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# Differential desensitization of μ- and δ- opioid receptors in selected neural pathways following chronic morphine treatment

[1]Florence Noble & [2]Brian M. Cox

Department of Pharmacology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799, U.S.A.

**1** Morphine produces a plethora of pharmacological effects and its chronic administration induces several side-effects. The cellular mechanisms by which opiates induce these side-effects are not fully understood. Several studies suggest that regulation of adenylyl cyclase activity by opioids and other transmitters plays an important role in the control of neural function.

**2** The aim of this study was to evaluate desensitization of μ- and δ- opioid receptors, defined as a reduced ability of opioid agonists to inhibit adenylyl cyclase activity, in four different brain structures known to be involved in opiate drug actions: caudate putamen, nucleus accumbens, thalamus and periaqueductal gray (PAG). Opiate regulation of adenylyl cyclase in these regions has been studied in control and morphine-dependent rats.

**3** The chronic morphine treatment used in the present study (subcutaneous administration of 15.4 mg morphine/rat/day for 6 days via osmotic pump) induced significant physical dependence as indicated by naloxone-precipitated withdrawal symptoms.

**4** Basal adenylyl cyclase in the four brain regions was not modified by this chronic morphine treatment. In the PAG and the thalamus, a desensitization of μ- and δ-opioid receptors was observed, characterized by a reduced ability of Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO; μ), Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE; δ) and [D-Ala$^2$]-deltorphin-II (DT-II; δ) to inhibit adenylyl cyclase, activity following chronic morphine treatment.

**5** The opioid receptor desensitization in PAG and thalamus appeared to be heterologous since the metabotropic glutamate receptor agonists, L-AP4 and glutamate, and the 5-hydroxytryptamine $(5\text{-HT})_{1A}$ receptor agonist, R(+)-8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT), also showed reduced inhibition of adenylyl cyclase activity following chronic morphine treatment.

**6** In the nucleus accumbens and the caudate putamen, desensitization of δ-opioid receptor-mediated inhibition without modification of μ-opioid receptor-mediated inhibition was observed. An indirect mechanism probably involving dopaminergic systems is proposed to explain the desensitization of δ-mediated responses and the lack of μ-opioid receptor desensitization after chronic morphine treatment in caudate putamen and nucleus accumbens.

**7** These results suggest that adaptive responses occurring during chronic morphine administration are not identical in all opiate-sensitive neural populations.

**Keywords:** Caudate-putamen; nucleus accumbens; thalamus; periaqueductal gray; opioid receptors; desensitization; morphine; chronic treatment; adenylyl cyclase

## Introduction

The wide distribution of opioid receptors in the central nervous system (CNS) probably accounts for the multiplicity of pharmacological responses elicited by acute administration of morphine, or its surrogates. Moreover, chronic treatment leads to adaptive changes including antinociceptive tolerance, and physical and psychic dependence, that create significant clinical problems in the use of opiate drugs. These changes may be related to prolonged activation of opioid receptors by opiate drugs in all brain areas (review in Nestler *et al.*, 1993).

The cellular mechanisms by which opiates induce tolerance and/or dependence are uncertain. Attention has been focused on post-receptor mechanisms, as it is generally accepted that alterations in opioid receptors *per se* cannot account for all aspects of opioid addiction. Accumulating evidence suggests a major role for adaptive changes in the regulation of adenylyl cyclase during long-term opiate exposure (Sharma *et al.*, 1975; Duman *et al.*, 1988; Puttfarcken *et al.*, 1988; Puttfarcken & Cox, 1989; De Vries *et al.*, 1993; review in Nestler *et al.*, 1993).

The locus coeruleus, a pontine nucleus containing the largest collection of noradrenergic neurones in the CNS, has been extensively examined. Several studies have related the locus coeruleus to the development of morphine-dependence, and have demonstrated that this structure is prominently associated with somatic symptoms of opiate withdrawal (Aghajanian, 1978; Maldonado *et al.*, 1992). Moreover, in this brain area, it has been shown that chronic morphine treatment induces an up-regulation of the cyclic AMP system (adenylyl cyclase, cyclic AMP-dependent protein kinase, $G_{1\alpha}$ and $G_{0\alpha}$) (Duman *et al.*, 1988; Nestler & Tallman, 1988; Nestler *et al.*, 1989; Matsuoka *et al.*, 1994), alterations that seem to be important for the expression of the various behavioural responses observed during withdrawal syndrome (Rasmussen *et al.*, 1990). Another brain structure widely examined is the nucleus accumbens, as dopaminergic neurones projecting from the ventral tegmental area to the nucleus accumbens might play a major role in the rewarding/reinforcing properties of opiates or other psychostimulant drugs (Phillips & Le Piane, 1982; Bozarth, 1986; Cador *et al.*, 1991). In this brain area, similar

[1]Present address: Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM-URA D 1500 CNRS, Faculte de Pharmacie, Université Rene Descartes, 4 ave. de l'Observatoire, 75270 Paris Cedex 06, France
[2]Author for correspondence.

biochemical modifications were observed in the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) system following chronic morphine treatment. These biochemical changes, which also seem to occur in a few other brain structures (amygdala or thalamus), are not a generalized response exhibited by all brain regions (Terwilliger *et al.*, 1991).

Nevertheless, desensitization of $\mu$- and /or $\delta$-opioid receptors (defined as a reduced ability of the agonist to inhibit the enzyme adenylyl cyclase) in different brain regions from animals treated chronically with morphine has not been extensively investigated. The objective of the current study was to compare the desensitization of $\mu$- and $\delta$-opioid receptors in brain regions involved in the motor and reinforcing properties of opiates (caudate putamen, nucleus accumbens) with desensitization in brain regions participating in the antinociceptive actions of opiates (thalamus and periaqueductal gray; PAG). For this purpose, possible changes in inhibition of adenylyl cyclase activity by the highly selective $\mu$-opioid agonist, Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO; Handa *et al.*, 1981), and the $\delta$-opioid agonists, Tyr-D-P*en-Gly-Phe-D-Pe*n (DPDPE) and [D-Ala$^2$]deltorphin-II (DT-II; Mosberg *et al.*, 1983; Erspamer *et al.*, 1989), were investigated in these rat brain regions following chronic morphine treatment.

## Methods

### *Animals and surgery*

Male Sprague-Dawley rats (Taconic, Germantown, NY, U.S.A.) weighing 220–250 g at the start of the experiment, were used. Rats were group-housed in standard laboratory cages and kept in a temperature- and humidity-controlled colony room at least one week before the surgery. Food and water were available *ad libitum*.

Osmotic pumps (2ML1, Alza Corporation, Palo Alto, CA) which deliver 10 $\mu$l h$^{-1}$, were used to administer saline or morphine by continuous s.c. infusion. The pumps were filled either with 64 mg ml$^{-1}$ morphine sulphate in saline or with saline alone. The pumps were surgically implanted s.c., caudal to the dorsum of the neck, under halothane anaesthesia. Then, rats were housed in single-animal cages with free access to food and water.

### *Assessment of physical dependence*

On the sixth day of morphine treatment a behavioural withdrawal syndrome was precipitated by injection of naloxone hydrochloride (1 mg kg$^{-1}$, s.c.). Immediately after naloxone injection, the rats were placed individually in test chambers, and withdrawal symptoms were evaluated over a 20 min period. The numbers of jumps, and of episodes of wet-dog shakes, paw tremor, teeth chattering, and mastication were each counted independently during the 20 min observation period. Tremor, ptosis, and diarrhoea were evaluated over four 5 min periods with one point being given for the presence of each of these symptoms during each period (maximum score, 4).

### *Membrane preparation*

On the seventh day rats were killed by decapitation. Their brains were rapidly removed. The periaqueductal gray region (PAG) was excised from brain sections by punch-out, using the rat brain atlas of Pellegrino *et al.* (1979) as a guide. Caudate putamen, nucleus accumbens and thalamus were obtained by gross dissection. Dissected tissue from isolated brain regions of a single rat was homogenized and diluted into buffer [20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM $MgCl_2$ and 250 mM sucrose] and centrifuged at 27,000 *g* for 15 min at 4°C. The pellet was resuspended in fresh buffer and centrifuged again for 15 min. The supernatant was discarded and the tissue was homogenized in 30% (wt/vol) of ice-cold buffer [2 mM Tris-HCl (pH 7.4) and 2 mM EGTA] for determination of adenylyl cyclase activity.

### *Determination of adenylyl cyclase activity*

Tissue homogenate (15–30 $\mu$g of protein in 10 $\mu$l) was added on ice to assay tubes (final volume 60 $\mu$l) containing 80 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM $MgSO_4$, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM ATP, 0.01 mM GTP and either the drug being tested or water. Triplicate samples for each treatment were incubated at 30°C for 5 min. Adenylyl cyclase activity was terminated by placing the tubes in boiling water for 2 min. The amount of cyclic AMP formed was determined by a [$^3$H]-cyclic AMP protein binding assay (Brown *et al.*, 1971; Noble & Cox, 1995). [$^3$H]-cyclic AMP (final concentration 4 nM) in citrate-phosphate buffer (pH 5.0) followed by binding protein prepared from bovine adrenal glands were added to each sample. Additional samples were prepared, without tissue, containing known amounts of cyclic AMP and served as standards for quantification. The binding reaction was allowed to reach equilibrium by incubation for 90 min at 4°C, and the assay was terminated by the addition of charcoal and centrifugation (1000 *g* for 10 min, at 4°C) to separate the free tritiated cyclic AMP from that which was bound to the binding protein. Aliquots from the supernatant containing bound cyclic AMP were placed in scintillation vials to which Beckman Ready Value Scintillation Cocktail was added and radioactivity was determined by liquid scintillation spectrometry. Radioactivity was converted to pmol of cyclic AMP by comparison to the curve derived from the standards. Protein values were determined by a modification of Lowry procedure with bovine serum albumin used as standard (Peterson, 1977). Results are expressed as percentage of the respective basal activity (i.e. naive or morphine-dependent rats) measured in the absence of opioid.

Adenylyl cyclase activity in the various brain regions studies from saline- or morphine-treated rats did not differ across days, and between rats that had received an acute s.c. injection of naloxone or saline during the behavioural study. Thus, all data from replicate experiments were pooled according to brain region for adenylyl cyclase assay analysis.

### *Chemicals*

DAMGO (Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol), DPDPE (Tyr-D-P*en-Gly-Phe-D-Pe*n), [D-Ala$^2$]deltorphin II (DT-II), L-glutamic acid monosodium salt (glutamate) and L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.), naloxone hydrochloride, naltrindole hydrochloride, **R**(+)-8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT), (±)-$\alpha$-methyl-4-carboxyphenylglycine (MCPG) and pindobind-5-$HT_{1A}$ from Research Biochemicals Inc. (Natick, MA), CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$) from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.) and morphine sulphate from Merck Chemical Division (Rahway, NJ, U.S.A.). The other reagents were obtained from the following sources: [$^3$H]-cyclic AMP (ammonium salt; specific activity 28:1 Ci mmol$^{-1}$) from Du Pont NEN Research Products (Boston, MA, U.S.A.); ATP (disodium salt), GTP (lithium salt), cyclic AMP (sodium salt), theophylline, EGTA from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### *Statistical analysis*

Differences among treatment groups for each withdrawal sign were analyzed by one-way analysis of variance (ANOVA). Comparisons of concentration-response curves from adenylyl cyclase assays were analyzed with a two-way ANOVA. If a significant treatment effect was observed, a one-way ANOVA was used, followed by a Newman-Keuls test, to determine the significance at each concentration. Antagonist studies were analyzed with a one-way ANOVA, followed by a Newman-

Keuls test for multiple comparisons. The level of significance was set at $P<0.05$.

## Results

### *Behavioural changes during opioid withdrawal*

Rats given continuous morphine infusions for six days were tested for physical dependence by the administration of naloxone (1 mg $kg^{-1}$, s.c.). The withdrawal signs observed over a 20 min period immediately after injection of naloxone are shown in Figure 1. One-way ANOVA revealed significant effects for all signs observed. Administration of naloxone had a significant effect on the number of teeth chattering events [$F(1,15)=45.76$, $P<10^{-4}$], mastication [$F(1,15)=41.80$, $P<10^{-4}$], wet dog shakes [$F(1,15)=54.75$, $P<10^{-4}$] and paw tremor [$F(1,15)=5.76$, $P<0.05$]. For tremor and ptosis, significant differences were also observed: $F(1,15)=47.82$, $P<10^{-4}$ and $F(1,15)=336.19$, $P<10^{-4}$, respectively. A significant effect was also observed in the case of diarrhoea: $F(1,15)=48.12$, $P<10^{-4}$. Moreover, an increase in jumping occurred in morphine-treated rats following naloxone administration (number of events observed: $1.4\pm0.5$), while this withdrawal sign was not observed in saline-treated rats (number of events: $0\pm0$) (data not shown).

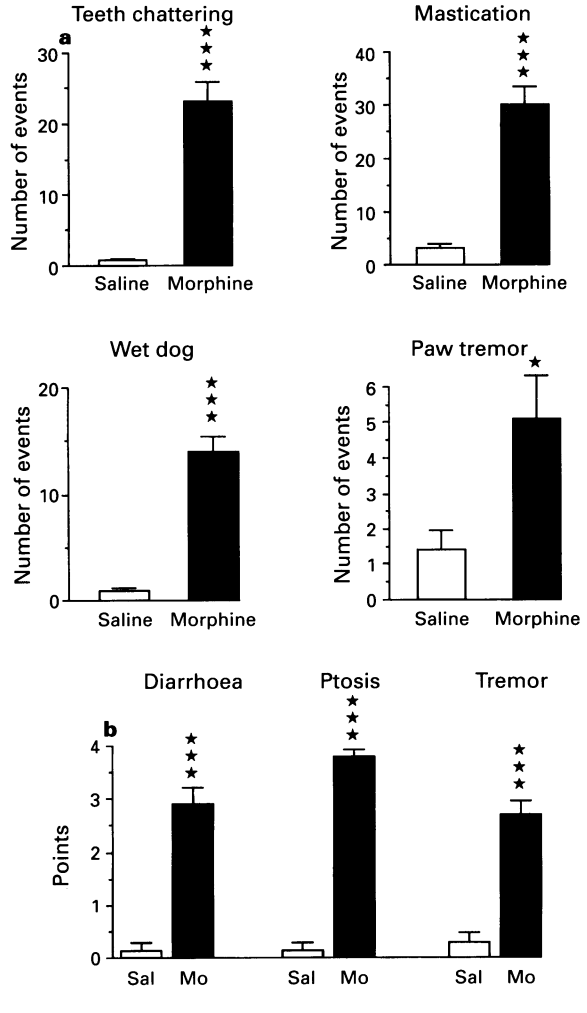


**Figure 1** Effects of naloxone administration (1 mg $kg^{-1}$, s.c.) on behaviour (teeth chattering, mastication, wet dog shakes, paw tremor diarrhoea, ptosis and tremor) in rats chronically treated with morphine for 6 days. Results are expressed as means ± s.e.mean, (a) of the number of episodes counted during the 20 min period of observation immediately after naloxone injection, or (b) on an arbitrary point scale: one point was given for the presence of each sign over 5 min periods during the 20 min of observation (maximum score: 4). Number of animals per group: 7–8. $\star P<0.05$, $\star\star\star P<0.001$ vs. saline-treated group (Student's $t$ test).

### *Effects of chronic morphine treatment on basal adenylyl cyclase activity*

The amounts of cyclic AMP produced by the membrane preparations under basal conditions (i.e., without added stimulator or inhibitor of enzyme activity) in the four brain regions studied, nucleus accumbens, caudate putamen, thalamus and PAG, were not statistically different in rats chronically treated with morphine or with saline.

In animals chronically treated with saline, basal cyclic AMP production was $19\pm3$, $25\pm4$, $18\pm5$ and $31\pm4$ pmol $mg^{-1}$ protein $min^{-1}$ in the nucleus accumbens, caudate putamen, thalamus and PAG, respectively. Following chronic morphine treatment, the amounts of basal cyclic AMP produced were $25\pm3$ (nucleus accumbens), $31\pm1$ (caudate putamen), $14\pm1$ (thalamus) and $23\pm3$ (PAG) pmol $mg^{-1}$ protein $min^{-1}$.

It should be pointed out that any residual morphine that might be present in the membrane preparation from the different brain structures studied did not influence the determination of adenylyl cyclase activity, since the addition of naloxone (100 $\mu$M) to the assays did not affect adenylyl cyclase activity in membranes from control or morphine-treated rats (data not shown).

### *Effects of chronic morphine treatment on inhibition of adenylyl cyclase activity by δ-opioid agonists*

The selective δ-opioid agonists, DPDPE and DT-II, inhibited basal adenylyl cyclase activity in the four brain areas studied: caudate putamen, nucleus accumbens, thalamus and PAG, of saline-treated rats. These inhibitory effects were dose-dependent.

DPDPE inhibited adenylyl cyclase activity with a maximal inhibition of $30\pm4\%$, $30\pm2\%$, $34\pm5\%$, and $35\pm4\%$ in the caudate putamen, nucleus accumbens, thalamus and PAG, respectively, in saline-treated rats. Continuous subcutaneous

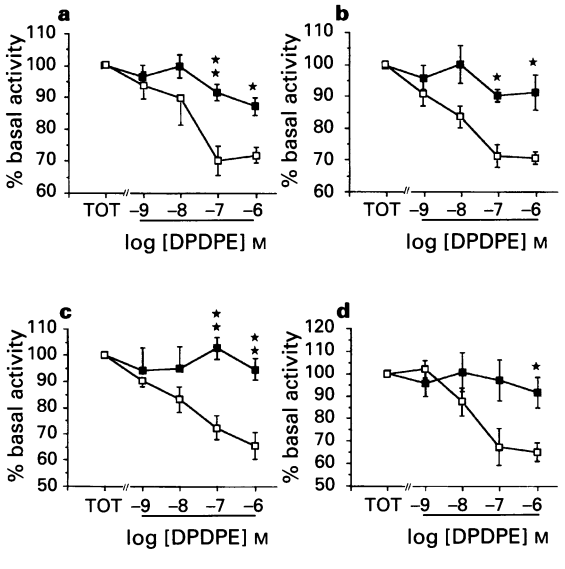


**Figure 2** Effect of DPDPE on basal adenylyl cyclase activity in the caudate putamen (a), nucleus accumbens (b), thalamus (c) and PAG (d) in saline-treated rats (□) or morphine-treated rats (■). Results are expressed as means ± s.e.mean from three or more independent experiments, each performed in triplicate. $\star P<0.05$ and $\star\star P<0.01$ show significant differences at the same dose of opioid agonist (Newman Keuls test).

administration of morphine for six days significantly attenuated the ability of DPDPE to inhibit adenylyl cyclase activity. Two-way ANOVA revealed significant differences between dose-response curves obtained with DPDPE in saline-treated rats as compared to morphine-treated animals: $F(1,27)=5.640$, $P<0.05$; $F(1,30)=28.529$, $P<0.001$; $F(1,34)=17.186$, $P<0.001$ and $F(1,28)=9.135$, $P<0.01$ in the caudate putamen, nucleus accumbens, thalamus and PAG, respectively (Figure 2).

Similar results were obtained with another $\delta$-receptor-selective agonist; DT-II inhibited adenylyl cyclase activity in a concentration-dependent manner with maximal inhibition of $28\pm1\%$, $32\pm6\%$, $35\pm4\%$, and $33\pm2\%$, in the caudate putamen, thalamus, PAG and nucleus accumbens, respectively, in saline-treated rats. Two-way ANOVA revealed significant differences between dose-response curves obtained with DT-II in saline-treated rats as compared to morphine-treated animals: $F(1,24)=13.958$, $P<0.001$; $F(1,31)=28.749$, $P<0.001$; $F(1,31)=24.269$, $P<0.001$ and $F(1,31)=11.443$, $P<0.01$ in the caudate putamen, nucleus accumbens, thalamus and PAG, respectively (Figure 3).

### *Effects of chronic morphine treatment on inhibition of adenylyl cyclase activity by μ-opioid agonists*

The highly selective $\mu$-agonist, DAMGO (a full agonist), inhibited basal adenylyl cyclase activity in the four brain structures studied: caudate putamen, nucleus accumbens, thalamus and PAG in saline-treated rats. The $\mu$-preferential agonist morphine (a partial agonist) also inhibited basal adenylyl cyclase activity in the caudate putamen and the nucleus accumbens. These inhibitory effects were dose-dependent.

DAMGO inhibited adenylyl cyclase activity with a maximal inhibition of $32\pm3\%$, $33\pm4\%$, $43\pm3\%$, and $44\pm3\%$ in the caudate putamen, nucleus accumbens, thalamus and PAG respectively, in saline-treated rats. Continuous s.c. infusion of morphine significantly attenuated the ability of DAMGO to inhibit adenylyl cyclase activity in the PAG and thalamus. Two-way ANOVA revealed significant differences between dose-response curves obtained with DAMGO in saline-treated rats as compared to morphine-treated animals: $F(1,23)=44.192$, $P<0.001$ and $F(1,28)=8.799$, $P<0.01$ in the thalamus and the PAG, respectively. In contrast, two-way ANOVA revealed no differences in the ability of DAMGO to inhibit adenylyl cyclase activity in the caudate putamen and the nucleus accumbens in morphine-treated animals as compared to saline-treated rats: $F(1,25)=0.149$, $P>0.05$ and $F(1,35)=0.001$, $P>0.05$, respectively (Figure 4). It should be noted that the caudate putamen and nucleus accumbens preparations, which showed no loss of the inhibitory activity of DAMGO, were taken from the same rats that showed significant loss of DAMGO inhibition in thalamus and PAG.

In order to determine if any loss of agonist-induced inhibitory activity could be detected if a partial agonist instead of a full agonist (DAMGO) were used, the inhibitory effect of morphine was also tested in these two brain regions. Morphine inhibited adenylyl cyclase activity in a concentration-dependent manner with maximal inhibition of $30\pm2\%$ and $32\pm4\%$ produced by 10 $\mu$M, in the caudate putamen and the nucleus accumbens, respectively, in saline-treated rats. A small rightwards shift of the dose-response curves obtained with the alkaloid on the inhibition of adenylyl cyclase activity were observed in both structures following chronic morphine treatment. Nevertheless, two-way ANOVA analysis revealed a significant chronic morphine-treatment effect in the caudate putamen: $F(1,21)=4.557$, $P<0.05$ but not in the nucleus accumbens: $F(1,21)=4.234$, $P>0.05$ (Figure 5).

### *Effects of naloxone, CTOP and naltrindole on inhibition of adenylyl cyclase activity induced by DAMGO or morphine in saline- or morphine-treated rats in caudate putamen and nucleus accumbens*

Studies with opioid-receptor type-selective antagonists indicated that the nature of the receptor activated by DAMGO and morphine to regulate adenylyl cyclase activity was not altered by the chronic morphine treatment. DAMGO (1 $\mu$M)-induced inhibition of adenylyl cyclase activity in saline-treated rats was selectively antagonized by naloxone (0.1 $\mu$M) and by the highly selective $\mu$-antagonist, CTOP (0.1 $\mu$M), in the caudate putamen and the nucleus accumbens. In contrast the selective $\delta$-opioid receptor antagonist, naltrindole (10 nM), did

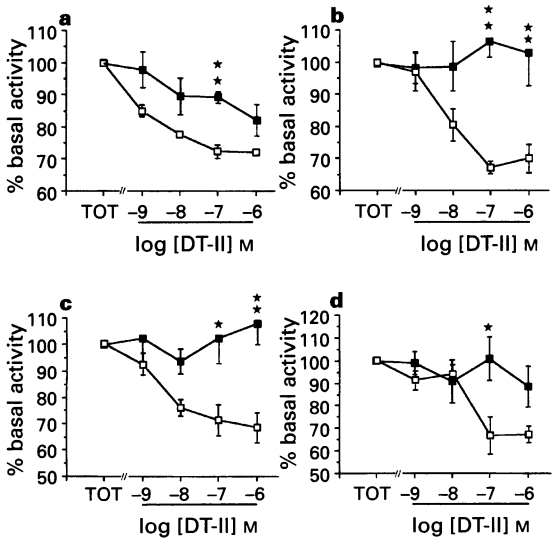


**Figure 3** Effect of DT-II on basal adenylyl cyclase activity in the caudate putamen (a), nucleus accumbens (b), thalamus (c) and PAG (d) in saline-treated rats (□) or morphine-treated rats (■). Results are expressed as means±s.e.mean from three or more independent experiments, each performed in triplicate. $\star P<0.05$ and $\star\star P<0.01$ show significant differences at the same dose of opioid agonist (Newman Keuls test).

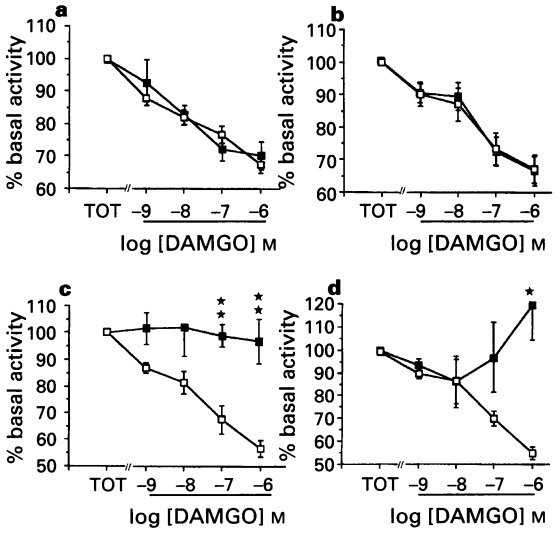


**Figure 4** Effect of DAMGO on basal adenylyl cyclase activity in the caudate putamen (a), nucleus accumbens (b), thalamus (c) and PAG (d) in saline-treated (□) or morphine-treated rats(■). Results are expressed as means±s.e.mean from three or more independent experiments, each performed in triplicate. $\star P<0.05$ and $\star\star P<0.01$ show significant differences at the same dose of opioid agonist (Newman Keuls test).

not modify the inhibition induced by DAMGO, in either structure (Figure 6). Similar results were obtained in morphine-treated rats: naloxone and CTOP antagonized the inhibition induced by the $\mu$-selective agonist, DAMGO, in both the caudate putamen and the nucleus accumbens, while NTI did not modify the inhibition induced by DAMGO (Figure 7).

Inhibitions of adenylyl cyclase activity induced by morphine (1 $\mu$M) in the caudate putamen and the nucleus accumbens of saline-treated rats were also selectively antagonized by naloxone (1 $\mu$M) and CTOP (0.1 $\mu$M), but not by the $\delta$-selective antagonist, naltrindole (10 $\mu$M). Similar results were obtained in both brain structures following chronic morphine treatment (data not shown).

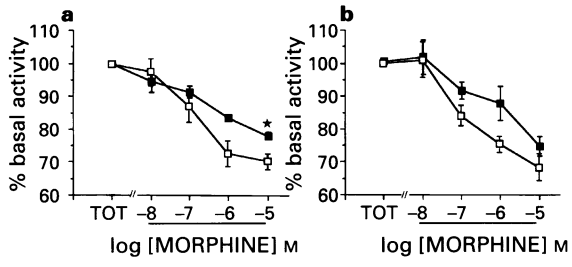


**Figure 5** Effect of morphine on basal adenylyl cyclase activity in the caudate putamen (a) and nucleus accumbens (b) in saline-treated rats (□) or morphine-treated rats (■). Results are expressed as means±s.e.mean from three or more independent experiments, each performed in triplicate. ★$P<0.05$ shows significant differences at the same dose of opioid agonist (Newman Keuls test).

### *Effects of chronic morphine treatment on inhibition of adenylyl cyclase activity by 5-HT$_{1A}$ receptor agonist and metabotropic glutamate receptor agonists*

In the thalamus and PAG, chronic exposure to the moderately $\mu$-selective agonist, morphine, induced loss of agonist response for agents acting selectively at both $\mu$- and $\delta$-receptors. This suggested that the desensitization was heterologous. To confirm this, the effects of morphine treatment on the activities of agents regulating adenylyl cyclase in these brain regions through different receptors was evaluated. In the thalamus, metabotropic glutamate receptor (mGluR) agonist, glutamate and L-AP4, and the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, inhibited basal adenylyl cyclase activity in a dose-dependent manner in saline-treated rats (Figure 8). These inhibitions were antagonized by the mGluR antagonist MCPG (0.1 mM and 1 mM) and by the 5-HT$_{1A}$ receptor antagonist, pindobind-5-HT$_{1A}$ (100 nM), respectively. Continuous administration of morphine significantly attenuated the ability of these three agonists to inhibit adenylyl cyclase in the thalamus. Thus, two-way ANOVA revealed significant differences between dose-response curves obtained with glutamate ($F(1,19)=13.331$, $P<0.01$), L-AP4 ($F(1,22)=37.250$, $P<0.001$) and 8-OH-DPAT ($F(1,22)=31.246$, $P<0.001$), in control rats as compared to morphine-treated animals (Figure 8).

In the PAG, 8-OH-DPAT also inhibited basal adenylyl cyclase activity in a dose-dependent manner in control rats

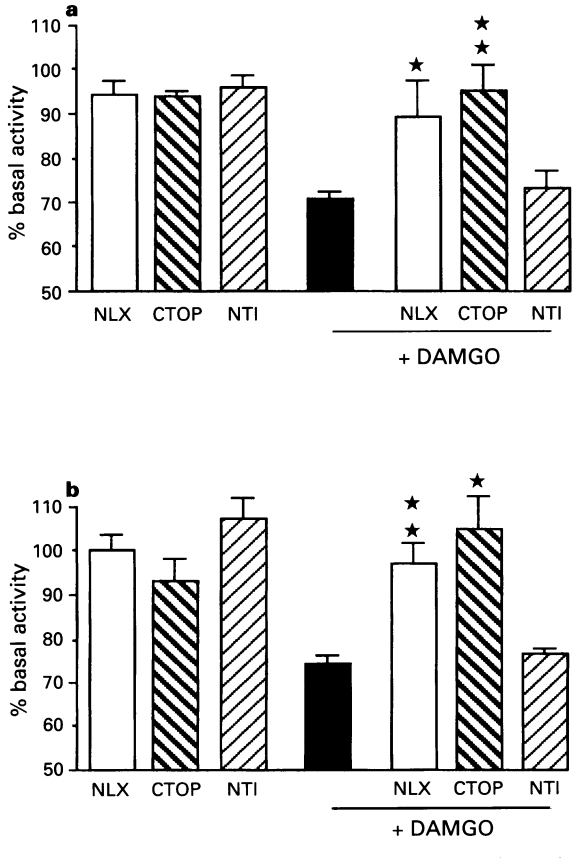


**Figure 6** Effects of naloxone (NLX; 0.1 $\mu$M), CTOP (0.1 $\mu$M) and naltrindole (NTI; 10 nM) on the inhibitory effect of DAMGO ($10^{-7}$ M) on basal adenylyl cyclase activity in the nucleus accumbens (a) and caudate putamen (b) in saline-treated rats. Results are expressed as means±s.e.mean from three or more independent experiments, each performed in triplicate. ★$P<0.05$, ★★$P<0.01$ as compared to DAMGO alone (Newman Keuls test).

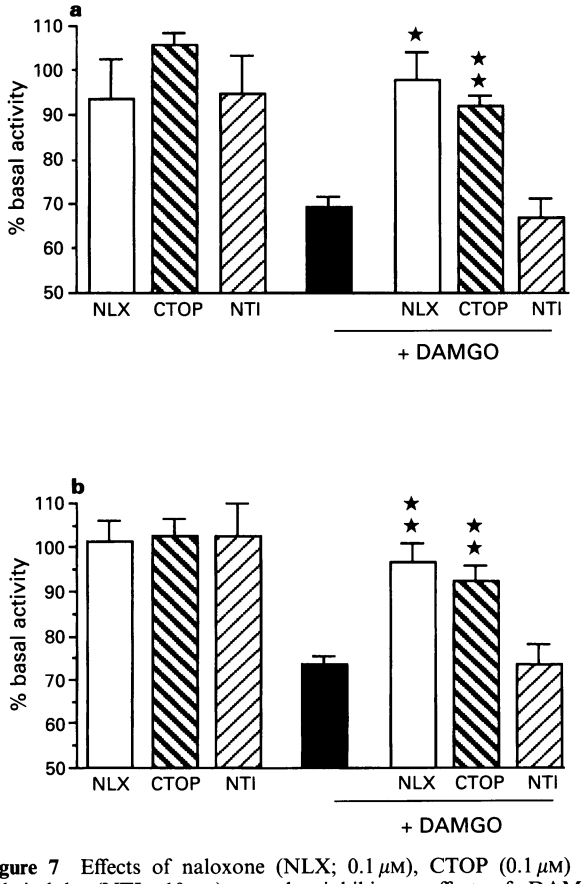


**Figure 7** Effects of naloxone (NLX; 0.1 $\mu$M), CTOP (0.1 $\mu$M) and naltrindole (NTI; 10 nM) on the inhibitory effect of DAMGO ($10^{-7}$ M) on basal adenylyl cyclase activity in the nucleus accumbens (a) and caudate putamen (b) in morphine-treated rats. Results are expressed as means±s.e.mean from three or more independent experiments, each performed in triplicate. ★$P<0.05$, ★★$P<0.01$ as compared to DAMGO alone (Newman Keuls test).

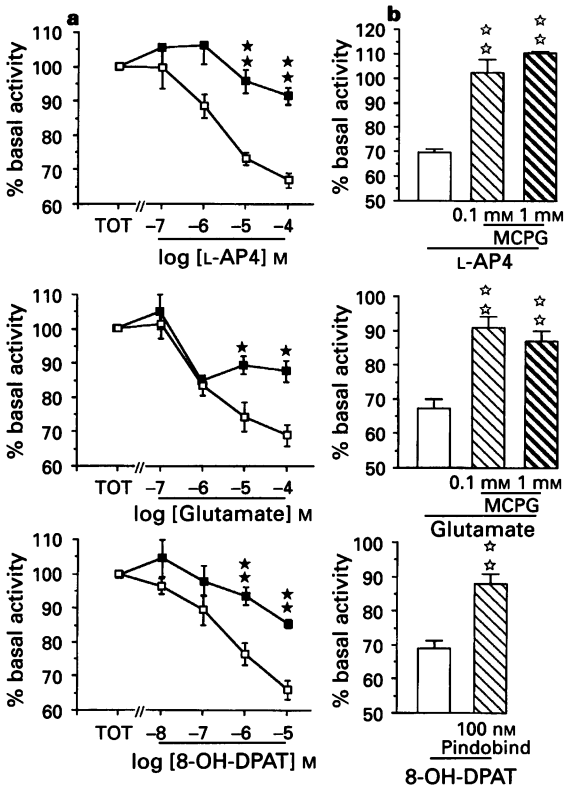


**Figure 8** (a) Effect of metabotropic gluatmate receptor (mGluR) agonists, L-AP4 and glutamate, and of 5-$HT_{1A}$ agonist, 8-OH-DPAT, on basal adenylyl cyclase activity in the thalamus in saline-treated rats (□) or morphine-treated rats (■). (b) Effects of the mGluR antagonist, MCPG, or 5-$HT_{1A}$ antagonist, pindobind, on the inhibitory effects of L-AP4 ($10^{-4}$ M), glutamate ($10^{-4}$ M) or 8-OH-DPAT ($10^{-5}$ M) on basal adenylyl cyclase activity in the thalamus in saline-treated rats. Results are expressed as means ± s.e.mean from three or more independent experiments, each performed in triplicate. $*P<0.05$, $**P<0.01$ show significant difference at the same dose. $\dot{*}\dot{*}P<0.01$ as compared to the agonist alone (Newman Keuls test).

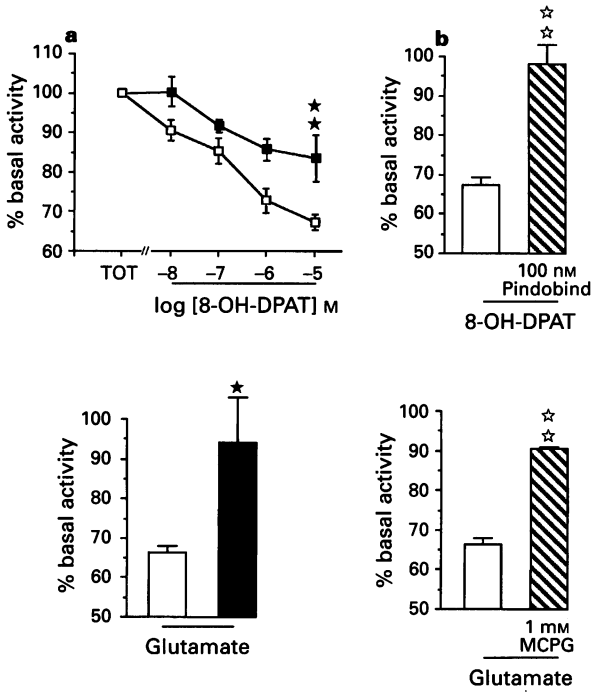


**Figure 9** (a) Effect of 5-$HT_{1A}$ agonist, 8-OH-DPAT, and of metabotropic glutamate receptor (mGluR) agonist, glutamate, on basal adenylyl cyclase activity in the PAG in saline-treated rats (□) or morphine-treated rats (■). (b) Effects 5-$HT_{1A}$ antagonist, pindobind, or of the mGluR antagonist, MCPG, on the inhibitory effects 8-OH-DPAT ($10^{-5}$ M) or of glutamate ($10^{-4}$ M) on basal adenylyl cyclase activity in the PAG in saline-treated rats. Results are expressed as means ± s.e.mean from three or more independent experiments, each performed in triplicate. $*P<0.05$, $**P<0.01$ show significant difference at the same dose; $\dot{*}\dot{*}P<0.01$ as compared to the agonist alone (Newman-Keuls test).

($F(3,16)=14.431$, $P<0.001$), and was selectively antagonized by the 5-$HT_{1A}$ antagonist, pindobind-5-$HT_{1A}$ (Figure 9). A rightward shift of the dose-response curve obtained with the 5-$HT_{1A}$ agonist on the inhibition of adenylyl cyclase activity was observed following chronic morphine treatment ($F(1,26)=22.430$, $P<0.001$). In this structure, glutamate (0.1 mM) also inhibited basal adenylyl cyclase activity, an effect that was antagonized by the selective mGluR antagonist, MCPG, in control animals. A two-way ANOVA revealed significant differences between inhibition induced by the metabotropic glutamate receptor agonist in saline-treated rats as compared to morphine-treated animals ($F(1,6)=6.103$, $P<0.05$) (Figure 9).

## Discussion

Adenylyl cyclase is an important regulator of neural function. The enzyme is subject to positive and negative regulation through several types of neurotransmitter and neuromodulator receptors, and by other factors (Mons & Cooper, 1994; Noble & Cox, 1995). In the present study, we have evaluated the effects of chronic morphine treatment on the regulation of adenylyl cyclase by opioids in brain regions associated with the motor and reinforcing properties of opiate drugs (i.e., the caudate putamen and nucleus accumbens) and in brain regions participating in opiate-induced antinociception (i.e., the thalamus and PAG).

Morphine-dependence was induced by morphine delivery at a constant rate for six days from implanted osmotic pumps. This procedure is known to induce narcotic tolerance and dependence as demonstrated by behavioural and electrophysiological criteria in this study and by others (Schaefer & Michael, 1986; Malin *et al.*, 1987; Huston-Lyons *et al.*, 1989; Adams & Holtzman, 1990; Paronis & Holtzman, 1992). Thus, a strong naloxone-precipitated abstinence syndrome was observed in rats treated with morphine. The major signs of withdrawal, widely accepted as being the most reliable indices for quantifying the degree of physical dependence, i.e. teeth chattering, mastication, paw tremor, wet-dog shakes, diarrhoea or ptosis, were triggered by injection of the opioid antagonist in morphine-treated animals, but not in saline-treated rats. An increase in jumping, another sign of abstinence syndrome which appears when morphine dependence is severe, occurred only weakly in the present study.

Addiction following chronic treatment with opiates, is probably the consequence of still unknown neuronal adaptations produced by repeated drug exposure and is due to multiple cellular events. One of the well documented mechanisms implicated in these side effects is the development of tolerance to the acute inhibitory actions of opiates. The major finding of this study is the occurrence of a differential $\mu$- and $\delta$-opioid receptor desensitization in different brain structures, as measured by the decline in inhibition of adenylyl cyclase activity in $\mu$- or $\delta$-opioid agonists, following chronic morphine treatment.

Basal adenylyl cyclase activity remained at levels comparable to those observed in saline-treated rats after chronic morphine treatment in the four brain regions studied: nucleus accumbens, caudate putamen, thalamus and PAG. These results are not in agreement with those previously reported by Terwilliger *et al.* (1991), who found that while most regions

(including caudate putamen, thalamus and PAG) showed no change in the regulation of adenylyl cyclase activity in response to chronic morphine treatment, the nucleus accumbens showed an increase in the ability of forskolin to stimulate adenylyl cyclase activity. Several factors including the use of forskolin as an activator, a different assay methodology and a different tolerance-inducing treatment regimen, may all contribute to the discrepancies between the two studies.

The thalamus and the PAG are known to be involved in acute and chronic effects of morphine. It is well established that the thalamus is strongly implicated in the nociceptive pathway (Basbaum & Fields, 1984; Kayser *et al.*, 1984; Mogil *et al.*, 1994; Roberts & Dong, 1994), as is the PAG (Sharpe *et al.*, 1974; Jacquet & Lajtha, 1976; Keay *et al.*, 1994; Urban & Smith, 1994; review in Bandler & Shipley, 1994). The PAG also plays an important role in the development and/or expression of physical dependence (Laschka *et al.*, 1976; Maldonado *et al.*, 1992; Bozarth, 1994). In both brain structures, the results obtained in the current study show an impairment of the ability of $\delta$-opioid agonists and $\mu$-opioid agonists to inhibit adenylyl cyclase activity in morphine-treated rats as compared to saline-treated animals. This opioid receptor desensitization observed in thalamus and PAG could be relevant to the phenomenon underlying opioid antinociceptive tolerance and/or development of physical dependence.

5-$HT_{1A}$ receptors and the metabotropic glutamate receptors, mGluR4, and mGluR7, are coupled to inhibition of cyclic AMP synthesis (via $G_i$ proteins) and are expressed in the rat thalamus (Tanabe *et al.*, 1993; Okamoto *et al.*, 1994; Martin & Humphrey, 1994; Boess & Martin, 1994). Dose-dependent inhibitions of adenylyl cyclase activity were observed in the thalamus with selective agonists of these receptor types (8-OH-DPAT, L-AP4; glutamate), and their inhibitory actions were antagonized by the selective 5-$HT_{1A}$ antagonist, pindobind-5-$HT_{1A}$ (Liau *et al.*, 1991), and by the mGluR antagonist, MCPG (selective to mGluR negatively coupled to adenylyl cyclase activity) (Kemp *et al.*, 1994), respectively. In the PAG, where 5-$HT_{1A}$ receptors have also been characterized (Boess & Martin, 1994), a dose-dependent inhibition of adenylyl cyclase activity was observed with 8-OH-DPAT, and this was antagonized by the 5-$HT_{1A}$ antagonist. A reduction of cyclic AMP production was also obtained with glutamate, this was selectively antagonized by the mGluR antagonist, MCPG. In both brain regions, chronic morphine treatment impaired the ability of both 5-$HT_{1A}$ receptor and mGluR agonists to inhibit basal adenylyl cyclase activity. Taken together with the loss of inhibitory activity of $\mu$- and $\delta$-opioid agonists in these brain regions after chronic morphine treatment, these results demonstrate that the morphine-induced desensitization is heterologous.

In a number of cell types, opiates and other neurotransmitters may produce some of their physiological responses via a common intracellular mechanism that involves receptor coupling through $G_i$ proteins regulating adenylyl cyclase. Thus, decreased levels of the G-protein could account for the heterologous desensitization. This hypothesis is emphasized by previous results obtained in dorsal root ganglion/spinal cord cultures where heterologous desensitization has been reported following chronic opiate treatment, associated to a decrease in levels of $G_{i\alpha}$ immunoreactivity (Crain *et al.*, 1982; Attali *et al.*, 1989). However, Terwilliger *et al.* (1991) were unable to observed any changes in levels of ADP-ribosylation of $G_iG_o$ proteins in the thalamus and the PAG following chronic morphine treatment. Nevertheless, this determination does not necessarily reflect alterations in the total amount of these proteins (Nestler *et al.*, 1989). Moreover, the combined presence of several $G_{i\alpha}$ subunit isoforms and of $G_{o\alpha}$ may mask possible changes in the amounts of functional activities of one or more of the $G_i$ or $G_o$ species.

The caudate putamen and the nucleus accumbens are also known to be involved in acute and chronic effects of opiates, and both structures contain high concentrations of opioid binding sites. The nucleus accumbens is prominently implicated in the reinforcing effects of abused drugs, and is an important site for mediating aversive properties of opiate withdrawal (Koob *et al.*, 1989; Stinus *et al.*, 1990; Acquas & Di Chiara, 1992; review in Nestler *et al.*, 1993), while the caudate putamen seems to be more involved in the motor responses to opiates. The results obtained in the present study show that chronic morphine treatment did not affect DAMGO-induced inhibition of adenylyl cyclase in either brain structure. DAMGO effects were selectively antagonized by the $\mu$-selective antagonist CTOP, suggesting that $\mu$-receptor function was not significantly impaired by the chronic morphine treatment in this brain region. Surprisingly, a decreased ability of the $\delta$-selective opioid agonists, DPDPE or DT-II, to inhibit cyclic AMP production was observed after morphine treatment. In the caudate putamen, this desensitization appeared to be only partial, while in the nucleus accumbens $\delta$-opioid agonists no longer inhibited adenylyl cyclase activity. This apparently selective impairment of $\delta$-receptor function might be explained by a higher receptor reserve (more spare receptors) for $\mu$-receptors as compared to $\delta$-receptors, resulting in little attenuation of the inhibitory effect of the $\mu$-opioid agonist, DAMGO, while the inhibitory effect of $\delta$-agonist was significantly reduced. However, the inhibitory activity of morphine, a partial agonist, was reduced only slightly in the caudate putamen and not significantly changed in the nucleus accumbens after chronic morphine treatment, suggesting that differences in agonist efficacy *per se* are not primary factors in the selective loss of $\delta$-receptor function in caudate putamen and nucleus accumbens. These results emphasize the resistance to adaptive changes in $\mu$-receptor function in these two brain regions.

Both brain regions receive major dopaminergic innervation, the caudate putamen from nigrostriatal pathway and the nucleus accumbens from the mesolimbic tract. Moreover, it has been reported that chronic cocaine treatment also induces a selective impairment of $\delta$-opioid receptor-mediated effector function in the caudate putamen and the nucleus accumbens without change in $\mu$-receptor-mediated inhibitory activity (Unterwald *et al.*, 1993). Thus the involvement of the dopaminergic system is strongly suggested. Previous studies have reported that chronic cocaine treatment and chronic morphine treatment induced common regulation in the mesolimbic system through actions at different levels: G proteins, protein kinase activity, neurofilament proteins (Terwilliger *et al.*, 1991; Beitner-Johnson & Nestler, 1991; Beitner-Johnson *et al.*, 1992).

In summary, the results of the present study indicate that desensitization of opioid receptors represent a general response of specific neuronal cell types to chronic morphine treatment. Nevertheless, a differential desensitization of $\mu$- and $\delta$-opioid receptors was observed in the four brain regions studied. This could be explained by a direct effect of the alkaloid on $\mu$-opioid receptors leading either to a desensitization of this receptor, and consequently to other receptors that have a common intracellular mechanism (PAG and thalamus), or to alteration of other neurotransmitter systems, such as dopaminergic system (nucleus accumbens and caudate putamen). In the latter case, modification in dopamine release might alter the function of dopamine receptors and opioid receptors if they are coupled to the same messenger system. This hypothesis is the subject of further experiments in the laboratory.

In conclusion, it appears that chronic morphine treatments, via a ubiquitous stimulation of opioid receptors, induce functional alterations in numerous brain structures, both in the opioid system itself, and in other neurotransmitter systems.

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## References


ACQUAS, E. & DI CHIARA, G. (1992). Depression of mesolimbic dopamine transmission and sensitization to morphine during opiate abstinence. *J. Neurochem.*, **58,** 1620–1625.

ADAMS, J.U. & HOLTZMAN, S.G. (1990). Tolerance and dependence after continuous morphine infusion from osmotic pumps measured by operant responding in rats. *Psychopharmacology*, **100,** 451–458.

AGHAJANIAN, G.K. (1978). Tolerance of locus coeruleus neurons to morphine and suppression of withdrawal response by clonidine. *Nature*, **276,** 186–188.

ATTALI, B., SAYA, D. & VOGEL, Z. (1989). $\kappa$-opiate agonists inhibit adenylate cyclase and produce heterologous desensitization in rat spinal cord. *J. Neurochem.*, **52,** 360–369.

BANDLER, R. & SHIPLEY, M.T. (1994). Columnar organization in the midbrain periaqueductal gray: modules for emotional expression. *Trends Neurosci.*, **17,** 379–389.

BASBAUM, A.I. & FIELDS, H.L. (1984). Endogenous opioid pain control systems: Brainstem spinal pathways and endorphin circuitry. *Annu. Rev. Neurosci.*, **7,** 309–338.

BEITNER-JOHNSON, D., GUITART, X. & NESTLER, E.J. (1992). Neurofilament proteins and the mesolimbic dopamine system: common regulation by chronic morphine and chronic cocaine in the rat ventral tegmental area. *J. Neurosci.*, **12,** 2165–2176.

BEITNER-JOHNSON, D. & NESTLER, E.J. (1991). Morphine and cocaine exert common chronic actions on tyrosine hydroxylase in dopaminergic brain reward regions. *J. Neurochem.*, **57,** 344–347.

BOESS, F.G. & MARTIN, I.L. (1994). Review: molecular biology of 5-HT receptors. *Neuropharmacology*, **33,** 275–317.

BOZARTH, M.A. (1986). Neural basis of psychomotor stimulant and opiate reward: evidence suggesting the involvement of a common dopaminergic system. *Behav. Brain Res.*, **22,** 107–116.

BOZARTH, M.A. (1994). Physical dependence produced by central morphine infusions: an anatomical mapping study. *Neurosci. Behav. Rev.*, **18,** 373–387.

BROWN, B.L., ALBANO, J.D.M., EKINS, R.P., SGHERZI, A.M. & TAMPION, W. (1971). A simple and sensitve saturation assay method for measurement of adenosine 3′,5′-cyclic monophosphate. *Biochem. J.*, **121,** 561–562.

CADOR, M., TAYLOR, J.R. & ROBBINS, T.W. (1991). Potentiation of the effects of reward-related stimuli by dopaminergic-dependent mechanisms in the nucleus accumbens. *Psychopharmacology*, **104,** 377–385.

CRAIN, S.M., CRAIN, B. & PETERSON, E.R. (1982). Development of cross-tolerance to 5-hydroxytryptamine in organotypic cultures of mouse spinal cord-ganglia during chronic exposure to morphine. *Life Sci.*, **31,** 241–247.

DE VRIES, T.J., TJON TIEN RIL, G.H.K., VAN DER LAAN, J.W., MULDER, A.H. & SCHOFFELMEER, A.N.M. (1993). Chronic exposure to morphine and naltrexone induces changes in catecholaminergic neurotransmission in rat brain without altering $\mu$-opioid receptor sensitivity. *Life Sci.*, **52,** 1685–1693.

DUMAN, R.S., TALLMAN, J.F. & NESTLER, E.J. (1988). Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. *J. Pharmacol. Exp. Ther.*, **246,** 1033–1039.

ERSPAMER, V., MELCHIORRI, P., FALCONIERI-ERSPAMER, G., NEGRI, L., CORSI, R., SEVERINI, C., BARRA, D., SIMMACO, M. & KREIL, G. (1989). Deltorphins; a family of naturally occurring peptides with high affinity and selectivity for $\delta$ opioid binding sites. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 5188–5192.

HANDA, B.K., LANE, A.C., LORD, J.A.H., MORGAN, B.A., RANCE, M.J. & SMITH, C.F.C. (1981). Analogues of $\beta$-LPH$^{61-64}$ possessing selective agonist activity at $\mu$-opiate receptors. *Eur. J. Pharmacol.*, **70,** 531–540.

HUSTON-LYONS, D., BAIN, G.T. & KORNETSKY, C. (1989). Opiate dependence alters central reward of nalbuphine or pentazocine plus tripelennamine. *Eur. J. Pharmacol.*, **169,** 153–157.

JACQUET, Y.F. & LAJTHA, A. (1976). The periaqueductal gray: site of morphine analgesia and tolerance as shown by 2-way cross-tolerance between systemic and intracerebral injection. *Brain Res.*, **103,** 501–513.

KAYSER, V., BENOIST, J.M., GAUTRON, M. & GUILBAUD, G. (1984). Effects of ES52, an enkephalinase inhibitor, on response of ventrobasal thalamic neurons in rat. *Peptides*, **5,** 1159–1165.

KEAY, K.A., CLEMENT, C.I., OWLER, B., DEPAULIS, A. & BANDLER, R. (1994). Convergence of deep somatic and visceral nociceptive information onto a discrete ventrolateral midbrain periaqueductal gray region. *Neuroscience*, **61,** 727–732.

KEMP, M., ROBERTS, P., POOK, P., JANE, D., JONES, A., SUNTER, D., UDVARHELYI, P. & WATKINS, J. (1994). Antagonism of presynaptically mediated depressant responses and cyclic AMP-coupled metabotropic glutamate receptors. *Eur. J. Pharmacol. Mol. Pharmacol. Sec.*, **266,** 187–192.

KOOB, G.F., WALL, T.L. & BLOOM, F.E. (1989). Nucleus accumbens as a substrate for the aversive stimulus effects of opiate withdrawal. *Psychopharmacology*, **98,** 530–534.

LASCHKA, E., TESCHEMACHER, P., MEHRAIN, P. & HERZ, A. (1976). Sites of action of morphine involved in the development of physical dependence in rats. II. Morphine withdrawal precipitated by application of morphine-antagonists into restricted parts of the ventricular system and by microinjection into various brain areas. *Psychopharmacologia*, **46,** 141–147.

LIAU, L.M., SLEIGHT, A.J., PITHA, J. & PEROUTKA, S.J. (1991). Characterization of a novel and potent 5-hydroxytryptamine$_{1a}$ receptor antagonist. *Pharmacol. Biochem. Behav.*, **38,** 555–559.

MALDONADO, R., STINUS, L., GOLD, L.H. & KOOB, G.F. (1992). Role of different brain structures in the expression of the physical morphine withdrawal syndrome. *J. Pharmacol. Exp. Ther.*, **261,** 669–677.

MALIN, D.H., HARTER, L., JENKINS, P.D., MONFORT, R.D., BRUCE, P.D., FARLEY, P.A., FEREBEE, R., THRASHER, K.L. & MARULLO, D.S. (1987). Cerebrospinal fluid from morphine-dependent rats precipitates opiate abstinence syndrome. *Life Sci.*, **41,** 377–383.

MARTIN, G.R. & HUMPHREY, P.P.A. (1994). Classification review: receptors for 5-hydroxytryptamine: current perspectives on classification and nomemclature. *Neuropharmacology*, **33,** 261–273.

MATSUOKA, I., MALDONADO, R., DEFER, N., NOËL, F., HANOUNE, J. & ROQUES, B.P. (1994). Chronic morphine administration causes region-specific increase of brain type VIII adenylyl cyclase mRNA. *Eur. J. Pharmacol. Mol. Pharmacol. Sec.*, **268,** 215–221.

MOGIL, J.S., MAREK, P., OTOOLE, L.A., HELMS, M.L., SADOWSKI, B., LIEBESKIND, J.C. & BELKNAP, J.K. (1994). Mu opiate receptor binding is up-regulated in mice selectively bred for high stress-induced analgesia. *Brain Res.*, **653,** 16–22.

MONS, N. & COOPER, D.M.F. (1994). Selective expression of one $Ca^{2+}$-inhibitable adenylyl cyclase in dopaminergically innervated rat brain regions. *Mol. Brain. Res.*, **22,** 236–244.

MOSBERG, H.I., HURST, R., HRUBY, V.I., GEE, K., YAMAMURA, H.I., GALLIGAN, J.J. & BURKS, T.F. (1983). Bis-penicillamine enkephalins show pronounced delta receptor selectivity. *Proc. Natl. Acad. Sci. USA*, **80,** 5871–5874.

NESTLER, E.J., ERDOS, J.J., TERWILLIGER, R., DUMAN, R.S. & TALLMAN, J.F. (1989). Regulation of G proteins by chronic morphine in the rat locus coeruleus. *Brain. Res.*, **476,** 230–239.

NESTLER, E.J., HOPE, B.T. & WIDNELL, K.L. (1993). Drug addiction: a model for the molecular basis of neural plasticity. *Neuron.*, **11,** 995–1006.

NESTLER, E.J. & TALLMAN, J.F. (1988). Chronic morphine treatment increases cyclic AMP-dependent protein kinase activity in the rat locus coeruleus. *Mol. Pharmacol.*, **33,** 127–132.

NOBLE, F. & COX, B.M. (1995). Differential regulation of $D_1$ dopamine and $A_{2a}$ adenosine receptor-stimulated adenylyl cyclase by $\mu$, $\delta_1$, and $\delta_2$-opioid agonists in rat caudate putamen. *J. Neurochem.*, **65,** 125–133.

OKAMOTO, N., HORI, S., AKAZAWA, C., HAYASHI, Y., SHIGEMOTO, R., MIZUNO, N. & NAKANISHI, S. (1994). Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. *J. Biol. Chem.*, **269,** 1231–1236.

PARONIS, C.A. & HOLTZMAN, S.G. (1992). Development of tolerance to the analgesic activity of mu agonists after continous infusion of morphine, meperidine or fentanyl in rats. *J. Pharmacol. Exp. Ther.*, **262,** 1–9.

PELLEGRINO, L.J., PELLEGRINO, A.S. & CUSHMAN, A.J. (1979). *A Stereotaxic Atlas of the Rat Brain.* New York: Plenum Press.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.*, **83,** 346–356.

PHILLIPS, A.G. & LE PAINE, F.G. (1982). Reward produced by microinjection of (D-Ala$^2$), Met$^5$-enkephalinamide into the ventral tegmental area. *Behav. Brain Res.*, **5,** 225–229.

PUTTFARCKEN, P.S. & COX, B.M. (1989). Morphine-induced densensitization and down-regulation at mu-receptors in 7315c pituitary tumor cells. *Life Sci.*, **45,** 1937–1942.

PUTTFARCKEN, P.S., WERLING, L.L. & COX, B.M. (1988). Effects of chronic morphine exposure on opioid inhibition of adenylyl cyclase in 7315c cell membranes: a useful model for the study of tolerance at $\mu$ opioid receptors. *Mol. Pharmacol.*, **33,** 520–527.

RASMUSSEN, K., BEITNER-JOHNSON, D.B., KRYSTAL, J.H., AGHAJANIAN, G.K. & NESTLER, E.J. (1990). Opiate withdrawal and the rat locus coeruleus: behavioural, electrophysiological, and biochemical correlates. *J. Neurosci.*, **10,** 2308–2317.

ROBERTS, V.J. & DONG, W.K. (1994). The effect of thalamic nucleus submedius lesions on nociceptive responding in rats. *Pain*, **57,** 341–349.

SCHAEFER, G.J. & MICHAEL, R.P. (1986). Changes in response rates and reinforcement thresholds for intracranial self-stimulation during morphine withdrawal. *Pharmacol. Biochem. Behav.*, **25,** 1263–1269.

SHARMA, S.K., KLEE, W.A. & NIRENBERG, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. USA*, **72,** 3092–3096.

SHARPE, L.G., GARNETT, J.E. & CICERO, T.J. (1974). Analgesia and hyperreactivity produced by intracranial microinjections of morphine into the periaqueductal gray matter of the rat. *Behav. Biol.*, **11,** 303–313.

STINUS, L., LE MOAL, M. & KOOB, G.F. (1990). Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. *Neuroscience*, **37,** 767–773.

TANABE, Y., NOMURA, A., MASU, M., SHIGEMOTO, R., MIZUNO, N. & SHIGETADA, N. (1993). Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. Neurosci.*, **13,** 1372–1378.

TERWILLIGER, R.Z., BEITNER-JOHNSON, D., SEVARINO, K.A., CRAIN, S.M. & NESTLER, E.J. (1991). A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res.*, **548,** 100–110.

UNTERWALD, E.M., COX, B.M., KREEK, M.J., COTE, T.E. & IZENWASSER, S. (1993). Chronic repeated cocaine administration alters basal and opioid-regulated adenylyl cyclase activity. *Synapse*, **15,** 33–38.

URBAN, M.O. & SMITH, D.J. (1994). Nuclei within the rostral ventromedial medulla mediating morphine antinociception from the periaqueductal gray. *Brain Res.*, **652,** 9–16.

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