In vivo homologous regulation of μ-opioid receptor gene expression in the mouse

Fatima Sehba, Alokesh Duttaroy, Sukrut Shah, Billy Chen, Joanne Carroll, Byron C. Yoburn *

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Queens, NY 11439, USA

Abstract

Regulation of the μ-opioid receptor gene by opioid analgesic drugs has not been observed in rats and mice following in vivo treatments that produce tolerance. Although in vivo heterologous regulation of μ-opioid receptor mRNA by non-opioid compounds has been reported, the failure to observe changes in μ-opioid receptor mRNA levels in vivo after treatment with opioid agonists raised the possibility that in vivo homologous regulation by agonists may not occur. Therefore, in the present study, the effect of a high intrinsic efficacy opioid receptor agonist on opioid receptor density, gene expression and tolerance was determined. Mice were infused with etorphine for 7 days using an osmotic minipump, then the pump was removed and studies conducted 16–168 h later. Etorphine (50–250 μg/kg/day) infusion produced significant dose-dependent tolerance to the analgesic (tailflick) effects of etorphine, as well as dose-dependent μ-opioid receptor downregulation in brain at 16 h following the end of the infusion. μ-opioid receptor density returned to control levels over a 168 h period following the end of etorphine (250 μg/kg/day) infusion. Similarly, the magnitude of tolerance decreased over the same period. Evaluation of changes in brain μ-opioid receptor mRNA 16 h following etorphine infusion indicated that there was dose-dependent increase in steady-state levels, with no significant change in GAPDH mRNA. The increase in μ-opioid receptor mRNA was ∼ 55–65% over control at the highest etorphine infusion dose. μ-opioid receptor mRNA returned to control levels over a 168 h period following the end of etorphine (250 μg/kg/day) infusion. These data suggest that the increase in μ-opioid receptor mRNA following the termination of etorphine treatment may drive the recovery of μ-opioid receptors. These data are the first demonstration of in vivo homologous regulation of μ-opioid receptor gene expression in the mouse by an opioid receptor agonist that produces tolerance and receptor downregulation. © 1997 Elsevier Science B.V.

Keywords: μ-Opioid receptor; Regulation of mRNA; Etorphine; Receptor downregulation; Analgesia; Tolerance

1. Introduction

An extensive literature indicates that in vivo opioid receptor density can be regulated by homologous (i.e. opioid) agents. Both opioid receptor agonists and antagonists can produce significant changes in opioid receptor density (μ, δ and κ). For example, in vivo treatment with an opioid receptor antagonist such as naloxone or naltrexone increases the density of opioid receptors (i.e. upregulation) without altering affinity and produces a coordinate increase in the potency of opioid receptor agonists (i.e. functional supersensitivity) (Zukin et al., 1982; Moudy et al., 1985; Tempel et al., 1985; Yoburn et al., 1989, 1995; Giordano et al., 1990). However, the source of new opioid binding sites following chronic antagonist exposure in vivo is not well-understood, since upregulation is not associated with increases in opioid (μ and δ) receptor gene expression (Chan et al., 1995; Jenab et al., 1995; Unterwald et al., 1995; Castelli et al., 1997; however, see Brodsky et al., 1995). Similar to chronic opioid receptor antagonist treatment, in vivo treatment with opioid receptor agonists does not appear to regulate opioid receptor gene expression (Kest et al., 1994, 1995; Brodsky et al., 1995; Buzas et al., 1996; Castelli et al., 1997; however, see Rønnekleiv et al., 1996). Taken together, data from studies using both opioid receptor agonists and antagonists indicate that there is little evidence of homologous regulation of opioid receptor mRNA using in vivo models.

* Corresponding author. Tel.: + 1-718-9901623; fax: + 1-718-9906036; e-mail: yoburn@mail.stjohns.edu.

Although homologous regulation of opioid receptor gene expression has not been reliably demonstrated in vivo, there are several reports that non-opioids (e.g. cocaine, haloperidol and other dopaminergic compounds) can regulate μ- and κ-opioid receptor density and mRNA in a variety of in vivo models (Hammer, 1989; Unterwald et al., 1992, 1994; Delfs et al., 1994; Azaryan et al., 1996a,b; Spangler et al., 1996). These data suggest that homologous agents do not, but heterologous agents do, regulate opioid receptor gene expression in vivo. In contrast, results from cell culture studies indicate that both homologous and heterologous agents regulate μ- and δ-opioid receptor mRNA and density (Charness et al., 1993; Jenab and Inturrisi, 1994; Abood and Tao, 1995; Kim et al., 1995; Beczkowska et al., 1996).

While treatment with opioid receptor agonists can produce both tolerance and receptor density changes (e.g. downregulation), opioid tolerance does not require the development of receptor downregulation (Law et al., 1983; Loh et al., 1988; Yoburn et al., 1993; Kest et al., 1994). Studies show that the magnitude of receptor regulation and tolerance produced by opioid receptor agonists is dependent upon agonist dose, dosing protocol and intrinsic efficacy (Stevens and Yaksh, 1989; Paronis and Holtzman, 1992; Yoburn et al., 1993; Duttaroy and Yoburn, 1995) where intrinsic efficacy is a measure of the effect produced by a single agonist–drug receptor interaction (Kenakin, 1993). In general, high intrinsic efficacy opioid receptor agonists (e.g. etorphine) produce less tolerance than equieffective doses of a lower intrinsic efficacy agonist (e.g. morphine). On the other hand, continuous exposure to a high intrinsic efficacy opioid receptor agonist is more likely to produce receptor downregulation than a lower intrinsic efficacy agonist (Law et al., 1983; Tao et al., 1987; Yoburn et al., 1993; Kim et al., 1995). Consequently, it might be predicted that a high intrinsic efficacy opioid receptor agonist would be more effective in regulating opioid receptor gene expression in vivo. Consistent with this suggestion are reports that morphine does not induce μ- and δ- opioid receptor internalization, but etorphine does (von Zastrow et al., 1994; Keith et al., 1996; Sternini et al., 1996).

In the present study, we have used an established in vivo model of receptor downregulation (Yoburn et al., 1993; Duttaroy and Yoburn, 1995) to examine the effect of chronic opioid receptor agonist treatment on μ-opioid receptor mRNA, receptor density and tolerance. We examined changes in binding, mRNA and analgesic potency at least 16 h following drug treatment since previous results (Yoburn et al., 1993) indicate that residual drug bound to membranes is eliminated by then. We now report the first instance of in vivo homologous regulation of μ-opioid receptor gene expression following chronic treatment with an opioid receptor agonist in the mouse. These results demonstrate that μ-opioid receptor mRNA can be regulated in in vivo studies in mice.

2. Materials and methods

2.1. Subjects

Male Swiss–Webster mice (22–35 g) (Taconic Farms, Germantown, NY) were used throughout the study. Mice were housed 10–11 per cage for at least 24 h prior to experimentation with free access to food and water.

2.2. Procedure

Mice were infused with etorphine (50–250 μg/kg/day) for seven days. Etorphine was administered via s.c. implanted osmotic pumps (ALZET model # 2001, Alza Corp., Palo Alto, CA) that delivered pump contents at a rate of 1.0 μl/h. Control mice were implanted with an inert placebo pellet in order to minimize costs. At the end of seven days (168 h following implantation), the pumps and pellets were removed and 16–168 h later mice were either sacrificed (binding or mRNA experiments) or tested in dose–response studies. Pumps and pellets were implanted and removed while mice were anesthetized with halothane:oxygen (96:4). Mice were anesthetized prior to sacrifice.

2.3. μ-Opioid receptor binding studies

Binding studies were performed as described by Yoburn et al. (1993). Mice ($n = 2$/group/treatment) were sacrificed and whole brain rapidly removed, weighed and then homogenized in 80 volumes of ice cold 50 mM Tris buffer (pH 7.4). Homogenates were then centrifuged at 15,000 rpm for 15 min, the supernatant discarded and the pellet resuspended in buffer, centrifuged again and the pellet frozen ($-80°C$) until analysis. The pellets were thawed, resuspended in 50 mM Tris buffer, incubated (30 min at 25°C), centrifuged and finally resuspended in 20–80 volumes of 50 mM phosphate buffer (pH 7.2). An aliquot (200 μl) of homogenate was then assayed in triplicate in tubes containing 0.04–5.0 nM [^{3}H] [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAMGO). Nonspecific binding was determined in the presence of 1000 nM levorphanol. Homogenates were incubated for 90 min at 25°C. Incubation was terminated by the addition of ice-cold phosphate buffer and the samples were filtered over GF/B glass fiber filters with a cell harvester. Filters were washed three times with cold buffer, transferred to vials with scintillation cocktail and then counted in a liquid scintillation analyzer. Cpms were converted to dpms using the external standard method. Specific binding was the difference between binding determined in the absence of cold ligand and the presence of cold ligand. Protein was determined using a microassay technique based on the method of Bradford (1976) using reagent purchased from BIO-RAD (Richmond, CA).
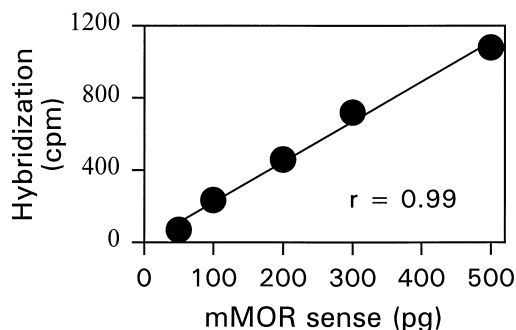
Fig. 1. A typical standard curve for mMOR mRNA. The mMOR plasmid was linearized with *Hind*III and transcribed with *T7* polymerase to produce a 668 nt (not full-length) sense transcript. Known amounts of sense transcripts were included in each assay and subjected to the same ribonuclease protection procedure as RNA extracted from mouse brain. A standard curve was included in each assay for mMOR mRNA. Specific hybridization is measured in cpms for each sense concentration, which represents the trichloracetic acid precipitated hybrids collected on filters minus background (yeast tRNA alone) cpms. A linear relationship between sense and cpms was observed in all assays. A representative standard curve is shown.

2.4. Measurement of μ-opioid receptor and GAPDH mRNA

Total RNA was extracted from brain using TRIzol reagent (GIBCO, Gaithersburg, MD). RNA was precipitated using 100% isopropyl alcohol and washed with 75% EtOH. The pellet was dried briefly, resuspended in diethyl pyrocarbonate treated water and stored at $-20°C$ until analyzed. The yield of RNA extraction was determined using UV spectrophotometry (260 nm).

An antisense riboprobe directed at mRNA for the mouse μ-opioid receptor (mMOR; Kaufman et al., 1995) was prepared. To construct the mMOR riboprobe, a 668 nt *Pvu*II–*Bam*H1 fragment from the full length mouse μ-opioid receptor cDNA (163–831 bp) was subcloned into pGem3Zf (Promega, Madison, WI). This fragment includes 93 bp upstream of the translation start site and coding region extending through the first 6 residues of transmembrane domain 4 of mMOR. The riboprobe is prepared by linearizing purified mMOR plasmid with *Eco*R1 endonuclease and transcribing with *Sp6* RNA polymerase using [^{32}P]-labeled CTP and purified over a Sephadex G-25 column. Riboprobe specific activity was typically 2×10^8 cpm/μg. A single 668 nt band was observed when an aliquot of riboprobe was analyzed by urea-polyacrylamide gel electrophoresis (PAGE) and auto-radiography.

Total (25–50 μg) RNA (10–25 μg brain + 15–25 μg yeast tRNA) was co-precipitated with riboprobe ($\sim$ 2–6 × 10^4 cpms), suspended in 20 μl of hybridization buffer (RPA II kit, Ambion, Austin, TX), allowed to hybridize for $\approx$ 16 h (42°C) and then treated with 200 μl of 1:1000 dilution of RNase (0.5 mg/ml of RNase A; 10,000 units/ml RNase T1) for 30 min (37°C). The RNase-resistant hybrids were precipitated with trichloroacetic acid

(5%) and collected on glass fiber filters followed by liquid scintillation counting. A single 668 nt band was observed when RNase-resistant hybrids were analyzed using urea-PAGE and autoradiography. Specific hybridization was measured in cpms, which represents the trichloracetic acid precipitated hybrids collected on filters minus cpms precipitated in the absence of brain RNA. To construct a standard curve, mMOR plasmid was linearized with *Hind*III and transcribed with *T7* polymerase to produce a 668 nt (not full-length) sense transcript. A standard curve for mMOR was included in each assay using known amounts of this sense transcript (Fig. 1). Samples of mouse brain RNA from untreated mice typically contained $\approx$ 200 pg of mMOR, which was within the linear portion of the standard curve (Fig. 1). The specificity of the riboprobe for mMOR was examined using RNA from NG108-15 cells which only express δ-opioid receptors (e.g. Charness et al., 1993) and SH-SY5Y cells (e.g. Yu et al., 1986) which express both μ and δ-opioid receptors. No hybridization was observed for NG108-15 RNA, whereas detectable levels of mMOR mRNA were found in the SH-SY5Y cells (data not shown).

Linearized plasmids containing rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sequence was purchased (Ambion) and transcribed by *T7* polymerase to make antisense riboprobe (316 nt). The specific activity
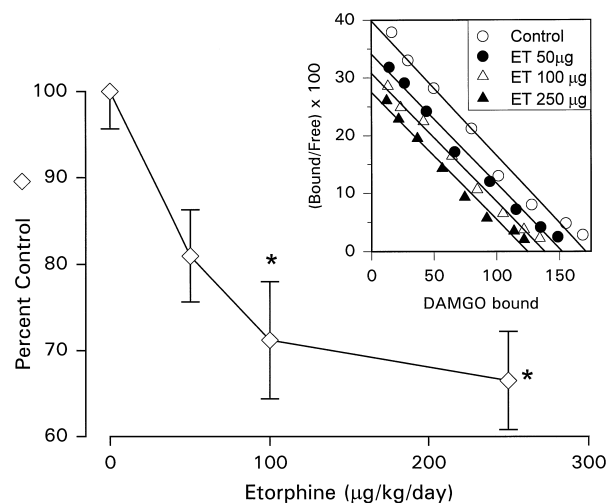

Fig. 2. The dose-dependent effect of etorphine (ET) treatment on μ-opioid receptor density in mouse brain determined 16 h following a 7 day infusion. Mice ($N = 2$/treatment) were infused, s.c. with etorphine (50–250 μg/kg/day) using an osmotic minipump or implanted with an inert placebo pellet (control) for 168 h. Pumps and pellets were then removed and 16 h later mice sacrificed and saturation binding ([^{3}H] DAMGO) studies for μ-opioid receptor density in whole brain homogenate preparations were conducted. Data from individual experiments were analyzed by nonlinear regression which estimated B_{max} and K_d values. At least 4 independent experiments were conducted for each dose. All data were converted to means ± S.E.M. percent control values (Control B_{max} mean ± S.E.M. = 182 ± 8 fmol/mg protein). * Indicates significantly different from control ($P < 0.05$). The inset shows a representative Scatchard plot from a single experiment.

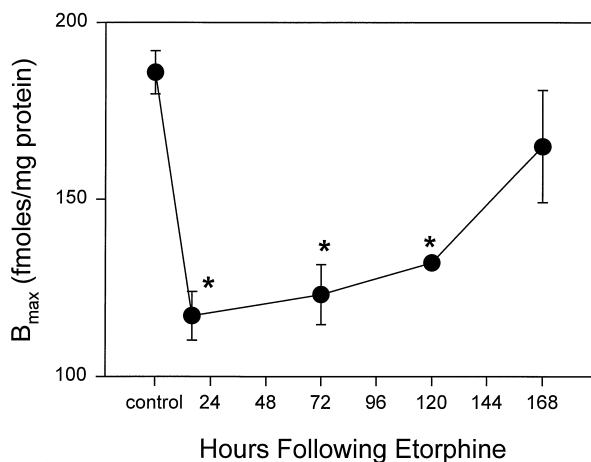Fig. 3. The recovery of μ-opioid receptor density following etorphine treatment in mouse brain. Mice ($N = 2$/treatment) were infused, s.c. with etorphine (250 μg/kg/day) using an osmotic minipump or implanted with an inert placebo pellet (control) for 168 h. Pumps and pellets were then removed and 16–168 h later mice sacrificed and saturation binding ([^{3}H] DAMGO) studies for μ-opioid receptor density in whole brain homogenate preparations were conducted. Data from individual experiments were analyzed by nonlinear regression which estimated B_{max} and K_d values. At least 4 independent experiments were conducted for each dose. * Indicates significantly different from control ($P < 0.05$).
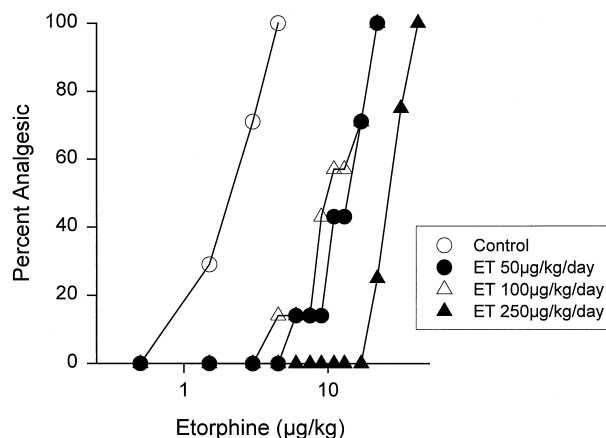
Fig. 4. The dose-dependent effect of etorphine (ET) infusion on the analgesic potency of etorphine was determined 16 h following a 7 day infusion. Mice ($N = 7$/treatment) were infused, s.c. with etorphine (50–250 μg/kg/day) using an osmotic minipump or implanted with an inert placebo pellet (control) for 168 h. Pumps and pellets were then removed and 16 h later analgesic (tailflick) dose–response studies were conducted. The etorphine ED_{50} (95% CL) in μg/kg for control was 2.06 (1.51–2.76). Etorphine infusion shifted (increased) the ED_{50} by 6.0*, 5.1* and 13.4*-fold for the 50, 100 and 250μg/kg/day etorphine infusions, respectively. * Indicates significantly different from control ($P < 0.05$) by probit analysis. Representative results from one of two studies are shown.

was typically 2×10^8 cpm/μg. A sense curve was not constructed for GAPDH, although a linear relationship between cpms and increasing concentration of total brain RNA was demonstrated. The slope of this linear relationship for GAPDH mRNA was used to calculate the percent change from control.

2.5. Dose–response protocol

At selected times (16–168 h) following removal of the osmotic pump or placebo pellet, mice were tested for

etorphine-induced analgesia (tailflick, see below) using a cumulative dose–response protocol. A parallel control group was included for all treatments and shifts in ED_{50}s are determined relative to that group. All mice ($N = 7–9$/group/treatment) in a treatment group were injected s.c. with a starting dose of etorphine (0.5 μg/kg) and tested for analgesia 15 min later. Mice that were not analgesic were given a another dose (increment dose) of etorphine within 3 min of testing and then tested for analgesia again 15 min later. This cumulative dose–re-
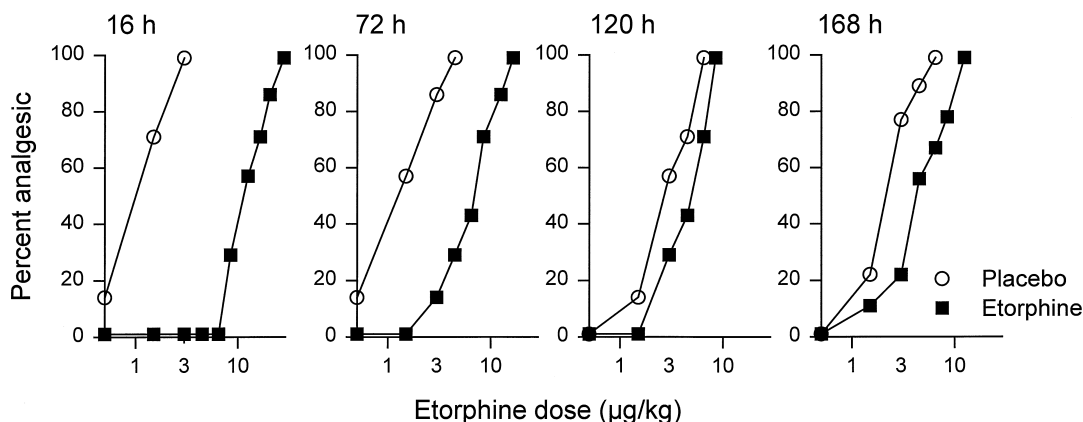

Fig. 5. The recovery of analgesic potency of etorphine following 7 day etorphine infusion. Mice ($N = 7–9$/treatment) were infused, s.c. with etorphine (250 μg/kg/day) using an osmotic minipump or implanted with an inert placebo pellet (control) for 168 h. Pumps and pellets were then removed and 16–168 h later analgesic (tailflick) dose–response studies were conducted. Separate groups of control and treated mice were used for each time point. The mean ($\pm$S.E.M.) etorphine ED_{50} for controls was 1.79 ± 0.36 μg/kg. The ED_{50} for the etorphine-infused mice was shifted 12.3*, 5.0*, 1.6* and 2.1*-fold at 16, 72, 120 and 168 h, respectively. * Indicates significantly different from control ($P < 0.05$) by probit analysis. Results from one study are shown.

sponse protocol was continued (increment doses ranged from 1.0–5.0 μg/kg) until 100% of mice were analgesic (cumulative etorphine dose range = 0.5–42.0 μg/kg). The actual etorphine doses used in the cumulative dose–response protocol were determined in a previous study (Duttaroy and Yoburn, 1995). Data from cumulative dosing are presented such that the percent of mice that are analgesic are plotted against the total (cumulated) dose administered.

2.6. Analgesia assay

Analgesia (antinociception) was determined using the tailflick assay in which a beam of light was focused on the dorsal tail surface approximately 2 cm from the tip of the tail. The intensity of the light was adjusted so that baseline flick latencies determined prior to etorphine administration were 2–4 s. If a mouse failed to flick by 10 s following etorphine administration, the test was terminated and mice were defined as analgesic. Mice were tested for analgesia 15 min following etorphine. All testing was conducted in a blind manner.

2.7. Drugs and reagents

Etorphine hydrochloride and inert placebo pellets were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of the National Institute on Drug Abuse. Etorphine was dissolved in 0.9% saline and doses were calculated as the base. Reagents were purchased from Sigma Chemical (St. Louis, MO) unless noted above. Isotopes were purchased from Amersham Life Science (Arlington Heights, IL).

2.8. Data analysis

In vivo dose–response data were analyzed by Probit Analysis (Finney, 1973) using a computerized program (BLISS 21, Department of Statistics, University of Edinburgh) that estimates ED_{50}s, 95% confidence limits, relative potencies and significant differences among ED_{50}s. B_{max}'s and K_d's were determined from saturation studies using nonlinear regression (Prism ver 1.03, Graphpad Software, San Diego, CA). In all cases binding data were fit best by a one-site model. Significant differences for data from binding and mRNA studies were analyzed using analysis of variance with appropriate post hoc comparisons.

3. Results

3.1. Binding

Etorphine produced a dose-dependent reduction in the density of μ-opioid receptors ($F(3, 17) = 8.6$, $P < 0.05$)

at 16 h following the removal of the osmotic pump (Fig. 2). The density of μ-opioid receptors was significantly reduced for the 100 and 250 μg/kg/day dosing groups relative to the control. There was no significant effect of etorphine treatment on K_d ($F(3, 17) < 1.0$, $P > 0.05$; not shown, mean K_d range = 0.5–0.6 nM).

Downregulation of μ-opioid receptors was examined over a 168 h period following the end of a 7 day infusion of 250 μg/kg/day etorphine (Fig. 3). There was a significant time-dependent recovery of μ-opioid receptor density
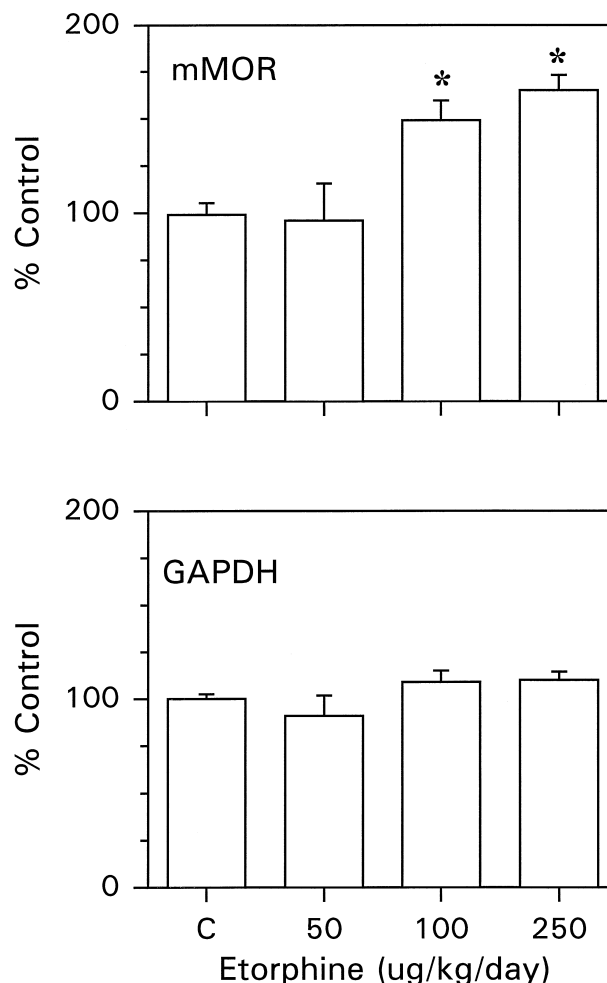

Fig. 6. The dose-dependent effect of etorphine infusion on mouse μ-opioid receptor (mMOR) gene expression determined 16 h following a 7 day infusion. Mice ($N = 4$–10/treatment) were infused, s.c. with etorphine (50–250 μg/kg/day) using an osmotic minipump or implanted with an inert placebo pellet (control) for 168 h. Pumps and pellets were then removed and 16 h later brains removed, total RNA extracted and processed for determination of mMOR mRNA or GAPDH mRNA using a ribonuclease protection assay. The mMOR mRNA data were converted to percent of control values for each experiment (mean $\pm$ S.E.M. mMOR mRNA in mouse brain for control was 6.1 $\pm$ 0.25 pg/μg total RNA). For GAPDH, the slope of the relationship between varying total RNA concentration and cpms for the GAPDH riboprobe was used to calculate percent of control for each experiment. Data are means $\pm$ S.E.M. of 3–7 independent experiments. * Indicates significantly different from control $P < 0.05$.

($F(4, 33) = 14.3$, $P < 0.05$), with no effect on K_d ($F(4, 33) = 2.0$, $P > 0.05$, not shown; mean K_d range = 0.6–0.7 nM). Receptor density was significantly less than control at 16–120 h following the end of the 250 μg/kg/day etorphine infusion and had returned to control levels by 168 h.

3.2. Dose–response

Etorphine infusion (50–250 μg/kg/day) produced significant ($P < 0.05$) dose-dependent tolerance to the analgesic effects of etorphine at 16 h following the end of the infusion (Figs. 4 and 5). Recovery from tolerance following the 250 μg/kg/day etorphine infusion was observed for 168 h following treatment (Fig. 5). Although etorphine (250 μg/kg/day) produced significant tolerance from 16 to 168 h following the 7 day infusion, the magnitude of tolerance at 16 h following infusion was $\approx$ 12–13-fold and declined to $\approx$ 2-fold at 120–168 h. The placebo ED_{50} tended to increase from 16–168 h (open circles, Fig. 5). However, the ED_{50} for etorphine-treated mice should be compared to the corresponding control group, since each
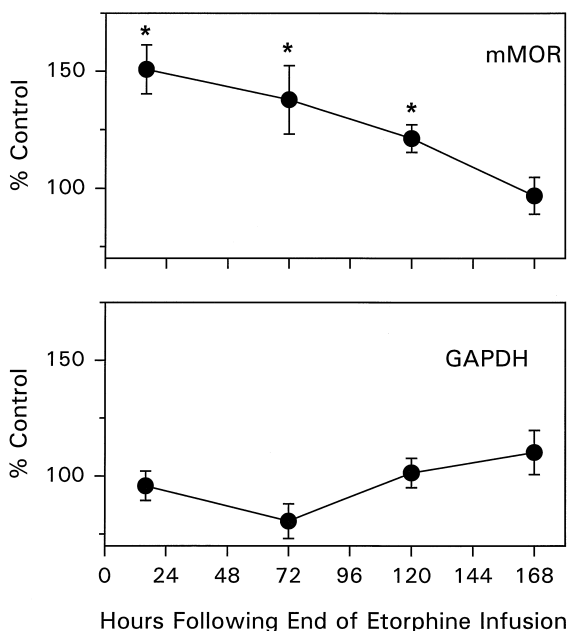

Fig. 7. The recovery of mouse μ-opioid receptor (mMOR) mRNA following etorphine treatment in brain. Mice ($N = 5$–8/treatment) were infused, s.c. with etorphine (250 μg/kg/day) using an osmotic minipump or implanted with an inert placebo pellet (control) for 168 h. Pumps and pellets were then removed and 16–168 h later brains removed, total RNA extracted and processed for determination of mMOR mRNA or GAPDH mRNA using a ribonuclease protection assay. mMOR mRNA data were converted to percent of control values for each experiment (mean $\pm$ S.E.M. mMOR mRNA in mouse brain for control was 8.6$\pm$0.6 pg/μg total RNA). For GAPDH, the slope of the relationship between varying total RNA concentration and cpms for the GAPDH riboprobe was used to calculate percent of control for each experiment. Data are means $\pm$ S.E.M. of 3–4 independent experiments. * Indicates significantly different from control $P < 0.05$.

time-point study was conducted as an independent experiment.

3.3. mRNA

Etorphine dose-dependently increased μ-opioid receptor mRNA (Fig. 6) at 16 h following a 7 day infusion ($F(3, 59) = 13.9$, $P < 0.05$). Post hoc tests indicated that mMOR mRNA was increased by both 100 and 250 μg/kg/day etorphine. There was no significant effect of etorphine on GAPDH at 16 h following a 7 day infusion ($F(3, 97) = 2.04$, $P > 0.05$). Next, the time course of changes in mMOR gene expression following a 7 day infusion of 250 μg/kg/day etorphine was determined (Fig. 7). There was a significant effect of etorphine treatment on mMOR mRNA ($F(1, 129) = 23.1$, $P < 0.05$), followed by a significant time-dependent return to control levels ($F(3, 129) = 5.22$, $P < 0.05$). Post hoc tests indicated that etorphine-treated mice had higher steady-state levels of mMOR mRNA at 16–120 h following the 7 day infusion of 250 μg/kg/day etorphine. mMOR mRNA had returned to control levels by 168 h. There was no significant effect of treatment on GAPDH mRNA ($F(1, 123) = 0.4$, $P > 0.05$).

4. Discussion

It is well-established that opioid receptor density can be regulated by in vivo and in vitro treatment with opioid receptor agonists or antagonists (e.g. Law et al., 1983; Tempel et al., 1985; Loh et al., 1988; Yoburn et al., 1989, 1995; Belcheva et al., 1991). Although homologous regulation of opioid receptor gene expression has been reported in in vitro studies (Jenab and Inturrisi, 1994; Kim et al., 1995), in vivo regulation by opioid receptor agonists has not been demonstrated. The present results are the first report, to our knowledge, that chronic in vivo treatment with an opioid receptor agonist can coordinately regulate μ-opioid receptor density, mRNA and agonist potency (i.e. tolerance).

In the present study, a 7 day infusion of the high intrinsic efficacy opioid receptor agonist etorphine produced dose-dependent downregulation of μ-opioid receptor density and increases in mMOR mRNA. These effects were associated with dose-dependent tolerance to the analgesic effects of etorphine. The density of receptors and the increase in mRNA returned to control levels by 7 days following the end of the infusion. Similarly the magnitude of tolerance showed a significant time-dependent decrease. There were no significant changes in the gene expression of GAPDH, a finding which suggests that the effects of etorphine are specific.

Previous studies have shown that a wide variety of heterologous agents can effectively regulate opioid receptor density and mRNA in in vitro and in vivo systems (Hammer, 1989; Unterwald et al., 1992, 1994; Charness et al., 1993; Delfs et al., 1994; Jenab and Inturrisi, 1994; Abood and Tao, 1995; Azaryan et al., 1996a,b; Beczkowska et al., 1996; Spangler et al., 1996). Thus, although those data made it clear that opioid receptor gene expression and density were subject to regulation, in vivo treatments with opioid receptor agonists and antagonists were relatively ineffective in modifying levels of opioid receptor mRNA. Most investigators have reported no change in μ- or δ-opioid receptor mRNA following chronic in vivo opioid receptor antagonist treatment (Jenab et al., 1995; Unterwald et al., 1995; Castelli et al., 1997), although there has been one report of modest decreases (21–28%) in the levels of μ-opioid receptor mRNA in nucleus raphe magnus, hypothalamus and media thalamus, but not in spinal cord, periaqueductal grey or cortex (Brodsky et al., 1995). Similarly, chronic treatment with a variety of tolerance-inducing opioid receptor agonists in vivo has generally not been found to alter opioid receptor mRNA in the CNS (Kest et al., 1994, 1995; Brodsky et al., 1995; Buzas et al., 1996; Castelli et al., 1997; however see Rønnekleiv et al., 1996). Finally, treatment with an irreversible μ-opioid receptor antagonist that depletes μ-opioid receptors, followed by recovery of receptors, does not produce any changes in μ-opioid receptor mRNA (Chan et al., 1995). Taken together, these earlier findings suggested that homologous agents could not regulate in vivo opioid receptor gene expression.

There are several reasons that may account for previous failures to observe changes in opioid receptor gene expression in vivo following agonist treatment. Robust downregulation of opioid receptor density typically requires high intrinsic efficacy opioid receptor agonists (Law et al., 1983; Tao et al., 1987; Yoburn et al., 1993; Duttaroy and Yoburn, 1995). Both in vitro and in vivo studies tend to concur that morphine is not an effective agonist for opioid receptor downregulation or internalization (Law et al., 1982, 1983; Loh et al., 1988; Yoburn et al., 1993; Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996). In agreement with this suggestion is a recent report showing that exposure of NG108-15 cells to morphine does not regulate δ-opioid receptor mRNA, whereas etorphine does (Kim et al., 1995).

In the present study, etorphine produced a significant dose-dependent increase in mMOR mRNA 16 h following the end of a 7 day infusion. At this time point, significant dose-dependent μ-opioid receptor downregulation and analgesic tolerance were observed. We chose to examine changes in binding, mRNA and analgesic potency at this time since previous studies indicated significant tolerance and downregulation of brain μ-opioid receptor density (e.g., Yoburn et al., 1993). Our earlier in vivo studies indicated that receptor binding studies must be conducted at least 16 h following exposure to etorphine since residual drug bound to membranes could contaminate estimates of μ-opioid receptor density and affinity. We continued to use this 16 h time point in the present study so that parallel estimates of effects on binding, tolerance and mRNA could be determined.

Our findings extend results obtained in cell culture studies that show that chronic etorphine treatment regulates δ-opioid receptor density and mRNA (Kim et al., 1995). While Kim et al. (1995) observed a decrease in δ-opioid receptor density and its mRNA following etorphine, we found an increase in mMOR mRNA at a time when μ-opioid receptor density was decreased. The apparent discrepant effects of etorphine in vivo and in vitro are most probably related to the different assay times following etorphine exposure. In the in vitro study, mRNA was examined in the presence of etorphine, not during the start of the recovery phase, as in the present study. The increase in mMOR mRNA in the present study probably represents an adaptive response that mediates the recovery of μ-opioid receptors over the 7 day period following the termination of the etorphine infusion. At present we do not know if mMOR mRNA is downregulated during treatment with etorphine since we only determined the effects following an infusion. However, based on the results for other G-protein coupled receptors (e.g. Collins et al., 1991; Hadcock and Malbon, 1993) and the results of Kim et al. (1995), it seems likely that mMOR mRNA would be decreased during the course of the infusion. We are in the process of examining that issue now.

In the present experiment, decreased opioid receptor density was associated with the development of tolerance and changes in receptor gene expression. Although it is established that tolerance need not require receptor regulation (see for discussion Yoburn et al., 1993) these data provide a clear instance of in vivo changes in gene expression, receptor function and density in response to chronic agonist. That changes in receptor density are not a requirement for tolerance is demonstrated in the present study in which mice showed small but significant tolerance to etorphine-induced analgesia on the seventh day of recovery in the absence of significant changes in receptor density. At present, it is not known what the contribution of receptor downregulation is to tolerance, but based on receptor theory (Kenakin, 1993), it is likely that it further decreases the potency of agonists.

In summary, we have shown that the μ-opioid receptor gene can be regulated in mouse brain by a high intrinsic efficacy opioid receptor agonist. Chronic etorphine treatment may represent a good model for evaluating factors that modify μ-opioid receptor gene expression in vivo. Finally, these results demonstrate coordinate in vivo changes in μ-opioid receptor mRNA and receptor density and indicate that in vivo regulation of μ-opioid receptor gene expression is not confined to treatment protocols using heterologous agents.

Acknowledgements

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