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REGULATION OF μ-OPIOID RECEPTORS, G-PROTEIN-COUPLED RECEPTOR KINASES AND β-ARRESTIN 2 IN THE RAT BRAIN AFTER CHRONIC OPIOID RECEPTOR ANTAGONISM

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Abstract—The aim of this study was to analyse the biochemical and behavioural consequences of chronic treatment with opioid receptor antagonists in rats. We have evaluated the respiratory depressant and antinociceptive effects of the μ-opioid agonist sufentanil, the density of brain μ-opioid receptors, and the expression of G-protein-coupled receptor kinases and β-arrestin 2 in cerebral cortex and striatum, following sustained opioid receptor blockade. Our results demonstrate that 24 h after interruption of 7 days chronic infusion of naltrexone (120 μg/h), the respiratory depressant potency of the μ-opioid receptor agonist sufentanil was increased to a similar extent as the antinociceptive potency (about three-fold). This was accompanied by μ-opioid receptor up-regulation in several areas of the rat brain associated with opioid control of pain perception and breathing. Moreover, chronic treatment with either naltrexone (120 μg/h) or naloxone (120 μg/h) caused significant increases in the expression levels of G-protein-coupled receptor kinases types 2, 3, and 6, and of β-arrestin 2 in brain cortex and striatum.

Together our data suggest an increased constitutive receptor activity secondary to μ-opioid receptor up-regulation following chronic antagonist treatment. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: autoradiography, analgesia, breathing, naloxone, naltrexone.

Opioid receptors (μ, δ, and κ) are G-protein-coupled receptors (GPCRs) that, like other GPCRs (see Fergusson, 2001), undergo adaptations such as desensitisation, internalisation, and down-regulation in response to agonist activation (Díaz et al., 2000; see Law et al., 2000). It is known that the molecular mechanisms responsible for agonist-dependent desensitisation of μ-opioid receptors expressed in several cell lines involve G-protein-coupled receptors kinase (GRK)-mediated phosphorylation, and β-arrestin-dependent sequestration in endocytotic vesicles (Kovoor et al., 1997, 1998; Zhang et al., 1998; Wang, 2000). In living animals this regulatory mechanism contributes to the receptor down-regulation and loss of analgesic potency observed after long-term opioid treatment, and to the addictive potential of this family of drugs (Terwilliger et al., 1994; Ozaita et al., 1998; Bohn et al., 1999, 2000; Hurlé, 2001). Conversely to what occurs under the influence of agonists, opioid receptors undergo up-regulation and supersensitivity following the interruption of long-term treatment with opioid receptor antagonists such as naloxone and naltrexone, in both cultured cells and living animals (Morris et al., 1988; Yoburn et al., 1986, 1989; Unterwald et al., 1995). To date, there are not available data on the regulation of GRKs and β-arrestin expression associated to these phenomena.

The respiratory depressant effect of opioids is a severe and life-threatening adverse reaction when these drugs are used as therapeutic agents in analgesia. Apnoea is also known to be the most frequent cause of death after opiate overdose in heroin addicts (see Flórez and Hurlé, 1993). Since naltrexone is being used as anti-craving agent for opiate addictive processes (Kreek, 1996; Schaffer and Naranjo, 1998), it is deemed of interest to assess whether the chronic administration of opioid receptor antagonists could modify the sensitivity to the respiratory depressant effect of opioid receptor antagonists as it occurs with the analgesia.

In this study we have chronically administered opioid receptor antagonists in order to assess the changes in the expression levels of brain GRK2, GRK3, GRK6 and β-arrestin 2 that are associated with μ-opioid receptor up-regulation. We have also evaluated the ability of chronic naltrexone to potentiate the respiratory depressant effect of the μ-opioid receptor agonist sufentanil in comparison with its antinociceptive effect.

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Abbreviations: ANOVA, analysis of variance; DAMGO, [D-Ala(2)-MePhe(4)-Gly(5)-ol]enkephalin; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; TBST, Tris-buffered saline with Tween 20.

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EXPERIMENTAL PROCEDURES

Animals

Male albino Wistar rats (Charles River, Criffa, Barcelona, Spain), weighing 250–300 g, were used. They were housed in a room kept at 22°C with a 12:12 h light–dark cycle. Food and water were provided *ad libitum*. This study was approved by the Cantabria University Institutional Laboratory Animal Care and Use Committee and carried out in accordance with the Declaration of Helsinki and the European Communities Council Directive of 24 November (86/609/EEC).

Experimental protocols

The chronic delivery of drug-containing solution was carried out with Alzet 2001 osmotic minipumps (Alza Corporation, Palo Alto, CA, USA), implanted subcutaneously, under light ether anaesthesia. These pumps deliver solutions at a constant rate of 1 μl/h, during 7 days, and drug solutions were appropriately composed for the desired dosing regime. The control group of rats received a 7 days chronic infusion of saline (1 μl/h). The opioid antagonists naltrexone or naloxone (Sigma-Aldrich, Spain) were administered at a dose of 120 μg/h, for 7 days. On the 7th day minipumps were removed, and the *in vivo* and *in vitro* assays were carried out 24 h after saline or opioid antagonist withdrawal. The antinociceptive and respiratory depressant potencies of the μ-opioid receptor agonist sufentanil were determined in awake animals. For autoradiographic and western blot immunodetection experiments, animals were killed by decapitation under light ether anaesthesia in order to obtain the brain samples.

Measurement of respiration

Ventilation was measured in awake rats by means of the whole body plethysmographic technique (Barlett and Tenney, 1970). In brief, the rat was placed in a home-made air-tight 7-l chamber and allowed to move freely while the chamber was flushed with humidified air at a flow rate of about 5 l per min. After an adaptation period of about 1 h, the rat was taken out of the plethysmograph to receive drug treatment and then returned to the plethysmograph. Respiration was assessed by recording changes in pressure in the animal chamber, with reference to the pressure in a second, identical chamber, by means of a differential pressure transducer (Validyne MP45-871, Europavia, Madrid, Spain). In the course of each measurement, 0.25 and 0.50 ml of air were extracted from and reinjected rapidly into the animal chamber at the end of an expiration. The pressure changes caused by the injections were the references for tidal volume (ml) calculation. Respiratory frequency (breaths/min) was directly counted in the record. The minute volume (V_E; ml/min/100 g) was corrected for the body weight. The control value is the mean of four recordings measured along a 15-min pre-drug period. Changes in minute volume after drug treatment are expressed for each animal as percentage of change with respect to the pre-drug control value. Each animal received a single dose of the opioid. The time-course of the respiratory depressant effect induced by 10, 20, 40 and 80 μg/kg of subcutaneous sufentanil was constructed from the percent changes of minute volume at 15, 30, 45, and 60 min after injection of the drug. At each time point, two measurements of ventilation were made. The dose–response curve was constructed with the maximal depressant effect on minute volume obtained at any time.

Evaluation of nociception

The tail-flick test was used to assess nociceptive threshold. Tail-flick response was elicited by applying radiant heat to the surface of the tail (mod. Letica 7100, Panlab, Barcelona, Spain). The intensity of the stimulus was adjusted so that the control latency was within 3–5 s. A cut-off time of 10 s was set to prevent skin blistering. Tail-flick latencies were measured before drug injection, and 30 min after subcutaneous administration of each sufentanil dose (0.5, 1, 2, 4, 8, and 16 μg/kg), when the maximal antinociceptive effect of this opioid has been demonstrated to occur (Dierssen et al., 1990). Analgesic endpoint was defined as an increase of 100% in the individual reaction time in relation to the pre-drug reaction latency. The antinociceptive effect was expressed as the percentage of animals which reached the analgesic endpoint. The cumulative dose–response curve was performed with the antinociceptive response obtained 30 min after the administration of each dose of the opioid.

Ligand binding autoradiography

Rats were killed by decapitation under light ether anaesthesia. The brain and spinal cord were rapidly removed and frozen at −70°C. Ten μm thick coronal sections were cut on a cryostat (Frigocut 2800 E, Reichert-Jung, Germany), thaw-mounted onto pre-cleaned and gelatin-coated microscope slides, and then stored at −20°C until the autoradiographic labelling of μ-opioid receptors was carried out. Brain sections were selected at rostrocaudal levels corresponding to bregma coordinates 0.2, −2.8, −4.3, −6.8, −8.3, −10.3 and −13.8 mm (according to Paxinos and Watson, 1982, atlas). Transversal spinal cord sections at lumbar-sacral level were also selected.

μ-Opioid receptors were labelled with [^{3}H]DAMGO (Tyr-[D-Ala(2)-MePhe(4)-Gly(5)-ol]enkephalin) (55 Ci/mmol; New England Nuclear/Du Pont, Madrid, Spain). Sections were firstly preincubated for 30 min at room temperature in Tris–HCl buffer (50 mM, pH 7.4) to remove endogenous ligands and/or administered drugs. Then, incubation was carried out in the same buffer, for 45 min, at room temperature, and in the presence of 5 nM [^{3}H]DAMGO. Non-specific binding was assessed on adjacent sections exposed to 10 μM naloxone. Slides were then rinsed twice for 5 min in ice-cold buffer, dipped in cold distilled water, and dried under a stream of cold air. Twenty-four h after incubation, dried sections were placed in X-ray cassettes along with tritium standards ([^{3}H]Microscales, Amersham Pharmacia-Biotech, Spain) and exposed to tritium sensitive films (Hyperfilm ^{3}H, Amersham Pharmacia-Biotech) at 4°C for 8 weeks. Films were developed in D-19 (Kodak, Spain), fixed and dried. The autoradiograms generated were quantified with a computer-assisted analysis system (Scion Image, Scion Corporation, MD, USA). Optical density values of each region from each rat were averaged over at least two bilateral readings, and subsequently converted to nCi/mg of tissue equivalent (wet weight) with reference to the standards. Autoradiographic values were finally expressed in fmol/mg of tissue equivalent.

Western blot immunodetection

Animals were killed by decapitation under light ether anaesthesia. The brains were rapidly removed, and the cerebral cortices were dissected and homogenised in phosphate-buffered saline (PBS) with 1% sodium dodecyl sulphate (SDS). Whole cell lysates were boiled 10 min, and then centrifuged at 10 000 × *g* for 5 min, at 4°C. Total protein content of the samples was determined by the method of Lowry et al. (1951). The optimal amount of protein to be loaded (in the middle range of detection) was determined in preliminary experiments by loading gels with increasing protein contents (25–125 μg) from duplicate samples of two different individuals from each experimental group. 100 μg of whole cell protein from each sample were loaded on a 10% SDS–polyacrylamide gel (SDS–PAGE), electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini trans-blot electrophoresis transfer cell (Bio-Rad Lab., CA, USA). Equal loading and transfer of proteins was confirmed by Ponceau S straining of the membranes. Non-specific binding of antibodies was prevented by incubating membranes in 10% dried milk powder in Tris-buffered saline with Tween 20 (TBST) buffer: 10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20. The membranes were incubated overnight, at 4°C, with polyclonal primary antibodies anti-GRK2, anti-GRK3, anti-GRK6, or anti-β-arrestin 2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution and with monoclonal anti-α-tubulin (Sigma-Aldrich)

at 1:1000 dilution in TBST with 2% dried milk. After extensive washings with TBST, the membranes were incubated for 1 h, at room temperature, with peroxidase-labelled secondary antibodies at 1:5000 dilution. After washing, immunoreactivity was detected with an enhanced chemiluminescence western blot detection system (ECL, Amersham Pharmacia-Biotech) and visualised by Amersham Hyperfilm-ECL. After film scanning, the integrated optical density of the bands was estimated (Scion Image software, Scion Corporation, MD, USA), and normalised to the background values. The same blots used to measure proteins that are drug regulated were also used to measure levels of the structural protein α-tubulin that was not drug regulated. Relative variations between the bands of the problem samples and the control samples were calculated in the same image. Duplicate measurements in three different gels for each individual sample were performed. Measurements were in the linear range.

Drugs and reagents

Naltrexone hydrochloride and naloxone hydrochloride were purchased from Sigma-Aldrich and dissolved in saline; sufentanil was kindly provided by Janssen Cylag, S.A. (Madrid, Spain) and was also dissolved in saline; SDS, PAGE and the PVDF membrane were obtained from Bio-Rad Lab. (Teknovas, Bilbao, Spain); all other chemicals were of reagent grade and obtained from Sigma-Aldrich.

Evaluation of the results and statistical analysis

Results on respiration are expressed as percentage changes relative to the values obtained during the pre-drug control period. The dose–response curves were performed from the maximal depressant effects on minute volume induced by each dose of the drug. The values are expressed as mean ± S.E.M. The ED_{50} and confidence limits were calculated with non-linear regression analyses. Results on analgesia are expressed as percentage of animals that achieved the analgesic endpoint. The percent values were transformed in probits before performing statistical assay. The cumulative quantal dose–response curves were performed from the effects obtained 30 min after injection of each dose of the drug, when the maximal antinociceptive effect has been demonstrated to occur (Dierssen et al., 1990). The ED_{50} and confidence limit determinations were done by the method of Lichtfield and Wilcoxon (1949) and Finney (1964).

Data on radioligand binding are reported as means ± S.E.M. of a minimum of four different sample values determined in duplicate. Comparison was done using unpaired *t*-test. Data on western blot immunodetection are reported as means ± S.E.M. of a minimum of four different sample values determined in triplicate. Statistical comparisons were done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The statistical analysis was done with the aid of the GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). A $P < 0.05$ was considered to be statistically significant.

RESULTS

Respiratory depressant effect of sufentanil

The values of minute volume determined during the control period for all groups of rats were within normal limits (data not shown). No significant differences between groups were observed. Subcutaneous administration of 10 ($n = 8$), 20 ($n = 8$), 40 ($n = 8$), or 80 μg/kg ($n = 8$) of sufentanil to awake rats induced an immediate and dose-dependent depression of minute volume (Fig. 1A) which was accounted for by a reduction in both respiratory frequency and tidal volume (data not shown). The dose–response curve performed with the maximal depressant effect on minute volume induced by each dose of sufentanil leads to an $ED_{50} = 45.7$ μg/kg (95% confidence limits 30 and 69). As shown in Fig. 1, the respiratory depressant effect of sufentanil was significantly potentiated by the pre-treatment with naltrexone (120 μg/h) for 7 days. The dose–response curve performed with the maximal depressant effect on minute volume induced by each dose of sufentanil was shifted to the left, the ED_{50} being significantly reduced to 15.3 μg/kg (95% confidence limits 4.4 and 50) ($P < 0.05$). Therefore the respiratory depressant potency of sufentanil was potentiated by three-fold.

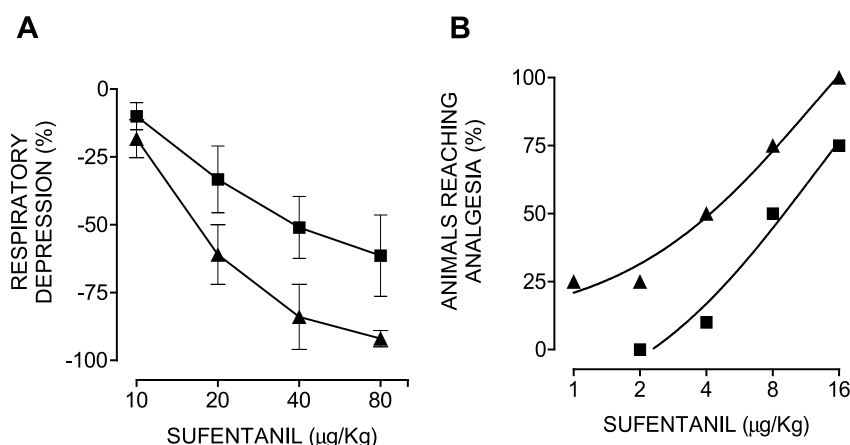

Fig. 1. Behavioural effects of sufentanil 24 h after chronic saline and naltrexone treatment withdrawal. (A) Dose–response curves of the maximal depressant effect of sufentanil on minute volume in control animals (■) and in animals chronically treated with naltrexone (120 μg/h) for 7 days (▲). Results are expressed as percentages of change relative to the values obtained during the pre-drug control period. The values are the means ± S.E.M. The ED_{50} of sufentanil was significantly decreased after chronic naltrexone treatment ($P < 0.05$, unpaired *t*-test). (B) Antinociceptive dose–response curves of sufentanil in control animals (■) and in animals chronically treated with naltrexone (120 μg/h) for 7 days (▲). The results are expressed as percentages of animals reaching the analgesic endpoint in the tail-flick test. A significant shift to the left of the dose–response curve was observed in the animals treated with naltrexone as assessed by the probit analysis.

Analgesic effect of sufentanil

Subcutaneous sufentanil (1–16 μg/kg) ($n = 8$), administered 24 h after 7 days chronic saline, induced a dose-dependent antinociceptive effect (Fig. 1B). The ED_{50}, calculated from the cumulative dose–response curve, was 9.4 μg/kg (95% confidence limits 5.6 and 15.8). Pre-treatment with naltrexone (120 μg/h) for 7 days ($n = 8$) did not modify the nociceptive threshold, but significantly increased the analgesic potency of sufentanil, as measured 24 h after naltrexone withdrawal. The dose–response curve was shifted leftward, and the ED_{50} was significantly ($P < 0.01$) reduced to 3.1 μg/kg (95% confidence limits 1.6 and 6.1). Therefore the analgesic potency of sufentanil was potentiated by three-fold.

Autoradiography

In control animals, the specific binding of [^{3}H]DAMGO in coronal brain sections reached up to 95% of total binding as determined in the presence of 10 μM naloxone. Chronic administration of naltrexone induced significant increases in the density of μ-opioid receptors in several brain regions (Fig. 2). As shown in Table 1, clear regional differences in the degree of receptor up-regulation were observed all throughout the brain. The highest increments were quantified within basal ganglia, substantia nigra and the dorsal horn of the spinal cord. High to moderate increases were observed in somatosensory cortex, central grey and locus coeruleus.

G-protein-coupled receptor kinases and β-arrestin 2 immunodetection in cortex and striatum

In the western blot immunoassays, performed in cerebral cortex and striatum lysates, the different kinases were detected in bands located at ~80 kDa for GRK2 (Fig. 3A), ~80 kDa for GRK3 (Fig. 3B), ~66 kDa for GRK6 (Fig. 3C), and ~47 kDa for β-arrestin 2 (Fig. 3D). These sizes are consistent with recent studies (see Premont et al., 1995).

In brain cortices, chronic treatment with naltrexone (120 μg/h) ($n = 5$) significantly increased the cortical levels of total GRK2 (+100 ± 23%), GRK3 (+222 ± 23.8%), GRK6 (+171 ± 55%), and β-arrestin 2 (+92 ± 22%). In striatal lysates, chronic treatment with naltrexone (120 μg/h) for 7 days ($n = 5$) also increased the striatal levels of total GRK2 (+50 ± 20%), GRK3 (+219 ± 18%), GRK6 (+80 ± 19%), and β-arrestin 2 (+132 ± 29%). Similar results were observed with naloxone (120 μg/h) ($n = 4$) in both brain cortices (GRK2: +85 ± 5%;

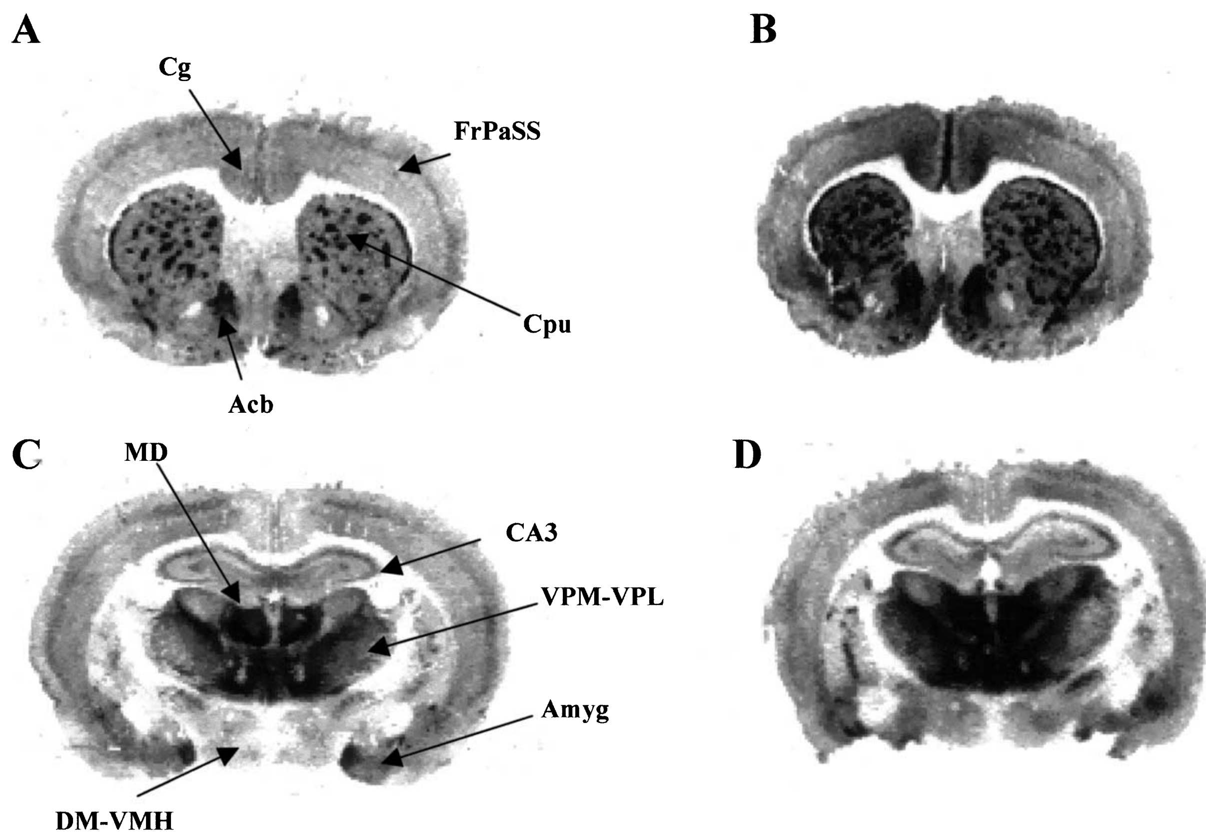

Fig. 2. Autoradiograms illustrating [^{3}H]DAMGO binding in coronal brain sections from representative animals of the different experimental groups. (A) and (C) Animals treated with saline for 7 days. (B) and (D) Animals treated with naltrexone (120 μg/h) for 7 days. Abbreviations: Cg, cingular cortex; FrPaSS; frontoparietal cortex-somatosensory area; Cpu, caudate-putamen; Acb, accumbens; CA_3, field CA_3 hippocampus; VPM-VPL, ventroposterolateral-ventroposteromedial thalamic nuclei; MD, mediodorsal thalamic nuclei; DM-VMH, dorsomedial and ventromedial hypothalamic nuclei; Amyg, amygdaloid nuclei. The images were scanned from the autoradiographic film. In the final image composition, brightness/contrast was adjusted to clear background.

Table 1. Autoradiographic densities of μ-opioid receptors in rat brain and spinal cord from animals treated chronically (7 days) with saline (control) or naltrexone (120 μg/h) using osmotic minipumps

Regions	Control ($n=5$)	Naltrexone ($n=4$)	% of change vs. control
Cerebral cortex			
Entorrinal cortex	73.15 ± 4.47	103.89 ± 7.44**	+42%
Cingular cortex	49.38 ± 3.28	84.56 ± 12.85*	+71%
Frontoparietal cortex			
Layers I–III	30.48 ± 1.95	43.87 ± 3.81*	+44%
Layer IV	48.64 ± 1.40	60.96 ± 6.99	+25%
Layers V–VI	38.27 ± 1.70	52.15 ± 5.66*	+36%
Basal ganglia			
Globus pallidus	32.15 ± 1.82	52.26 ± 6.27*	+63%
Caudate-putamen (patches)	173.45 ± 19.98	351.52 ± 42.20**	+103%
Accumbens nucleus	49.15 ± 7.32	109.37 ± 16.66**	+122%
Amygdala and hippocampus			
Lateral amygdala	70.25 ± 9.53	107.81 ± 3.49*	+53%
Basal amygdaloid nuclei	76.01 ± 6.80	85.91 ± 12.98	+13%
CA_3 stratum pyramidal, hippocampus	53.99 ± 3.88	66.58 ± 6.01	+23%
Hypothalamus			
Dorsomedial-ventromedial nuclei	15.19 ± 2.82	28.89 ± 5.52**	+90%
Lateral hypothalamic area	22.84 ± 2.63	40.72 ± 6.67*	+78%
Thalamus			
Laterodorsal nucleus	85.92 ± 11.01	79.61 ± 6.76	−7%
Ventrolateral-ventromedial nuclei	218.53 ± 12.15	275.75 ± 9.39*	+26%
Ventroposterolateral-ventroposteromedial nuclei	85.42 ± 2.20	109.90 ± 9.72*	+29%
Centromedial-dorsomedial nuclei	207.88 ± 14.24	311.57 ± 29.35*	+50%
Posterior nuclear group	192.88 ± 10.48	189.37 ± 7.27	−2%
Lateroposterior nucleus	146.34 ± 6.49	152.45 ± 14.17	+4%
Midbrain-hindbrain			
Substantia nigra	39.42 ± 6.04	81.63 ± 18.28*	+107%
Central grey	35.92 ± 4.44	59.47 ± 14.21	+66%
Locus coeruleus	76.63 ± 7.02	133.12 ± 14.68**	+74%
Tractus solitarii nucleus	49.20 ± 5.08	73.23 ± 9.68*	+49%
Spinal trigeminus nucleus	11.78 ± 2.64	19.23 ± 3.01	+63%
Spinal cord (lumbar level)			
Ventral horn (layers 7–9)	17.63 ± 1.44	16.22 ± 1.36	−8%
Dorsal horn (layers 1–2)	40.38 ± 2.15	83.71 ± 4.63**	+107%

Autoradiograms were generated using 5 nM [^{3}H]DAMGO. Non-specific binding was defined in the presence of 10 μM naloxone. Data are reported in fmol/mg tissue and expressed as the means ± S.E.M. of average measurements from n animals with at least two bilateral readings per brain in each animal. The percentage of change versus control values is also given. Significance was assessed with unpaired t-test. $*P<0.05$ and $**P<0.01$ vs. control.

GRK3: +174 ± 58%; GRK6: +330 ± 61%; β-arrestin 2: +50 ± 20%) and striatal lysates (GRK2: +54 ± 14%; GRK3: +210 ± 55%; GRK6: +70 ± 20%; β-arrestin 2: +100 ± 10%). The level of α-tubulin, a cytoskeletal protein used as control, was not changed (naltrexone: +9 ± 5% for cortex and −1 ± 10% for striatum, naloxone: +1 ± 10% for brain cortices and −2 ± 9% for striatum). This lack of change rules out any possible non-specific effect of opioid antagonists on GRKs and β-arrestin 2 regulation.

DISCUSSION

The present results demonstrate that 7 days chronic treatment with naltrexone enhances the respiratory depressant effect of the selective μ-opioid receptor agonist sufentanil, as measured 24 h after naltrexone treatment withdrawal. In the control group of rats the ED_{50} of sufentanil for depressing respiration was about five-fold that needed to produce analgesia in the tail-flick test, and the maximal respiratory depression attained was −61.4 ± 15.2%. It is known that awake rodents are very resistant to the respiratory effect of opioids, and that breathing arrest is very improbable to occur with these drugs, due to the counteracting mechanisms brought into action by the neural network controlling breathing in the unanaesthetised animal (Ruiz et al., 1993; Flórez and Hurlé, 1993). In our study, a relevant finding is that chronic naltrexone treatment potentiated the sufentanil depressant effect on breathing in a similar extent to the analgesic effect (three-fold). Furthermore, apnoea was induced in three out of eight animals with the highest dose of the opioid tested. In the clinical practice, respiratory depression following opioid drug administration is a troublesome secondary effect. Indeed, small acute analgesic doses of morphine and other opiates are able to depress ventilation in humans, being respiratory arrest responsible for fatal opioid overdose (Flórez and Hurlé, 1993). Considering that naltrexone is used as anti-craving agent for opiate and alcohol addictive processes (Kreek, 1996; Schaffer and Naranjo, 1998), the potential risk of overdose following a period of naltrexone administration, due to supersensitivity to opioids and/or other respiratory depressants such as alcohol and benzodiazepines, warrants further investigation.

The present study confirms and extents that supersensitivity to the behavioural opioid effects is accompanied

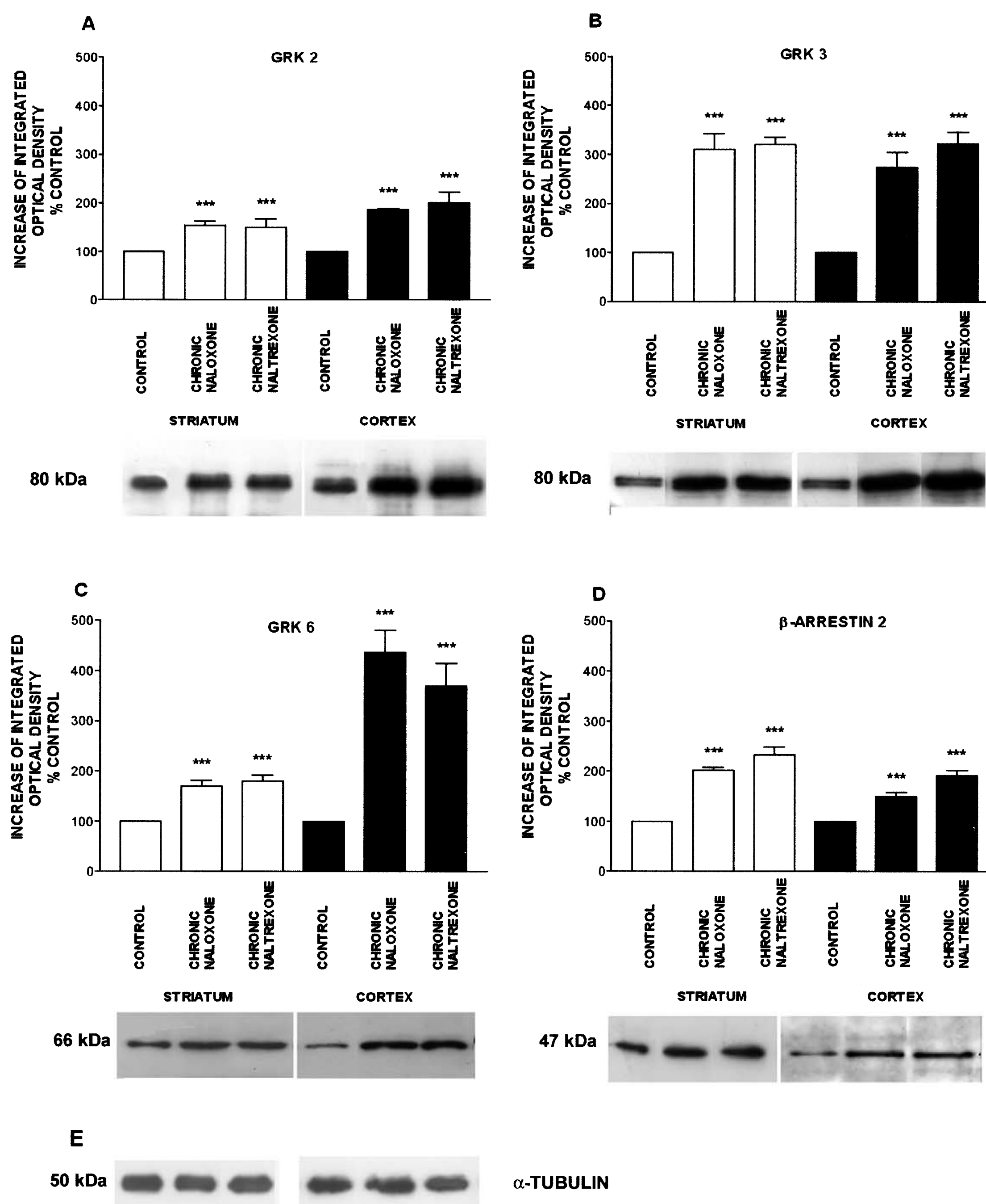

Fig. 3. Western blotting analysis of GRK2 (A), GRK3 (B), GRK6 (C), and β-arrestin 2 (D) and α-tubulin (E) immunoreactivity levels in striatum and cortex from rats of the different experimental groups. Control animals received a 7 days chronic infusion of saline (1 μl/h). Chronic naltrexone or naloxone were administered during 7 days at a dose of 120 μg/h. Immunoblots were performed 1 day after opioid antagonists or saline pump withdrawal. Blots were digitised, and the integrated optical density was estimated using densitometric analysis (Scion Image software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100% value). Data are means ± S.E.M. Statistical analysis was carried out by one-way ANOVA followed by Dunnett's multiple comparison test; $^{***}P < 0.001$. Representative immunoblots directly scanned from the films, without any image manipulation, are shown.

by up-regulation of μ-opioid receptors in several areas of the CNS, as determined by an increase in [^{3}H]DAMGO binding sites (Millan et al., 1988; Morris et al., 1988; Yoburn et al., 1986, 1989). Consistent with the functional data on analgesia, significant increases in the number of μ-opioid receptors were found in regions involved in opioidergic modulation of pain perception. It is also noteworthy the increase in receptor density observed in the nucleus of the solitary tract, an area involved in the control of breathing. Another interesting area of receptor up-regulation is the dopaminergic mesolimbic system because all drugs of abuse increase dopaminergic neurotransmission in this system, a property that has been related to their addictive potential (Maldonado et al.,

1997). Therefore, it is not unlikely that such a naltrexone dosing regime could potentiate the rewarding properties of opioids and other abused drugs after treatment discontinuation.

Recent studies involve GRKs and β-arrestins in the molecular mechanisms responsible for agonist-dependent desensitisation and endocytosis of μ-opioid receptors expressed in several cell lines (Kovoor et al., 1997, 1998; Zhang et al., 1997, 1998; Wang, 2000). In living animals, increased brain cortical levels of GRKs and β-arrestin 2 have been reported after acute or chronic treatment with opioid agonists such as morphine, heroin, and sufentanil (Hurlé, 2001; Ozaita et al., 1998; Terwilliger et al., 1994). Furthermore, in mice lacking β-arrestin 2 the analgesic effectiveness of morphine is enhanced, and these animals fail to develop morphine tolerance (Bohn et al., 1999, 2000).

Our present results clearly demonstrate that chronic treatment with two different opioid antagonists, naloxone and naltrexone, induces significant increases in the expression levels of GRK2, GRK3, GRK6 and β-arrestin 2 in brain cortex and striatum. According to the accepted theory on GPCRs regulation (see Leurs et al., 1998), receptor overexpression leads to a proportional increase in the number of spontaneously active receptors. Therefore, a possible explanation for our findings is that constitutive or agonist-independent signalling would be displayed after naltrexone/naloxone withdrawal, as a consequence of μ-opioid receptor up-regulation. Thus, auto-activated receptors would recruit GRKs and β-arrestins leading to an increase in the expression of these elements, as it occurs after agonist-dependent activation (Hurlé, 2001). However, this interpretation does not explain how the number of μ-opioid receptors is increased after exposure to antagonists. An alternative explanation would imply that naltrexone and naloxone were acting as inverse agonists at the μ-opioid receptor. Inhibition of the basal constitutive activity by inverse agonists would prevent constitutive desensitisation and receptor endocytosis, which would be seen as receptor up-regulation. This increased receptor density in membrane surface would, in turn, contribute to the observed increased agonist responses upon prolonged treatment with naltrexone and naloxone. Several experimental evidences support this interpretation. First, there is growing evidence indicating that such a pattern of GPCR regulation can be induced by inverse agonists but not by antagonists lacking negative intrinsic activity, as it has been described for μ-, δ- and κ-opioids, β_2-adrenergic and cannabinoid receptors, etc. (Bouaboula et al., 1999; Leurs et al., 1998; MacEwan and Milligan, 1996; Morris and Millan, 1991; Nagaraja et al., 1999). Second, various *in vivo* and *in vitro* studies have reported experimental evidence indicating that naloxone and naltrexone could exhibit negative intrinsic activity at opioid receptors (Costa and Herz, 1989; Wang et al., 1994; Chiu et al., 1996; Morris and Millan, 1991; Neilan et al., 1999; Burford et al., 2000; Zaki et al., 2000). Finally, a recent report has demonstrated a similar phenomenon to that reported herein for the β_2-adrenergic receptor (Nagaraja et al., 1999). Sustained treatment with inverse agonists of mice overexpressing β_2-adrenergic receptors induced sensitisation to agonists associated with increased expression levels of GRK2 in rat heart (Nagaraja et al., 1999).

However, when we compare the present data with previous results obtained under identical experimental conditions and, in some cases, from the same gels (Hurlé, 2001), strong quantitative differences are observed between agonist- and antagonist-treated animals. All GRKs and β-arrestin 2 suffered higher increases in the naltrexone- and naloxone-treated groups. Particularly, GRK3 remained unmodified in the agonist-treated group but displayed the highest up-regulation in the opioid antagonist groups. In our opinion, it is unlikely that the constitutive activity derived from a moderate receptor up-regulation, as seen in this study, could account for such an increase in GRKs an β-arrestin 2. In fact, this increase is even higher than that induced by chronic sufentanil, an extremely potent opioid agonist (Hurlé, 2001). Although we cannot give a plausible explanation for these differences, it is obvious that additional mechanisms must be involved in the effects of chronic exposure to naloxone and naltrexone on GRKs and β-arrestin 2 regulation. *In vitro* studies have demonstrated that GRK-mediated phosphorylation and β-arrestin binding are obligatory events for receptor recycling and resensitisation (Zhang et al., 1997; Cao et al., 1999; Oakley et al., 1999). The endogenous expression levels of GRKs and β-arrestins determine the capacity of some GPCRs, i.e. β-adrenergic receptors, to be recycled. Furthermore, the recycling and resensitisation of a sequestration-defective β_2-adrenergic receptor mutant was reestablished following the overexpression of either GRK2 or β-arrestin (Zhang et al., 1997). On the basis of these data, we could speculate that, somehow, the increases in GRKs and β-arrestin induced by opioid antagonists were involved in sorting receptor to the recycling pathway. Thus, it would be very interesting to assess in knock-out mice, lacking β-arrestin 2, if opioid receptor up-regulation and supersensitivity are prevented as it occurs with tolerance development (Bohn et al., 2000).

In summary, our results demonstrate that discontinuation of chronic treatment with naltrexone results in a similar potentiation of both the antinociceptive and respiratory depressant effects of the μ-opioid agonist sufentanil. This functional supersensitivity towards agonists is associated with an increase in μ-opioid receptor density in rat CNS. The immunoreactivity levels of GRK2, GRK3, GRK6 and β-arrestin 2 are also augmented, suggesting an increased constitutive activity secondary to μ-opioid receptor up-regulation.

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REFERENCES

Barlett, D., Tenney, S.M., 1970. Control of breathing in experimental anaemia. Respir. Physiol. 10, 384–395.
Bohn, L.M., Gainetdinov, R.R., Lin, F.T., Lefkowitz, R.J., Caron, M.G., 2000. Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature 408, 720–723.
Bohn, L.M., Lefkowitz, R.J., Gainetdinov, R.R., Peppel, K., Caron, M.G., Lin, F.T., 1999. Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 286, 2495–2498.
Bouaboula, M., Dussossoy, D., Casellas, P., 1999. Regulation of peripheral cannabinoid receptor CB_2 phosphorylation by the inverse agonist SR 144528. Implications for receptor biological responses. J. Biol. Chem. 274, 20397–20405.
Burford, N.T., Wang, D., Sadee, W., 2000. G-protein coupling of mu-opioid receptors (OP3): elevated basal signalling activity. Biochem. J. 348, 531–537.
Cao, T.T., Deacon, H.W., Reczek, D., Bretscher, A., von Zastrow, M., 1999. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. Nature 401, 286–290.
Chiu, T.T., Yung, L.Y., Wong, Y.H., 1996. Inverse agonistic effect of ICI-174,864 on the cloned delta-opioid receptor: role of G protein and adenylyl cyclase activation. Mol. Pharmacol. 50, 1651–1657.
Costa, T., Herz, A., 1989. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc. Natl. Acad. Sci. USA 86, 7321–7325.
Díaz, A., Pazos, A., Flórez, J., Hurlé, M.A., 2000. Autoradiographic mapping of mu-opioid receptors during opiate tolerance and supersensitivity in the rat central nervous system. Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 101–109.
Dierssen, M., Flórez, J., Hurlé, M.A., 1990. Calcium channel modulation by dihydropyridines modifies sufentanil-induced antinociception in acute and tolerant conditions. Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 559–565.
Fergusson, S.S., 2001. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signalling. Pharmacol. Rev. 53, 1–24.
Finney, D.J., 1964. Statistical Method in Biological Assay. Griffen, London.
Flórez, J., Hurlé, M.A., 1993. Opioids in respiration and vomiting. In: Herz, A., Akil, H., Simon, E. (Eds.), Handbook of Experimental Pharmacology: Opioids. Springer-Verlag, Berlin, pp. 263–292.
Hurlé, M.A., 2001. Changes in the expression of g protein-coupled receptor kinases and β-arrestin-2 in rat brain during opioid tolerance and supersensitivity. J. Neurochem. 77, 486–492.
Kovoor, A., Celver, J.P., Wu, A., Chavkin, C., 1998. Agonist induced homologous desensitization of mu-opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. Mol. Pharmacol. 54, 704–711.
Kovoor, A., Nappey, V., Kieffer, B.L., Chavkin, C., 1997. Mu and delta opioid receptors are differentially desensitized by the coexpression of beta-adrenergic receptor kinase 2 and beta-arrestin 2 in xenopus oocytes. J. Biol. Chem. 272, 27605–27611.
Kreek, M.J., 1996. Opiates, opioids and addiction. Mol. Psychiatry 1, 232–254.
Law, P.Y., Wong, Y.H., Loh, H.H., 2000. Molecular mechanisms and regulation of opioid receptor signalling. Annu. Rev. Pharmacol. Toxicol. 40, 389–430.
Leurs, R., Smith, M.J., Alewijnse, A.E., Timmerman, H., 1998. Agonist-independent regulation of constitutively active G-protein-coupled receptors. Trends Biochem. Sci. 23, 418–422.
Lichtfield, J.T., Wilcoxon, F., 1949. A simplified method for evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96, 99–113.
Lowry, O-H., Roseborough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275.
MacEwan, D.J., Milligan, G., 1996. Up-regulation of a constitutively active form of the beta2-adrenoceptor by sustained treatment with inverse agonists but not antagonists. FEBS Lett. 399, 108–112.
Maldonado, R., Saiardi, A., Valverde, O., Samad, T.A., Roques, B.P., Borrelli, E., 1997. Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. Nature 388, 586–589.
Millan, M.J., Morris, B.J., Herz, A., 1988. Antagonist-induced opioid receptor up-regulation. I. Characterization of supersensitivity to selective mu and kappa agonists. J. Pharmacol. Exp. Ther. 247, 721–728.
Morris, B.J., Millan, M.J., 1991. Inability of an opioid antagonist lacking negative intrinsic activity to induce opioid receptor up-regulation *in vivo*. Br. J. Pharmacol. 102, 883–886.
Morris, B.J., Millan, M.J., Herz, A., 1988. Antagonist-induced opioid receptor up-regulation. II. Regionally specific modulation of mu, delta and kappa binding sites in rat brain revealed by quantitative autoradiography. J. Pharmacol. Exp. Ther. 247, 729–736.
Nagaraja, S., Iyer, S., Liu, X., Eichberg, J., Bond, R.A., 1999. Treatment with inverse agonists enhances baseline atrial contractility in transgenic mice with chronic beta2-adrenoceptor activation. Br. J. Pharmacol. 127, 1099–1104.
Neilan, C.L., Akil, H., Woods, J.H., Traynor, J.R., 1999. Constitutive activity of the delta-opioid receptor expressed in C6 glioma cells: identification of non-peptide delta-inverse agonists. Br. J. Pharmacol. 128, 556–562.
Oakley, R.H., Laporte, S.A., Holt, J.A., Barak, L.S., Caron, M.G., 1999. Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. J. Biol. Chem. 274, 32248–32257.
Ozaita, A., Escriba, P.V., Ventayol, P., Murga, C., Mayor, F., Garcia-Sevilla, J.A., 1998. Regulation of G protein-coupled receptor kinase 2 in brains of opiate-treated rats and human opiate addicts. J. Neurochem. 70, 1249–1257.
Paxinos, G., Watson, C., 1982. The Rat Brain in Stereotaxic Coordinates. Academic Press, New York.
Premont, R.T., Inglese, J., Lefkowitz, R.J., 1995. Protein kinases that phosphorylate activated G protein coupled receptors. FASEB J. 9, 175–182.
Ruiz, F., Dierssen, M., Flórez, J., Hurlé, M.A., 1993. Potentiation of acute opioid-induced respiratory depression and reversal of tolerance by the calcium antagonist nimodipine in awake rats. Naunyn-Schmiedeberg's Arch. Pharmacol. 348, 633–637.
Schaffer, A., Naranjo, C.A., 1998. Recommended drug treatment strategies for the alcoholic patient. Drugs 56, 571–585.
Terwilliger, R.Z., Ortiz, J., Guitart, X., Nestler, E.J., 1994. Chronic morphine administration increases beta-adrenergic receptor kinase (beta ARK) levels in the rat locus coeruleus. J. Neurochem. 63, 1983–1986.
Unterwald, E.M., Rubenfeld, J.M., Imai, Y., Wang, J.B., Uhl, G.R., Kreek, M.J., 1995. Chronic opioid antagonist administration upregulates mu opioid binding without altering mu opioid receptor mRNA levels. Brain Res. Mol. Brain Res. 33, 2351–2355.
Wang, H.L., 2000. A cluster of Ser/Thr residues at the C-terminus of μ-opioid receptor is required for G protein-coupled receptor kinase 2-mediated desensitization. Neuropharmacology 39, 353–363.
Wang, Z., Bilsky, E.J., Porreca, F., Sadee, W., 1994. Constitutive mu opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. Life Sci. 54, 339–350.
Yoburn, B.C., Kreuscher, S.P., Inturrisi, C.E., Sierra, V., 1989. Opioid receptor upregulation and supersensitivity in mice: effect of morphine sensitivity. Pharmacol. Biochem. Behav. 32, 727–731.
Yoburn, B.C., Nunes, F.A., Adler, B., Pasternak, G.W., Inturrisi, C.E., 1986. Pharmacodynamic supersensitivity and opioid receptor upregulation in the mouse. J. Pharmacol. Exp. Ther. 239, 132–135.

Zaki, P.A., Keith, D.E., Brine, G.A., Carroll, F.I., Evans, C.J., 2000. Ligand-induced changes in surface mu-opioid receptor number: Relationship to G protein activation. J. Pharmacol. Exp. Ther. 292, 2705–27014.

Zhang, J., Barak, L.S., Winkler, K.E., Caron, M.G., Ferguson, S.S., 1997. A central role for β-arrestins and clathrin-coated vesicle-mediated endocytosis in β_2-adrenergic receptor resensitization. J. Biol. Chem. 272, 193–199.

Zhang, J., Ferguson, S.S., Barak, L.S., Bodduluri, S.R., Laporte, S.A., Law, P.Y., Caron, M.G., 1998. Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. Proc. Natl. Acad. Sci. USA 95, 7157–7162.

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