Acceleration by chronic treatment with clorgyline of the turnover of brain α_2 -adrenoceptors in normotensive but not in spontaneously hypertensive rats

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1 The aim of this study was to quantitate and compare the turnover of α_2 -adrenoceptors in the cerebral cortex of normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats, and its modulation during chronic treatment with the monoamine oxidase (MAO) inhibitor, clorgyline.

2 In SHR, the specific binding of the agonist [³H]-UK 14304 and of the antagonist [³H]-RX 821002 was significantly reduced in the brain ($B_{\max}$ 15–19% lower) as compared to that in sex- and age-matched WKY rats. In contrast, no significant changes in the K_d values for both radioligands were found between WKY and SHR rats. Therefore, SHR rats offer a genetic model with a lower density of α_2 -adrenoceptors in the brain.

3 Chronic treatment (21–35 days) with clorgyline (1 mg kg⁻¹, i.p.) markedly decreased the density of brain α_2 -adrenoceptors ([³H]-UK 14304 binding) in Sprague-Dawley ($B_{\max}$ reduced by 50%) and in WKY ($B_{\max}$ reduced by 30%) rats without any apparent change in the affinity of the radioligand. In contrast, the density of brain α_2 -adrenoceptors in SHR was not down-regulated by chronic clorgyline treatment.

4 The recovery of [³H]-UK 14304 binding after irreversible inactivation by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; 1.6 mg kg⁻¹) (an alkylating agent for the α_2 -adrenoceptor) was assessed in control and clorgyline-treated (1 mg kg⁻¹, i.p. for 7–21 days) WKY and SHR rats to study the process of α_2 -adrenoceptor repopulation and to calculate receptor turnover parameters.

5 The simultaneous analysis of receptor recovery curves revealed that the turnover of brain α_2 -adrenoceptors in SHR rats was accelerated ($k = 0.141$ day⁻¹; $t_{1/2} = 4.9$ days; $r/k = 40$ fmol mg⁻¹ protein) compared to that in WKY rats ($k = 0.085$ day⁻¹; $t_{1/2} = 8.1$ days; $r/k = 54$ fmol mg⁻¹ protein) and that the reduced density of cortical α_2 -adrenoceptors ($B_{\max}$ or r/k values) in SHR was probably due to an abnormal higher receptor degradation ($\Delta k = 66\%$) and not to a decreased receptor synthesis which in fact showed a slight increase ($\Delta r = 24\%$).

6 Treatment with clorgyline (1 mg kg⁻¹, i.p. for 21 days) accelerated the turnover of brain α_2 -adrenoceptors in WKY rats ($k = 0.328$ days⁻¹; $t_{1/2} = 2.1$ days; $r/k = 29$ fmol mg⁻¹ protein) and the greater increase in receptor degradation ($\Delta k = 286\%$) over receptor synthesis ($\Delta r = 109\%$) led to down-regulation of receptor density ($r/k = 46\%$ lower). In contrast, treatment with clorgyline did not modify significantly the turnover of brain α_2 -adrenoceptors in SHR ($k = 0.192$ days⁻¹; $t_{1/2} = 3.6$ days; $r/k = 39$ fmol mg⁻¹ protein), indicating that in this genetic model of hypertension, the desensitized α_2 -adrenoceptors cannot be further down-regulated by clorgyline treatment and that they lack the expected adaptative increase in receptor synthesis.

Keywords: Spontaneously hypertensive rats (SHR); brain α_2 -adrenoceptors; [³H]-UK 14304; [³H]-RX 821002; N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ); receptor turnover; clorgyline

Introduction

Biochemical and functional studies have suggested that endogenous depression is related to α_2 -adrenoceptor supersensitivity (García-Sevilla *et al.*, 1986; 1990) and desensitization of these inhibitory receptors has been involved in the mechanism of action of various classes of antidepressant treatments such as tricyclic drugs and monoamine oxidase (MAO) inhibitors (Meana *et al.*, 1992 and other references therein). However, the basic biochemical mechanisms involved in antidepressant-induced down-regulation of the steady state expression of brain α_2 -adrenoceptors are poorly understood. Recently long-term treatment with desipramine has been shown to down-regulate the density of α_2 -adrenoceptors in the rat brain and to accelerate the turnover of the receptor with a marked increase in its degradation rate (Barturen & García-Sevilla, 1992). The increased α_2 -

adrenoceptor degradation, which appears to be the result of prolonged receptor activation by endogenous noradrenaline (NA) after inhibition of neuronal uptake by desipramine, could explain the induction of down-regulation of brain α_2 -adrenoceptors. Therefore, accelerated α_2 -adrenoceptor degradation could be the first change in the metabolism of the receptor induced by specific antidepressant drugs.

The genetic model of the spontaneously hypertensive rats (SHR) of the Wistar-Kyoto (WKY) strain has been associated with dysfunctions of α_2 -adrenoceptors which appear to have pathophysiological implications (see Michel *et al.*, 1990). In SHR, the specific binding of [³H]-clonidine to brain α_2 -adrenoceptors as well as clonidine-induced mydriasis are reduced when compared to those in sex- and age-matched normotensive WKY rats (Olmos *et al.*, 1991). Therefore, in contrast to the antidepressant-induced receptor down-regulation, the SHR offers a genetic model of down-regulation of α_2 -adrenoceptors in the brain (receptor number and function) to assess further biochemical events associated with this relevant receptor regulatory mechanism.

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In this context, the present study was designed to quantify and compare the turnover of brain α_2 -adrenoceptors in WKY and SHR rats, and its modulation during treatment with the antidepressant drug, clorgyline, a MAO inhibitor which induces down-regulation of brain α_2 -adrenoceptors (Giralt & Garcia-Sevilla, 1989; Menargues *et al.*, 1990), most probably through an indirect mechanism, similar to that described above for desipramine, which involves an increased availability of intraneuronal and/or synaptic NA (L'Heureux *et al.*, 1986; Routledge & Marsden, 1987; Giralt & Garcia-Sevilla, 1989). A preliminary report of this work was given at a meeting of the British Pharmacological Society (London Meeting, September 1992) (Ribas *et al.*, 1992).

Methods

α_2 -Adrenoceptor turnover

The irreversible blockade/inactivation of receptors, followed by assessment of the kinetics of reappearance of receptor binding to control levels has been the approach most widely used to study the metabolism of adrenoceptors *in vivo* and *in vitro* (Mahan *et al.*, 1987). The peptide-coupling agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) at a dose of 1.6 mg kg^{-1} was selected as the irreversible antagonist for brain α_2 -adrenoceptors (see Barturen & Garcia-Sevilla, 1992 for specific details of EEDQ as a tool to study the turnover of α_2 -adrenoceptors). EEDQ appears to alkylate the carboxyl group of an acylamino acid, forming a mixed carbonic anhydride and free quinoline. This mixed carbonic anhydride reacts with a sterically accessible nucleophilic group to inactivate the receptor (Belleau *et al.*, 1969).

Animals and treatments

Male normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats (200–300 g) (R. Janvier, Le Genest, France) and adult male Sprague-Dawley (SD) rats (250–300 g) were used. The systolic blood pressure, measured by the tail-cuff method, of SHR ($196 \pm 1 \text{ mmHg}$, $n = 64$) was significantly higher than those of age-matched normotensive WKY ($142 \pm 1 \text{ mmHg}$, $n = 68$; $P < 0.001$, two-tailed *t* test) and SD ($132 \pm 1 \text{ mmHg}$, $n = 12$; $P < 0.001$, two-tailed *t* test) rats. The animals received a standard diet with water freely available and were housed at $20 \pm 2^\circ\text{C}$ with a 12 h light/dark cycle. The animals received *i.p.* every 24 h either 0.9% saline vehicle or the MAO inhibitor, clorgyline (1 mg kg^{-1}) for 7–35 days (SD rats) or 21 days (WKY and SHR rats). The rats were killed 24 h after the last injection. This dose of clorgyline was considered optimal for induction of brain α_2 -adrenoceptor down-regulation (Giralt & Garcia-Sevilla, 1989). EEDQ (1.6 mg kg^{-1}) was dissolved in ethanol and then diluted sequentially with propyleneglycol and purified water (final ratio, 1:1:2, v/v/v) and it was administered *i.p.* in a single dose. Rats were killed 0.25, 1, 2, 4, 7, 9, 14, 21 and 30 days after EEDQ administration to evaluate the recovery of brain α_2 -adrenoceptor density, which allowed estimation of receptor turnover parameters. To study the modulation of α_2 -adrenoceptor turnover by clorgyline, EEDQ was injected into rats treated with the antidepressant for 7 days, and treatment was then continued until day 21 (phase 7–21 days, presence or absence of receptor down-regulation in WKY and SHR rats, respectively; see Table 2). The animals were killed by decapitation, the brains rapidly removed and the parietooccipital cortex dissected on ice and stored at -70°C until assay.

Preparation of membranes

Cortical membranes (P_2 fractions) were prepared by established methods (Giralt & Garcia-Sevilla, 1989) from the parietooccipital cortex. Briefly, after thawing, the tissue sam-

ples were homogenized in 5 ml of ice-cold Tris-sucrose buffer (5 mM Tris-HCl, 250 mM sucrose, 1 mM MgCl_2 ; pH 7.4). The homogenates were centrifuged at $1,100 \text{ g}$ for 10 min, and the supernatants were then recentrifuged at $40,000 \text{ g}$ for 10 min. The resulting pellet was washed twice with 2 ml of fresh incubation buffer (50 mM Tris-HCl, 0.1 mM MnCl_2 , 0.1% ascorbic acid, pH 7.7 for [^3H]-UK 14304 binding assays, or 50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5 for [^3H]-RX 821002 binding assays) (Meana *et al.*, 1989; Miralles *et al.*, 1993). The final pellet was resuspended in an appropriate volume of the incubation buffer to a final protein content of $800\text{--}1000 \mu\text{g ml}^{-1}$. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

[^3H]-UK 14304 and [^3H]-RX 821002 binding assays

Total [^3H]-UK 14304 binding was measured in 1.1 ml-aliquots (50 mM Tris-HCl, 0.1 mM MnCl_2 , 0.1% ascorbic acid, pH 7.7) of the cortical membranes which were incubated for 60 min at 25°C with eight concentrations of [^3H]-UK 14304 ($6 \times 10^{-11} \text{ M}$ to $8 \times 10^{-9} \text{ M}$). Total [^3H]-RX 821002 binding was also measured in 1.1 ml-aliquots (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) of the membranes which were incubated for 30 min at 25°C with eight concentrations of [^3H]-RX 821002 ($6 \times 10^{-11} \text{ M}$ to $8 \times 10^{-9} \text{ M}$). For both radioligands, nonspecific binding was determined in presence of 10^{-5} M (–)-adrenaline. Specific binding (about 85% at K_d values) was defined as the difference between total binding and nonspecific binding and was plotted as a function of increasing concentrations of the radioligands. Incubations were terminated by diluting the samples with 5 ml of ice-cold Tris incubation buffer (4°C). Membrane-bound [^3H]-UK 14304 or [^3H]-RX 821002 was measured by vacuum filtration, using a Brandel 48R cell harvester (Biomedical Research & Development Laboratories, U.S.A.), through Whatman GF/C glass fibre filters, which had been presoaked with 0.5% polyethylenimine (Bruns *et al.*, 1983). Then the filters were rinsed twice with 5 ml of incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, England) and counted for radioactivity by liquid scintillation spectrometry at 50% efficiency (Packard model 300C).

Analyses of binding data and statistics

Analyses of saturation isotherms (K_d , dissociation constant; B_{max} , maximum density of binding sites) as well as the fitting of data to the appropriate binding model were performed by computer-assisted nonlinear regression using the EBDA-LIGAND programmes (Munson & Rodbard, 1980; McPherson, 1985). The results were expressed as means $\pm$ s.e.mean. One- and two-way analysis of variance (ANOVA), followed by Fisher's LSD or Scheffé's test; and Student's two-tailed *t* test were used for the statistical evaluations. The level of significance was $P = 0.05$. To strengthen the comparison between WKY and SHR rats, saturation curves for [^3H]-UK 14304 and [^3H]-RX 821002 were also simultaneously analysed for best fit using the LIGAND programme. First, the two sets of data (WKY and SHR groups) were analysed separately. Next, they were pooled and analysed simultaneously, and constrained to share two or only one common parameter (K_d and/or B_{max}). The statistical significance of the improvement was determined by the extra sum of squares principle (*F*-test) as outlined by Munson & Rodbard (1980), with a level of significance of $P = 0.05$. A *P* value less than 0.05 indicated that the separate fit was much better than the pooled fit (i.e. the fit with one or two shared parameters).

Analyses of α_2 -adrenoceptor turnover functions and statistics

For analysis of α_2 -adrenoceptor turnover, data from the recovery of brain α_2 -adrenoceptor density after irreversible

inactivation by EEDQ were analysed as previously described (Barturen & García-Sevilla, 1992) according to a monoexponential model based on two implicit assumptions (Mauger *et al.*, 1982), that (1) the rate of receptor appearance is constant during the repopulation period (i.e. zero-order process) and (2) receptor disappearance is proportional to the density of receptors at any time (i.e. first-order process). Exponential recovery data were fitted, using the simple nonlinear least squares fitting programme Grafit (Leatherbarrow, 1990), to the equation:

$$[Rt] = r/k (1 - e^{-kt}) \quad (\text{Eq. 1})$$

where $[Rt]$ is expressed as fmol mg⁻¹ protein and represents the receptor number at a given discrete time t ; r is the appearance ('synthesis') rate constant of the receptor expressed as fmol mg⁻¹ protein day⁻¹, and k is the disappearance ('degradation') rate constant of the receptor, in units of day⁻¹, that allows to estimate the apparent half-life for the receptor ($t_{1/2} = \ln 2/k$). In the present model the ratio r/k represents the density of receptors at steady state towards the system tends after irreversible inactivation of the receptors.

Receptor turnover parameters are expressed as the best fit values $\pm$ standard error determined by the matrix inversion method, using the nonlinear regression programme GraFit (Leatherbarrow, 1990). Standard error values determined by nonlinear regression were not used in further formal statistical calculations. Comparisons of experimental data sets for the recovery of α_2 -adrenoceptor density were performed by comparing the goodness of fit of a model with and without a set of constraints by means of an F -test. First, the sets of data were analysed separately (with no constraints), the overall value for the sum of squares was the sum of the individual values from each fit and, similarly, the number of degrees of freedom. Next, the sets of data were pooled analysed simultaneously, and constrained to share one or more common parameters, which gave values for the sum of squares and degrees of freedom. The analysis that permitted one or more of the parameters to be shared without a significant increase in the residual variance was taken as the best fit. For further details see Barturen & García-Sevilla (1992) and other references therein.

Drugs

[³H]-UK 14304 (bromoxidine; specific activity, 60–87 Ci mmol⁻¹) was purchased from New England Nuclear/Du Pont (U.S.A.) and it was stored at -30°C . [³H]-RX 821002 (2-methoxyidazoxan; specific activity, 56 Ci mmol⁻¹) was purchased from Amersham International plc (U.K.) and it was stored at 2°C . For the binding assays appropriate amounts of the stock solutions were diluted with distilled and purified water (Milli-Q) containing 2.5 mM HCl and 6% ethanol. Other drugs (and their sources) included: (–)-adrenaline bitartrate (Sigma Chemical Co., U.S.A.); EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) (Sigma); clorgyline HCl (Sigma). Other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Results

Density of α_2 -adrenoceptors in the cerebral cortex of WKY and SHR rats

Saturation experiments with the α_2 -adrenoceptor agonist [³H]-UK 14304 and the antagonist [³H]-RX 821002 were performed in order to quantitate accurately the density of brain α_2 -adrenoceptors in normotensive WKY and spontaneously hypertensive (SHR) rats. In both strains, the specific binding of [³H]-UK 14304 and that of [³H]-RX 821002 were saturable processes of high affinity that revealed the existence of single populations of sites (Table 1, Figure 1). As expected, the

density of sites for the antagonist [³H]-RX 821002 (total population of α_2 -adrenoceptors) was higher than that obtained for the agonist [³H]-UK 14304 (high-affinity state of the α_2 -adrenoceptor; e.g. Meana *et al.*, 1989) (Table 1).

In SHR, the specific binding of [³H]-UK 14304 was reduced in the cerebral cortex (B_{max} 19% lower, $P < 0.01$) as compared to that in sex- and age-matched WKY rats (Table 1). Moreover, the simultaneous analysis of multiple saturation isotherms from the WKY and SHR rats further indicated the existence of a decreased number of binding sites for [³H]-UK 14304 ($F[1,12] = 4.98$; $P = 0.045$) with no difference in affinity (K_d values) ($F[1,12] = 0.36$; $P = 0.56$) in the cerebral cortex of SHR (Figure 1a). In SHR, the specific binding of [³H]-RX 821002 was also decreased in the cortex although to a lesser extent (B_{max} 15% lower, $P = 0.056$), and the simultaneous analysis of multiple saturation isotherms from the WKY and SHR rats clearly indicated in this latter strain the existence of a decreased density of sites for [³H]-RX 821002 ($F[1,42] = 8.83$; $P = 0.005$) with no change in receptor affinity ($F[1,42] = 0.16$; $P = 0.69$) (Figure 1b).

Effect of chronic treatment with clorgyline on α_2 -adrenoceptors in the cerebral cortex of SD, WKY and SHR rats

Analyses of saturation curves for [³H]-UK 14304 binding to cortical membranes from saline and clorgyline-treated SD rats (1 mg kg⁻¹; i.p. every 24 h for 7, 14, 21, 28, and 35 days) revealed the existence of a time-dependent down-regulation of α_2 -adrenoceptor density induced by the antidepressant drug. Thus, short-term treatment for 7 days with clorgyline did not reduce significantly the density of brain α_2 -adrenoceptors. In contrast, long-term treatment for 14–35 days clearly induced significant decreases in receptor density with maximal reductions at 21–28 days (B_{max} reduced by 51–54%, respectively; $P < 0.001$) (Table 2). Chronic treatment with clorgyline did not alter significantly the binding affinity (K_d values) of [³H]-UK 14304 for the α_2 -adrenoceptor (Table 2).

Similarly, long-term treatment of WKY rats with the MAO inhibitor, clorgyline, for 21 days induced down-regulation of the density of brain α_2 -adrenoceptors (B_{max} reduced by 30%, $P < 0.05$) with no change in receptor

Table 1 Specific binding of [³H]-UK 14304 and [³H]-RX 821002 to brain cortical membranes of normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats

Radioligand/strain	Binding parameters		
	K_d (nM)	B_{max} (fmol mg ⁻¹ protein)	n
[³H]-UK 14304			
WKY	1.5 $\pm$ 0.1	57 $\pm$ 3	10
SHR	1.1 $\pm$ 0.3	46 $\pm$ 3*	5
[³H]-RX 821002			
WKY	0.5 $\pm$ 0.03	81 $\pm$ 3†	3
SHR	0.5 $\pm$ 0.03	69 $\pm$ 4†	3

Neural membranes were incubated at 25°C for 60 min ([³H]-UK 14304) or 30 min ([³H]-RX 821002) with eight concentrations of the radioligands (6×10^{-11} M to 8×10^{-9} M). The specific binding of the radioligands 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 nonlinear analysis from untransformed data using the EBDA-LIGAND programmes. Each value represents the mean $\pm$ s.e.mean of n experiments per group with an animal per experiment.

* $P < 0.01$ when compared with the corresponding value in WKY rats (two-tailed t test).

† $P < 0.01$ when compared with the corresponding value for [³H]-UK 14304 (two-tailed t test).

Figure 1 Specific binding of (a) $[^3\text{H}]\text{-UK 14304}$ and (b) $[^3\text{H}]\text{-RX 821002}$ to brain cortical membranes of normotensive Wistar-Kyoto (WKY) (O) and spontaneously hypertensive (SHR) (●) rats as a function of increasing concentrations of the radioligand. Data shown are means $\pm$ s.e.mean derived from 3–10 experiments per group. See Table 1 for changes in binding parameters. Saturations isotherms also were analysed for best fit as described in Methods. For each radioligand, the simultaneous analysis (LIGAND programme) of the two sets of data (WKY and SHR rats: common K_d and $B_{\max}$) indicated that saturation isotherms were different from each other for both $[^3\text{H}]\text{-UK 14304}$ ($F[2,12] = 17.54$; $P < 0.0001$) and $[^3\text{H}]\text{-RX 821002}$ ($F[2,42] = 16.98$; $P < 0.0001$). In both strains of rat, the constraint of the curves to share a common K_d value did not yield a significant increase in the sum of squares ($F[1,12] = 0.36$; $P = 0.56$ for $[^3\text{H}]\text{-UK 14304}$; and ($F[1,42] = 0.16$; $P = 0.691$ for $[^3\text{H}]\text{-RX 821002}$) whereas sharing a common $B_{\max}$ significantly increased the sum of squares of the fit ($F[1,12] = 4.98$; $P = 0.045$ for $[^3\text{H}]\text{-UK 14304}$; and ($F[1,42] = 8.83$; $P = 0.005$ for $[^3\text{H}]\text{-RX 821002}$), which indicated that WKY and SHR rats differ significantly as to $B_{\max}$ but not as to K_d whatever the radioligand used. Inset: Scatchard plots (same data) showing K_d and $B_{\max}$ values similar to those reported in Table 1.

affinity (Table 2). In contrast, the density of brain α_2 -adrenoceptors in SHR rats was not down-regulated by chronic 21 days clorgyline treatment (Table 2).

α_2 -Adrenoceptor turnover in the cerebral cortex of WKY and SHR rats

Treatment of WKY and SHR rats with a single dose of EEDQ (1.6 mg kg^{-1} , i.p.) induced an almost complete reduction in the density of α_2 -adrenoceptors in the cerebral cortex ($B_{\max}$ for $[^3\text{H}]\text{-UK 14304}$ reduced more than 95% at 6 h), that was followed by a progressive recovery of the receptor density, as revealed by $[^3\text{H}]\text{-UK 14304}$ saturation isotherms performed at different times after *in vivo* EEDQ administration (1, 2, 4, 7, 9, 14, 21 and 30 days). The binding affinity of $[^3\text{H}]\text{-UK 14304}$ for the α_2 -adrenoceptor was unaltered during recovery from EEDQ-induced receptor inactivation. These

Table 2 Effects of chronic treatments with clorgyline on the specific binding of $[^3\text{H}]\text{-UK 14304}$ to brain cortical membranes of normotensive Sprague-Dawley (SD) and Wistar-Kyoto (WKY), and spontaneously hypertensive (SHR) rats

Strain/Treatment	Duration (days)	$[^3H]\text{-UK 14304}$		n
		K_d (nM)	B_{max} (fmol mg^{-1} protein)	
SD				
Saline		2.1 ± 0.2	70 ± 3	8
Clorgyline	7	2.7 ± 0.2	66 ± 2	3
	14	2.5 ± 0.1	$56 \pm 2^*$	3
	21	2.0 ± 0.4	$34 \pm 2^{**}$	3
	28	1.7 ± 0.3	$32 \pm 2^{**}$	3
	35	1.5 ± 0.1	$41 \pm 1^*$	3
WKY				
Saline		1.46 ± 0.2	60 ± 4	5
Clorgyline	21	2.10 ± 0.1	$42 \pm 3^*$	3
SHR				
Saline		1.37 ± 0.1	45 ± 4	6
Clorgyline	21	2.30 ± 0.3	48 ± 5	3

Clorgyline (1 mg kg^{-1}) was administered i.p., every 24 h for various periods of time. The rats were killed 24 h after the last injection. Other details as for Table 1. Each value represents the mean $\pm$ s.e.mean of n experiments per group with an animal per experiment. Two-way ANOVA for $B_{\max}$, but not for K_d values, detected significant differences between strains ($F[2,34] = 3.39$, $P = 0.042$), treatments ($F[1,34] = 9.73$, $P = 0.003$) and for the interaction between strains and treatment ($F[2,34] = 4.10$, $P = 0.025$). One-way ANOVA followed by a multiple comparison test detected a significant decrease in $B_{\max}$ after chronic treatment with clorgyline in SD and WKY rats but not in SHR rats ($F[9,30] = 11.92$, $P = 0.0001$).

* $P < 0.05$; ** $P < 0.01$ when compared with the corresponding saline-treated group (ANOVA followed by Fisher's LSD or Sheffé's test).

Figure 2 Recovery of α_2 -adrenoceptor density in the rat cerebral cortex of normotensive Wistar-Kyoto (WKY) rats after EEDQ-induced receptor inactivation. Data shown are means $\pm$ s.e.mean derived from 4 experiments. Other details as for Tables 1 and 3. The $B_{\max}$ values were determined, for each time, from complete saturation experiments for $[^3\text{H}]\text{-UK 14304}$ binding using the nonlinear programme LIGAND. Range for K_d values was 0.6 ± 0.1 to $1.6 \pm 0.6 \text{ nM}$. ANOVA for K_d values gave $F[9,22] = 1.18$ and $P = 0.35$. The solid line represents the computer-assisted curve fitting of experimental data to the monoexponential model described by the equation $R_t = r/k(1 - e^{-kt})$. Vehicle-treated controls ($B_{\max} = 60 \pm 4 \text{ fmol mg}^{-1}$ protein; $K_d = 1.5 \pm 0.2 \text{ nM}$). Inset: Semilogarithmic plot of the time course for the assessment of α_2 -adrenoceptor turnover according to the equation (Mauger *et al.*, 1982): $\ln([R_{ss}]/[R_t]) = kt$, where $[R_{ss}]$ and $[R_t]$ are the density of α_2 -adrenoceptors in vehicle- and EEDQ-treated rats at time t , respectively. In this linear transformation the slope of the line gives the rate constant of disappearance of the receptor ($k = 0.079 \text{ days}^{-1}$). The coefficient of correlation is $r = 0.94$. See Table 3 for turnover parameters.

experiments provided the $B_{\max}$ values for the analysis of the exponential recovery function (Figure 2).

The quantitative evaluation of the process of α_2 -adrenoceptor repopulation in the brain of WKY rats (Figure 2) yielded estimates for the appearance (r) and disappearance (k) rate constants of 4.6 ± 0.5 fmol mg⁻¹ protein day⁻¹ and 0.085 ± 0.015 day⁻¹, respectively; and a half life ($t_{1/2}$) for the receptor of 8.1 ± 1.5 days (Table 3). The estimated value for the limit of the recovery function (eventual steady state density of α_2 -adrenoceptors after complete recovery) was very similar ($r/k = 54$ fmol mg⁻¹ protein) to the density of α_2 -adrenoceptors obtained in control rats ($B_{\max} = 57$ –60 fmol mg⁻¹ protein) (Tables 1–3 and Figure 3a). Similarly, the quantitative evaluation of α_2 -adrenoceptor repopulation in the brain of SHR rats yielded estimates for r and k of 9.6 ± 2.2 fmol mg⁻¹ protein day⁻¹ and 0.328 ± 0.098 day⁻¹, respectively. The $t_{1/2}$ of the receptor in SHR rats was 2.1 ± 0.7 days and the estimated value for the limit of the recovery function was also very similar ($r/k = 40$ fmol mg⁻¹ protein) to the density of α_2 -adrenoceptors in control rats ($B_{\max} = 45$ –46 fmol mg⁻¹ protein) (Tables 1–3 and Figure 3b). The simultaneous analysis of experimental data obtained for WKY and SHR rats according to a sole monoexponential function (same r and k values) compared with the analysis without constraints (i.e. independent r and k values for each strain), revealed that the turnover of brain α_2 -adrenoceptors in SHR rats was accelerated (increased k value and shorter $t_{1/2}$) compared to that in WKY rats ($F[2,54] = 3.76$, $P = 0.03$) and that the reduced density of cortical α_2 -adrenoceptors ($B_{\max}$ or r/k values) in hypertensive rats (Tables 1–3) was probably due to an abnormal higher receptor degradation ($\Delta k = 66\%$, $F[1,54] = 3.19$, $P = 0.08$) and not to a decreased receptor synthesis which in fact showed a slight increase ($\Delta r = 24\%$, $F[1,54] = 1.34$, $P = 0.25$) (Table 3 and Figure 3a,b).

Effect of chronic treatment with clorgyline on α_2 -adrenoceptor turnover in cerebral cortex of WKY and SHR rats

Long-term treatment with clorgyline for 21 days induced down-regulation of brain α_2 -adrenoceptor density in WKY

($B_{\max}$ reduced by 30%, $P < 0.05$) but not in SHR rats (Table 2). Since clorgyline modulated the steady state expression of brain α_2 -adrenoceptors in WKY rats with a pattern of receptor down-regulation, such a pattern might be expected to be the consequence of altered rates of receptor appearance (synthesis) and/or disappearance (degradation). In SHR rats, however, the apparent lack of receptor down-regulation induced by clorgyline might reflect an altered pattern of these receptor regulatory mechanisms. Therefore, α_2 -adrenoceptor turnover was assessed during clorgyline treatment (7–21 days) both in WKY and SHR rats.

Similarly to control rats, EEDQ (1.6 mg kg⁻¹, i.p.) treatment in clorgyline-treated WKY and SHR rats (1 mg kg⁻¹, i.p. every 24 h for 7 days) resulted in almost complete reductions (>90% at 6 h) in α_2 -adrenoceptor densities in the cerebral cortex ($B_{\max}$ for [³H]-UK 14304 binding). In both strains of rat the initial reductions of brain α_2 -adrenoceptors were followed by rapid and time-dependent recoveries in receptor density towards control values (i.e. receptor density after 21 days of clorgyline treatment) (Figure 3c,d). The binding affinity of [³H]-UK 14304 for the α_2 -adrenoceptor was unaltered during recovery from EEDQ-induced receptor inactivation (Figure 3, legend).

The simultaneous analysis of recovery functions in control and in clorgyline-treated WKY rats, revealed the existence of a marked modulation by the antidepressant of cortical α_2 -adrenoceptor turnover function ($F[2,42] = 7.32$, $P = 0.002$) (Table 3, Figure 3a,c). Clorgyline increased significantly the disappearance (degradation) rate constant of the receptor in the brain of WKY rats ($\Delta k = 286\%$, $F[1,42] = 12.7$, $P = 0.0009$), leading to a marked reduction in the half-life of the receptor (control, $t_{1/2} = 8.1$ days; clorgyline, $t_{1/2} = 2.1$ days). Moreover, the appearance (synthesis) rate constant was also increased by clorgyline in WKY rats ($\Delta r = 109\%$, $F[1,42] = 8.53$, $P = 0.0055$). In contrast, the simultaneous analysis of recovery functions in control and in clorgyline-treated SHR rats indicated that the MAO inhibitor did not modify significantly the turnover parameters of brain α_2 -adrenoceptors in hypertensive rats ($F[2,42] = 0.78$, $P = 0.46$) (Table 3 and Figure 3b,d). However, it should be noted that in SHR rats clorgyline also tended to accelerate the turnover of brain α_2 -adrenoceptors (Table 3).

Table 3 Effect of chronic treatment with clorgyline on α_2 -adrenoceptor turnover parameters in the cerebral cortex of normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats

Strain/ Treatment	Turnover parameters			
	k (day ⁻¹)	$t_{1/2}$ (days)	r (fmol mg ⁻¹ protein day ⁻¹)	r/k (fmol mg ⁻¹ protein)
WKY				
Saline	0.085 ± 0.015	8.1 ± 1.5	4.6 ± 0.5	54
Clorgyline	$0.328 \pm 0.098^{\dagger\dagger}$	$2.1 \pm 0.7^{\dagger\dagger}$	$9.6 \pm 2.2^{\dagger}$	29 [†]
SHR				
Saline	$0.141 \pm 0.024^*$	$4.9 \pm 0.9^*$	5.7 ± 0.7	40
Clorgyline	0.192 ± 0.049	3.6 ± 1.0	7.4 ± 1.2	39

Rats were injected with a single dose of the irreversible antagonist, EEDQ (1.6 mg kg⁻¹, i.p.) and killed after different periods of time to assess the recovery of the specific binding of [³H]-UK 14304 to cortical membranes, which was used as a biochemical index to quantitate the density of α_2 -adrenoceptors. Receptor turnover parameters were calculated from data shown in Figure 3 (see Methods). Receptor turnover was assessed in saline-treated rats and during clorgyline (1 mg kg⁻¹, i.p., every 24 h)-induced receptor down-regulation (phase 7–21 days of treatment) (see Table 2). Reappearance of receptors was assessed by nonlinear analysis according to the equation: $Rt = r/k (1 - e^{-kt})$, where r is the appearance (synthesis) rate constant, k is the disappearance (degradation) rate constant of the receptor, and the ratio r/k the density of receptors at steady state after irreversible inactivation. $t_{1/2}$ represents the apparent half-life of the receptor and was calculated by the equation: $t_{1/2} = \ln 2/k$. Turnover parameters are expressed as the best fit values $\pm$ standard error calculated by the matrix inversion method, using the nonlinear regression programme GraFit (Leatherbarrow, 1990). Statistical comparisons between strains and 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 recovery curves revealed significant differences in α_2 -adrenoceptor turnover parameters between WKY and SHR rats ($F[2,54] = 3.76$; $P = 0.03$), and also between saline and clorgyline-treated WKY rats ($F[2,42] = 7.32$, $P = 0.002$) but not between saline and clorgyline-treated SHR rats ($F[2,42] = 0.78$, $P = 0.46$).

* $P = 0.08$ when compared with the corresponding value in WKY rats (F -test).

[†] $P < 0.01$; ^{††} $P < 0.001$ when compared with the corresponding saline-treated group (F -test).

Figure 3 Recovery of α_2 -adrenoceptor density ($B_{\max}$ for [3 H]-UK 14304 binding) in the cerebral cortex of (a,c) normotensive Wistar-Kyoto (WKY) and (b,d) spontaneously hypertensive rats (SHR) after EEDQ-induced receptor inactivation in control rats (a,b) (○) and during clorgyline (1 mg kg^{-1} , i.p., every 24 h)-induced receptor down regulation in WKY but not in SHR rats (c,d) (●) (phase 7–21 days of treatment) (see Table 2). Clorgyline-treated rats were injected with EEDQ (1.6 mg kg^{-1} , i.p.) at day 7 and treatment with clorgyline was continued until 24 h before killing. The $B_{\max}$ values were determined from complete saturation experiments for [3 H]-UK 14304 using the nonlinear regression programme LIGAND. Other details as for Tables 1 and 3. Data shown are means $\pm$ s.e.mean derived from 3–5 experiments. The solid lines represent the computer-

Discussion

The results of this study are consistent with previous data that demonstrated the existence of a reduced density and sensitivity of brain α_2 -adrenoceptors in SHR compared to WKY rats (Olmos *et al.*, 1991). The decrease in the number of receptors detected with both the agonist [3 H]-UK 14304 and the antagonist [3 H]-RX 821002 was very similar (11 and 12 fmol mg^{-1} protein, respectively), suggesting that the observed alterations in the density and sensitivity of brain α_2 -adrenoceptors in SHR involve mainly the high affinity state of the receptor. A reduced α_2 -adrenoceptor-mediated autoinhibition of noradrenergic transmission has been demonstrated in the hypothalamus of SHR (Kubo *et al.*, 1986) which results in a potentiation of NA release (Tsuda *et al.*, 1990). This greater release of NA in SHR could explain (through desensitization processes) the decreases in the density and sensitivity of brain α_2 -adrenoceptors found in this model of genetic hypertension (Olmos *et al.*, 1991; present results). In this context, the quantitative evaluations of receptor repopulation after EEDQ in the cerebral cortex of WKY and SHR revealed that α_2 -adrenoceptor turnover is markedly accelerated (increased degradation and shorter half-life) in hypertensive rats. In contrast, receptor synthesis appears to be rather similar in both strains. These results are in agreement with the reported greater release of NA in the brain of SHR (Kubo *et al.*, 1986; Tsuda *et al.*, 1990) which through sustained activation of α_2 -adrenoceptors induces down-regulation by an increased receptor degradation. In SHR, this increased receptor degradation is not compensated with an adaptive increase in receptor synthesis which results in a permanent receptor down-regulation.

The time-dependent modulation of brain α_2 -adrenoceptor density by chronic treatment with clorgyline in SD rats is consistent with the reported decrease in the density of brain α_2 -adrenoceptors and desensitization of associated functional responses (e.g. clonidine-induced mydriasis) after long-term administration of various antidepressant drugs (including clorgyline), suggesting further that sustained stimulation of α_2 -adrenoceptors by the endogenous agonist NA results in down-regulation of the receptor (Smith & Hollingsworth, 1984; Giralt & García-Sevilla, 1989; Menargues *et al.*, 1990; Barturen & García-Sevilla, 1992). In line with these findings, chronic clorgyline treatment in WKY rats also resulted in down-regulation of brain α_2 -adrenoceptors. In contrast, the native lower density of brain α_2 -adrenoceptors in SHR was not down-regulated further by treatment with the MAO inhibitor. In this context, clorgyline treatment in WKY rats markedly accelerated the turnover of brain α_2 -adrenoceptors with the expected increased receptor degradation and shorter half life, but also with a greater receptor synthesis. The greater increase in receptor degradation over receptor synthesis could explain the down-regulation of brain α_2 -adrenoceptor density induced by clorgyline, as the greater receptor degradation in SHR explains the lower native brain receptor density in this strain. Similarly to the reported increase in the turnover of brain α_2 -adrenoceptors as a consequence of desipramine treatment (Barturen & García-Sevilla, 1992), as well as those of α_1 -adrenoceptors (Hughes & Insel, 1986), β -adrenoceptors (Snively *et al.*, 1985) and benzodiazepine receptors (Miller *et al.*, 1991) as a consequence of exposure to an agonist, one can suggest that sustained

assisted curve fitting of experimental data to the monoexponential model described by the equation $Rt = r/k(1 - e^{-kt})$. The dashed horizontal lines show the range (means $\pm$ s.e.mean) of receptor density at steady state (i.e. the densities of brain α_2 -adrenoceptors in saline- and clorgyline-treated WKY and SHR rats; Table 2). Range for K_d values during recovery was 0.9 ± 0.1 to $1.7 \pm 0.2 \text{ nM}$. ANOVA for K_d values (control and clorgyline groups) gave $F[9,99] = 1.70$ and $P = 0.098$. See Table 3 for clorgyline-induced changes in α_2 -adrenoceptor turnover parameters.

stimulation of α_2 -adrenoceptors by endogenous NA, after inhibition of MAO activity by clorgyline (present study) or neuronal uptake by desipramine (Barturen & García-Sevilla, 1992), increases receptor degradation, leading to an initial reduction in receptor number. Also, the increase in receptor synthesis induced by clorgyline treatment in WKY rats could be viewed as a later compensatory mechanism that would lead to restoration of brain α_2 -adrenoceptor density (see Discussion in Barturen & García-Sevilla, 1992). In marked contrast to WKY rats, chronic clorgyline treatment in SHR did not accelerate significantly the turnover of brain α_2 -adrenoceptors, indicating that these genetically desensitized α_2 -adrenoceptors cannot be further down-regulated by clorgyline treatment, probably due to the higher rate of receptor degradation in SHR compared to WKY rats and to the lack of the expected adaptative increase in receptor synthesis (see above). In this sense, it is noteworthy that chronic treatment with the imidazoline drugs, idazoxan and cirazoline, that in normotensive SD and WKY rats resulted in a significant up-regulation of brain I_2 -imidazoline sites, did not modify the density of these sites in the brain of SHR rats (Olmos *et al.*, 1992). Together these results could reflect the existence of a relevant abnormality in common receptor regulatory mechanisms controlling receptor synthesis in this genetic model of hypertension.

Little is known about the basic biochemical mechanisms of agonist (NA)-induced down-regulation of α_2 -adrenoceptors. In contrast, homologous desensitization and down-regulation of β_2 -adrenoceptors in cultured cells is a well characterized phenomenon (Schwinn *et al.*, 1992). Thus, two major mechanisms involved in down-regulation of β_2 -adrenoceptors have been elucidated: a decreased receptor synthesis (via destabilization of mRNA levels) and an increased receptor degradation (via receptor phosphorylation), and both mechanisms could explain a loss of receptors (Schwinn *et al.*, 1992). With respect to α_2 -adrenoceptors in brain tissue, previous studies with desipramine (Barturen & García-Sevilla, 1992) and the present results with clorgyline clearly demonstrate that only an increased receptor degradation is the relevant mechanism involved in down-regulation of brain α_2 -adrenoceptor density, indicating that this accelerated degradation is the first change in the metabolism of the receptor induced by these two prototypical antidepressant drugs. In this context, the consistent increase in receptor synthesis induced by chronic desipramine and clorgyline might represent a later compensatory mechanism that would lead to restoration of brain α_2 -adrenoceptor density (Barturen & García-Sevilla, 1992).

In the rat brain, α_2 -adrenoceptors are heterogeneous in nature and the existence of at least two receptor subtypes (α_{2A} and α_{2B}) has been documented in the various brain regions (Bylund, 1985; Brown *et al.*, 1990) with a preponderance of the α_{2A} subtype in the cerebral cortex (Uhlén & Wikberg, 1991; Barturen & García-Sevilla, 1992). In the present study, the radioligand used to quantify α_2 -adrenoceptors, [3 H]-UK 14304, is a mixed $\alpha_{2A/B}$ agonist and the alkylating agent EEDQ does not discriminate between α_2 -adrenoceptor subtypes (Barturen & García-Sevilla, 1992). Recently, however, the mechanisms of agonist-induced down-regulation of α_2 -adrenoceptors have been shown to be the same for α_{2A} - and α_{2B} -adrenoceptors (Eason & Liggett, 1992; Liggett *et al.*, 1992). Thus in cultured cells, adrenaline induces homologous desensitization through phosphorylation by β_2 -adrenoceptor kinase of α_{2A} - and α_{2B} -subtypes, which is followed by uncoupling from G_i proteins (rightward shifts for adrenaline-mediated inhibition of adenylyl cyclase and decreases in agonist high affinity binding), and in the long-term by a decrease in the density of cellular G_i proteins (important component of chronic desensitization), receptor sequestration (translocation from the cell surface to the interior) and finally receptor down-regulation (proteolytic receptor degradation) (Eason & Liggett, 1992; Liggett *et al.*, 1992). These same mechanisms, leading to α_2 -adrenoceptor down-regulation after sustained stimulation by endogenous NA, could operate in the rat brain after chronic treatment with desipramine (Barturen & García-Sevilla, 1992) and clorgyline (present study). Moreover, in line with the above mechanisms, preliminary results from this laboratory indicate that chronic treatment with the MAO inhibitor, clorgyline, decreases by about 20% the density of specific G_i protein subunits ($G_{\alpha_{i2}}$) in the rat cerebral cortex. In this context, the genetic model of down-regulation of brain α_2 -adrenoceptors in SHR together with an accelerated receptor turnover (increased receptor degradation), and an apparent lack of receptor down-regulation and turnover acceleration induced by clorgyline in these rats, suggest the existence of relevant altered patterns of α_2 -adrenoceptor regulatory mechanisms (e.g. increased receptor phosphorylation, abnormal G_i proteins and/or dysfunction of receptor synthesis) in this genetic model of hypertension.

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