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Brain α_2-adrenoceptors in monoamine-depleted rats: increased receptor density, G coupling proteins, receptor turnover and receptor mRNA

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1 This study was designed to assess the molecular and cellular events involved in the up-regulation (and receptor supersensitivity) of brain α_2-adrenoceptors as a result of chronic depletion of noradrenaline (and other monoamines) by reserpine.

2 Chronic reserpine (0.25 mg kg^{-1} s.c., every 48 h for 6–14 days) increased significantly the density (B_{max} values) of cortical α_2-adrenoceptor agonist sites (34–48% for [^{3}H]-UK14304, 22–32% for [^{3}H]-clonidine) but not that of antagonist sites (11–18% for [^{3}H]-RX821002). Competition of [^{3}H]-RX821002 binding by (−)-adrenaline further indicated that chronic reserpine was associated with up-regulation of the high-affinity state of α_2-adrenoceptors.

3 In cortical membranes of reserpine-treated rats (0.25 mg kg^{-1} s.c., every 48 h for 20 days), the immunoreactivities of various G proteins ($G\alpha i_{1/2}$, $G\alpha i_3$, $G\alpha o$ and $G\alpha s$) were increased (25–34%). Because the high-affinity conformation of the α_2-adrenoceptor is most probably related to the complex with $G\alpha i_2$ proteins, these results suggested an increase in signal transduction through α_2-adrenoceptors (and other monoamine receptors) induced by chronic reserpine.

4 After α_2-adrenoceptor alkylation, the analysis of receptor recovery (B_{max} for [^{3}H]-UK14304) indicated that the increased density of cortical α_2-adrenoceptors in reserpine-treated rats was probably due to a higher appearance rate constant of the receptor ($\Delta r = 57\%$) and not to a decreased disappearance rate constant ($\Delta k = 7\%$).

5 Northern- and dot-blot analyses of RNA extracted from the cerebral cortex of saline- and reserpine-treated rats (0.25 mg kg^{-1}, s.c., every 48 h for 20 days) revealed that reserpine markedly increased the expression of α_{2a}-adrenoceptor mRNA in the brain (125%). This transcriptional activation of the receptor gene expression appears to be the cellular mechanism by which reserpine induces up-regulation in the density of brain α_2-adrenoceptors.

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Abbreviations: ANOVA, analysis of variance; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline; G protein, guanine nucleotide-binding regulatory protein; IOD, integrated optical density; RX821002, 2-methoxy idazoxan or 2-[2-(2-methoxy-1,4-benzodioxanyl)] imidazoline; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; UK14304, bromoxidine or 5-bromo-6-(2-imidazolin-2-yl-amino) quinoxaline

Introduction

Reserpine, a depleter of brain monoamines (noradrenaline, serotonin, dopamine), induces in rats a biochemical and behavioural syndrome that has been extensively studied as a potential animal model of depression (Leith & Barrett, 1980; Cooper *et al*., 1983; Vetulani *et al*., 1986; Ugedo *et al*., 1993). In the past, reserpine treatment was commonly associated with the induction of depressive symptoms in patients who had histories of major depression (Goodwin & Bunney, 1971). Moreover, depletion of noradrenaline or serotonin in the brain of depressed patients, who were in clinical remission, has been recently shown to cause a rapid return to depressive symptoms (Berman *et al*., 1999; Delgado & Moreno, 1999; Ressler & Nemeroff, 1999).

In major depression α_2-adrenoceptors are of special interest because these receptors control, together with other mechanisms, the activity of noradrenergic and serotonergic neurones in the brain. Thus, a relevant proportion (20–40%) of α_2-adrenoceptors in the rat brain appears to be presynaptic autoreceptors playing a crucial role in the regulation of noradrenergic neurones (Heal *et al*., 1993; García-Sevilla *et al*., 1994). When these presynaptic inhibitory $\alpha_{2A/C}$-adrenoceptors are stimulated (autoreceptors on noradrenergic neurones and a smaller proportion of heteroreceptors on serotonergic neurones), release and synthesis of noradrenaline and serotonin are inhibited (Trendelenburg *et al*., 1994; Esteban *et al*., 1996); therefore, increased $\alpha_{2A/C}$-adrenoceptor

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density and/or sensitivity could result in insufficient neuronal release of noradrenaline/serotonin and lead to depression. Supersensitivity of $\alpha_{2A/C}$-adrenoceptors in specific brain regions could represent a common feature of the reduced monoaminergic (noradrenergic and/or serotonergic) function postulated in major depression (see Delgado & Moreno, 1999), and this receptor up-regulation has been observed in some (e.g. De Paermentier *et al*., 1997; Callado *et al*., 1998; García-Sevilla *et al*., 1999) but not all (e.g. Ferrier *et al*., 1986; Arango *et al*., 1993) postmortem studies in brains of depressed suicides.

Supersensitivity of α_2-adrenoceptors also appear to be associated with reserpine treatment. Thus, prolonged depletion of brain noradrenaline (and other monoamines) after treatment with low doses of reserpine is associated with marked increases in the density and/or affinity of agonist radioligands for brain $\alpha_{2A/C}$-adrenoceptors (U'Prichard & Snyder, 1978; Bylund & Martínez, 1980; Hong *et al*., 1988; Giralt & García-Sevilla, 1989; Ugedo *et al*., 1993). In line with these radioligand binding data, various functional *in vivo* responses mediated by central presynaptic and postsynaptic $\alpha_{2A/C}$-adrenoceptors were found to be potentiated after treatment with reserpine (Ugedo *et al*., 1993). Functional studies in peripheral tissues also have shown increases in α_{2A}-adrenoceptor sensitivity after reserpine treatment (Estan *et al*., 1990; Ugedo *et al*., 1993) and have suggested that there is an increase in the receptor reserve at α_{2A}-adrenoceptors (autoreceptors) in reserpine-treated rats (Pineda *et al*., 1997).

Therefore, prolonged depletion of brain noradrenaline (and other monoamines) in rats with reserpine can induce a neuronal presynaptic dysfunction (see Delgado & Moreno, 1999) and reproduce the relevant $\alpha_{2A/C}$-adrenoceptor supersensitivity detected in the postmortem brain of depressed suicide victims (e.g. García-Sevilla *et al*., 1999), suggesting that these receptor changes could indeed reflect some of the pathophysiology of major depression. As mentioned, however, other studies did not find up-regulation of α_2-adrenoceptors in brains of suicide victims (Ferrier *et al*., 1986; Arango *et al*., 1993). The supersensitivity of brain α_2-adrenoceptors induced by reserpine appears to be a homospecific adaptation of the noradrenergic pathway to the depletion of catecholamines. However, the cellular mechanisms underlying the reserpine-induced up-regulation of brain α_2-adrenoceptors are unknown and a better understanding of these mechanisms would be relevant to the pathophysiology of depressive disorders. Against this background, the present study was designed to re-assess in detail the changes induced by chronic treatment with reserpine on the density and affinity of $\alpha_{2A/C}$-adrenoceptors (agonist and antagonist binding sites), as well as on the immunoreactive levels of associated regulatory G proteins, and to assess whether reserpine-induced receptor supersensitivity is associated with an accelerated turnover of $\alpha_{2A/C}$-adrenoceptors and an increased α_{2a}-adrenoceptor gene expression in the rat brain. The α_{2A}-adrenoceptor is the predominant α_2-adrenoceptor subtype in the mammalian brain and it plays a crucial role as mediator of most of the inhibitory effects of α_2-adrenoceptor agonists (MacDonald *et al*., 1997). The α_2-adrenoceptor nomenclature recommended by the International Union of Pharmacology has been followed (Bylund *et al*., 1994).

Methods

Animals and treatments

Adult male Sprague-Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at $20\pm2^{\circ}$C with a 12 h light/dark cycle. The animals received s.c. every 48 h either 0.9% saline vehicle or reserpine (0.25 mg kg^{-1}) from day 1 to day 6–36 of treatment. The rats were killed by decapitation 48 h after the last injection of the corresponding treatment. The dose of reserpine (0.25 mg kg^{-1}) chosen did not induce weight loss (see also Fortin & Sundaresan, 1989). Chronic treatment with this dose of reserpine (0.25 mg kg^{-1}, s.c., every 48 h, for 20 days) resulted in prolonged depletion of noradrenaline (>80%), serotonin (>75%) and dopamine (>85%) in various rat brain regions (data not shown). EEDQ (1.6 mg kg^{-1}) was dissolved in ethanol and then diluted sequentially with propilenglycol and purified water (final ratio 1:1:2, v/v/v) and it was administered i.p. in a single dose. To study the modulation of α_2-adrenoceptors turnover by reserpine, EEDQ was injected only once in saline-treated rats and in rats treated with the monoamine depleter drug for 6 days, and treatment with saline or reserpine was then continued for various periods of time. Thus, rats were killed 6 h, and 1, 2, 4, 7, 9, 14, 18, 21, 26 and 30 days after EEDQ administration to evaluate the recovery of brain α_2-adrenoceptor density, which allowed estimation of receptor turnover parameters. The brains were rapidly removed and the frontal and parieto-occipital cortex dissected on ice and stored at -70°C until assay. These experiments on rats were performed according to the guidelines of the University of the Balearic Islands.

[^{3}H]-UK14304, [^{3}H]-clonidine and [^{3}H]-RX821002 binding assays

The specific binding of [^{3}H]-UK14304 (full agonist), [^{3}H]-clonidine (partial agonist) and that of [^{3}H]-RX821002 (antagonist) to parieto-occipital cortical membranes were used as biochemical indexes to quantify the density of α_2-adrenoceptor binding sites ($\alpha_{2A/C}$-subtypes). Preparation of cortical membranes (P_2 membrane fraction) and [^{3}H]-radioligand binding assays were carried out as described previously (Olmos *et al*., 1993; Ribas *et al*., 1993). Briefly, total [^{3}H]-UK14304, [^{3}H]-clonidine and [^{3}H]-RX821002 binding was measured with eight concentrations of radioligand (6×10^{-11} M to 8×10^{-9} M, for [^{3}H]-UK14304 and [^{3}H]-RX821002; 1.25×10^{-10} M to 1.6×10^{-8} M, for [^{3}H]-clonidine). For all radioligands, nonspecific binding was determined in the presence of 10^{-5} M (−)-adrenaline. Agonist competition studies also were performed in cortical membranes, which were incubated with [^{3}H]-RX821002 (1×10^{-9} M), in the absence or presence of various concentrations of (−)-adrenaline (3.3×10^{-11} M to 10^{-3} M, 22 concentrations). Membrane-bound [^{3}H]-radioligands were quantified as described previously (Ribas *et al*., 1993). Protein was determined by the method of Lowry *et al*. (1951) with bovine serum albumin as the standard. Analyses of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate

binding model were performed by computer-assisted non-linear analysis from untransformed data, using the EBDA-LIGAND programs (McPherson, 1985; Munson & Rodbard, 1980).

Analyses of the recovery of $\alpha_{2A/C}$-adrenoceptor binding sites after EEDQ

For analysis of α_2-adrenoceptor turnover, data from the recovery (all individual experiments together) of brain α_2-adrenoceptor density (B_{max} for the full agonist [^{3}H]-UK14304 binding) after irreversible inactivation by EEDQ, during saline and reserpine treatments, were analysed according to the standard mono-exponential model (Mauger *et al*., 1982; Barturen & García-Sevilla, 1992; Ribas *et al*., 1993). Exponential recovery data were fitted, using the simple nonlinear least-squares fitting program GraFit (Leatherbarrow, 1990), to the equation:

$$R_t = r/k(1 - e^{-kt}) \qquad \text{(Eq.1)}$$

where R_t is expressed as fmol mg^{-1} protein and represents the receptor number at a given discrete time t; r is the rate constant of receptor appearance expressed as fmol mg^{-1} protein day^{-1}, and k is the rate constant of receptor disappearance (in units of day^{-1}) which allows estimation of the apparent half-life of the receptor ($t_{1/2} = \ln 2/k$). The ratio r/k represents the density of receptors at steady state.

In saline-treated rats, but not in reserpine-treated rats, the recovery of [^{3}H]-UK14304 binding after EEDQ also fitted well to a newly proposed biphasic model for the recovery of α_2-adrenoceptor agonist binding sites (Ribas *et al*., 1998) (data not shown). For this reason α_2-adrenoceptor turnover parameters in saline- and reserpine-treated rats were calculated and compared only according to the monoexponential model (Equation 1).

Immunoblot analysis of G protein subunits

Groups of rats were treated s.c. with saline ($n=4$) or reserpine (0.25 mg kg^{-1}) ($n=4$), every 48 h for 20 days. The rats were killed 48 h after the last injection. Preparation of cortical membranes (P_2 membrane fraction), immunoblot analysis of specific G protein subunits and quantitation of specific immunoreactivity were performed as described previously (Escribá *et al*., 1994; Ribas *et al*., 1998). Briefly, solubilized G proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes (Western blotting) and then labelled with specific antibodies: anti-G$\alpha i_{1/2}$ (AS/7) at a dilution of 1 : 7000, anti-Gαi_3 (EC/2) at a dilution of 1 : 3000, anti-Gαo (GC/2) at a dilution of 1 : 4000 and anti-Gαs (RM/1) at a dilution of 1 : 3000. The secondary antibody, horseradish peroxidase-labelled donkey anti-rabbit immunoglobulin G, was incubated at a dilution of 1 : 5000. Immunoreactivity was detected with the Enhanced Chemiluminescence Western Blot Detection system (Amersham International), followed by exposure to Hyperfilm ECL film for 1–10 min. The film was scanned in the image analyser Bio Image (Millipore, Ann Arbor, MI, U.S.A.). The quantitation of specific immunoreactivity was done as described previously (Escribá *et al*., 1994), using appropriate standard curves (i.e., total protein loaded versus Integrated Optical Density, IOD), which consisted of at least four different protein contents (the protein was from naïve control rats), all loaded on the same gel, resulting in linear relationships in the range of protein content used (for further details see Escribá *et al*., 1994).

Northern and dot-blot analyses of α_{2a}-adrenoceptor mRNA

Total RNA was extracted from rat brain specimens by use of a single-step RNA isolation system (TRIzol reagent, GIBCO–BRL, Berlin, Germany) which is based on the method of Chomczynski & Sacchi (1987) and quantitated spectrophotometrically by measuring the absorbance at 260 nm. Total cerebral cortex RNA yields were routinely 0.5–1 μg mg^{-1} tissue. The Northern and dot-blot procedures for the quantitation of α_{2a}-adrenoceptor mRNA have been described elsewhere (Busquets *et al*., 1997). The plasmid containing the cDNA encoding the human platelet α_{2A}-adrenoceptor was kindly provided by Dr Robert J. Lefkowitz (Department of Medicine, Duke University, Durham, NC, U.S.A.). In the rat cerebral cortex, however, the α_{2a}-adrenoceptor probe also was shown to cross-hybridize weakly with the α_{2c}-adrenoceptor mRNA (Lorenz *et al*., 1990). This was confirmed in preliminary experiments which demonstrated that the α_{2a}-adrenoceptor probe cross-hybridized with the α_{2c}- but not with the α_{2b}-adrenoceptor cDNA (cDNAs provided by R.J. Lefkowitz) in dot-blot analyses (see Busquets *et al*., 1997). The weak cross hybridization of the probe used suggests that also α_{2c}-mRNA can be quantitated, but this contamination appears to be of minor relevance. In the rat cerebral cortex, both α_{2a}- and α_{2c}-adrenoceptor mRNAs are expressed (Nicholas *et al*., 1993). For the routine quantitation of α_{2a}-adrenoceptor mRNA levels dot-blot analyses were performed (see Busquets *et al*., 1997 for details). Quantitative analysis of dot-blot densities was performed by scanning densitometry in the image analyser Bio Image (Millipore, Ann Arbor, MI, U.S.A.). The relative levels of α_{2a}-adrenoceptor mRNA signals in dot-blots (IOD) were normalized to correct for any loading discrepancies of RNA by scanning the ethidium bromide fluorescence of total RNA signals from the same dot blots after u.v. light irradiation of the nylon membranes (Sambrook *et al*., 1989). The IOD of the α_{2a}-adrenoceptor probe hybridization signal (α_{2a} mRNA signal) and the IOD of the ethidium bromide fluorescence (total RNA signal) were concentration-dependent in a linear fashion with respect to the total RNA content. In saline- and reserpine-treated rats, the results are expressed as the ratio α_{2a} mRNA/total RNA (IODs).

Statistics

Radioligand binding, immunoblot and dot-blot data are expressed as means ± s.e.mean. Two-way analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison test, and Student's two-tailed t-test were used for the statistical evaluations. The level of significance was chosen as $P=0.05$. Radioligand binding experiments were analysed initially assuming a one-site model of radioligand binding and then assuming two or three-site binding models.

Receptor turnover parameters are expressed as the best fit values ± s.e. determined by the matrix inversion method, using the non-linear regression program GraFit (Leatherbarrow, 1990). Comparisons of experimental data sets for the recovery of α_2-adrenoceptor binding sites were performed by comparing the goodness of fit of a model with and without a set of constraints by means of an *F*-test. The selection between the different binding and recovery models was made statistically using the extra sum of squares principle (*F*-test) as outlined by Munson & Rodbard (1980). The more complex model was accepted if the *P*-value resulting from the *F*-test was less than 0.05.

Drugs and chemicals

[^{3}H]-UK14304 (bromoxidine or brimonidine, specific activity, 60–87 Ci mmol^{-1}) and [^{3}H]-clonidine (specific activity, 66–68 Ci mmol^{-1}) were purchased from New England Nuclear Du Pont (U.S.A.). [^{3}H]-RX821002 (2-methoxy-idazoxan, specific activity, 48–56 Ci mmol^{-1}) was purchased from Amersham International (U.K.). Rabbit anti-G protein subunits polyclonal antisera raised against specific C-terminal peptides or the N-terminus (Gαo) were purchased from New England Nuclear Du Pont: anti-G$\alpha i_{1/2}$ (AS/7), anti-Gαi_3 (EC/2), anti-Gαo (GC/2) and anti-G$\alpha$is (RM/1). Horseradish peroxidase-labelled donkey anti-rabbit immunoglobulin G, Enhanced chemiluminescence reagents and Hyperfilm ECL film for Western blot autoradiography were obtained from Amersham International. Single-step RNA isolation system, TRIzol reagent, and restriction endonucleases were purchased from GIBCO–BRL (Germany). Redivue [α-^{32}P]-dCTP (deoxycytidine triphosphate, 3000 Ci mmol^{-1}), Megaprime DNA labelling systems, Rapid-hibridization buffer and Hyperfilm-MP film for Northern and dot-blot autoradiography were obtained from Amersham International. Reserpine, EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline), (−)-adrenaline bitartrate and other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Results

Effect of chronic treatment with reserpine on brain $\alpha_{2A/C}$-adrenoceptors

Saturation experiments with the α_2-adrenoceptor agonists [^{3}H]-UK14304 and [^{3}H]-clonidine and the antagonist [^{3}H]-RX821002 were performed in order to accurately quantitate the density of rat brain α_2-adrenoceptors after chronic treatment with reserpine. Non-linear analysis of the saturation isotherms indicated in all cases the existence of single populations of high-affinity sites for [^{3}H]-UK14304, [^{3}H]-clonidine and [^{3}H]-RX821002. In cortical membranes of saline-treated rats the density of binding sites (B_{max}) for the three radioligands differed. The lowest density was for the full agonist [^{3}H]-UK14304 and the greatest density for the antagonist [^{3}H]-RX821002 (Figure 1 and Table 1).

Analysis of saturation curves for the full agonist [^{3}H]-UK14304 binding to cortical membranes from saline and reserpine-treated rats (0.25 mg kg^{-1}, i.p., every 48 h for 6–14 days) revealed the induction of up-regulation of α_2-adrenoceptor density induced by the monoamine depleter (B_{max} increased by 34–48%, $P<0.001$) (Table 1 and Figure 1). Moreover, chronic treatment (6–14 days) with reserpine also led to marked reductions (34–47%, $P<0.001$) of the dissociation constants (K_d) of [^{3}H]-UK14304 (increased affinity) for the α_2-adrenoceptor (Table 1). More prolonged treatments with reserpine (28 and 36 days) were also effective in increasing both the density (B_{max} increased by 40 and 44%, $n=2$ for each treatment) and the affinity (K_d reduced by 30 and 37%, $n=2$ for each treatment) of [^{3}H]-UK14304 binding to cortical membranes. Analysis of saturation curves for the partial agonist [^{3}H]-clonidine also showed a significant increase in receptor density (B_{max} increased by 22–31%, $P<0.001$) and receptor affinity (K_d reduced by 24–29%, $P<0.05$) induced by chronic reserpine (6–14 days) (Table 1 and Figure 1). By contrast, analysis of saturation curves for the antagonist [^{3}H]-RX821002 did not reveal significant

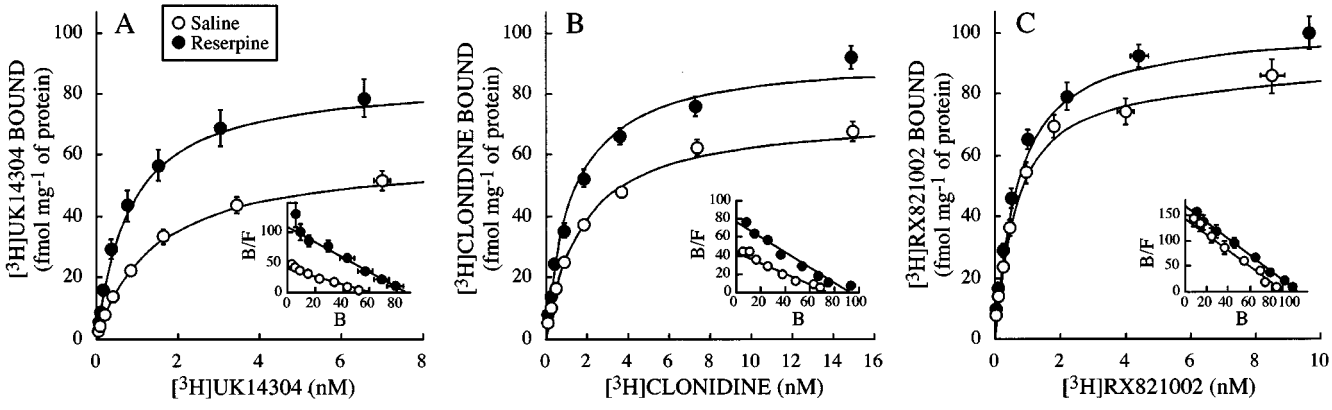

Figure 1 Specific binding of [^{3}H]-UK14304 (A), [^{3}H]-clonidine (B), and [^{3}H]-RX821002 (C) to brain cortical membranes of saline- and reserpine (0.25 mg kg^{-1}, s.c., every 48 h for 6–14 days)-treated rats as a function of increasing concentrations of the radioligands. Data shown are mean ± s.e.mean derived from 7–11 experiments per group. Binding parameters (K_d in nM and B_{max} in fmol mg^{-1} of protein) for pooled saline- and reserpine-treated rats (6 and 14 days) were as follows: [^{3}H]-UK14304, saline: $K_d=1.4\pm0.2$, $B_{max}=60\pm4$, $n=10$; reserpine: $K_d=0.8\pm0.1$, $B_{max}=85\pm6$, $n=10$; [^{3}H]-clonidine, saline: $K_d=1.7\pm0.2$, $B_{max}=60\pm4$, $n=7$; reserpine: $K_d=1.2\pm0.1$, $B_{max}=92\pm2$, $n=10$; [^{3}H]-RX821002, saline: $K_d=0.62\pm0.05$, $B_{max}=88\pm5$, $n=10$; reserpine: $K_d=0.64\pm0.04$, $B_{max}=101\pm4$, $n=11$. Insets: Scatchard plots of the same data.

Table 1 Effect of chronic treatments with reserpine on the specific binding of [^{3}H]-UK14304, [^{3}H]-clonidine and [^{3}H]-RX821002 to rat brain cortical membranes

		[^{3}H]-UK14304			*[^{3}H]-Clonidine*			*[^{3}H]-RX821002*		
Treatment	*Duration* (days)	K_d (nM)	B_{max} (fmol mg^{-1} of protein)	n	K_d (nM)	B_{max} (fmol mg^{-1} of protein)	n	K_d (nM)	B_{max} (fmol mg^{-1} of protein)	n
Saline	6	1.5±0.2	58±3	5	1.7±0.3	72±5	3	0.57±0.03	92±3	5
	14	1.2±0.2	62±6	5	1.7±0.3	73±4	4	0.67±0.10	85±9	5
Reserpine	6	0.8±0.1**	86±7*	5	1.3±0.1	88±1**	5	0.61±0.03	102±4	5
	14	0.8±0.1*	83±10*	5	1.2±0.1	96±3***	5	0.67±0.07	100±7	6

Saline or reserpine (0.25 mg kg^{-1}) was administered s.c. every 48 h for 6 and 14 days. The rats were killed 48 h after the last injection. Cortical membranes were incubated with eight concentrations of the radioligands (see Figure 1), and the specific binding to α_2-adrenoceptors was defined as the difference between total binding and binding in the presence of 10^{-5} M (−)-adrenaline (non-specific binding). Binding parameters (K_d, B_{max}) were determined directly by computer-assisted non-linear analysis from untransformed data using the EBDA–LIGAND programmes. Each value represents the mean±s.e.mean of n experiments per group with an animal per experiment. Two-way ANOVA for K_d and B_{max} values, with duration and treatment as independent factors, revealed significant differences for the binding parameters of the agonist radioligands between treatments ([^{3}H]-UK14304; $F[1,16]=14.23$, $P=0.0017$ for K_d values, and $F[1,16]=11.83$, $P=0.0034$ for B_{max} values; [^{3}H]-clonidine: $F[1,13]=5.92$, $P=0.0301$ for K_d values, and $F[1,13]=41.21$, $P<0.0001$ for B_{max} values), but not with respect to duration. A similar analysis did not detect differences (duration or treatment) for the antagonist [^{3}H]-RX821002. *$P<0.05$; **$P<0.01$; ***$P<0.001$ when compared with the corresponding saline-treated group (ANOVA followed by Fisher's LSD test).

changes in binding parameters after chronic treatment with reserpine (6–14 days) (Table 1 and Figure 1)

Competition experiments with (−)-adrenaline against [^{3}H]-RX821002 binding in both saline- and reserpine-treated (14 days) rats resulted in shallow curves, and non linear analysis indicated that a three-site binding model for the agonist fitted the data best (Figure 2). The estimated K_i values for each site were assumed to correspond to the high-affinity ($K_i=4.5$–7 nM) and low-affinity ($K_i=168$–183 nM) states of the α_2-adrenoceptor, and to a third binding site ($K_i=3006$–4050 nM) previously identified as binding to the 5-HT$_{1A}$ receptor (Vauquelin *et al*., 1990; Grijalba *et al*., 1996) for which (−)-adrenaline has very low affinity (Figure 2). The simultaneous analysis of these competition curves (i.e. the two mean curves together) according to the more complex model (i.e. the three site binding model) yielded significant differences compared to the analysis without constraints (i.e. the mean curves separately) ($F[7,32]=46.6$, $P<0.0001$). The main difference was the estimated density for the high-affinity state of the α_2-adrenoceptor (B_{max}: 49 and 62 fmol mg^{-1} of protein for saline- and reserpine-treated rats, respectively), because the estimated B_{max} values for the second (α_2-adrenoceptor low affinity) and third (5-HT$_{1A}$ receptor) binding sites were not affected by reserpine treatment (31 versus 34, and 10 versus 14, fmol mg^{-1} of protein, respectively).

Together the radioligand binding data indicated that chronic treatment with reserpine was associated with up-regulation of high affinity α_2-adrenoceptor agonist binding sites.

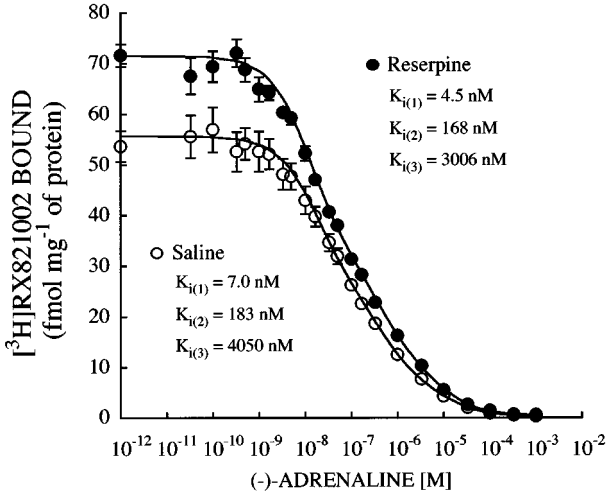

Figure 2 Competition curves for (−)-adrenaline against [^{3}H]-RX821002 (1 nM) binding to brain cortical membranes from saline- and reserpine (0.25 mg kg^{-1}, s.c., every 48 h for 14 days)-treated rats. Data shown are mean±s.e.mean derived from 5–6 experiments per group. Computer-assisted curve fitting demonstrated that a three-site fit was significantly better than a two-site binding model for both saline- and reserpine-treated rats ($P<0.001$, F-test). Binding parameters (K_i values) were determined directly by simultaneous analysis of 5–6 independent experiments for each group using the EBDA–LIGAND programs. Simultaneous analysis of both set of data yielded significant differences between the two curves ($F[7,32]=46.6$, $P<0.0001$), indicating that reserpine increased the density of the high-affinity state of the α_2-adrenoceptor.

Effect of chronic treatment with reserpine on brain regulatory G protein subunits

To assess for a possible relation between the increased agonist binding sites and parallel increases of specific G proteins in brain, the densities of various regulatory Gα protein subunits were assessed after chronic treatment with reserpine. In cortical membranes of reserpine-treated rats (0.25 mg kg^{-1}, every 48 h for 20 days), the immunoreactivities of the inhibitory subunits G$\alpha i_{1/2}$, Gαi_3 and Gαo were increased (25–34%, $0.5>P>0.01$) compared with those in saline-treated rats (Figure 3). Also the immunoreactivity of the stimulatory Gαs protein subunit was increased (29%, $P<0.01$) in cortical membranes of reserpine-treated rats (Figure 3). These results suggested an increase in signal

transduction through α_2-adrenoceptors (and other monoamine receptors) induced by chronic reserpine.

Effect of chronic treatment with reserpine on the turnover of brain $\alpha_{2A/C}$-adrenoceptors

In saline- and reserpine-treated rats (0.25 mg kg^{-1}, s.c., every 48 h, for 6 days), EEDQ (1.6 mg kg^{-1}, i.p., for 6 h) induced an almost complete reduction in the density of α_2-adrenoceptors in the cerebral cortex (B_{max} for [^{3}H]-UK14304 reduced more than 95%) (Figure 4). The initial loss of receptor density was followed by a progressive and time-dependent recovery towards control values (i.e., saline receptor density or receptor density after 36 days of reserpine treatment). These experiments provided the B_{max} values for the analysis of the exponential recovery function (Figure 4), whose parameters are summarized in Table 2.

The simultaneous analysis of the monoexponential recovery functions (Equation 1) in saline and reserpine-treated rats, revealed the existence of a marked modulation by chronic reserpine of brain α_2-adrenoceptor turnover function ($F[2,96]=31.36$; $P<0.0001$). This analysis also indicated that the increased density of cortical α_2-adrenoceptor binding sites

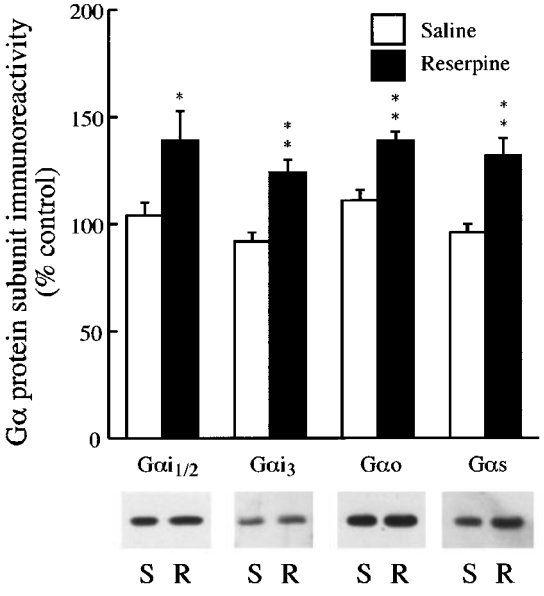

Figure 3 Effect of chronic treatment with reserpine on the immunoreactivity of regulatory Gα protein subunits in the rat frontal cortex. Groups of rats ($n=4$) were treated s.c. with saline or reserpine (0.25 mg kg^{-1}) every 48 h for 20 days. The rats were killed 48 h after the last injection. The levels of Gα protein subunits were quantified by immunoblotting (Western blotting) of cortical membranes as described in Methods. Upper panel: data are the mean ± s.e.mean of four experiments per group performed in triplicate with an animal per experiment, and expressed as percentage of control (standard curves from naïve rats). Lower panel: representative immunoblots for brain Gα protein subunits from the saline (S) and reserpine (R) groups. The amount of total protein loaded on the gels was 3.2–3.4 μg for both groups of rats. Note that the immunoreactive densities of Gα protein subunits were significantly increased after chronic reserpine treatment. *$P<0.05$, **$P<0.01$ when compared with the corresponding saline-treated group (two-tailed Student's *t*-test).

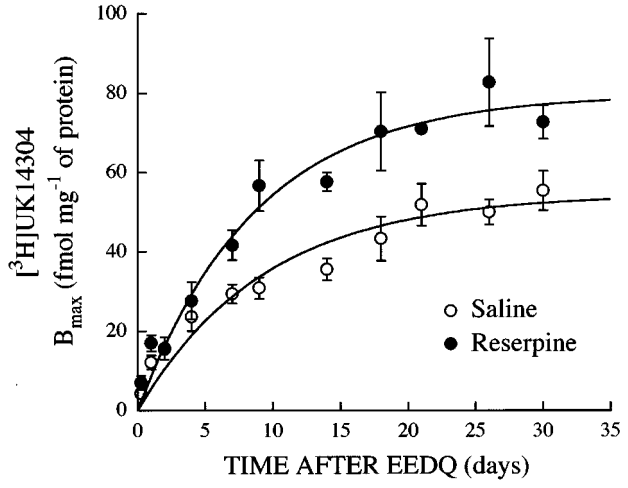

Figure 4 Recovery of α_2-adrenoceptor density (B_{max} for [^{3}H]-UK14304) in the cerebral cortex after EEDQ-induced receptor inactivation in saline-treated rats and during reserpine treatment (0.25 mg kg^{-1}, s.c., every 48 h for 6 to 36 days). Rats were killed 6 h and 1, 2, 4, 7, 9, 14, 18, 21, 26, and 30 days after the administration of EEDQ (1.6 mg kg^{-1}, i.p.). Reserpine treated rats were injected with EEDQ at day 6 and treatment with reserpine was continued until 48 h before killing. The B_{max} values were determined from complete saturation experiments for the agonist [^{3}H]-UK14304 using the non-linear regression programme LIGAND. Other details as for Tables 1 and 2. Data shown are mean ± s.e.mean derived from 3–6 experiments. The solid lines represent the computer-assisted curve fitting of experimental data to the monoexponential model described by the equation $R_t=r/k$ (1-e^{-kt}). See Table 2 for reserpine-induced changes in α_2-adrenoceptor turnover parameters.

Table 2 Turnover parameters of α_2-adrenoceptors labelled with the agonist [^{3}H]-UK14304 in the cerebral cortex of saline- and reserpine-treated rats

	Turnover parameters			
Treatment	r (fmol mg^{-1} of protein day^{-1})	k (day^{-1})	$t_{1/2}$ (days)	r/k (fmol mg^{-1} of protein)
Saline	5.8 ± 0.7	0.107 ± 0.019	6.4 ± 1.2	54 ± 4
Reserpine	9.1 ± 1.2*	0.115 ± 0.020	6.1 ± 1.1	80 ± 5**

Turnover parameters were assessed in saline-treated rats and during reserpine treatment (0.25 mg kg^{-1}, s.c. every 48 h for 6–36 days) (see Figure 4). Reappearance of receptors was assessed according to the equation: $R_t=(r/k)$ (1 − e^{-kt}) (see text for details). Turnover parameters are expressed as the best fit values ± s.e. calculated by the matrix inversion method, using the program GraFit. Statistical comparison between treatments were made by comparing the goodness of fit of simultaneous analysis with and without a set of constraints (same or different *r* and *k* values) by means of an *F*-test. The simultaneous analysis of the recovery curves revealed significant differences in α_2-adrenoceptor turnover parameters between saline- and reserpine-treated rats ($F[2,96]=31.36$; $P<0.0001$). *$P<0.05$, **$P<0.01$ when compared with the saline-treated group (*F*-test).

in reserpine-treated rats (B_{max} or r/k values, see Tables 1 and 2) was probably due to a higher appearance (synthesis) rate constant of the receptor ($\Delta k = 57\%$, $P < 0.05$) and not to a decreased disappearance (degradation) rate constant ($\Delta k = 7\%$, $P > 0.05$) (Table 2 and Figure 4). Thus, chronic reserpine treatment did not modify the $t_{1/2}$ of brain α_2-adrenoceptors (Table 2). These results suggested that an increased receptor synthesis might explain the up-regulation of α_2-adrenoceptors during reserpine treatment.

Effect of chronic treatment with reserpine on the expression of brain α_{2a}-adrenoceptor mRNA

In the rat cerebral cortex, Northern blot analysis demonstrated the presence of $\sim$3.8-kb band corresponding to the α_{2a}-adrenoceptor mRNA transcript (Figure 5A), which was in agreement with previous studies (see Lorenz *et al*., 1990).

Northern blot analysis of RNA extracted from the cerebral cortex of saline- and reserpine-treated rats (0.25 mg kg^{-1}, s.c., every 48 h for 20 days) clearly revealed that chronic treatment with reserpine markedly increased the expression of α_{2a}-adrenoceptor mRNA in the brain (Figure 5A). This up-regulation in the levels of α_{2a}-adrenoceptor mRNA was confirmed and quantitated by dot-blot experiments. In these experiments, chronic treatment with reserpine, compared with saline administration, was also associated with a marked up-regulation in the expression of α_{2a}-adrenoceptor mRNA in the cerebral cortex (125%, $P < 0.01$) (Figure 5B). Therefore, chronic treatment with reserpine is associated with an increased α_{2a}-adrenoceptor gene expression in the rat brain.

Discussion

The main finding of this study is the demonstration that chronic treatment with reserpine is associated with an accelerated turnover of α_2-adrenoceptors (increased appearance rate of agonist binding sites) and with a transcriptional activation of the α_{2a}-adrenoceptor mRNA (and probably also of the α_{2c}-mRNA because of cross hybridization of the probe used) in the rat cerebral cortex. This increased receptor synthesis appears to be the cellular mechanism by which chronic reserpine treatment induces up-regulation in the density of α_2-adrenoceptors (probably of the $\alpha_{2A/C}$-subtypes) in the rat brain (U'Prichard & Snyder, 1978; current results). Moreover, chronic reserpine treatment also induced an increase in the abundance of α_2-adrenoceptor-coupled inhibitory G proteins, which would lead to an improvement in the efficiency of the stimulus-response coupling (Kenakin, 1997) resulting in receptor supersensitivity (e.g. Ugedo *et al*., 1993; current results). In reserpine-treated rats the discrepancy between $\alpha_{2a/c}$-adrenoceptor mRNA levels (125% increase after 20 days of treatment) and α_2-adrenoceptor density (B_{max} for [^{3}H]-RX821002: 18% increase after 14 days of treatment; r/k value: 48% increase after 36 days of treatment) suggests that the membrane content of α_2-adrenoceptors is post-transcriptionally regulated. A similar discrepancy between the increases in $\alpha_{2a/c}$-adrenoceptor mRNA levels and $\alpha_{2A/C}$-adrenoceptor densities was previously reported in morphine-withdrawn rats (Busquets *et al*., 1997).

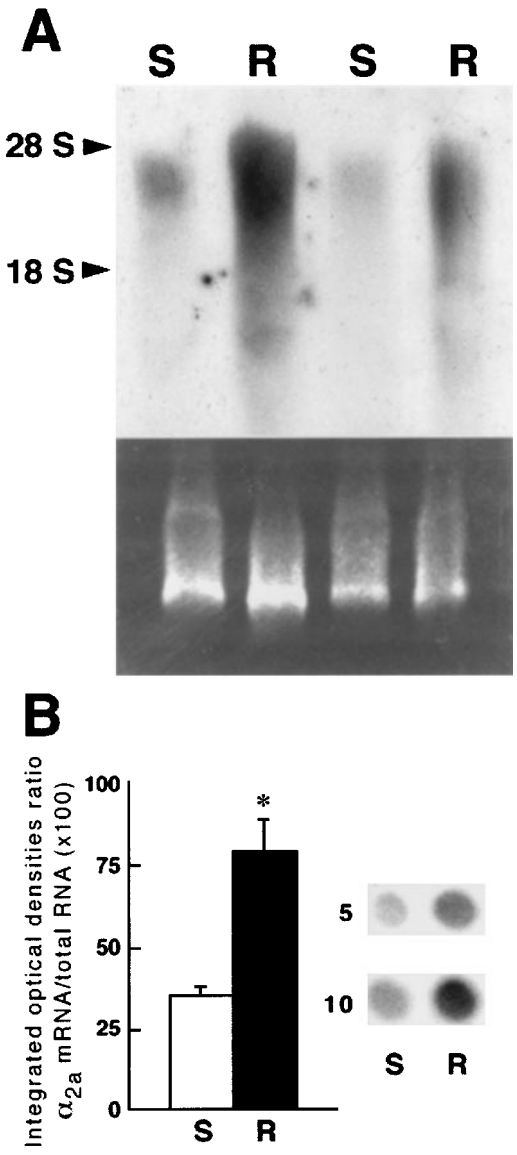

Figure 5 (A) Autoradiogram of a representative Northern blot analysis of brain RNA examining the expression of α_{2a}-adrenoceptor mRNA in saline-treated rats (S) and in reserpine-treated (0.25 mg kg^{-1}, s.c., every 48 h for 20 days) rats (R). Northern blot analysis was performed as described in Methods. Each lane was loaded with 20 μg of total RNA extracted from the cerebral cortex of one animal (two different S and R are shown). The size of the α_{2a}-adrenoceptor mRNA (approximately 3.8 kb) was estimated by comparison with 28S (5.0 kb) and 18S (2.0 kb) RNAs (arrow heads). Bottom panel: ethidium bromide-stained RNA agarose gel showing the 28S and 18S ribosomal bands for size marker comparison. (B) Effect of chronic reserpine treatment on the relative amount of α_{2a}-adrenoceptor mRNA in the cerebral cortex from saline (S) and 20-day reserpine-treated (R) rats. Receptor mRNA levels were assessed by dot-blot analysis as described in Methods. Left panel: The relative levels of α_{2a}-adrenoceptor mRNA signal in dot-blots (integrated optical density) were normalized to correct any loading discrepancies of RNA by scanning the acridine orange fluorescence signal from the same dot-blots after u.v. light irradiation of the nylon membranes (see Methods). Ratios of α_{2a}mRNA/total RNA ($\times 100$); S: 35 ± 3, R: 79 ± 10. Each value represents the mean $\pm$ s.e.mean of six experiments per group. $^*P < 0.01$ when compared with the saline group (two-tailed Student's *t*-test). Right panel: A representative experiment (dot-blots) is shown for saline (S) and reserpine (R) groups. Duplicate dot-blots of total RNA (5 and 10 μg) were performed for each treatment.

The increased density of α_2-adrenoceptor agonist binding sites in reserpine-treated rats appears to be related to a selective enhancement in the high-affinity conformation of the receptor rather than to an increase of the total α_2-adrenoceptor population. Previous studies have shown that chronic treatments with low doses of reserpine increase the affinity of agonist radioligands ([^{3}H]-clonidine, [^{3}H]-p-aminoclonidine, [^{3}H]-UK14304) for the brain α_2-adrenoceptor, with no change in receptor density (Hong *et al*., 1988; Giralt & García-Sevilla, 1989; Ugedo *et al*., 1993), whereas acute treatments with high doses of reserpine have been associated with decreases in brain α_2-adrenoceptor density, with no change in receptor affinity (Kuno *et al*., 1990). It is currently accepted that agonist radioligands label preferentially the high-affinity state of α_2-adrenoceptors, whereas antagonist radioligands label both the high- and low-affinity states of this group of receptors (Asakura *et al*., 1985). Therefore, the increase in α_2-adrenoceptor density observed in reserpine-treated rats with the agonist radioligands [^{3}H]-clonidine and [^{3}H]-UK14304 as compared with the antagonist [^{3}H]-RX821002 could be related to an enhanced expression of the high-affinity conformation of the receptor. In this sense, the competition experiments with the agonist (−)-adrenaline against the binding of [^{3}H]-RX821002 to α_2-adrenoceptors demonstrated that the enhanced receptor density in reserpine-treated rats was related to a selective increase in the density of the high-affinity state of the receptor. Moreover, these results agree well with the increased immunodensity of inhibitory G proteins (Gαi/o) detected in the brain of reserpine-treated rats. Because the high-affinity conformation of the α_2-adrenoceptor is most probably related to the complex with $G\alpha i_2$ proteins (Sastre & García-Sevilla, 1994; Ribas *et al*., 1998), an enhanced expression of $G\alpha i_{1/2}$ proteins linked to a higher abundance of α_2-adrenoceptor agonist binding sites was expected in brains of reserpine-treated rats. Similarly, the increased immunodensity of stimulatory Gαs proteins could be related to the well-known supersensitivity of β-adrenoceptors induced by chronic reserpine (U'Prichard & Snyder, 1978; Grassby & Broadley, 1986).

Also in line with these results, the accelerated turnover of α_2-adrenoceptors during reserpine treatment indicated that the increased density of [^{3}H]-UK14304 binding sites was due to an apparent higher rate of receptor appearance and not to a decreased receptor disappearance. A similar α_2-adrenoceptor turnover study in morphine-dependent rats also demonstrated that the up-regulation of cortical α_2-adrenoceptors ([^{3}H]-clonidine agonist binding sites) observed during morphine withdrawal (Ulibarri *et al*., 1987) was probably due to an enhanced rate of receptor appearance (Gabilondo & García-Sevilla, 1995). These results suggested that an increased receptor synthesis may explain the up-regulation of α_2-adrenoceptors. In fact, chronic reserpine treatment (current results) and morphine withdrawal (Busquets *et al*., 1997) are associated with a transcriptional activation of the α_{2a}-adrenoceptor mRNA in the rat brain. The molecular mechanism by which chronic reserpine induces a higher α_{2a}-adrenoceptor gene expression in the brain is not known, but it could be mediated through modulation of the adenylyl cyclase/cyclic AMP/protein kinase A system and cyclic AMP-responsive enhancer elements which are associated with changes in gene expression for these receptors (see Busquets *et al*., 1997 and other references therein).

In other receptor systems, such as the inhibitory D_2 dopamine receptor, similar cellular mechanisms seem to be involved in reserpine-induced receptor supersensitivity. Thus, in the striatal D_2 dopamine receptor system, reserpine-induced supersensitivity appears to be accompanied by increases of D_2 dopamine receptor density and mRNA (Rubinstein *et al*., 1990; Jaber *et al*., 1992; Butkerait & Friedman, 1993). Moreover, turnover studies also indicated that the up-regulation of D_2 dopamine receptor is an active process resulting from an increase in the rate of receptor appearance (Norman *et al*., 1987). Furthermore, the receptor up-regulation was paralleled by a significant increase of the high-affinity population of the D_2 dopamine receptor (Rubinstein *et al*., 1990), that could be associated with the increases of specific G proteins ($G\alpha i_2$ and Gαo) mRNAs (Butkerait & Friedman, 1993) and of the dopamine-stimulated guanine nucleotide binding to Gαi/o (Butkerait *et al*., 1994). In contrast to the current results, reserpine treatment did not change the striatal membrane content of Gαs, $G\alpha i_{1/2}$ or Gαo as assessed by immunoblotting or by toxin-catalysed ADP ribosylation (Butkerait *et al*., 1994).

The current findings further support the concept that depletion of brain monoamines in rats with reserpine is a relevant biochemical model of major depression, as far as the induction of up-regulation of α_{2A}-adrenoceptors and associated regulatory proteins and mechanisms is concerned. Postmortem studies in human brain have shown an enhancement of α_2-adrenoceptor density in depressed suicide victims when labelled with agonist (Meana *et al*., 1992; González *et al*., 1994; Ordway *et al*., 1994) or antagonist (De Paermentier *et al*., 1997) radioligands. In other studies with agonist or antagonist radioligands, however, this receptor up-regulation was not observed (Ferrier *et al*., 1986; Arango *et al*., 1993; Ordway *et al*., 1994). In a recent study, the simultaneous analysis of agonist and antagonist binding sites indicated that a selective increase in the high-affinity conformation of the α_{2A}-adrenoceptor was present in the prefrontal cortex of depressed suicide victims (Callado *et al*., 1998). Moreover, recent data have shown that the immunodensities of α_{2A}-adrenoceptors, $G\alpha i_{1/2}$ proteins, and G protein-coupled receptor kinase 2 are up-regulated in brains of suicides and depressed suicides (García-Sevilla *et al*., 1999). Clinical studies in depressed patients also indicate that depletion of brain noradrenaline or serotonin induces a presynaptic dysfuntion in the brain, which is relevant for the understanding of the pathophysiology of major depression and the therapeutic effects of antidepressant drugs (for a review see Delgado & Moreno, 1999).

In summary, the results of this study indicate that chronic reserpine treatment in rats induces up-regulation of brain $\alpha_{2A/C}$-adrenoceptors (agonist binding sites), which is associated with an increase in the abundance of inhibitory G coupling proteins ($G\alpha i_{1/2}$ subunits), an acceleration of receptor turnover (increase in the rate of agonist binding sites appearance) and a transcriptional activation of the $\alpha_{2a/c}$-adrenoceptor mRNA. Together, the results provide a better understanding of the cellular events associated with reserpine-induced supersensitivity of α_2-adrenoceptors in the brain.

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