the efficacy of fentanyl. $\tau$ is equal to the ratio of the total concentration of receptors ($R_o$) and the concentration of receptor needed to be occupied by an agonist to provoke half maximal response ($K_E$). The irreversible μ-opioid receptor antagonist CCAM was used to deplete the fraction of receptors available for an agonist to interact which can be denoted as $q$ (Furchgott, 1996). The $q$ value can be obtained by dividing the $\tau$ value of a CCAM treated group by the $\tau$ value of control. According to Black et al., 1985, non-rectangular hyperbolic curves relate agonist dose [$A$] to the effect $E$:

$$E = \frac{E_m \times \tau^n \times [A]^n}{(K_A + [A])^n + \tau^n \times [A]^n}$$

Where $E$ is the graded effect (mean tailflick latency), $E_m$ is the maximum attainable response (analgesia) (10 s). [$A$] is the dose of agonist, $K_A$ is the apparent dissociation constant, and $n$ is the slope factor of the transducer function. A semi-logarithmic form of the above equation (Zernig et al., 1995) is used that incorporates a new constant $c$, which is the baseline response (base line tailflick latency in the present case), and replaces $\tau$ with ($q \times \tau_{Control}$):

$$E = E_m \Big/ \left( \left( \left( 10^{(\log(K_A) - \log[A])} + 1 \right) / (q^* \tau_{control}) \right)^n + 1 \right) + c$$

Using a non linear fitting program developed by Zernig et al. (1995), and a general mathematical software package (Mathematica, Wolfram Research, Champaign, USA, Wolfram 1991), all fentanyl dose–response curves obtained from controls and CCAM treated groups were simultaneously fit to the above equation.

Binding data from saturation studies were analyzed by Prism ver 5.01 (Graph Pad Software, San Diego, CA) using nonlinear regression. All binding data were best fit by a one-site model.

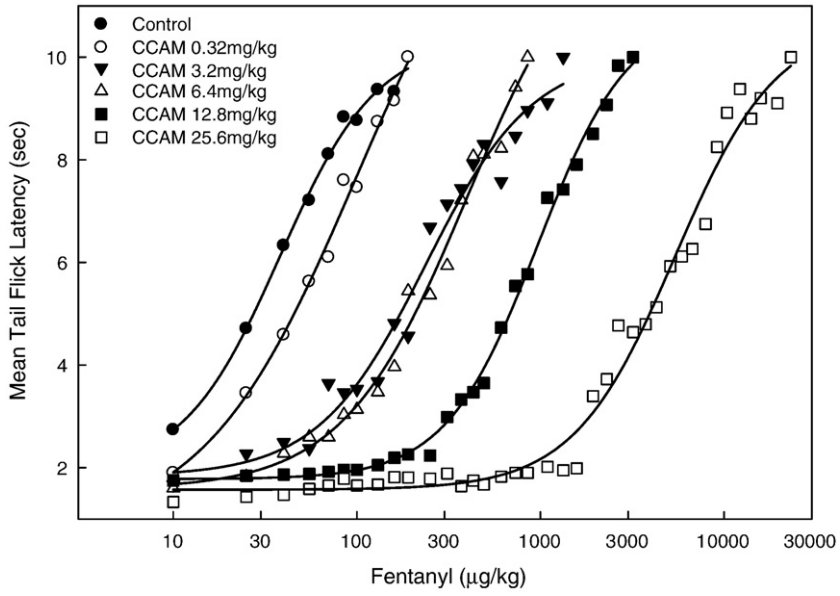


**Fig. 2.** The effect of clocinnamox (CCAM) pretreatment on fentanyl analgesia. Mice were injected with saline ($N$=8–10) or 0.32–25.6 mg/kg CCAM ($N$=5–10/CCAM dose) i.p., and 24 h later, fentanyl cumulative dose–response studies (see methods) were conducted. Each CCAM dose was evaluated with a corresponding saline treated group (control). The results for each CCAM dose were determined 1–3 times. The data presented are the combined mean tail flick latencies from all experiments. Dose–response data were fit using nonlinear regression (4 parameter logistic) using Sigmaplot (version 10; Systat Software, Inc., San Jose, CA).

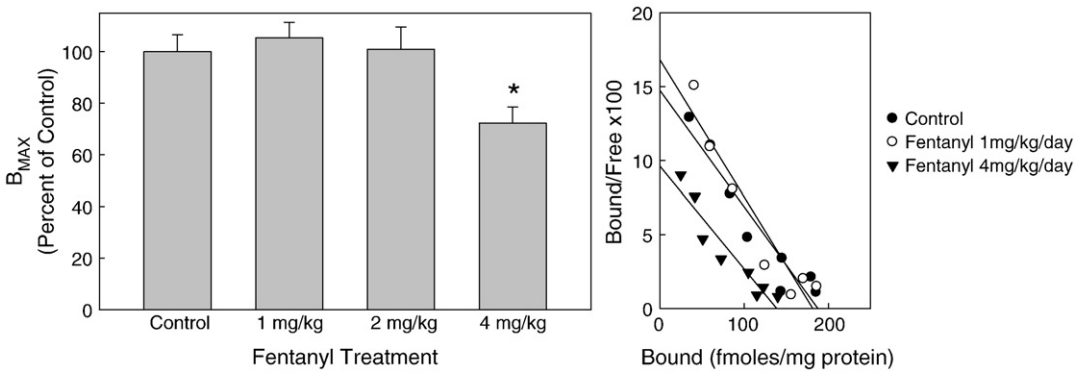


**Fig. 3.** The effect of chronic fentanyl treatment on μ-opioid receptor density in mouse spinal cord. Mice (10/group) were infused s.c. with fentanyl (1, 2 or 4 mg/kg/day) for 7 days. Controls were implanted with placebo pellets. At the end of treatment, pumps and pellets were removed and 16–24 h later mice were sacrificed. Spinal cords were removed and assayed in [$^3$H] DAMGO saturation binding studies (see materials and methods). The histogram (left panel) presents the mean $B_{MAX}$ (±sem) as percent change from control from 8 independent experiments. A representative scatchard plot for control and fentanyl (1 or 4 mg/kg/day) is shown to the right of the histogram. * Significantly different from control ($p<0.05$).

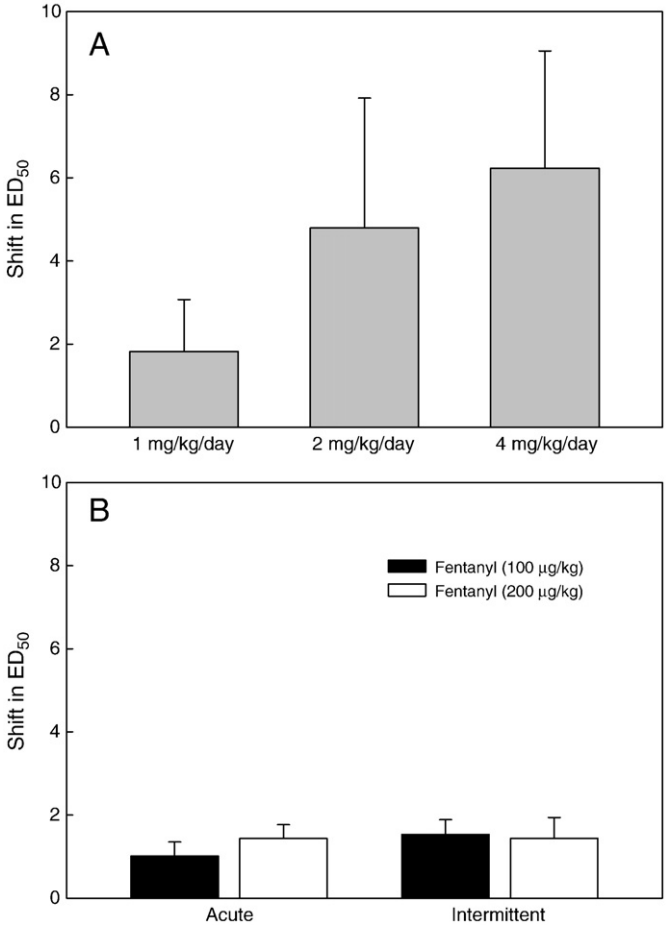


**Fig. 4.** The effect of fentanyl treatment on morphine analgesic potency. A. The effect of 7 days fentanyl infusion on morphine potency. Mice (8–10/group) were infused with fentanyl (1, 2 or 4 mg/kg/day) for 7 days. Controls were implanted with placebo pellets. At the end of treatment, pumps and pellets were removed and 16 h later morphine cumulative dose–response studies were conducted. The histograms show the shift relative to control in morphine's $ED_{50}$ (+upper 95%CL) for each fentanyl dose from one experiment for each dose. B. Effect of acute (single dose) or 7 days intermittent fentanyl treatment on morphine potency. Mice (8–10/group) were injected s.c. once (acute) or once a day for seven days (intermittent) with fentanyl (100 and 200 μg/kg). Controls were injected s.c. with saline. Twenty four hours after the last injection, morphine cumulative dose–response studies were conducted. The histogram shows the shift in morphine's $ED_{50}$ (+sem) for each dose based on 3 experiments for each fentanyl dose.

## 3. Results

Both standard and cumulative dosing protocols were used to determine the $ED_{50}$ for fentanyl (Fig. 1). The $ED_{50}$ for fentanyl in two independent studies using the standard dose protocol was 18.1±0.07 and 22.0±0.06 μg/kg (±SE from Probit Analysis). The mean (±sem) $ED_{50}$ for fentanyl in 8 independent studies for controls using the cumulative dosing protocol was 40.8±5.2 μg/kg (Fig. 1). Next, the efficacy of fentanyl was determined using CCAM (Fig. 2). Mice were injected with CCAM and 24 h later fentanyl cumulative dose–response studies were conducted. CCAM dose dependently shifted the fentanyl dose–response function to the right. The apparent efficacy ($\tau$) of fentanyl based on the operational model of agonism was estimated as 58 (54–62, 95% CL).

To examine the effect of fentanyl on μ-opioid receptor density in spinal cord, mice were infused with fentanyl (1, 2 or 4 mg/kg/day) for 7 days or injected once/day for 7 days (200 μg/kg/day). Chronic infusion with fentanyl (4 mg/kg/day) significantly reduced μ-opioid receptor density by 28% without altering affinity, whereas 1 or 2 mg/kg/day fentanyl infusions had no significant effect (Fig. 3). When mice were injected daily for 7 days with fentanyl (200 μg/kg) there were no significant changes in $B_{MAX}$ or $K_D$ (data not shown). Daily injections of fentanyl at doses higher than 200 μg/kg could not be used because of lethality (≈10–20% lethality at 500 μg/kg).

The effect of 7 day fentanyl treatment on morphine potency was examined (Fig. 4). Fentanyl infusions (1, 2 or 4 mg/kg) dose dependently decreased morphine potency with the highest fentanyl dose reducing morphine potency by ≈6 fold. However, neither acute nor 7 days of intermittent fentanyl (100 or 200 μg/kg) injections had any significant effect on morphine potency.

## 4. Discussion

Opioid agonist efficacy has been proposed to be an important factor in μ-opioid receptor regulation and in determining the magnitude of opioid tolerance. Previous studies have shown that higher efficacy opioid agonists downregulate μ-opioid receptors in vivo and, when infused at equi-effective doses, produce less tolerance compared to lower efficacy agonists (e.g., Duttaroy and Yoburn, 1995; Pawar et al., 2007). To most directly address the potential role of opioid agonist efficacy in tolerance and receptor regulation, it is important to accurately quantitate efficacy. In the current study, we used a modification of the operational model of agonism (Zernig et al., 1995; 1996) to estimate the efficacy ($\tau$) of fentanyl to produce analgesia in the tailflick assay in the mouse. We have previously used this approach to estimate analgesic efficacy for morphine, etorphine and oxycodone (Pawar et al., 2007). In that study, the analgesic efficacy of these agonists was predictive of the

magnitude of tolerance and opioid agonist induced downregulation of spinal μ-opioid receptors.

In the present study, the efficacy of fentanyl ($\tau$=58) in the mouse tailflick assay was found to be relatively high compared to oxycodone and morphine (Pawar et al., 2007). This outcome agrees with a study that examined the analgesic efficacy of fentanyl relative to morphine using the operational model (Zernig et al., 1995). Similarly, data from other studies that did not quantitatively estimate efficacy, suggest that fentanyl is a high analgesic efficacy agonist compared to morphine (e.g., Adams et al., 1990). On the other hand, fentanyl's relative efficacy in the GTPγS binding assay is generally similar to that of morphine, although this can vary depending on the cells or tissue examined (e.g., Clark et al., 2006; Peckham and Traynor, 2006; Selley et al., 1997, 1998). However, in a drug discrimination procedure in the pigeon, fentanyl was reported to have a relatively low $\tau$ value, although etorphine retained its high efficacy in that assay (Barrett et al., 2003). Taken together, these data indicate that relative efficacy of opioid agonists can vary depending on the outcome measure, which is consistent with the concept of functional selectivity (Mailman, 2007). Thus, it is important to estimate efficacy for each effect, rather than to assume that efficacy determined in one assay, or in one tissue source, accurately reflects efficacy for other effects.

Since the $\tau$ value for fentanyl indicated it is a high efficacy agonist in a model of analgesia in the mouse, we predicted that it would be effective in downregulating μ-opioid receptors. Consistent with previous studies demonstrating fentanyl induced μ-opioid receptor internalization and downregulation (Keith et al., 1998; Koch et al., 2005; Yoburn et al., 1993), fentanyl infusion was found to downregulate spinal μ-opioid receptors. We have previously shown that etorphine which has a comparable $\tau$ value (52) to fentanyl, also effectively downregulates spinal μ-opioid receptors (Pawar et al., 2007; Patel et al., 2002). In addition, we predicted that fentanyl would produce less tolerance than low analgesic efficacy opioids when infused at equi-effective doses (i.e., equi-multiples of the analgesic $ED_{50}$). To evaluate this prediction, Fig. 5 plots the shift in the $ED_{50}$ for fentanyl, as well as oxycodone and etorphine ($\tau$=20, 52, respectively; data from Pawar et al., 2007). As expected of a higher efficacy opioid agonist, fentanyl behaved similarly to etorphine and produced less tolerance than oxycodone, a lower efficacy agonist. Therefore, the current results with fentanyl are consistent with the inverse relationship between $\tau$ and tolerance; and the direct relationship between $\tau$ and μ-opioid receptor downregulation in vivo. Taken together, these results, and the results of previous studies (e.g., Keith et al., 1998; Pawar et al., 2007) are consistent with the concept of functional selectivity (Mailman, 2007) since opioid agonists can produce antinociception and receptor regulation (e.g., etorphine, fentanyl), while others, acting at the same receptor, produce only antinociception (e.g., morphine, oxycodone).

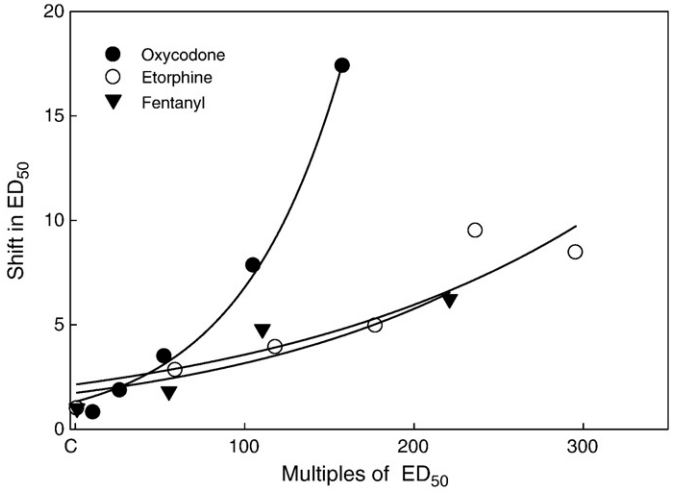


**Fig. 5.** The effect of continuous infusions with 3 opioid analgesics on morphine's analgesic potency. The results from the present experiments on fentanyl are compared with those of oxycodone and etorphine from previous studies from our lab (Pawar et al., 2007). The shift in $ED_{50}$ was calculated as $ED_{50}$ treated/$ED_{50}$ control (C). To compare data statistically each data set was fit using nonlinear regression (Prism ver 5.01, Graph Pad Software, San Diego, CA) with the equation:

$$y = ae^{kX}$$

where $y$ is the shift in the $ED_{50}$; $a$ is the $y$-intercept; and $k$ is the rate constant. $a$ values (±sem) for oxycodone, etorphine and fentanyl were 1.32±0.17, 2.14±0.71 and 1.74±0.72 respectively were not significantly different ($p>0.05$). The $k$ value (±sem) for oxycodone, etorphine and fentanyl were 0.016±0.001, 0.005±0.001 and 0.006±0.002. The $k$ for oxycodone was significantly ($p<0.05$) greater than for fentanyl and etorphine.

The $ED_{50}$ for fentanyl in the mouse tailflick assay was estimated using both standard and cumulative dosing protocols. The standard dosing protocol typically provides an accurate estimate of the $ED_{50}$. On the other hand, the $ED_{50}$ determined by cumulative dosing is dependent on the dosing schedule (starting dose and increment doses) and can vary substantially if different doses are used (Duttaroy et al., 1997). Therefore, we used the standard $ED_{50}$ value in this and previous studies (Duttaroy and Yoburn, 1995; Pawar et al., 2007) as the metric for determining equi-effective treatments as the multiple of the $ED_{50}$ (see Fig. 5). This allows relatively straight-forward comparison of dosing among various opioids. In addition, tolerance following fentanyl treatment was examined using morphine, which is primarily a μ-opioid receptor agonist that lacks activity in μ-opioid receptor knockout mice (Kieffer, 1999). By using morphine to assess tolerance, the effects of treatment with various opioid agonists can be directly compared.

Earlier work from our lab indicated that tolerance is most robust following continuous treatment. Intermittent administration of several opioid agonists produces moderate tolerance that appears to be unrelated to efficacy and is not dramatically affected by agonist dose (Duttaroy and Yoburn, 1995). In the present study, we intended to examine this issue using acute and intermittent fentanyl dosing. However, when doses of 25 times the fentanyl $ED_{50}$ were used, this was lethal for ≈10–20% of mice. Data were available for 5 and 10 times the fentanyl $ED_{50}$ but this produced no tolerance and minimal changes in μ-opioid receptors, as we have reported previously (Duttaroy and Yoburn, 1995). We are currently examining the role of intermittency in tolerance using other opioid agonists. Nevertheless, taken together, fentanyl infusions dose dependently produced substantial tolerance while acute or intermittent fentanyl injections produced minimal tolerance in all dosing groups, irrespective of duration of treatment.

Various hypotheses have been proposed to explain why higher efficacy opioid agonists produce less tolerance than lower efficacy agonists at equi-effective dosing. One hypothesis suggests that the lower efficacy agonists occupy more receptors compared to higher efficacy agonists to produce a desired effect. Thus, more receptors are desensitized by lower efficacy agonists (Waldhoer et al., 2004). Others have suggested that lower efficacy agonists don't regulate trafficking proteins, such as dynamins and GRK and hence do not induce receptor internalization (Patel et al., 2002; Stafford et al., 2001; Martini and Whistler, 2007). Internalization has been suggested to be a process by which a desensitized receptor undergoes endocytosis and subsequent dephosphorylation and resensitization (Ferguson et al., 1996, 1998). Failure of an agonist to promote receptor internalization may increase tolerance, as the densensitized receptor remains at the plasma membrane, and is not resensitized. Other explanations have focused on more robust development of compensatory signaling by lower efficacy agonists (Whistler et al., 1999). Recent studies raise the possibility that high efficacy opioids, including fentanyl, may engage different signaling pathways and that this may be important in inducing tolerance (e.g., Zheng et al., 2008). In any case, it is clear that a reduction in receptor density is not required for, but may contribute to, opioid tolerance (Stafford et al., 2001; Pawar et al., 2007). Thus, both "receptor-density independent mechanisms" (desensitization) and "receptor-density dependent mechanisms" (downregulation) can contribute tolerance (see

Stafford et al., 2001). The mechanism by which acute and intermittent treatment may produce reduced tolerance is not entirely clear. However, this may be related to periodic versus continuous opioid receptor occupancy. Relatively short periods of activation of receptors and downstream events might engage the mechanisms for opioid tolerance in a less than maximal manner.

In conclusion, the current study demonstrates that fentanyl is a higher efficacy opioid analgesic. Fentanyl infusion produced μ-opioid receptor downregulation and tolerance similar to that of other high efficacy opioid agonists. These results support suggestions that opioid analgesic efficacy is predictive of μ-opioid receptor regulation and tolerance.

## Acknowledgements

The authors are very grateful to H.H. Rajinder Singh Ji. Dr. M.T. Turnock provided continuous encouragement during this study. Supported in part by a grant from the National Institute on Drug Abuse (DA 19959, to BCY).

## References

Adams JU, Paronis CA, Holtzman SG. Assessment of relative intrinsic activity of mu-opioid analgesics in vivo by using beta-funaltrexamine. J Pharmacol Exp Ther 1990;255:1027–32.
Barrett AC, Smith ES, Picker MJ. Use of irreversible antagonists to determine the relative efficacy of mu-opioids in a pigeon drug discrimination procedure: comparison of beta-funaltrexamine and clocinnamox. J Pharmacol Exp Ther 2003;305:1061–70.
Black JW, Leff P, Shankley NP, Wood J. An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. Br J Pharmacol 1985;84:561–71.
Black JW, Leff P. Operational models of pharmacological agonism. Proc R Soc Lond B Biol Sci 1983;220:141–62.
Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, Barak LS. Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. Mol Pharmacol 2004;66:106–12.
Bradford MM. A refined and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
Burke TF, Woods JH, Lewis JW, Medzihradsky F. Irreversible opioid antagonist effects of clocinnamox on opioid analgesia and mu receptor binding in mice. J Pharmacol Exp Ther 1994;271:715–21.
Chan K, Brodsky M, Davis T, Franklin S, Inturrisi CE, Yoburn BC. The effect of the irreversible mu-opioid receptor antagonist clocinnamox on morphine potency, receptor binding and receptor mRNA. Eur J Pharmacol 1995;287:135–43.
Clark MJ, Furman CA, Gilson TD, Traynor JR. Comparison of the relative efficacy and potency of mu-opioid agonists to activate Galpha(i/o) proteins containing a pertussis toxin-insensitive mutation. J Pharmacol Exp Ther 2006;317:858–64.
Duttaroy A, Yoburn BC. The effect of intrinsic efficacy on opioid tolerance. Anesthesiol 1995;82:1226–36.
Duttaroy A, Kirtman R, Farrell F, Phillips M, Philippe J, Monderson T, et al. The effect of cumulative dosing on the analgesic potency of morphine in mice. Pharmacol Biochem Behav 1997;58:67–71.
Ferguson SS, Downey WE, Colapietro AM, Barak LS, Ménard L, Caron MG. Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 1996;271:363–6.
Ferguson SS, Zhang J, Barak LS, Caron MG. Role of beta-arrestins in the intracellular trafficking of G-protein-coupled receptors. Adv Pharmacol 1998;42:420–4.
Finney DJ. Probit analysis. 3rd ed. London: Cambridge University Press; 1973.
Furchgott RF, Bursztyn P. Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. Ann NY Acad Sci 1967;144:882–98.
Furchgott RF. The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor–agonist complexes. In: Harper NJ, Simmonds AB, editors. Advances in drug research, vol. 3. Academic Press; 1966. p. 21–55.
Galandrin S, Bouvier M. Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. Mol Pharmacol 2006;70:1575–84.
Haberstock-Debic H, Kim KA, Yu YJ, von Zastrow M. Morphine promotes rapid, arrestin-dependent endocytosis of μ-opioid receptors in striatal neurons. J Neurosci 2005;25:7847–57.
Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, et al. mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. Mol Pharmacol 1998;53:377–84.
Kenakin T. Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. Trends Pharmacol Sci 2007;28:407–15.
Kenakin T. Drug efficacy at G protein-coupled receptors. Annu Rev Pharmacol Toxicol 2002;42:349–79.
Kieffer BL. Opioids: first lessons from knockout mice. Trends Pharmacol Sci 1999;20:19–26.
Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, et al. Receptor endocytosis counteracts the development of opioid tolerance. Mol Pharmacol 2005;67:280–7.
Leff P, Prentice DJ, Giles H, Martin GR, Wood J. Estimation of agonist affinity and efficacy by direct, operational model-fitting. J Pharmacol Methods 1990;23:225–37.
Mailman RB. GPCR functional selectivity has therapeutic impact. Trends Pharmacol Sci 2007;28:390–6.
Martini L, Whistler JL. The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. Curr Opin Neurobiol 2007;17:556–64.
Motulsky HJ, Christopoulos A. Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting. New York: Oxford University Press; 2004.
Paronis CA, Holtzman SG. Development of tolerance to the analgesic activity of mu agonists after continuous infusion of morphine, meperidine or fentanyl in rats. J Pharmacol Exp Ther 1992;262:1–9.
Patel MB, Patel CN, Rajashekara V, Yoburn BC. Opioid agonists differentially regulate mu-opioid receptors and trafficking proteins in vivo. Mol Pharmacol 2002;62:1464–70.
Patel CN, Rajashekara V, Patel K, Purohit V, Yoburn BC. Chronic opioid antagonist treatment selectively regulates trafficking and signaling proteins in mouse spinal cord. Synapse 2003;50:67–76.
Pawar M, Kumar P, Sunkaraneni S, Sirohi S, Walker EA, Yoburn BC. Opioid agonist efficacy predicts the magnitude of tolerance and the regulation of μ-opioid receptors and dynamin-2. Eur J Pharmacol 2007;563:92–101.
Peckham EM, Traynor JR. Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague–Dawley rats. J Pharmacol Exp Ther 2006;316:1195–201.
Rodríguez-Muñoz M, de la Torre-Madrid E, Sánchez-Blázquez P, Garzón J. Morphine induces endocytosis of neuronal mu-opioid receptors through the sustained transfer of Galpha subunits to RGSZ2 proteins. Mol Pain 2007;3:19.
Selley DE, Liu Q, Childers SR. Signal transduction correlates of mu opioid agonist intrinsic efficacy: receptor-stimulated [35S]GTP gamma S binding in mMOR-CHO cells and rat thalamu. J Pharmacol Exp Ther 1998;285:496–505.
Selley DE, Sim LJ, Xiao R, Liu Q, Childers SR. Mu-opioid receptor-stimulated guanosine-5′-O-(gamma-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. Mol Pharmacol 1997;51:87–96.
Shah S, Duttaroy A, Davis T, Yoburn BC. Spinal and supraspinal effects of pertussis toxin on opioid analgesia. Pharmacol Biochem Behav 1994;49:773–6.
Stafford K, Gomes AB, Shen J, Yoburn BC. Mu-opioid receptor downregulation contributes to opioid tolerance in vivo. Pharmacol Biochem Behav 2001;69:233–7.
Waldhoer M, Bartlett SE, Whistler JL. Opioid receptors. Annu Rev Biochem 2004;73:953–90.
Walker EA, Young AM. Differential tolerance to antinociceptive effects of mu opioids during repeated treatment with etonitazene, morphine, or buprenorphine in rats. Psychopharmacology (Berl) 2001;154:131–42.
Whistler JL, von Zastrow M. Morphine-activated opioid receptors elude desensitization by beta-arrestin. Proc Natl Acad Sci USA 1998;95:9914–9.
Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 1999;23:737–46.
Yoburn BC, Billings B, Duttaroy A. Opioid receptor regulation in mice. J Pharmacol Exp Ther 1993;265:314–20.
Zernig G, Issaevitch T, Broadbear JH, Burke TF, Lewis JW, Brine GA, et al. Receptor reserve and affinity of mu opioid agonists in mouse antinociception: correlation with receptor binding. Life Sci 1995;57:2113–25.
Zernig G, Issaevitch T, Woods JH. Calculation of agonist efficacy, apparent affinity, and receptor population changes after administration of insurmountable antagonists: comparison of different analytical approaches. J Pharmacol Toxicol Methods 1996;35:223–37.
Zheng H, Loh HH, Law PY. Beta-arrestin-dependent mu-opioid receptor-activated extracellular signal-regulated kinases (ERKs) translocate to nucleus in contrast to G protein-dependent ERK activation. Mol Pharmacol 2008;73:178–90.