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Changes in [^{3}H]-UK14304 binding to α_2-adrenoceptors in morphine-dependent guinea-pigs

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1 The aim of this study was to investigate the effect of a noradrenergic input in the cortex of morphine-dependent animals. Binding of the α_1-adrenoceptor ligand [^{3}H]-prazosin did not change in cortical membranes taken from morphine-dependent as compared to control guinea-pigs. However, binding of the α_2-adrenoceptor ligand [^{3}H]-UK14304 showed decreased K_D (−30%) in the absence of significant changes in B_{max}, either in cortical membranes or in synaptosomes.

2 Several characteristics of this phenomenon were identified. First, it occurs in a time-dependent fashion, in that it takes 5 days of chronic morphine treatment to start developing. Second, it can be observed after acute administration of high doses of morphine (100 mg kg^{-1}). Third, it does not require a connection with the locus coeruleus or with other subcortical structures, in that it can be reproduced *in vitro* in isolated cortical slices. Fourth, it requires the integrity of cortical structures, since it cannot be reproduced *in vitro* in cortical synaptosomes.

3 Release studies were run to attempt identification of a functional correlate of the above observations. No changes were observed in the ability of the α_2-adrenoceptor agonist UK14304 to inhibit 35 mM K^+-evoked [^{3}H]-noradrenaline outflow from cortical synaptosomes taken from morphine-dependent as compared to control guinea-pigs. However, a large decrease in the IC_{50} of UK14304 for the inhibition of 35 mM K^+-evoked [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) outflow (41 vs. 501 nM) was observed in morphine-dependent as compared to control animals.

4 These data suggest that, in the guinea-pig, chronic morphine treatment is associated with a shift from a low to high affinity agonist state in α_2-adrenoceptors on cortical GABA terminals.

Keywords: Morphine addiction; α_2-adrenoceptors; UK14304; noradrenaline; GABA; neocortex; binding; release

Introduction

The molecular and cellular bases of opiate addiction are still elusive. An attempt to interpret this phenomenon in terms of alterations in opiate receptors number or affinity has proven disappointing (see Loh *et al.*, 1988). However, other studies suggest that changes in opiate receptor-regulated G proteins, adenosine 3':5'-cyclic monophosphate (cyclic AMP) and protein kinases in specific brain regions may account for important aspects of opiate addiction (Loh *et al.*, 1988; Nestler, 1992; Nestler *et al.*, 1993).

Among these regions, the locus coeruleus (LC) appears to be primarily implicated in the mechanism of dependence, and therefore of withdrawal (Koob & Bloom, 1988; Rasmussen *et al.*, 1990; Maldonado *et al.*, 1992). LC (a paired nucleus located on the floor of the fourth ventricle in the dorsal pons) contains more than 90% of the noradrenaline (NA) of the entire central nervous system (Foote *et al.*, 1983). Specific variations in LC firing rate take place after acute and chronic administration of morphine. The LC firing rate is decreased by acute treatment with the opiate, via activation of a K^+ conductance (Aghajanian & Wang, 1987; North *et al.*, 1987). Upon chronic treatment, it returns to baseline levels, i.e. tolerance develops (Aghajanian, 1978); furthermore, dependence also develops as demonstrated by an elevation in the firing rate during withdrawal (Aghajanian, 1978). Such events are thought to take place in several mammalian species, including primates (Redmond & Krystal, 1984; Rasmussen *et al.*, 1990).

Both extrinsic and intrinsic factors are believed to play a role in the changes of LC response during morphine addiction. As for the intrinsic factors, elegant studies suggest that an upregulation of the cyclic AMP system in the LC participates in the variations in the firing rate described above, and allow the identification of possible molecular mechanisms for the phenomenon (see Nestler *et al.*, 1993). As for the extrinsic factors, other studies suggest that a glutamatergic input from the nucleus paragigantocellularis accounts for approximately 50% of the increase in LC firing rate during withdrawal (Rasmussen & Aghajanian, 1989; Akaoka & Aston-Jones, 1991). Taken together with other studies, these findings begin to outline a neuronal network including the dorsal horn, the nucleus paragigantocellularis and the LC, each relay of which is the site of opiate receptors and of upregulation of the cyclic AMP system during chronic exposure to morphine. During withdrawal, this situation would lead to an escalating activation of the neurones in the network (Nestler, 1992).

In the above hypothesis, the consequences of the variations in LC firing rate should be viewed as a central question in the understanding of opiate addiction. Unfortunately, little information is presently available in this regard. In normal, awake guinea-pigs, noradrenaline (NA) injected in the lateral ventricles (i.c.v.) or electrical stimulation of the LC decreases the cortical release of acetylcholine (ACh) via α_2-adrenoceptors and increases the cortical outflow of γ-aminobutyric acid (GABA) via α_1-adrenoceptors (Beani *et al.*, 1978; Bianchi *et al.*, 1979; Moroni *et al.*, 1982). In morphine-dependent guinea-pigs, NA i.c.v. or electrical stimulation of the LC increases cortical ACh release via α_1-adrenoceptors and decreases the outflow of GABA via α_2-adrenoceptors (Beani *et al.*, 1988). This inverted response to NA of cortical cholinergic structures has been confirmed *in vitro* in cortical slices taken from morphine-tolerant animals (Tanganelli *et al.*, 1989), and that of GABAergic structures has been confirmed in cortical slices and synaptosomes (Beani *et al.*, 1991). The present study was undertaken to extend these findings.

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Methods

Animal and tissue preparation

Dunkin-Hartley guinea-pigs of either sex, average weight 400 to 500 g, were used in all experiments. Animals were housed under standard conditions: constant temperature (22–24°C) and humidity (55–65%), 12 h dark/light cycle, free access to food and water. Procedures involving animals were carried out in accordance with European Community and national laws and policies. Morphine tolerance and dependence was achieved by subcutaneous implants of three morphine base pellets (75 mg), one on the first day, two on the fourth (Antonelli *et al.*, 1986). Control animals were implanted with pellets not containing morphine. All experiments (unless otherwise stated) were carried out on the seventh day.

For membrane preparation, the cerebral cortices taken from control or morphine-dependent guinea-pigs were homogenized in 50 mM Tris HCl buffer (pH 7.4) and centrifuged at 40,000 *g* for 20 min. The resulting pellet was homogenized and centrifuged again as described above. In tolerant animals, 1 μM morphine was added in Tris HCl buffer to prevent possible withdrawal effects *in vitro* (Gillan *et al.*, 1979). The final pellets were resuspended in assay buffer and used for binding assay.

For synaptosome preparation, the cerebral cortices taken from control or morphine-dependent guinea-pigs were homogenized in cold 0.32 M sucrose buffered with 5 mM HEPES at pH 7.4. In tolerant animals, 1 μM morphine was added in all solutions from the onset. The homogenate was centrifuged (10 min, 1,000 *g*, 4°C) and the supernatant centrifuged again (20 min, 12,000 *g*, 4°C) to obtain a crude synaptosomal fraction in the pellet (P2).

Slices (400 μm thick) were prepared from fronto-parietal cortices and perfused as previously described (Beani *et al.*, 1978). Briefly, two or three slices (wet weight 50–60 mg each) were placed together in superfusion chambers (volume 0.9 ml), perfused at a rate of 0.5 ml min^{-1} with Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10) and bubbled with 95% O_2 and 5% CO_2. Some of the slices were perfused for 60 min with morphine (100 nM; 300 nM; 1 μM; 3 μM) added to the Krebs solution. In other experiments cortical slices were perfused with 1 μM morphine for 15 or 30 min. The slices were then collected and used to prepare synaptosomes.

Radioligand binding studies

α_1-Adrenoceptor binding assay was performed essentially according to Froldi *et al.* (1994) using [^{3}H]-1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine ([^{3}H]-prazosin, 79.2 Ci $mmol^{-1}$). Saturation binding experiments were carried out in 1 ml of buffer (Tris HCl 50 mM, NaCl 140 mM, pH 7.4 at 25°C) containing membranes or synaptosomes from 5 mg (wet weight) of cortex and increasing concentrations (0.002–2 nM) of [^{3}H]-prazosin. After 60 min incubation at 25°C, separation of bound from free radioligand was performed by rapid filtration through Whatman GF/C filters under reduced pressure using a cell harvester (Brandel, Geithersburg, MD, U.S.A.). Filters were washed three times with ice cold buffer, dried and treated with 5 ml Aquassure (NEN Research products, Boston, MA, U.S.A.). Radioactivity was determined using a LS1800 Beckman liquid scintillation counter. Non specific binding was defined in the presence of 10 μM phentolamine and was less than 10% of total binding. Data were analysed by using the Ligand computer program (Munson & Rodbard, 1980). Protein was assayed according to the method of Lowry *et al.* (1951).

α_2-Adrenoceptor binding assay was performed essentially according to Opocher *et al.* (1993) using [^{3}H]-5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline ([^{3}H]-UK14304, 95.1 Ci $mmol^{-1}$). Saturation binding experiments were carried out in 1 ml of buffer (Tris HCl 50 mM, $MgCl_2$ 10 mM, EDTA 0.8 mM) containing membranes or synaptosomes. Membranes in a final concentration of approximately 5 mg (wet weight) of cortex or synaptosomes were incubated for 120 min at 25°C with increasing concentrations of [^{3}H]-UK14304 over a range of 0.1–10 nM. Non-specific binding was defined as a binding in the presence of 10 μM phentolamine. Filters were washed and counted as described above for α_1-adrenoceptor binding assays; again data were analysed by means of the computer program Ligand (Munson & Rodbard, 1980). In homologous displacement experiments 1 nM [^{3}H]-UK14304 and 16 different concentrations of unlabelled UK14304 (0.03 nM–10 μM) were used.

Release experiments

The synaptosomal pellet was resuspended in oxygenated Krebs solution containing 200 nM [^{3}H]-NA or 40 nM [^{3}H]-GABA, and maintained at 33°C for 45 min in the case of [^{3}H]-NA or 20 min in the case of [^{3}H]-GABA. Aliquots of the suspension (containing 0.8–1.2 mg protein) were then slowly injected into nylon syringe filters (0.45 μm pore size, Micron Separation, Westboro, MA, U.S.A.) connected by tubing to a peristaltic pump and kept at 33°C in a thermostatic bath. Synaptosomes were perfused with a preoxygenated (95% O_2, 5% CO_2) Krebs solution at a flow rate of 0.5 ml min^{-1}. In the Krebs solution, ascorbic acid (60 μM) and EDTA (30 μM) were added for [^{3}H]-NA experiments; β-alanine (100 μM) and amino-oxyacetic acid (50 μM) were added for [^{3}H]-GABA experiments. Morphine (1 μM) was added in all solutions for synaptosomes taken from morphine-dependent guinea-pigs. This superfusion technique, based on that of Raiteri *et al.* (1974), has been described elsewhere (Simonato *et al.*, 1993).

Stimulations were applied by pulses (90 s duration) of 35 mM potassium-Krebs solution (equimolar substitution of KCl for NaCl) 45 and 75 min after starting the superfusion of synaptosomes. UK14304 was applied 15 min before the second stimulation, so that the first stimulation could be used as an internal control. The perfusate was collected at 5 min intervals, scintillation fluid added, and counted in a Beckman LS6500 counter. Protein concentration in the preparation was determined by the method of Lowry (Lowry *et al.*, 1951).

The 35 mM potassium-evoked overflow was calculated by subtracting the basal outflow from the total tritium found in the sample starting at the stimulation and in the subsequent 5 min period. The presumed basal outflow was obtained by interpolation between the sample before and after stimulation. Data were expressed as overflow evoked by the second stimulation (in the presence of the drug) divided by the overflow evoked by the first stimulation (the internal control). Under untreated conditions (i.e. no UK14304 in the second stimulation), this ratio was close to a value of 1 in all of the preparations employed (for [^{3}H]-NA: 0.92 ± 0.04 in control, 0.92 ± 0.13 in synaptosomes taken from morphine-dependent guinea-pigs; for [^{3}H]-GABA: 0.98 ± 0.05 in control, 1.01 ± 0.06 in synaptosomes taken from morphine-dependent guinea-pigs).

Drugs

All the salts used were purchased from Merck (Darmstadt, Germany). All other drugs were from Sigma (St. Louis, MO, U.S.A.), except for morphine (from Salars, Como, Italy) and UK14304 (from RBI, Natick, MA, U.S.A.). All radioactive compounds were from New England Nuclear (Boston, MA, U.S.A.).

Results

Affinity of α_2-adrenoceptors for [^{3}H]-UK14304 in the cerebral cortex of morphine-dependent guinea-pigs

In order to start investigating the effect of a noradrenergic input in the cerebral cortex of morphine-dependent animals, it

was decided to study whether changes in adrenoceptor binding occur in the neocortex during morphine dependence which could imply changes in the response to NA. α-Adrenoceptors were selected for study because of their direct implication in the changes induced by morphine addiction (Beani *et al.*, 1988, 1991; Nestler 1992). Considering the existence of multiple types of α-adrenoceptors (α_1 and α_2) and of subtypes of each (Bylund *et al.*, 1994; Watson & Girdlestone, 1995), it was decided to begin examining the binding of a ligand selective to α_1-relative to α_2-adrenoceptors, [^{3}H]-prazosin, and of a ligand selective for α_2-relative to α_1-adrenoceptors, [^{3}H]-UK14304 (Watson & Girdlestone, 1995).

Binding of [^{3}H]-prazosin to cortical membranes was not significantly changed in morphine-dependent as compared to control guinea-pigs (B_{max}: 68.9±2.0 fmol mg^{-1} protein in controls, 65.6±2.8 fmol mg^{-1} protein in morphine-dependent animals; K_D: 0.10±0.01 nM in controls, 0.13±0.02 in morphine-dependent animals). However, a decrease in the K_D (but not in the B_{max}) of [^{3}H]-UK14304 for α_2-adrenoceptors was observed in cortical membranes prepared from morphine-dependent guinea-pigs as compared to controls (Figures 1a and 2a). Binding of [^{3}H]-UK14304 in the concentration range used appeared to be monophasic (to a single site). However, since G protein coupled receptors are usually characterized by both a high and low affinity receptor population, we ran a homologous displacement experiment in an attempt to identify a low affinity site. Again, only a single binding site could be observed (Figure 2b): computer analysis failed to show a better fit to a two site than to a one site model. The K_D value obtained was not significantly different from the one calculated in saturation experiments (0.90±0.08 versus 0.91±0.09 nM).

These data suggest that an increased affinity of α_2, but not α_1, adrenoceptors for NA occurs in the neocortex of morphine-dependent guinea-pigs. It was decided to investigate whether this phenomenon took place presynaptically by attempting to replicate binding data in synaptosomes. In fact, binding of [^{3}H]-prazosin to cortical synaptosomes was not significantly changed in morphine-dependent as compared to control guinea-pigs (B_{max}: 53.4±2.5 fmol mg^{-1} protein in controls, 48.9±1.3 fmol mg^{-1} protein in morphine-dependent animals; K_D: 0.13±0.02 nM in controls, 0.14±0.02 in morphine-dependent animals), while binding of [^{3}H]-UK14304 showed a decreased K_D in the absence of significant changes in the B_{max} (Figure 1b). Importantly, the magnitude of the change in the K_D for [^{3}H]-UK14304 was quite similar in crude membranes and in synaptosomes (−27% in membranes, −30% in synaptosomes). The presence of morphine *per se* does not account for this phenomenon, since adding 1 μM morphine during the assay did not modify [^{3}H]-UK14304 binding (B_{max}: 31.7±1.3 fmol mg^{-1} protein in control synaptosomes, 32.2±0.9 fmol mg^{-1} protein in synaptosomes exposed to morphine; K_D: 0.85±0.02 nM in control synaptosomes, 0.97±0.02 in synaptosomes exposed to morphine).

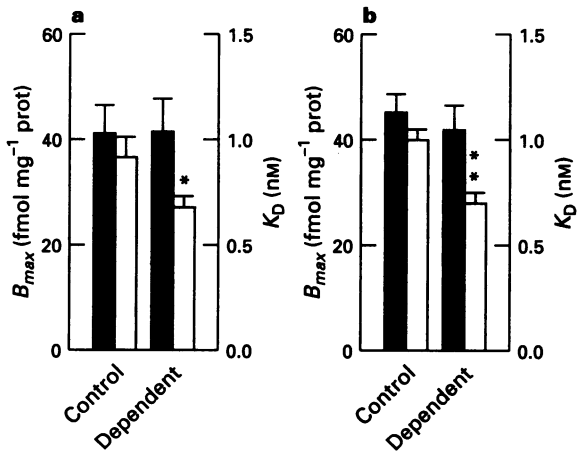

Figure 1 B_{max} (fmol mg^{-1} protein) (solid columns) and K_D (nM) (open columns) values of [^{3}H]-UK14304 binding to α_2-adrenoceptors in cortical membranes (a) and synaptosomes (b). K_D values in membranes were 0.91±0.09 nM in controls and 0.67±0.05 nM in morphine-dependent guinea-pigs; K_D values in synaptosomes were 1.00±0.04 nM in controls and 0.70±0.04 in morphine-dependent guinea-pigs. Means±s.e.mean of 8 experiments. Significantly different from control, Student's *t* test for non-paired data: $^*P<0.05$; $^{**}P<0.01$.

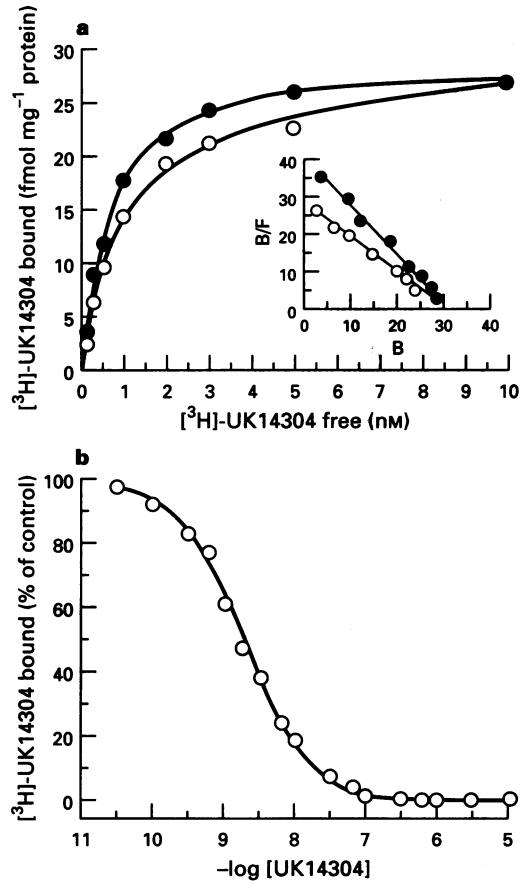

Figure 2 (a) Saturation of [^{3}H]-UK14304 binding to cortical membranes taken from control (○) and morphine-dependent (●) guinea-pigs; the inset is the corresponding Scatchard plot. Identical results were obtained in synaptosomes. (b) Homologous displacement of 1 nM [^{3}H]-UK14304 with cold UK14304 in cortical membranes taken from control guinea-pigs.

Time- and dose-dependency of the increased affinity of cortical α_2-adrenoceptors for [^{3}H]-UK14304

As a following step, the kinetics of development of the above phenomenon were investigated, and an attempt was made to parallel the development of morphine dependence with the development of increased α_2-adrenoceptor affinity. Dependence was tested by a naloxone test. Signs of withdrawal were recorded according to Bläsig *et al.* (1973). Naloxone-precipitated withdrawal was dose-dependent in guinea-pigs treated for 7 days with morphine, the dose of 3 mg kg^{-1} giving maximal effect (Table 1). This dose was, therefore, selected to check for the development of dependence after various durations of chronic morphine treatment. Although dependence was already present at day 3, it was fully developed only by day 5 (Table 1). Increased affinity of [^{3}H]-UK14304 to α_2-adrenoceptors could not be demonstrated at day 3, but had begun to develop at day 5, as shown by a 17% decrease in K_D as com-

Table 1 Withdrawal signs induced by naloxone in guinea-pigs treated with morphine or placebo

	Exploring	*Jumping*	*Wet dog shakes*	*Diarrhoea*
Naloxone 0.3 mg kg^{-1} i.p. Morphine day 7	19 ± 4	4 ± 3	4 ± 1	79%
Naloxone 1 mg kg^{-1} i.p. Morphine day 7	74 ± 19	17 ± 8	6 ± 1	100%
Naloxone 3 mg kg^{-1} i.p. Morphine day 3	42 ± 12	1 ± 1	1 ± 0	75%
Morphine day 5	104 ± 26	16 ± 8	3 ± 1	100%
Morphine day 7	126 ± 22	17 ± 7	9 ± 1	100%
Placebo day 7	4 ± 1	0 ± 0	1 ± 0	0%
Naloxone 10 mg kg^{-1} i.p. Morphine day 7	102 ± 15	14 ± 7	7 ± 2	100%
Naloxone 10 mg kg^{-1} i.p. Morphine 100 mg kg^{-1} i.p.	31 ± 13	4 ± 2	1 ± 0	20%

Means ± s.e.mean of 5–8 experiments. Signs of withdrawal have been identified and scored according to Bläsig *et al.* (1973). The incidence of the following signs was counted for 1 h after injection of naloxone: exploring (walking episodes, or standing up on hindlegs against the wall of the cage, or sniffing, or digging); jumping (vigorous jumps with all four feet off the ground); wet dog shakes (brief episodes of rapid repetitive shaking of the entire trunk). The presence of diarrhoea was checked and is shown here as % of responders.

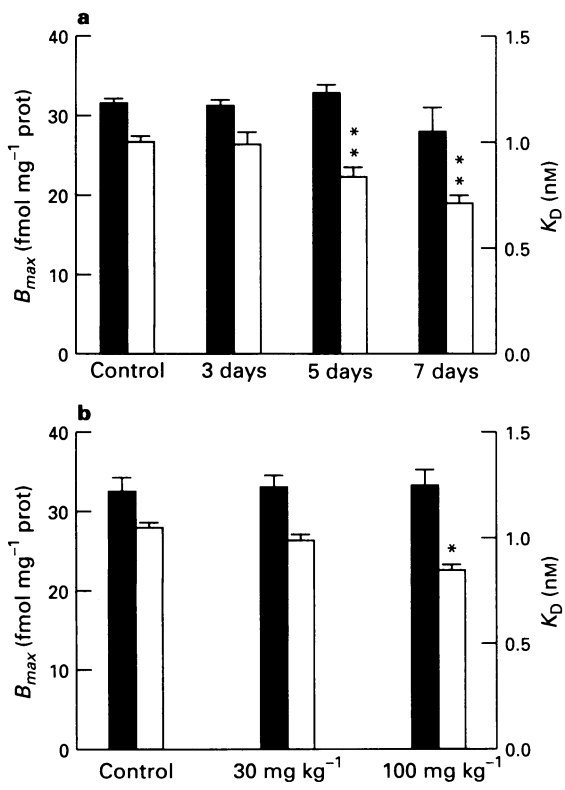

Figure 3 B_{max} (fmol mg^{-1} protein) (solid columns) and K_D (nM) (open columns) values of [^{3}H]-UK14304 binding to α_2-adrenoceptors after chronic (a) and acute (b) morphine administration. Means ± s.e.mean of 4–5 experiments. Significantly different from control, Student's *t* test for non-paired data: $^*P<0.05$; $^{**}P<0.01$.

pared to controls (Figure 3a). No significant change in the B_{max} was observed at any time-point.

Furthermore, to assess whether an acute morphine treatment was sufficient to increase the affinity of α_2-adrenoceptors for [^{3}H]-UK14304, other animals were killed 1 h after a single dose of morphine injected i.p. A small but significant decrease in K_D for [^{3}H]-UK14304 (approximately −15%) was observed after administration of morphine 100 mg kg^{-1} but not after morphine 30 mg kg^{-1} (Figure 3b), in the absence of changes in the B_{max}. Importantly, this dose of morphine was capable of inducing acute dependence, as shown by a naloxone test (Table 1).

In vitro *reproducibility of the findings*

One question raised by the above findings is whether they directly depend on the effects of morphine on the cerebral cortex or indirectly on the consequences of the variations in the firing pattern of the locus coeruleus in the course of morphine addiction (Aghajanian, 1978). In an attempt to address this point, cerebral cortex slices were prepared from naive guinea-pigs and divided in two groups: the first group was kept in perfusion for 15, 30 or 60 min with a Krebs solution containing morphine at concentrations in the range of those found in addicted animals (Goldstein & Schulz, 1973); the second group was perfused with normal Krebs. The [^{3}H]-UK14304 binding experiment was then run using synaptosomes separately prepared from each group of slices. The above observation that acute morphine administration is sufficient to induce a decrease in the K_D of [^{3}H]-UK14304 binding to α_2-adrenoceptors argues that synaptosomes prepared from slices of the first group should display an increased affinity for [^{3}H]-UK14304 if connections with the locus coeruleus are not necessary for that phenomenon to occur. In fact, this proved to be the case. The phenomenon was dependent on the concentration of morphine (Figure 4a), and a 30 min perfusion with 1 μM morphine was sufficient to induce a significant reduction in the K_D for [^{3}H]-UK14304 (Figure 4b).

Furthermore, in order to check whether isolated nerve terminals were sufficient for the expression of the phenomenon, the same experimental design was applied to synaptosomes, i.e. synaptosomes were prepared from naive animals and kept in morphine or in control Krebs solution for one hour before running the binding experiment. No significant changes in K_D or B_{max} were observed for [^{3}H]-UK14304 in synaptosomes treated for one hour with 1 μM morphine compared to controls (B_{max}: 30.1 ± 0.6 fmol mg^{-1} protein in control synaptosomes,

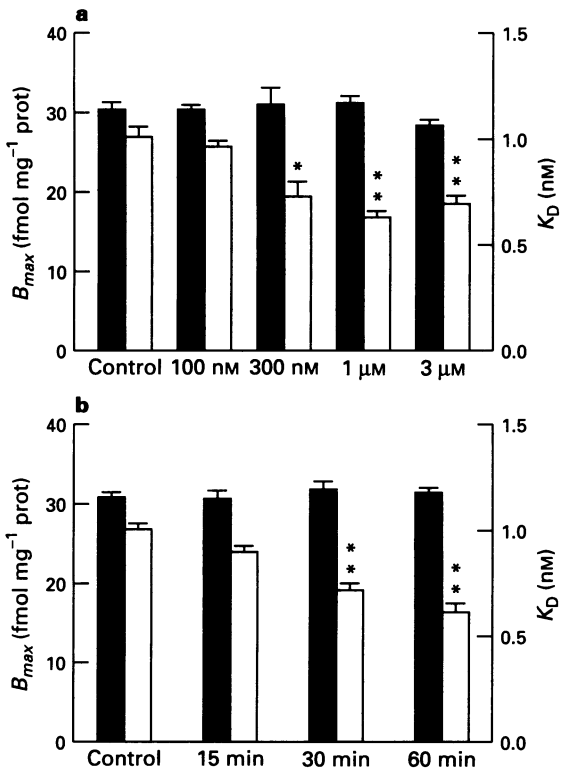

Figure 4 B_{max} (fmol mg^{-1} protein) (solid columns) and K_D (nM) (open columns) values of [^{3}H]-UK14304 binding to α_2-adrenoceptors in synaptosomes prepared from slices perfused for 1 h with a Krebs solution containing morphine at different concentrations (a), and in synaptosomes prepared from slices perfused with morphine 1 μM for different lengths of time (b). Means ± s.e.mean of 4–5 experiments. Significantly different from control, Student's t test for non-paired data: $^*P<0.05$; $^{**}P<0.01$.

28.1 ± 0.9 fmol mg^{-1} protein in synaptosomes treated with morphine; K_D: 1.15 ± 0.04 nM in control synaptosomes, 1.02 ± 0.04 in synaptosomes treated with morphine).

Shift to the left of the dose-response curve for α_2-adrenoceptor-mediated inhibition of cortical [^{3}H]-GABA (but not [^{3}H]-NA) outflow in morphine-dependent guinea-pigs

The above findings suggest that morphine-dependence is associated with an increased affinity of cortical presynaptic α_2-adrenoceptors for [^{3}H]-UK14304. These receptors are likely to be functionally involved in the inhibitory modulation of the release of neurotransmitters. Therefore, an attempt was made to identify whether the phenomenon has functional relevance, and on which neurotransmitters it applies. To this aim, the effect of the α_2-selective agonist UK14304 on the outflow of [^{3}H]-NA and [^{3}H]-GABA in synaptosomes prepared from control and morphine-dependent guinea-pigs was studied. We chose to study NA and GABA release because: (1) cortical noradrenergic terminals contain α_2-adrenoceptors exerting a feedback inhibition on the release of the neurotransmitters (Harsing & Vizi, 1991; Maura *et al.*, 1992; Tomasini *et al.*, 1992); (2) GABAergic terminals contain α_2-adrenoceptors which are implicated in the regulation of GABA release in morphine-dependent guinea-pigs (Beani *et al.*, 1991).

UK14304 concentration-dependently decreased 35 mM K^+-evoked [^{3}H]-NA overflow in cortical synaptosomes taken from control guinea-pigs, with an IC_{50} of 46 nM (Figure 5). Maximal effect (approximately −60%) was reached at a concentration of 1 μM. No significant change in the effects of UK14304 was observed in synaptosomes taken from morphine-dependent animals (Figure 5). Furthermore, no significant change in

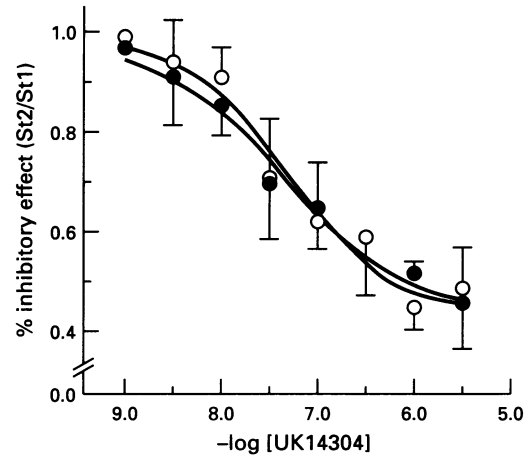

Figure 5 Effect of UK14304 on 35 mM K^+-evoked [^{3}H]-noradrenaline ([^{3}H]-NA) overflow in cortical synaptosomes taken from control (○) and morphine-dependent (●) guinea-pigs. Means ± s.e.mean of 8 experiments. IC_{50} value for control = 46 nM (27–78) and for morphine-dependent = 40 nM (26–62) with 95% confidence limits in parentheses.

maximal [^{3}H]-NA uptake (calculated after preincubation with a single concentration of [^{3}H]-NA (200 nM) and a 45 min washout) or in its 35 mM K^+-evoked overflow (first stimulation) were detected in morphine-dependent animals as compared to controls (uptake: 5.15 ± 0.25 pmol mg^{-1} protein in controls, 7.46 ± 1.04 pmol mg^{-1} protein in morphine-dependent animals; 35 mM K^+-evoked overflow: 4.16 ± 0.48% of content in controls, 4.06 ± 0.24% of content in morphine-dependent animals).

UK14304 also concentration-dependently decreased 35 mM K^+-evoked [^{3}H]-GABA overflow in cortical synaptosomes taken from control guinea-pigs, with an IC_{50} of 501 nM (Figure 6). Maximal inhibitory effect was approximately −25%. Concentrations of UK14304 higher than 3 μM occasionally produced a paradoxical, most likely non-specific, stimulating effect. A highly significant shift to the left in the concentration-response curve was observed in synaptosomes prepared from morphine-dependent guinea-pigs, in the absence of changes in

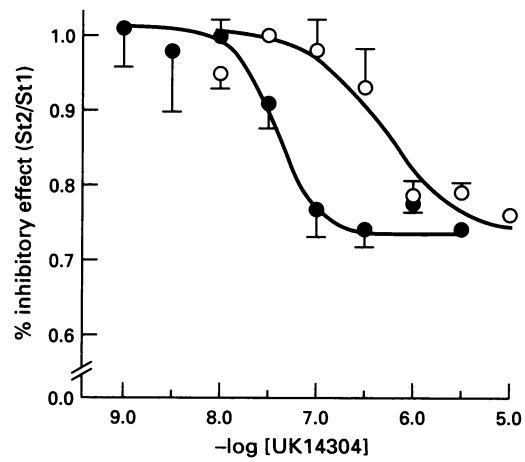

Figure 6 Effect of UK14304 on 35 mM K^+-evoked [^{3}H]-GABA overflow in cortical synaptosomes taken from control (○) and morphine-dependent (●) guinea-pigs. Means ± s.e.mean of 8 experiments. The mean IC_{50} of 8 separate dose-response experiments in synaptosomes taken from control animals (501 nM (219–1148)) significantly differs from the one calculated in synaptosomes taken from morphine-dependent animals (41 nM (30–56), 95% confidence limits shown in parentheses): $P<0.01$, Student's t test for non-paired data.

the maximal effect (Figure 6). The IC_{50} for the inhibitory effect of UK14304 on 35 mM K^+-evoked [^{3}H]-GABA overflow in morphine-dependent animals (41 nM) was similar to the one calculated for the effect on [^{3}H]-NA outflow. Finally, no significant change in [^{3}H]-GABA uptake (calculated after preincubation with a single 40 nM [^{3}H]-GABA concentration and a 45 min wash-out) or in its 35 mM K^+-evoked overflow (first stimulation) were detected in morphine-dependent animals as compared to controls (uptake: 1.76 ± 0.19 pmol mg^{-1} protein in controls, 1.46 ± 0.17 pmol mg^{-1} protein in morphine-dependent animals; 35 mM K^+-evoked overflow: $5.53 \pm 0.42\%$ of content in controls, $5.20 \pm 0.54\%$ of content in morphine-dependent animals).

Discussion

Binding to α_2-adrenoceptors in the cerebral cortex of morphine-treated guinea-pigs

Binding to α-adrenoceptors has been extensively studied in morphine-dependent rats. Consistent with the present study, no significant changes in cortical α_1-adrenoceptors have been obtained (Hamburg & Tallman, 1981). However, the absence of selective α_1-adrenoceptor agonists precludes the possibility of a more detailed investigation, and therefore variations in binding to these receptors cannot be ruled out on the basis of the data presented in this study.

In contrast, the cortical α_2-adrenoceptor number has been shown to be increased (Hamburg & Tallman, 1981), unchanged (Vicentini *et al.*, 1983; Carlson & Cooper, 1985) or decreased (Smith *et al.*, 1989) in morphine-dependent rats. The reasons for these discrepancies are not clear. As opposed to the present study, none of those studies could demonstrate a change in the affinity of α_2-adrenoceptors for [^{3}H]-clonidine, the ligand used in all cases. The present study is the first to employ a highly selective α_2-ligand ([^{3}H]-UK14304). Therefore, the low specificity of [^{3}H]-clonidine and/or species differences may account for this discrepancy.

It only proved possible for us to demonstrate a high affinity site for [^{3}H]-UK14304 binding both when approaching this problem by saturation or by homologous displacement experiments. In fact, the present saturation experiments are consistent with other studies in which this agonist has been used in the same concentration range in other species (Loftus *et al.*, 1984; Meana *et al.*, 1989; Gelbmann *et al.*, 1990). An implication of the homologous displacement experiment (Swillens *et al.*, 1995) is that, if a low affinity site for [^{3}H]-UK14304 exists (as demonstrated for other agonists binding G protein-coupled receptors – see for example Luthin *et al.*, 1995), this should be much less represented than the high affinity site and/or should have a K_D shifted no more than 1 log unit from the K_D of the high affinity site.

Several characteristics of the increased affinity of α_2-adrenoceptors for the selective ligand [^{3}H]-UK14304 have been identified in this study. First, this phenomenon can be observed in synaptosomes, suggesting an involvement of α_2-adrenoceptors located presynaptically. The participation of other receptors located on the cell body of neurones and/or on glial cells cannot be ruled out, and is indeed suggested by the observation that the change in affinity is identical in membranes and in synaptosomes. However, we decided to pursue the consequences of the changes in this subgroup of receptors (presynaptic), and all the subsequent data and discussion will focus on these.

Second, the increase in synaptosomal α_2-adrenoceptor affinity to [^{3}H]-UK14304 occurs in a time-dependent fashion, in that it takes 5 days of chronic morphine treatment to start developing. This observation, together with the other that morphine dependence in this model system is detectable around day 3 and is fully developed by day 5 of treatment, suggests that morphine dependence is a prerequisite for the increased α_2-adrenoceptor affinity to occur. This phenomenon can also be observed after a single administration of a high dose of morphine which induces acute dependence. Although it could be speculated that the same molecular mechanism underlies acute and chronic morphine-induced increases in α_2-adrenoceptor affinity, no direct evidence has been obtained in this regard in the present study. Nevertheless, it will be assumed that this is the case for the discussion of our *in vitro* data.

Third, the development of increased affinity of α_2-adrenoceptors for [^{3}H]-UK14304 does not require a connection with the LC or with other subcortical structures. One question raised by the findings discussed thus far is whether they depend directly upon the action of morphine on the cortex or indirectly on the effects of morphine on the LC and/or on other areas connected with the cortex. The observation that the phenomenon can be reproduced *in vitro* in isolated cerebral cortex slices perfused with morphine for more than 30 min argues in favour of the former hypothesis. The increase in α_2-adrenoceptor affinity for [^{3}H]-UK14304 occurs *in vitro* in a time- and concentration-dependent manner, and is quantitatively identical to the one observed *ex vivo*, suggesting that the same mechanism may underlie both events. Therefore, the effect of morphine on intracortical structures appears to be sufficient to explain the phenomenon. Indirect effects on areas connected with the cortex may be involved, but are not necessary for an explanation of the observed changes.

However, the integrity of cortical structures (presumably of intact neurones) is necessary for the occurrence of an increase in α_2-adrenoceptor affinity for [^{3}H]-UK14304. In fact, although this phenomenon appears to take place presynaptically, it could not be reproduced exposing isolated terminals (synaptosomes) to morphine at concentrations and for times identical to those employed in the above *in vitro* experiments, where synaptosomes were prepared from slices exposed to morphine. This observation argues that the preservation of an anatomical connection between the terminals and the cell body of intrinsic cortical neurones (for example, GABAergic neurones) during morphine treatment is required for the changes in presynaptic α_2-adrenoceptor affinity.

Further pharmacological studies will be needed in order to establish the subtype of α_2-adrenoceptor involved in the phenomenon described. However, it can be hypothesized they are homologous of the human α_{2A}-subtype, because this appears to be the predominant subtype in the cerebral cortex (Ordway *et al.*, 1993).

Pursuit of functional correlates for the findings

Cortical presynaptic α_2-adrenoceptors are known to be localized on multiple types of neurones, including autoreceptors on NA (Harsing & Vizi, 1991; Maura *et al.*, 1992; Raiteri *et al.*, 1992; Tomasini *et al.*, 1992), and heteroreceptors on GABA terminals (Beani *et al.*, 1991; Ferraro *et al.*, 1993). The present release studies identify a subpopulation of cortical terminals where a change in α_2-adrenoceptors occurs in morphine-treated animals. In fact, no changes were observed in the ability of the selective α_2-adrenoceptor agonist UK14304 to inhibit 35 mM K^+-evoked [^{3}H]-NA overflow from cortical synaptosomes of morphine-dependent as compared to control guinea-pigs. However, a large (1 log unit) decrease in the IC_{50} for the inhibition of [^{3}H]-GABA overflow was observed in morphine-dependent animals. This made the IC_{50} values overlap the one calculated for the inhibition of [^{3}H]-NA overflow.

It is difficult to parallel these release and binding data, unless one assumes that the receptor binding [^{3}H]-UK14304 with a K_D of approximately 1 nM is the same one that mediates inhibition of release with an IC_{50} of approximately 40 nM. This does not appear to be an unlikely hypothesis, since multiple explanations can be put forward to interpret the discrepancies between K_D and IC_{50} values: first, the K_D relates to the most simple event (binding of the ligand to the receptor) while the IC_{50} underlies a series of complex events connecting the activation of receptor-coupled transducing systems to the phenomenon of release; second, temperature, buffer composition,

times of exposure to the agonist and membrane potential of the preparation are different under the conditions of the binding and release experiments. Furthermore, such discrepancies are not unusual: for example, clonidine in the neocortex of the rat binds to its high affinity site with a K_D of 0.4 nM (U'Prichard *et al.*, 1979), but inhibits [^{3}H]-NA release with an IC_{50} of approximately 10 nM (Maura *et al.*, 1992). On the basis of these considerations, it will be assumed for the rest of this discussion that the same α_2-adrenoceptor binds [^{3}H]-UK14304 with a K_D of 1 nM and mediates inhibition of [^{3}H]-NA release with an IC_{50} of approximately 40 nM.

If this is the case, a receptor with low affinity for UK14304 should modulate GABA release in normal animals, since the IC_{50} for UK14304-mediated inhibition is approximately 500 nM. This receptor may speculatively be identified either as the low affinity site of the α_2-adrenoceptor described thus far or as a distinct subtype. This finding is in agreement with a previous study (Maura *et al.*, 1992) that clonidine inhibits [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) release approximately ten times less potently than [^{3}H]-NA release (i.e. it is more potent on auto- than on heteroreceptors). The fact that this hypothetical low affinity site could not be observed in binding experiments may depend on a relatively minor representation in the cortex and/or on a small difference in the K_D compared to the high affinity site (Swillens *et al.*, 1995, see above).

Therefore, these data suggest that a change in α_2-adrenoceptor agonist affinity or expression of a new α_2-adrenoceptor subtype (similar or identical to the one present on NA terminals) occurs on GABA terminals of morphine-dependent guinea-pigs. Other studies have shown a shift to the left in the dose-response curve for clonidine inhibition of electrically-evoked NA release from cerebral cortex slices (Tomasini *et al.*, 1992): this phenomenon could be observed only when stimulating at 1 Hz (not at 3 Hz), suggesting that other more subtle changes in α_2-adrenoceptor physiology may occur during morphine dependence.

Based on the assumption made above, [^{3}H]-GABA release studies are consistent with binding studies, in that (1) the maximal inhibition was unchanged, in agreement with the absence of variations in the B_{max}, while (2) a shift to the left in the concentration-response curve for the release of [^{3}H]-GABA was observed, which coincides with a decrease in the K_D for [^{3}H]-UK14304; moreover, the observation of such a shift in only one population of terminals is consistent with a relatively much smaller decrease in the K_D (which is calculated for all cortical synaptosomes).

The observation that the maximal inhibitory effect of UK14304 is higher for [^{3}H]-NA compared to [^{3}H]-GABA release is consistent with a greater impact of α_2-adrenoceptors on the modulation of NA than that of GABA terminals, as expected for an autoreceptor. It is important to stress that the above findings are most likely not an artifact due to changes in the uptake of the neurotransmitter and/or to changes in the responsivity of the terminals to depolarizing stimuli, which are identical under control and morphine-dependence conditions. Acute morphine treatments inhibit the release of both cortical NA (Montel *et al.*, 1974; Arbilla & Langer, 1978; Göthert & Wehking, 1980) and GABA (Antonelli *et al.*, 1986). Therefore, consistent with other studies (Antonelli *et al.*, 1986; Werling *et al.*, 1988), this observation suggests that tolerance has developed in the system.

In conclusion, these data suggest that chronic morphine treatment in the guinea-pig is associated with a shift from a low to a high affinity state or with expression of a new subtype of α_2-adrenoceptors on cortical GABA terminals. In the latter hypothesis, this new subtype should possess pharmacological characteristics similar to those of the α_2-adrenoceptor subtype located on noradrenergic terminals; however, the present data provide little support for this suggestion, and further studies will be needed to test the hypothesis. We propose that this phenomenon is functionally relevant, in that (1) in intact animals, NA i.c.v. or electrical stimulation of the LC increase cortical GABA outflow via α_1-adrenoceptors in controls (Moroni *et al*, 1982) but decrease it via α_2-adrenoceptors in morphine-dependent guinea-pigs (Beani *et al.*, 1988); (2) the same inverted response to NA can be observed *in vitro* in slices and synaptosomes taken from control vs. morphine-dependent guinea-pigs (Beani *et al.*, 1991).

Finally, the clinical relevance of the observation presented here may be suggested. It has been demonstrated that inhibitory α_2-adrenoceptors are present on human cortical GABAergic terminals, even in the prevalence of stimulating α_1-adrenoceptors (Ferraro *et al.*, 1993). This arrangement of the functional connections between a noradrenergic input and GABA is therefore similar in the human, in the rat and in the guinea-pig cortex (Ferraro *et al.*, 1993). Furthermore, an increase in the number of α_2-adrenoceptors has been observed in the platelets of heroin addicts (Garcia-Sevilla *et al.*, 1986): platelets have been proposed as a diagnostic and research tool for the study of biogenic amines in human neurologic disorders (Stahl, 1977). Increased α_2-adrenoceptor-mediated effects on cortical GABA terminals in heroin addicts may be implicated in some important aspects of heroin addiction, such as insomnia, anxiety and benzodiazepine abuse.

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