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Antiprogestins inhibit the binding of opioids to μ-opioid receptors in nervous membrane preparations

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Abstract

The present study showed that the glucocorticoid/progesterone antagonists, 17β-hydroxy-11β-(4-dimethylamino-phenyl-1)-17-(prop-1-ynyl)estra-4,9-dien-3-one (RU486) and 17β-hydroxy-11β-(4-dimethylamino-phenyl-1)-17-(propan-3-ol)estra-4,9-dien-3-one (ZK 98299), inhibit the binding of labeled dihydromorphine to μ-opioid receptors present on membrane preparations derived from rat and mouse brain, as well as from human neuroblastoma cells. The inhibitory effect of RU486 was dose-dependent and linked to a decrease of the affinity of labeled dihydromorphine to the μ-opioid receptors. Kinetic experiments have shown that RU486 induces a decrease of the association rate constant (k_{+1}) of dihydromorphine. RU486 also proved able to dissociate the dihydromorphine-μ-opioid receptor complex, although at a rate slower than that exhibited by unlabeled dihydromorphine. Finally, the addition of NaCl (100 mM) to the incubation buffer induced a 50% decrease of the inhibitory effect of RU486. A 6-day treatment of neuroblastoma cells with RU486 eliminated the inhibitory effect morphine exerts on the intracellular accumulation of cyclic AMP induced by prostaglandin E_1. These results indicate that RU-486 may interact with brain μ-opioid receptors in vitro, by decreasing the affinity of opioid ligands.

Keywords: Opioid receptor; Steroid; Nervous system; Neuroblastoma

1. Introduction

Evidence is accumulating which indicates that not all the actions of steroid hormones are mediated by genomic mechanisms (Celotti et al., 1992; Wehling, 1994). The best examples of this are provided by steroid effects occurring in the brain, where hormonal steroids modify the electrical activity of neurons (Ramirez et al., 1990; Saphier and Feldman, 1988), and induce sleep and/or anaesthesia (Gee et al., 1988; Sutanto et al., 1989) with latencies that are too short to imply an effect occurring through classical intracellular receptors. These observations suggest the possible existence of membrane-linked modes of action of steroid derivatives. In line with this hypothesis, direct binding of several steroid hormones has been found to occur in rat brain membrane preparations (Ramirez et al., 1990; Towle and Sze, 1983; Wehling, 1994). Moreover, some 5α-reduced metabolites of progesterone have been reported to interact with a specific binding site located on the γ-aminobutyric acid (GABA) type A receptor-chloride ionophore complex (Belelli et al., 1990), and to potentiate the actions of GABA on the $GABA_A$ receptor (Majewska, 1990; Majewska et al., 1986). Finally, steroids have been shown to be able to interfere with the binding of some neurotransmitters to their physiological receptors; progesterone, in particular, appears to modify the binding of specific ligands to the σ (Su et al., 1988) and the muscarinic receptors (Klangkalya and Chan, 1988). However, the chemical characteristics of steroidal compounds, and specially their hydrophobicity, suggest that the interference exerted by steroids on the binding of neurotransmitters to their receptors might result from indirect non-specific actions, for instance, influences exerted on the physical properties of the membranes anchoring these receptors (Sargent and Schwyzer, 1986).

There is a multifaceted interplay between steroid hormones and the brain opioid systems. For instance, sex steroids are believed to exert their effects on gonadotropin secretion at least in part through the modulation of hypothalamic opioids and/or their receptors (Bhanot and Wilkinson, 1984; Gabriel et al., 1983; Kalra, 1993; Limonta et al., 1986; Piva et al., 1985, 1986). In particular, it has

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been reported that, in the female rat, physiological as well as pharmacologically induced changes of the serum level of sex steroids are accompanied by variations of μ-opioid receptors binding characteristics in the whole brain and in the hypothalamus (Casulari et al., 1987; Dondi et al., 1992; Jacobson and Kalra, 1989; Maggi et al., 1989, 1993, 1994; Weiland and Wise, 1990).

The present work was performed to verify whether steroids might exert some direct actions on the binding characteristics of opioid receptors. To this purpose, the influence exerted by a number of steroidal compounds on the binding of labeled dihydromorphine to μ-opioid receptors was investigated. Membrane preparations obtained from the whole brain of adult female rats and adult male mice, as well as from a human neuroblastoma cell line (SH-SY5Y, known to be rich in μ-opioid receptors) (Yu et al., 1986) were used in the present study. Other experiments were performed on living SH-SY5Y neuroblastoma cells, to analyse whether the treatment with steroidal compounds might be followed by alterations of biochemical parameters known to be linked to μ-opioid receptor function (e.g., inhibition of cyclic AMP accumulation).

2. Materials and methods

2.1. Animals and cell cultures

Adult female rats of the Sprague-Dawley strain and male CD1 mice (Charles River, Calco, Italy) were used throughout the experiments. The animals were caged in groups of five, in rooms with controlled temperature and humidity. The light schedule was 14 h light–10 h darkness (lights on: 06.30 h). A standard pellet diet and water were available ad libitum. The animals were killed at 10.00 h in the morning by cervical dislocation and the brains were rapidly removed from the skulls and processed for membrane preparation.

SH-SY5Y cells (kindly provided by Dr. June Biedler, Sloan-Kettering Memorial Cancer Center, New York, USA) were grown in monolayer at 37°C in a humidified CO_2 incubator. The culture medium was minimum essential medium containing non-essential amino acids, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 IU/ml penicillin, 10 mg/l of phenol red (Biochrom, Berlin, Germany) and supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA). For the treatments, a 1000-fold concentrated stock solution of RU486 in 70% dimethyl sulfoxide (DMSO) was diluted in the culture medium to a final concentration of 10 μM of the steroid. The culture medium was replaced at 2-day intervals. Subconfluent cells were then washed twice and harvested in Dulbecco's phosphate-buffered saline solution, without calcium and magnesium salts, containing 0.4% EDTA (Biochrom, Berlin, Germany), then collected by centrifugation and processed for membrane preparation.

2.2. Membrane preparations

Animal brains and neuroblastoma cell pellets were individually homogenised (glass-Teflon homogeniser) in 10 volumes of sucrose 0.32 M. Homogenates were centrifuged at $1400 \times g$ for 10 min; the resulting supernatants were decanted and preincubated for 30 min at 37°C, to eliminate the endogenous ligand that might interfere with the assay of the μ-opioid binding sites. At the end of the incubation, the material was further centrifuged at $48\,000 \times g$ for 30 min. The pellets obtained (membrane preparations) were resuspended and homogenised in 3 volumes of assay buffer (Tris-HCl 50 mM, pH 7.4) and stored at −70°C until the time of the binding assay. In previous experiments, no loss of μ-opioid binding sites was observed up to 15-day storage at −70°C. The protein content of each membrane preparation was determined by a micro method (Bradford, 1976) using human serum albumin as standard.

2.3. Chemicals

17β-hydroxy-11β-(4-dimethylamino-phenyl-1)-17-(prop-1-ynyl) estra-4,9-dien-3-one (RU486) was kindly provided by Roussel UCLAF (France); 17β-hydroxy-11β-(4-dimethylamino-phenyl-1)-17-(propan-3-ol)estra-4,9-dien-3-one (ZK 98299) was provided by Schering (Berlin, Germany); (*Z*)-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethyl-ethanamine citrate (tamoxifen) was a generous gift of Zeneca (Milan, Italy). Other compounds of high chemical purity were obtained from commercial sources (Sigma Chemicals, St. Louis, MO, USA; Merck, Darmstadt, Germany).

[^{3}H]Dihydromorphine (87 Ci/mmol), [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin ([^{3}H]DAGO, 55 Ci/mmol) and [^{3}H]diprenorphine (31 Ci/mmol) were purchased from NEN-Dupont de Nemours (Florence, Italy).

2.4. Receptor binding assay

[^{3}H]Dihydromorphine was chosen as the ligand for binding experiments since this drug, at the doses required for the assay, binds specifically to the μ-opioid receptors (Pfeiffer and Herz, 1982).

The conditions of the receptor binding assay were optimised as previously described (Maggi et al., 1993). Aliquots from a pool of tissue membrane preparations (200 μg of protein) were incubated in the presence of labeled ligands (1 nM for single-point and kinetic experiments and from 0.1 to 100 nM for saturation curves). Incubations were carried out at 25°C for 30 min. The content of the tubes was then individually filtered through Whatman GF/B filters pre-soaked in assay buffer saturated with isoamyl alcohol; each filter was washed twice with 5 ml ice-cold assay buffer and counted in 7 ml Instagel scintillation cocktail (Packard Instruments, Milan, Italy). All the samples were assayed in duplicate. In order to minimise

inter-assay variation, different groups of experiments were performed in the same assay.

2.5. Cyclic AMP assay

Intracellular cyclic AMP accumulation was measured in subconfluent SH-SY5Y cells over a 15-min incubation period at 37°C in the presence of 1 μM of prostaglandin E_1 (Sigma Chemicals), as activator of adenylyl cyclase, after a 10-min preincubation with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (0.5 mM) (Sigma Chemicals). A commercial binding protein assay kit (Amersham, Milan, Italy) was used to evaluate cyclic AMP concentration in ethanol-extracted cells according to the manufacturer's instructions.

2.6. Statistical analysis

Comparisons of the competing effects of steroids were statistically evaluated by analysis of variance (ANOVA) using the SYSTAT program. The competition curves were analysed by means of a Macintosh version of the ALLFIT program (De Lean et al., 1978) kindly provided by Dr. V. Guardabasso (Cyanamid, Catania, Italy). The saturation curves were analysed by means of the LIGAND program (Munson and Rodbard, 1980) adapted for a Macintosh computer by Dr. G.E. Rovati (Inst. Pharmacol. Sciences, Milan, Italy). The 95% joint confidence regions for each pair of binding parameters were generated by the MacELLIPSE program developed by the authors (Maggi and Rovati, 1993). Data obtained from kinetic experiments were analysed by mean of the EXPFIT program (Guardabasso et al., 1988).

3. Results

3.1. Effect of several steroidal compounds on the binding of opioid ligands to rat brain membrane preparations

Table 1 illustrates the effect of a 10 μM concentration of several physiological steroids, of three synthetic steroid antagonists (RU486, ZK98299 and tamoxifen), and of the steroid ester, 17α-hydroxy-progesterone caproate on the binding of labelled dihydromorphine, [^{3}H]dihydromorphine, to μ-opioid receptors present in rat brain membrane preparations. First of all, it was evident that the two vehicles, DMSO and ethanol, at the concentrations used to dissolve the various compounds, did not interfere with the binding of [^{3}H]dihydromorphine to μ-opioid receptors. Among the natural steroids tested, only 17β-estradiol and 17α-hydroxy-progesterone were partially able to inhibit the binding of [^{3}H]dihydromorphine (41% and 33%, respectively). All other physiological steroids appeared to be devoid of any significant inhibitory activity (Table 1). Among the synthetic compounds tested, the progesterone antagonists, RU486 and ZK98299, were able to inhibit almost completely (> 90%) the specific binding of [^{3}H]dihydromorphine to rat brain membrane preparations, while the estrogen antagonist, tamoxifen (which does not possess a steroidal structure) was ineffective. Also, 17α-hydroxy-progesterone caproate, contrary to what was observed with 17α-hydroxy-progesterone, was totally inactive (Table 1).

Table 1
Effect of the in vitro addition of steroid hormones and synthetic analogs on the specific binding of [^{3}H]dihydromorphine to rat brain membrane preparations

Compounds	[^{3}H]DHM specific binding (% of control)	CV (%)
Ethanol 1%	93.1	3.4
DMSO 1%	98.1	2.0
Corticosterone	97.6	1.2
Progesterone	95.9	0.6
5α-Pregnane-3,20-dione (DHP)	94.3	4.6
5α-Pregnane-3α-ol-20-one (THP)	83.1	8.6
5α-Pregnane-3β-ol-20-one	90.4	1.0
5α-Pregnane-3α20α-diol	95.3	3.8
5β-Pregnane-3,20-dione	97.5	1.8
17α-Hydroxy-progesterone	66.8 [a]	5.0
17α-Hydroxy-progesterone caproate	105.0	3.8
RU486	5.6 [a]	3.4
ZK98299	8.4 [a]	2.4
Testosterone	85.9	7.4
5α-Androstan-17β-ol-3-one (DHT)	87.1	8.8
Androsten-3β,17β-diol	87.5	6.0
17β-Estradiol	59.2 [a]	4.0
Tamoxifen	75.7	4.8

The steroids and tamoxifen were used at 10 μM concentration. [a] Significant ($P < 0.05$) vs. DMSO or ethanol.

Subsequent experiments served to characterise the mode of action of the most effective progesterone antagonist, RU486.

To rule out the possibility that the effects of the progesterone antagonists might be linked to the particular opioid ligand chosen, the action of RU486 was investigated on rat brain membrane preparations using as ligands either the μ-opioid receptor agonist, [^{3}H]DAGO, or the non-selective opioid receptor antagonist, [^{3}H]diprenorphine. It is apparent from Table 2 that the competing effect of RU486 (10 μM) on the binding of 1 nM [^{3}H]DAGO was similar to

Table 2
Effect of the in vitro addition of RU486 (10 μM) on the specific binding of the μ-opioid receptor agonist, [^{3}H]DAGO, and the opioid receptor antagonist, [^{3}H]diprenorphine, to rat brain membrane preparations

Ligand	Specific binding (% of control)
DAGO	8.8 ± 0.2 [a]
Diprenorphine	73.2 ± 4.7 [a]

Values are means ± S.D. of quadruplicate determinations obtained from two independent experiments. [a] Significant ($P < 0.05$) vs. control samples containing 1% DMSO.

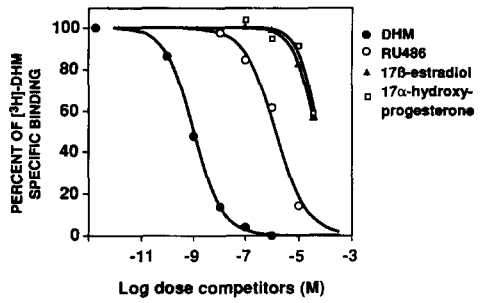

Fig. 1. Dose-dependent inhibition of [^{3}H]dihydromorphine binding to rat brain membrane preparations by unlabeled dihydromorphine (closed circles), RU486 (open circles), 17β-estradiol (triangles) and 17α-hydroxy-progesterone (squares). Incubations of membrane preparations were started by simultaneous addition of the labeled ligand and the competitors.

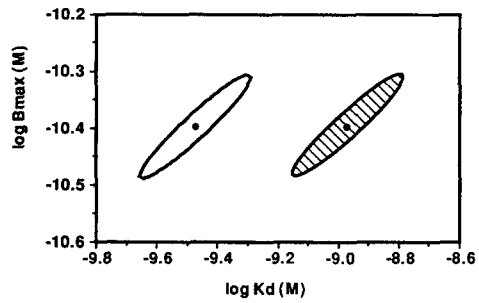

Fig. 2. 95% joint confidence regions of the binding parameters of [^{3}H]dihydromorphine to μ-opioid receptors present in rat brain membrane preparations obtained from saturation curves made in the absence (open ellipse) or in the presence (shaded ellipse) of a 5 μM concentration of RU486.

that exerted on the binding of [^{3}H]dihydromorphine. In contrast, the specific binding of 0.5 nM [^{3}H]diprenorphine was decreased by only about 25% in the presence of RU486.

Fig. 1 illustrates the competition curves for RU486, 17α-hydroxy-progesterone and 17β-estradiol on the binding of [^{3}H]dihydromorphine to rat brain membrane preparations. These curves reveal that RU486 inhibited the binding of [^{3}H]dihydromorphine in a dose-dependent manner, with a potency (expressed as IC_{50}) of 0.50 μM. It was not possible to evaluate the IC_{50} of 17α-hydroxy-progesterone and of 17β-estradiol. This was because it was difficult to reach the high concentrations of these steroids required for complete inhibition of [^{3}H]dihydromorphine binding, due to their limited solubility in the incubation buffer.

RU486 exerted an inhibitory effect on [^{3}H]dihydromorphine binding also when tested in membrane preparations derived from mouse rather than from rat brain, and from the human neuroblastoma cell line, SH-SY5Y (Table 3). The IC_{50} of RU486 in these different preparations was of the same order of magnitude (Table 3).

3.2. Effect of RU486 on the binding characteristics of [^{3}H]dihydromorphine to rat brain membrane preparations

3.2.1. Saturation curves

Analysis of saturation curves of the binding of [^{3}H]dihydromorphine to rat brain membrane preparations, in the absence or in the presence of 5 μM RU486, showed that RU486 induces a significant decrease of the affinity of [^{3}H]dihydromorphine (K_d values varying from 0.34 nM to 1.08 nM, $P < 0.05$ by F test performed with the program LIGAND), without any modification of the maximal binding capacity (B_{max}), suggesting a possible competitive effect of the steroid on the opioid receptor. This is seen in Fig. 2, where the results are presented as a log K_d − log B_{max} plot obtained from the analysis of these curves; the ellipses represent the 95% joint confidence regions of the two binding parameters.

Table 3
Inhibitory effect of RU486 (10 μM) on the binding of [^{3}H]dihydromorphine to membrane preparations obtained from rat brain, mouse brain and SH-SY5Y human neuroblastoma cells

Tissue	IC_{50} (μM)	CV (%)
Rat brain	0.5	5.4
Mouse brain	0.76	6.2
SH-SY5Y cells	1.73	12.5

Competition curves were analysed with the program ALLFIT. CV is the coefficient of variation of the estimated IC_{50} value.

3.2.2. Kinetic studies

To further characterise the mode of action of RU486, its effects were studied on the rates of association and dissociation of [^{3}H]dihydromorphine to rat brain membrane preparations. To this purpose, aliquots of membrane samples were incubated with [^{3}H]dihydromorphine (1 nM), and the reaction was stopped at various times of incubation; this approach permits the determination of the rate constant of association (k_{+1}) of [^{3}H]dihydromorphine. In order to obtain the value for the rate constant of dissociation (k_{-1}), some of the samples incubated, which had reached a stable binding equilibrium (at 60 min), were exposed to unlabeled dihydromorphine (1 μM final concentration). The experiment was performed in the absence or in the presence of RU486 (5 μM). The results, shown in Fig. 3, indicate that RU486 induced a significant decrease of the association rate constant (k_{+1}) of [^{3}H]dihydromorphine (from 0.072 min^{-1} in control membranes to 0.002 min^{-1} in RU486-treated membranes, $P < 0.05$) without significantly altering the dissociation rate constant (k_{-1}; 0.030 min^{-1} and 0.038 min^{-1}, respectively).

In the binding experiments so far described, the steroids had been added to the membrane preparations simultaneously with the labelled ligands; it was then necessary to test the ability of RU486 to dissociate the complex, [^{3}H]dihydromorphine-μ-opioid receptors, when it was already formed. Membranes were incubated with [^{3}H]dihydromorphine until binding equilibrium was reached; at this point unlabeled dihydromorphine (1 μM final concentra-

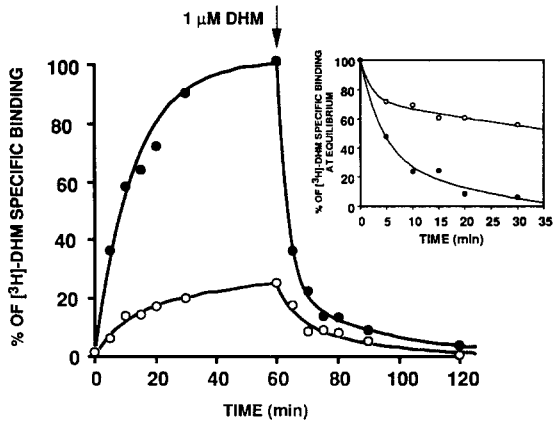

Fig. 3. Association and dissociation curves of the [^{3}H]dihydromorphine binding to rat brain membrane preparations, in the absence (close circles) or presence (open circles) of 5 μM RU486. Aliquots of membrane preparations were incubated in the presence of 1 nM [^{3}H]dihydromorphine and the incubation was stopped at different times until a stable equilibrium was reached. Some of the incubated membranes were left to reach equilibrium and the [^{3}H]dihydromorphine bound to membranes was dissociated by the addition of 1 μM unlabeled dihydromorphine (arrow). Inset: Dissociation of [^{3}H]dihydromorphine bound to rat brain membrane preparations by unlabeled dihydromorphine and RU486. Aliquots of membrane preparations were incubated in the presence of 1 nM [^{3}H]dihydromorphine until equilibrium was reached (60 min). The [^{3}H]dihydromorphine bound to membranes was then displaced by unlabeled dihydromorphine (1 μM, closed circles) or RU486 (10 μM, open circles).

tion) or RU486 (10 μM) was added and the reaction was stopped at different times. Fig. 3 (inset) shows that, as expected, unlabeled dihydromorphine dissociates completely the [^{3}H]dihydromorphine from its binding sites. In contrast, the dose of RU486 that was able to inhibit almost completely the binding of [^{3}H]dihydromorphine when added at the beginning of the incubation period dissociated only partially the [^{3}H]dihydromorphine-μ-opioid receptor complex (maximum inhibition 40%), with a significantly slower rate of dissociation than unlabeled dihydromorphine (k_{-1} = 0.0121 min^{-1} vs. 0.0556 min^{-1}).

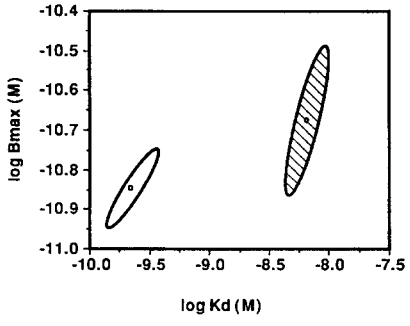

Fig. 4. 95% joint confidence regions of the binding parameters of [^{3}H]dihydromorphine to μ-opioid receptors present in SH-SY5Y human neuroblastoma cells after a 6-day treatment with RU486. The cells were maintained for 6 days in the culture medium in the absence (open ellipse) or in the presence of a 10 μM concentration of RU486 (shaded ellipse). The binding characteristics of [^{3}H]dihydromorphine to μ-opioid receptors were then evaluated on SH-SY5Y membrane preparations as described in Materials and methods.

Table 4
Effect of RU486 (10 μM) on [^{3}H]dihydromorphine binding to rat brain membrane preparations in the presence or in the absence of NaCl

Group	[^{3}H]DHM specific binding (% of control)
RU486	5.4 ± 0.2
RU486 + 100 mM NaCl	52.3 ± 5.8 [a]

Values are means ± S.D. of quadruplicate determinations obtained from two independent experiments. [a] Significant ($P > 0.05$) vs. RU486.

3.2.3. Effect of sodium ions

It is well known that the binding of opiate agonists to opioid receptors is influenced by monovalent ions. Opioid receptor agonists, in particular, show a reduced binding affinity to the μ-opioid receptor in the presence of sodium ions. Consequently, it was of interest to verify whether the decrease of affinity of [^{3}H]dihydromorphine for the μ-opioid receptor induced by RU486 was due to mechanisms similar to those operating in the case of sodium ions. As expected, the addition of NaCl (100 mM) to the incubation buffer induced a 6-fold decrease of the affinity of [^{3}H]dihydromorphine for the μ-opioid receptors (K_d 0.17 nM for control and 1.05 nM in the presence of NaCl) present in rat brain membrane preparations. Table 4 shows that the inhibition exerted by a 10 μM concentration of RU486 on the binding of 1 nM [^{3}H]dihydromorphine to brain membrane preparation was significantly decreased in the presence of sodium ions.

3.3. Evaluation of [^{3}H]dihydromorphine binding and opioid-mediated inhibition of cyclic AMP accumulation in SH-SY5Y neuroblastoma cells after RU486 treatment

Further evaluation of the effects of RU486 on μ-opioid receptors was performed on living cells in a defined in vitro system taking advantage of the availability of the human neuroblastoma cell line, SH-SY5Y, which is rich in μ-opioid receptors. In a first series of experiments, the binding characteristics of [^{3}H]dihydromorphine were analysed in membrane preparations obtained from SH-SY5Y cells cultured for 6 days in the presence of 10 μM RU486. Five minutes before harvesting, RU486 (10 μM final concentration) was added to control untreated cells, to eliminate the possibility that the effects exerted by RU486 in the treated cells might be linked to the presence of a residual amount of the steroid, which could obviously alter the results of the receptor binding assay. All the cells were then processed, as described in Materials and methods, to obtain membrane preparations that were washed extensively to remove any residual RU486.

Fig. 4, which presents the 95% joint confidence ellipses of the binding parameters, shows that a 6-day treatment of SH-SY5Y cells with RU486 induced a significant decrease of the affinity of [^{3}H]dihydromorphine for the μ-opioid receptor (K_d values: 0.22 nM for control cells, and 6.59

Table 5
Effect of the in vitro addition of RU486 (10 μM) on the morphine inhibition of prostaglandin E_1-induced cyclic AMP accumulation in intact SH-SY5Y human neuroblastoma cells

Treatments	Cyclic AMP accumulation (% of basal levels)	Inhibitory effect of morphine (%)
Prostaglandin E_1 + vehicle	183 ± 0.9	
Prostaglandin E_1 + vehicle + morphine	137 ± 2.1 [a]	−25
15 min		
Prostaglandin E_1 + RU486	182 ± 1.8	
Prostaglandin E_1 + RU486 + morphine	138 ± 3.2 [a]	−24
6 days		
Prostaglandin E_1 + RU486	180 ± 1.3	
Prostaglandin E_1 + RU486 + morphine	182 ± 0.8	−1

Values are means ± S.D. of quadruplicate determinations obtained from two independent experiments. [a] Significant ($P < 0.05$) vs. prostaglandin E_1-stimulated cells.

nM for RU486-treated cells; $P < 0.05$), but does not induce any significant change of the B_{max}.

It is well known that, in opioid-responsive systems, opioid receptor agonists (e.g., morphine) inhibit the accumulation of cyclic AMP induced by prostaglandin E_1. In order to evaluate whether RU486 might influence a biochemical parameter associated with μ-opioid receptor function, the accumulation of cyclic AMP induced by prostaglandin E_1 was evaluated in intact SH-SY5Y cells. In these experiments, the basal content of intracellular cyclic AMP was 60 pmol/mg protein; this was increased by 183% by the treatment with prostaglandin E_1. The presence of 10 μM RU486 (during the 15 min of incubation necessary to observe the prostaglandin E_1 stimulation, or with chronic 6-day treatment) did not modify either the basal levels of intracellular cyclic AMP (data not shown) or the prostaglandin E_1-stimulated levels of intracellular cyclic AMP (Table 5). In the 15-min experiments, RU486 was also unable to modify the inhibition of cyclic AMP accumulation induced by morphine in prostaglandin E_1-stimulated cells (Table 5). However, the chronic 6-day treatment with RU486 eliminated the inhibitory effect of morphine on the accumulation of cyclic AMP induced by prostaglandin E_1 (Table 5).

4. Discussion

The present study analysed first the effects of different steroids on the in vitro binding of [^{3}H]dihydromorphine to μ-opioid receptors present in rat brain membrane preparations. From the results obtained it appears that, among the 12 physiological steroids examined, only 17β-estradiol and 17α-hydroxy-progesterone partially inhibited the binding of [^{3}H]dihydromorphine. The results also indicate that other naturally occurring steroids (e.g., corticosterone, testosterone, progesterone and some of their metabolites) do not interfere with the binding of [^{3}H]dihydromorphine to brain μ-opioid receptors. These observations are in partial agreement with those of a recent study by Schwarz and Pohl (1994), who reported that elevated concentrations of 17β-estradiol alter with similar potencies the binding of different opioids to the μ-, δ- and κ-opioid receptors. In the same study, the IC_{50} of 17β-estradiol was greater than 10 μM (Schwarz and Pohl, 1994), i.e. close to that expected from the data presented here. In agreement with the present data, it has also been found that testosterone, dihydrotestosterone and progesterone are practically ineffective to displace opioid ligands (Schwarz and Pohl, 1994). However, at variance with the results of Schwarz and Pohl (1994), in the present experiments it was found that 17α-hydroxy-progesterone competes with [^{3}H]dihydromorphine binding with a potency similar to that of 17β-estradiol. The reasons for this small discrepancy are probably linked to some methodological differences.

The fact that high doses of a wide range of natural steroids and of their metabolites (with the exception of 17β-estradiol and 17α-hydroxy-progesterone) do not directly modify the binding of opioid ligands to the μ-opioid receptors suggests that the effects observed after in vivo treatments with hormonal steroids on the binding characteristics of opioid receptors (Dondi et al., 1992; Jacobson and Kalra, 1989; Maggi et al., 1989) are due to indirect mechanisms, for instance, modulation of the synthesis of the opioid receptors, or an effect on the opioid-producing neurons resulting in down- or up-regulation of the receptors because of the increased or decreased amounts of the endogenous ligand released.

The results presented here have also shown that the progesterone antagonist, RU486, inhibits with a high potency (> 90%) the binding of [^{3}H]dihydromorphine to all the preparations containing the μ-opioid receptors studied (rat brain, mouse brain, neuroblastoma cell membrane preparations). The progesterone antagonist, ZK98299, which possesses a chemical structure similar to that of RU486 (Fig. 5), showed a similar inhibitory effect in the preparations in which it was studied. The observation that the competition of RU486 for [^{3}H]dihydromorphine binding was evident in all situations examined rules out the possibility that the phenomenon observed might be linked only to a particular tissue preparation. In addition, RU486 was able to inhibit the specific binding not only of [^{3}H]dihydromorphine but also of another μ-opioid receptor agonist ([^{3}H]DAGO) emphasising that this synthetic steroid is able to specifically affect the μ subtype of opioid receptors. This is also suggested by the fact that RU486 inhibited only partially (25%) the binding of the opioid non-selective antagonist [^{3}H]diprenorphine which binds with

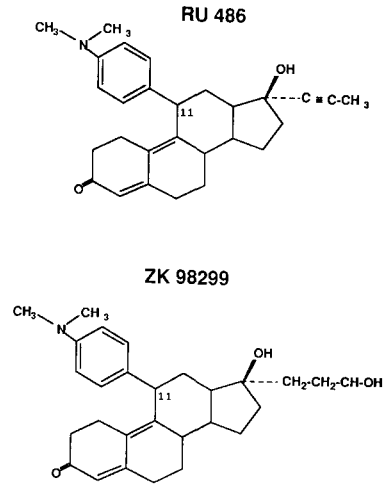

Fig. 5. Structure of the progesterone antagonists RU486 and ZK98299.

similar affinities to μ-, δ- and κ-opioid receptors (Leslie, 1987; Magnan et al., 1982; Paterson et al., 1983).

From the results reported here, it appears that RU486 inhibits the binding of [^{3}H]dihydromorphine in a dose-dependent fashion, with an IC_{50} ranging from 0.50 to 1.73 μM for the different tissues or cell preparations used. These values are lower than those reported for the effect of other steroids on the σ and the muscarinic receptors (Klangkalya and Chan, 1988; Su et al., 1988). It is interesting that these values are similar to those found for the binding of some protein-linked steroids (e.g., bovine serum albumin-progesterone) to their putative membrane binding sites (0.1 μM) (Ke and Ramirez, 1990; Ramirez et al., 1990). The present results also indicate that the effect of RU486 on μ-opioid receptors results in a decrease of the affinity of opioid receptor agonists for their receptors, and that this decrease of affinity is secondary to a diminished association rate of the ligand to the μ-opioid receptors, rather than to an increase of its dissociation rate. The present observations also support the view that steroids can convert the μ-opioid receptors to a low affinity state, as has been reported for the effects exerted by some steroids on other neurotransmitter (e.g., the dopamine D_2 receptor), and peptide (e.g., the receptors for insulin and tumour necrosis factor) receptors (Cull, 1988; Kahn et al., 1978; Levesque and Di Paolo, 1988). The decrease of the affinity of [^{3}H]dihydromorphine, observed in the present study after treatment with RU486, might suggest competitive antagonism of the steroid on μ-opioid binding sites. However, RU486, at the dose that almost completely inhibited [^{3}H]dihydromorphine binding when added simultaneously with the labelled ligand, was much less effective to displace [^{3}H]dihydromorphine when the ligand-receptor complex is already formed. This seems to rule out the possibility of a simple competitive interaction of RU486 on the μ-opioid receptor.

It has been reported that steroids may interact with cell membrane structures; it has also been proposed that the anchoring of steroids at membrane interfaces may produce membrane perturbation (Makriyannis et al., 1990). In addition, it has been found that exposure of rat brain membranes to highly hydrophobic molecules (e.g., fatty acids) may result in alteration of membrane fluidity, which may modify the binding characteristics of opioid ligands (Remmers et al., 1990). These facts suggest the hypothesis that, because of its hydrophobic nature, RU486 might interfere with the binding of [^{3}H]dihydromorphine by an interaction with membrane constituents. However, it is difficult to ascribe the potent action of RU486 on μ-opioid binding only to the hydrophobicity linked to its steroidal structure, since none of the other steroids tested showed similar activity. This hypothesis is also supported by the observation that 17α-hydroxy-progesterone becomes ineffective when conjugated with a caproic fatty acid residue. It must also be considered that RU486 and ZK98299, which are both capable of inhibiting almost completely the binding of [^{3}H]dihydromorphine, have a strictly similar chemical structure, characterised by the addition of a dimethylaminophenyl group in position 11β (Fig. 5). It is probable that the inhibitory effect on [^{3}H]dihydromorphine binding shown by these two steroids might be due to this particular functional group.

Opioid receptors are members of a superfamily of membrane-linked binding sites coupled to GTP-binding proteins (G proteins) (Leslie, 1987). The binding of opioid receptor agonists (but not of opioid receptor antagonists) to opioid receptors is decreased in the presence of sodium ions (Pert and Snyder, 1974; Puttfarcken et al., 1986), which are fundamental to the coupling of opioid receptors to G proteins. The apparent decrease of the affinity observed in the presence of sodium seems to be secondary to conformational changes of the receptor, which also modify its coupling to G proteins (Law et al., 1983). In the present work, it was found that the inhibitory effect of RU486 on the binding of dihydromorphine to μ-opioid receptors was significantly decreased in the presence of NaCl, a result which suggests that the effect of RU486 is, at least in part, mediated by some mechanisms similar to those which operate in the case of sodium ions. This hypothesis agrees with the evidence now reported, showing the decreased ability of RU486 to inhibit the binding of the non-selective antagonist [^{3}H]diprenorphine; as previously mentioned, the binding of opioid receptor antagonists is insensitive to the low affinity state of the receptors induced by sodium ions (Pert and Snyder, 1974; Puttfarcken et al., 1986). These considerations lead to the speculation that RU486 may interact with membrane structures involved in the control of the coupling of opioid receptors with G proteins. This hypothesis is not overly speculative, considering the recent findings showing that some natural steroids may interact with other GTP-binding protein coupled receptors (Minami et al., 1990; Petitti and Etgen, 1992; Wehling, 1994).

The results of the experiments on intact living neuroblastoma cells have shown that a chronic, 6-day, treatment

with RU486 induces a decrease of the affinity of [^{3}H]dihydromorphine to opioid receptors and a block of the inhibitory effect of morphine on the accumulation of cyclic AMP induced by prostaglandin E_1. In the same experiment it was found that acute treatment (15 min) with RU486 was unable to modify the inhibitory effect of morphine on prostaglandin E_1-induced cyclic AMP accumulation. This is not surprising considering the decreased effect of RU486 on [^{3}H]dihydromorphine binding in the presence of sodium, an ion that is obviously present in the incubation buffer.

Several in vitro and in vivo aspects of the biological profile of RU486 previously reported cannot be explained by its antiprogesterone activity; some might be linked to the effects on the opioid system reported here. For instance, it has been shown that RU486 may decrease the proliferation of immune cells in vitro even in the absence of cortisol or progesterone (Bradley et al., 1989; Monterroso and Hansen, 1993); it is well known that opioids may induce immunosuppression (Donahoe and Falek, 1988). It has also been reported that RU486 is able to block pituitary FSH secretion through a mechanism that does not involve progesterone and/or its receptors (Knox et al., 1993); opioids are known to exert inhibitory control on pituitary gonadotropin secretion. There are some data which support the biological relevance of the findings reported here. It has been found that RU486 partially blocks the increase of methallothionein occurring in the rat liver under the influence of morphine (Hidalgo et al., 1991). Moreover, recent results from this laboratory have shown that 2-day treatment of mice with RU486 reduces the increase in pain threshold induced by morphine, as measured by the hot plate test; this effect does not appear to be antagonised by either progesterone or dexamethasone (Bianchi et al., submitted).

One usual criticism of studies showing effects of steroids on the binding characteristics of neurotransmitter and peptide receptors is that physiological steroids cannot reach adequate concentrations in biological fluids to interact with binding sites characterised by an affinity in the micromolar range (Schwarz et al., 1989; Schwarz and Pohl, 1994). However, it is relevant to mention that an almost 10 μM concentration of RU486 is achieved in biological fluids of women under abortifacient treatment (Liu et al., 1988). It is probable that, at these concentrations, the steroid influences peripheral μ-opioid receptors. It is known that RU486 does not easily cross the blood-brain barrier. However, it is possible that the steroid, at the highest in vivo concentrations, might penetrate in the brain areas where the blood-brain barrier does not exist (median eminence, circumventricular organs, etc.). In conclusion, the present data show that physiological steroids (e.g., 17β-estradiol and 17α-hydroxy-progesterone) as well as synthetic progesterone antagonists may interfere with the binding of specific ligands to the μ-opioid receptor. These findings support the hypothesis that, at least in the nervous cells, steroids may act through non-genomic mechanisms also.

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References

Belelli, D., N.C. Lan and K.W. Gee, 1990, Anticonvulsant steroids and the GABA/benzodiazepine receptor-chloride ionophore complex, Neurosci. Biobehav. Rev. 14, 315.

Bhanot, R. and M. Wilkinson, 1984, The inhibitory effect of opiates on gonadotrophin secretion is dependent upon gonadal steroids, J. Endocrinol. 102, 133.

Bradford, M.M., 1976, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248.

Bradley, J.V.V., D.J. Anderson and J.A. Hill, 1989, The effects of RU486 on immune function and steroid-induced immunosuppression in vitro, J. Clin. Endocrinol. Metab. 69, 1195.

Casulari, L.A., R. Maggi, D. Dondi, P. Limonta, F. Piva, M. Motta and L. Martini, 1987, Effect of oestrus cyclicity on the number of brain opioid μ receptors in the rat, Horm. Metab. Res. 19, 549.

Celotti, F., R.C. Melcangi and L. Martini, 1992, The 5α-reductase in the brain: molecular aspects and relation to brain function, Front. Neuroendocrinol. 13, 163.

Cull, F.C.J., 1988, Reduction in tumor necrosis factor receptor affinity and cytotoxicity by glucocorticoids, Biochem. Biophys. Res. Commun. 153, 402.

De Lean, A., P.J. Munson and D. Rodbard, 1978, Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves, Am. J. Physiol. 235, 97.

Donahoe, R.M. and A. Falek, 1988, Neuroimmunomodulation by opiates and other drugs of abuse: relationship to HIV infection and AIDS, in: Psychological Neuropsychiatric and Substance Abuse Aspects of AIDS, eds. T.P. Bridge et al. (Raven Press, New York) p. 145.

Dondi, D., P. Limonta, R. Maggi and F. Piva, 1992, Effects of ovarian hormones on brain opioid μ binding sites in castrated female rats, Am. J. Physiol. 263, 507.

Gabriel, S.M., J.W. Simpkins and S.P. Kalra, 1983, Modulation of endogenous opioid influence on luteinizing hormone secretion by progesterone and estrogen, Endocrinology 113, 1806.

Gee, K.W., M.B. Bolger, R.E. Brinton, H. Coirini and B.S. McEwen, 1988, Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action, J. Pharmacol. Exp. Ther. 246, 803.

Guardabasso, V., P.J. Munson and D. Rodbard, 1988, EXPFIT: a program for simultaneous analysis of families of exponential decay curves, Comput. Methods Progr. Biomed. 27, 55.

Hidalgo, J., M. Giralt, J.S. Garvey and A. Armario, 1991, Effect of morphine administration on rat liver metallothionein and zinc metabolism, J. Pharmacol. Exp. Ther. 259, 274.

Jacobson, W. and S.P. Kalra, 1989, Decreases in mediobasal hypothalamic and preoptic area opioid ([^{3}H]naloxone) binding are associated with the progesterone-induced luteinizing hormone surge, Endocrinology 124, 199.

Kahn, C.R., I.D. Goldfine, D.M.J. Neville and P. De Meyts, 1978, Alterations in insulin binding induced by changes in vivo in the levels of glucocorticoids and growth hormone, Endocrinology 103, 1054.

Kalra, S.P., 1993, Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge, Endocr. Rev. 14, 507.

Ke, F.-C. and V.D. Ramirez, 1990, Binding of progesterone to nerve cell membranes of rat brain using progesterone conjugated to ^{125}I-bovine serum albumin as a ligand, J. Neurochem. 54, 467.

Klangkalya, B. and A. Chan, 1988, Inhibition of hypothalamic and pituitary muscarinic receptor binding by progesterone, Neuroendocrinology 47, 294.

Knox, K.L., S.J. Ringstrom and N.B. Schwartz, 1993, RU486 blocks the effect of inhibin antiserum or luteinizing hormone on the secondary follicle-stimulating hormone surge, Endocrinology 133, 277.

Law, P.Y., D.S. Hom and H.H. Loh, 1983, Opiate receptor down-regulation and desensitization in neuroblastoma × glioma NG 108-15 hybrid cells are two separate cellular adaptation processes, Mol. Pharmacol. 24, 413.

Leslie, F.M., 1987, Methods used for the study of opioid receptors, Pharmacol. Rev. 39, 197.

Levesque, D. and T. Di Paolo, 1988, Rapid conversion of high into low striatal D_2-dopamine receptor agonist binding state after an acute physiological dose of 17beta-estradiol, Neurosci. Lett. 88, 113.

Limonta, P., F. Piva, R. Maggi, D. Dondi, M. Motta and L. Martini, 1986, Morphine stimulates prolactin release in normal but not in castrated male rats, J. Reprod. Fertil. 76, 745.

Liu, J.H., V.G. Garzo and S. Yen, 1988, Pharmacodynamics of the antiprogesterone RU 486 in women after oral administration, Fertil. Steril. 50, 245.

Maggi, R. and E. Rovati, 1993, MacEllipse, a graphical aid to the problem of the joint confidence region: a practical example for ligand binding experiments, Pharmacol. Res. 28, 351.

Maggi, R., P. Limonta, D. Dondi and F. Piva, 1989, Effect of ovarian steroids on the concentration of mu opiate receptors in different regions of the brain of the female rat, Pharmacol. Res. 21, 91.

Maggi, R., D. Dondi, E. Rovati, L. Martini, F. Piva and P. Limonta, 1993, Binding characteristics of hypothalamic μ opioid receptors throughout the estrous cycle in the rat, Neuroendocrinology 58, 366.

Maggi, R., L. Martini and F. Piva, 1994, Effect of ovariectomy on the binding characteristics of hypothalamic mu opioid receptors in the rat, Endocr. Regul. 28, 171.

Magnan, J., S. Paterson, A. Tavani and H. Kosterlitz, 1982, The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties, Naunyn-Schmied. Arch. Pharmacol. 319, 197.

Majewska, M.D., 1990, Steroid regulation of the $GABA_A$ receptor: ligand binding, chloride transport and behaviour, in: Steroids and Neuronal Activity, eds. D. Chadwick and K. Widdows, Ciba Foundation Symposium 153 (John Wiley and Sons, Chichester) p. 83.

Majewska, M.D., N.L. Harrison and R.D. Schwartz, 1986, Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor, Science 232, 1004.

Makriyannis, A., Y. De-Ping and T. Mavromoustakos, 1990, The molecular features of membrane perturbation by anesthetic steroids: a study using differential scanning calorimetry, small angle X-ray diffraction and solid state ^{2}H NMR, in: Steroids and Neuronal Activity, eds. D. Chadwick and K. Widdows, Ciba Foundation Symposium 153 (John Wiley and Sons, Chichester) p. 172.

Minami, T., Y. Oomura, J. Nabekura and A. Fukuda, 1990, 17β-Estradiol depolarization of hypothalamic neurons is mediated by cyclic AMP, Brain Res. 519, 301.

Monterroso, V.H. and P.J. Hansen, 1993, Regulation of bovine and ovine lymphocyte proliferation by progesterone: modulation by steroid receptor antagonists and physiological status, Acta Endocrinol. 129, 532.

Munson, P.J. and D. Rodbard, 1980, LIGAND: a versatile computerized approach for characterization of ligand-binding system, Anal. Biochem. 107, 220.

Paterson, S.J., L.E. Robson and H.W. Kosterliz, 1983, Classification of opioid receptors, Br. Med. Bull. 39, 31.

Pert, C.B. and S.H. Snyder, 1974, Opiate receptor binding of agonists and antagonists affected differentially by sodium, Mol. Pharmacol. 10, 868.

Petitti, N. and A.M. Etgen, 1992, Progesterone promotes rapid desensitization of α_1-adrenergic receptor augmentation of cAMP formation in rat hypothalamic slices, Neuroendocrinology 55, 1.

Pfeiffer, A. and A. Herz, 1982, Different types of opiate agonists interact distinguishably with mu, delta and kappa opiate binding sites, Life Sci. 31, 1355.

Piva, F., R. Maggi, P. Limonta, M. Motta and L. Martini, 1985, Effect of naloxone on luteinizing hormone, follicle-stimulating hormone and prolactin secretion in the different phases of the estrus cycle, Endocrinology 117, 766.

Piva, F., P. Limonta, R. Maggi and L. Martini, 1986, Stimulatory and inhibitory effects of the opioids on gonadotropin secretion, Neuroendocrinology 42, 504.

Puttfarcken, P., L.L. Werling, S.R. Brown, T.E. Cote and B.M. Cox, 1986, Sodium regulation of agonist binding at opioid receptors. I. Effects of sodium replacement on binding at μ- and δ-type receptors in 7315c and NG108-15 cells and cell membranes, Mol. Pharmacol. 30, 81.

Ramirez, V.D., D.E. Dluzen and F.C. Ke, 1990, Effect of progesterone and its metabolites on neuronal membranes, in: Steroids and Neuronal Activity, eds. D. Chadwick and K. Widdows, Ciba Foundation Symposium 153 (John Wiley and Sons, Chichester) p. 125.

Remmers, A.E., G.L. Nordby and F. Medzihradsky, 1990, Modulation of opioid receptor binding by *cis* and *trans* fatty acids, J. Neurochem. 55, 1993.

Saphier, D. and S. Feldman, 1988, Iontophoretic application of glucocorticoids inhibit identified neurones in the paraventricular nucleus, Brain Res. 453, 183.

Sargent, D.F. and R. Schwyzer, 1986, Membrane lipid phase as catalyst for peptide receptor interactions, Proc. Natl. Acad. Sci. USA 83, 5774.

Schwarz, S. and P. Pohl, 1994, Steroid and opioid receptors, J. Steroid Biochem. Mol. Biol. 48, 391.

Schwarz, S., P. Phol and G.Z. Zhou, 1989, Steroid binding at σ-'opioid' receptors, Science 246, 1635.

Su, T.P., E.D. London and J.H. Jaffe, 1988, Steroid binding at σ receptors suggests a link between endocrine, nervous and immune system, Science 240, 219.

Sutanto, W., G. Handelmann, F. De Bree and R. De Kloet, 1989, Multifacet interaction of corticosteroids with the intracellular receptors and with membrane GABA-A receptor complex in the rat brain, J. Neuroendocrinol. 1, 243.

Towle, A.C. and P.Y. Sze, 1983, Steroid binding to synaptic plasma membrane: differential binding of glucocorticoids and gonadal steroids, J. Steroid Biochem. 18, 135.

Wehling, M., 1994, Nongenomic actions of steroid hormones, Trends Endocrinol. Metab. 5, 347.

Weiland, N.G. and P.M. Wise, 1990, Estrogen and progesterone regulate opiate receptor density in multiple brain regions, Endocrinology 126, 804.

Yu, C.V., M.L. Richards and W. Sadée, 1986, A human neuroblastoma cell line expresses μ and δ opioid receptor sites, J. Biol. Chem. 261, 1065.