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Long Term Treatment with Desipramine Increases the Turnover of α_2-Adrenoceptors in the Rat Brain

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SUMMARY

The aim of this study was to quantitate the turnover of α_2-adrenoceptors in different regions of the rat brain and its modulation during desipramine (a cyclic antidepressant drug)-induced receptor down-regulation. The recovery of [^{3}H]clonidine (a mixed $\alpha_{2A/B}$-adrenoceptor agonist) binding after irreversible inactivation by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (an alkylating agent for both α_2-adrenoceptor subtypes) was assessed in control and desipramine-treated (3 mg/kg, intraperitoneally, every 12 hr for 7–35 days) rats to study the process of α_2-adrenoceptor repopulation and to calculate receptor turnover parameters. In control rats, the turnover of brain α_2-adrenoceptors showed marked regional differences. The fastest receptor turnover rate was found in the hypothalamus and corpus striatum (receptor half-life, $t_{½}$ = 2.1 days), compared with that in the brainstem ($t_{½}$ = 2.6 days), cerebral cortex ($t_{½}$ = 3.9 days), and hippocampus ($t_{½}$ = 4.3 days). In the cerebral cortex and other brain regions, desipramine induced a time-dependent modulation of α_2-adrenoceptors, with significant decreases in the number of receptors (40–71%; $p < 0.01$) during the first 7–14 days of treatment and regulation to base-line values by 21–35 days. In the cerebral cortex, α_2-adrenoceptor turnover evaluated during desipramine-induced receptor down-regulation (phase of 7–14 days of treatment) revealed an increase in both the disappearance (degradation) (Δk = 122%; $p < 0.05$; $t_{½}$ = 1.7 days) and appearance (synthesis) (Δr = 68%; $p < 0.05$) rates of the receptor. In other noradrenergic brain regions (hippocampus, brainstem, and hypothalamus) but not in the corpus striatum, desipramine (7–14 days) also increased α_2-adrenoceptor degradation (Δk = 97–144%) and shortened the half-life of the receptor, and it tended to increase the rate of synthesis (Δr = 51–83%). Similar results, but with a higher appearance rate, were obtained in the cerebral cortex during the phase of treatment (21–35 days) without apparent receptor down-regulation (Δk = 160%; $p < 0.01$; $t_{½}$ = 1.5 days; Δr = 128%; $p < 0.001$). It is proposed that sustained stimulation of α_2-adrenoceptors by endogenous norepinephrine, after inhibition of neuronal uptake, increases their disappearance rate (degradation), leading to a reduction in receptor number. The increase in the appearance (synthesis) rate could be viewed as a later compensatory mechanism that would lead to restoration of brain α_2-adrenoceptor density.

α_2-Adrenoceptors play an important physiological role in the regulation of transmitter release from noradrenergic nerve endings (1, 2). Biochemical and functional studies have suggested that endogenous depression is related to α_2-adrenoceptor supersensitivity (3, 4), and desensitization of these receptors has been implicated in the mechanism of action of antidepressant drugs (5). Thus, long term, but not acute, administration of cyclic antidepressant drugs, monoamine oxidase inhibitors, lithium, or electroshock has been associated with a decrease in the density and/or sensitivity of central and peripheral α_2-adrenoceptors (6–14). This receptor desensitization process disappears with time in spite of continued antidepressant treatment (15).

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Little is known about the basic biochemical mechanisms involved in antidepressant-induced down-regulation of the steady state expression of brain α_2-adrenoceptors. The density of a membrane receptor can be understood as the end result of a number of cellular events involved in the receptor appearance on, and disappearance from, the cell surface (16). Thus, variations in the receptor number would imply perturbations in one or more steps of its metabolism and finally in a decrease or increase in the receptor appearance or disappearance rate. The role of these processes in the regulation of the density of α_2-adrenoceptors is obscure, and relevant information may come from studies on receptor turnover. Most data on the cellular metabolism of α_2-adrenoceptors have resulted from studies performed, under basal conditions, using irreversible inactivation of the receptors by the antagonists phenoxybenzamine (17, 18), EEDQ (19), and benextramine (20) and measuring the

ABBREVIATIONS: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; ARC 239, 2-[2-[4-(*o*-methoxyphenyl)piperazine-1-yl]ethyl]-4,4-dimethyl-1,3(2*H*, 4*H*)-isoquinolinedione; ANOVA, analysis of variance; 5-HT, 5-hydroxytryptamine; IBS, imidazoline binding sites.

subsequent recovery of binding sites with a selective radioligand. The apparent half-life for the α_2-adrenoceptor estimated from these studies varied from 1.6 to 6.1 days in rabbit spleen (17) and cerebral cortex (18), respectively, suggesting tissue differences. However, relevant pharmacological modulations of α_2-adrenoceptor turnover have not been studied.

The aim of the present study was to measure dynamic characteristics (receptor turnover parameters) of α_2-adrenoceptors in different regions of the rat brain and their modulation during chronic treatment with the cyclic antidepressant drug desipramine. The main objective was to obtain information about the cellular events associated with desipramine-induced modulation (time course of induction of down-regulation) of the steady state density of brain α_2-adrenoceptors. For this purpose, the kinetics of reappearance of rat brain α_2-adrenoceptors (mixed population of α_{2A} and α_{2B} subtypes) after irreversible inactivation by EEDQ were assessed in control rats and were compared with those obtained during the different phases of treatment with desipramine.

Experimental Procedures

Materials. Adult male Sprague-Dawley rats, weighing 300 ± 50 g, were housed 2–6/cage and maintained at 22° on a 12-hr light/12-hr dark cycle, with a standard diet and water available *ad libitum*. Animals were killed by decapitation, the brains were rapidly removed, and brain areas were dissected on ice. Tissues for binding studies were stored at −70° until used.

[^{3}H]Clonidine (41.5 and 46.4 Ci/mmol) was purchased from New England Nuclear/DuPont. Other drugs and their sources were EEDQ (Sigma Chemical Co.), ARC 239 HCl and clonidine HCl (Boehringer Ingelheim España, Barcelona, Spain), desipramine HCl (USV Laboratories, New York, NY), oxymetazoline HCl (Pensa, Barcelona, Spain), and phentolamine HCl (Ciba-Geigy, Barcelona, Spain). Other reagents were obtained from Sigma.

Drug treatments. The peptide-coupling agent EEDQ (4 mg/kg) 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 intraperitoneally in a single dose. Rats were killed 0.25, 1, 2, 4, 7, 9, or 13 days after EEDQ administration to evaluate the recovery of brain α_2-adrenoceptor density, which allowed estimation of receptor turnover parameters. Desipramine (3 mg/kg every 12 hr) was dissolved in purified water and administered intraperitoneally for 1–5 weeks. Rats were killed 36 hr after the last injection. This dose of desipramine was considered optimal for induction of receptor down-regulation (11). To study the modulation of α_2-adrenoceptor turnover by desipramine, EEDQ was injected into rats treated with the antidepressant for 7 or 21 days, and treatment was then continued until days 14 and 35, respectively (phase 7–14 days, presence of receptor down-regulation; phase 21–35 days, absence of receptor down-regulation).

In some experiments, selective *in vitro* drug protection of α_2-adrenoceptor subtypes (α_{2A} and α_{2B}) from inactivation by EEDQ was performed to assess whether the alkylating agent can discriminate between receptor subtypes. Oxymetazoline (100-fold selective α_{2A}-adrenoceptor agonist) (used at 3.3×10^{-7} M), ARC 239 (100-fold selective α_{2B}-adrenoceptor antagonist) (3.3×10^{-7} M), and clonidine (mixed $\alpha_{2A/B}$-adrenoceptor agonist) (10^{-7} M) were used as selective and nonselective drugs at concentrations 20 times their K_i values against the rat brain α_2-adrenoceptor,[1] which represented about 95% protection of the corresponding receptor subtype with minimal occupancy (16%) of the other subtype.

[^{3}H]Clonidine binding to brain membranes. The specific binding of the agonist [^{3}H]clonidine to brain membranes was used as a biochemical index to quantify α_2-adrenoceptor density in the high affinity state and the affinity of the radioligand for the receptor. Neural membranes were isolated from the following brain areas: parieto-occipital cortex, hypothalamus, hippocampus, brainstem, and corpus striatum. The area that is referred to as brainstem consisted of a section 1-mm thick medial and inferior to the superior cerebellar peduncle and it contained, among other nuclei, both loci coerulei. Some of these brain regions appear to possess similar proportions of α_{2A}- and α_{2B}-adrenoceptors (e.g., cerebral cortex, 60:40%; hippocampus, 50:50%), although the precise distribution of the subtypes in the brain has yet to be determined. Brain areas isolated from two to six rats were pooled for each experiment.

Preparation of neural membranes (P_2 membrane fraction) and [^{3}H]clonidine binding assays were done as described previously (8), with minor modifications. Briefly, total [^{3}H]clonidine binding was measured at equilibrium in 1-ml aliquots (50 mM Tris·HCl, 10 mM $MgCl_2$, pH 7.5) of the neural membranes (protein content: cortex, 831 ± 3 μg/ml; hypothalamus, 599 ± 18 μg/ml; hippocampus, 914 ± 14 μg/ml; brainstem, 566 ± 13 μg/ml; striatum, 751 ± 21 μg/ml), which were incubated at 25° for 30 min with various concentrations (0.25–16 nM, cortex) or with a single concentration (16 nM, other brain regions) of [^{3}H]clonidine. Nonspecific binding was determined in the presence of phentolamine (10^{-5} M). Specific binding ranged from 97 ± 2% to 81 ± 3% at 0.25–16 nM [^{3}H]clonidine. Both clonidine and phentolamine are imidazoline drugs that can detect not only α_2-adrenoceptors but also other norepinephrine-insensitive, nonadrenoceptor sites, the so-called IBS (21, 22). In the rat brain, however, clonidine is more selective for the α_2-adrenoceptor (K_i = 2.6 nM) than for IBS (K_i = 973 nM). Moreover, phentolamine, used to define nonspecific binding, is a very weak imidazoline drug with a K_i of 16.6 μM for IBS, being about 4000 times more selective for the α_2-adrenoceptor (K_i = 5.2 nM).[1] Therefore, phentolamine can be used to define the specific binding of [^{3}H]clonidine to rat brain α_2-adrenoceptors, especially in the cerebral cortex where a very small proportion of IBS sensitive to clonidine has been detected (23). Moreover, similar turnover parameters for the α_2-adrenoceptor were obtained when the specific binding of [^{3}H]UK 14304 (bromoxidine; a mixed $\alpha_{2A/B}$ agonist) and [^{3}H]RX 821002 (methoxyidazoxan; a mixed $\alpha_{2A/B}$ antagonist) to neural membranes was defined with 10^{-5} M (−)-epinephrine.[2]

Data analyses. For analysis of binding data, the weighted nonlinear least-squares curve-fitting program LIGAND (24) was used for the analysis of complete saturation curves for [^{3}H]clonidine to estimate the apparent dissociation constant (K_d) and the maximum number of binding sites (B_{max}). In the range of concentrations used (0.25–16 nM), only a single population of binding sites was present on the isolated membranes (cortex). When a single saturating concentration (16 nM) of [^{3}H]clonidine was used (other brain regions), the B_{max} was estimated using the K_d value obtained from previous saturation curves, according to the equation $B_{max} = B(K_d + F)/F$, where B represents the amount of [^{3}H]clonidine specifically bound at 16 nM and F is the corresponding amount of free radioligand.

For analysis of α_2-adrenoceptor turnover, data from the recovery of brain α_2-adrenoceptor density after irreversible inactivation by EEDQ were analyzed according to a monoexponential model based on two implicit assumptions (25), 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). Experimental recovery data were fitted, using the simple nonlinear least squares fitting program GraFit (26), to the equation:

[1] A. Miralles, G. Olmos, M. Sastre, F. Barturen, I. Martin, and J. A. García-Sevilla. Discrimination and pharmacological characterization of I_2-imidazoline sites with [^{3}H]idazoxan and α_2-adrenoceptors with [^{3}h]RX 821002 (2-methoxyidazoxan) in the human and rat brains. Submitted for publication.

[2] C. Ribas, A. Miralles, and J. A. García-Sevilla, unpublished observations.

$$R_t = \frac{r}{k}(1 - e^{-kt}) \quad (1)$$

where R_t is expressed as fmol/mg of 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 of protein/day, and k is the disappearance ("degradation") rate constant of the receptor, in units of day^{-1}, that allows estimation of the apparent half-life of the receptor ($t_{1/2} = \ln 2/k$). In the present model the ratio r/k represents the density of receptors at steady state to which the system tends after irreversible inactivation of the receptors. To assess the experimental uncertainty of this parameter (r/k) for further statistical comparisons, the experimental data were also fitted to the equation:

$$R_t = \text{limit}\ (1 - e^{-kt}) \quad (2)$$

where the limit corresponds to the r/k value, understood as a constant to be estimated. The possible incomplete irreversible inactivation of the α_2-adrenoceptor by EEDQ was assessed by fitting the experimental data to the more general equation:

$$R_t = \frac{r}{k}(1 - e^{-kt}) + R_0 e^{-kt} \quad (3)$$

where R_0 represents the residual number of receptors at time zero. The selection between the simple (eq. 1) or general model (eq. 3) was made statistically by means of an F test (27). Similarly, the possible existence of subpopulations or subtypes of receptors with different turnover parameters was assessed by fitting the data to a biexponential model, as follows:

$$R_t = \frac{r_1}{k_1}(1 - e^{-k_1 t}) + \frac{r_2}{k_2}(1 - e^{-k_2 t}) \quad (4)$$

and comparing the goodness of fit with the monoexponential model defined by eq. 1.

Statistics. Radioligand binding data are expressed as means ± standard errors. One-way ANOVA followed by Scheffé's test was used for the statistical evaluations. The level of significance was chosen as $p = 0.05$. Receptor turnover parameters are expressed as the best fit values (mean ± standard error) determined by the matrix inversion method, using the nonlinear regression program GraFit (26). 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 analyzed separately (with no constraints), and 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, analyzed 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 (28, 29). In the present model, equality was tested during the simultaneous analysis by inspecting the consequences, in terms of increasing residual variance, of forcing the parameters to be equal. The statistical significance of the improvement was determined with an F test at a level of significance of $p = 0.05$. This approach is statistically optimal, because experimental errors are not systematically distorted (29), and implies fewer assumptions than linearizing transformations (25). Thus, this procedure is applicable when variations in the steady state density of the receptor along the recovery process are expected, without the necessity of estimating basal receptor densities at each time point. Moreover, the proposed analysis technique allows the statistical significance of differences in turnover function and parameters between different experimental conditions to be determined, using a powerful method based on the principle of extrasum of squares (27).

Results

Effect of EEDQ *in vitro* on α_2-adrenoceptor subtypes in the rat brain. Pharmacological heterogeneity of α_2-adrenoceptors has led to the subclassification of this receptor into the well defined α_{2A} and α_{2B} subtypes, which has been confirmed by the molecular cloning of these (and possibly other) receptor subtypes in the brain. In the present study, the radioligand used to quantify α_2-adrenoceptors, [^{3}H]clonidine, is a mixed $\alpha_{2A/B}$ agonist, and in the rat brain various regions appear to possess a mixed population of α_{2A}- and α_{2B}-adrenoceptors. Therefore, it appeared relevant to assess whether the alkylating agent EEDQ is equally effective against these two brain α_2-adrenoceptors.

Selective *in vitro* drug protection of α_{2A}- and α_{2B}-adrenoceptor subtypes from inactivation by EEDQ indicated that the alkylating agent can inactivate the two receptor subtypes in the various brain regions (Fig. 1). Thus, incubation with EEDQ (10^{-5} M; 30 min) resulted in marked reductions in the specific binding of [^{3}H]clonidine (mixed $\alpha_{2A/B}$ agonist) to neural membranes from the cerebral cortex (71%), hypothalamus (62%), brainstem (53%), and striatum (71%). Preincubation with oxymetazoline (selective α_{2A} agonist) (3.3×10^{-7} M, 30 min) or ARC 239 (selective α_{2B} antagonist) (3.3×10^{-7} M, 30 min) induced similar degrees of protection of both receptor subtypes (but with a preponderance of the α_{2A} subtype) in the cerebral cortex (α_{2A}, 29%; α_{2B}, 18%) and hypothalamus (α_{2A}, 20%; α_{2B}, 15%) but not in the brainstem (α_{2A}, 17%; α_{2B}, 48%) and striatum (α_{2A}, 16%; α_{2B}, 44%), where the higher degree of protection for the α_{2B} subtype suggested a relatively higher proportion of this adrenoceptor subtype in these latter brain regions. In all brain regions, preincubation with clonidine (mixed $\alpha_{2A/B}$ agonist) (10^{-7} M, 30 min) resulted in greater degrees of receptor protection from inactivation by EEDQ (cortex, 51%; hypothalamus, 28%; brainstem, 49%; striatum, 59%), and at least in the cerebral cortex and striatum the degree of protection induced by clonidine was equivalent to that afforded by oxymetazoline and ARC 239 together (Fig. 1).

These selective drug protection experiments against EEDQ and the *in vivo* effects of the alkylating agent in the different brain regions (inactivation of 87–96% of α_2-adrenoceptors in regions with mixed populations of α_{2A} and α_{2B} subtypes) (see below) clearly indicated that EEDQ is able to inactivate α_{2A}- and α_{2B}-adrenoceptors. Because EEDQ cannot discriminate between α_2-adrenoceptor subtypes and [^{3}H]clonidine is a mixed $\alpha_{2A/B}$ agonist, the quantitated receptors are simply termed α_2-adrenoceptors.

α_2-Adrenoceptor turnover in the rat brain. Treatment of rats with a single dose of EEDQ (4 mg/kg, intraperitoneally) induced an almost complete reduction (>90% at 6 hr) in the density of α_2-adrenoceptors in the cerebral cortex (B_{max} for [^{3}H]clonidine, 7 ± 3% of control) that was followed by a progressive recovery of receptor density, as revealed by [^{3}H]clonidine saturation isotherms performed at different times after *in vivo* EEDQ administration (1, 2, 4, 7, 9, and 13 days). The binding affinity of [^{3}H]clonidine for the α_2-adrenoceptor was unaltered during recovery from EEDQ-induced receptor inactivation (Fig. 2A).

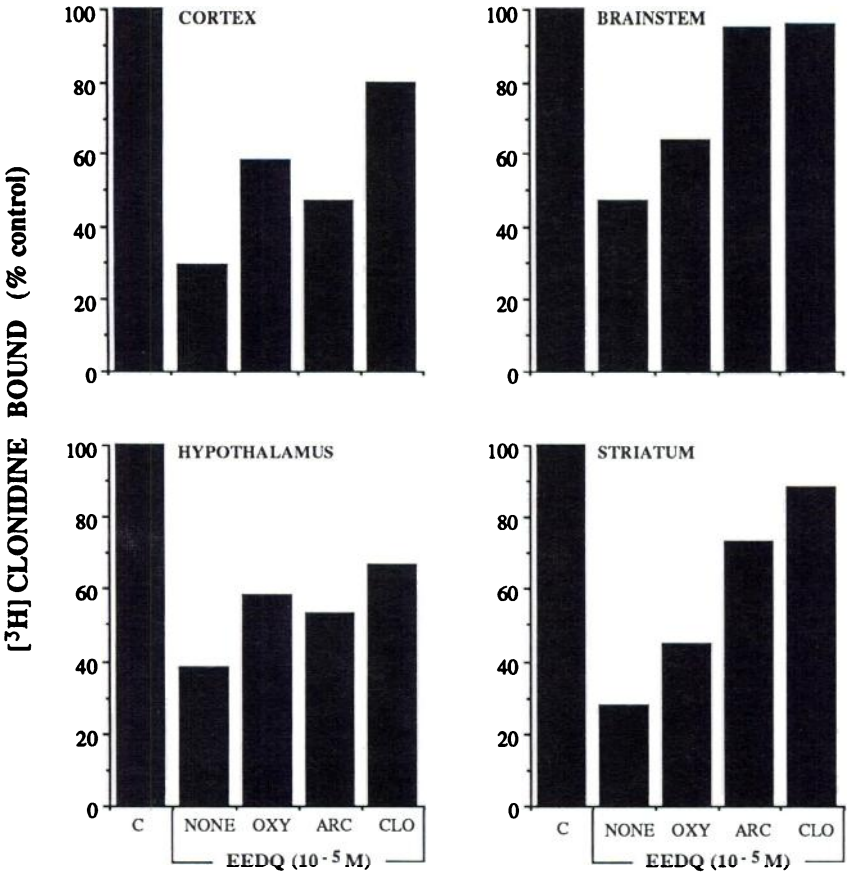

Fig. 1. *In vitro* protection from the EEDQ-induced inactivation of the specific binding of [^{3}H]clonidine by oxymetazoline (selective α_{2A}-adrenoceptor agonist), ARC 239 (selective α_{2B}-adrenoceptor antagonist), and clonidine (mixed $\alpha_{2A/B}$-adrenoceptor agonist) in various rat brain regions. Neural membranes were preincubated at 25° for 30 min in the absence (*NONE*) or presence of oxymetazoline (*OXY*) (3.3×10^{-7} M), ARC 239 (*ARC*) (3.3×10^{-7} M), or clonidine (*CLO*) (10^{-7} M), further incubated with EEDQ (10^{-5} M) for another 30 min, and then washed several times and prepared for [^{3}H]clonidine binding. *Bars*, means of duplicate/triplicate determinations from a representative experiment. Control (*C*) values for [^{3}H]clonidine binding (16 nM) were 83 ± 5 (cortex), 70 ± 8 (brainstem), 93 ± 8 (hypothalamus), and 59 ± 7 (striatum) fmol/mg of protein.

The quantitative evaluation of the process of α_2-adrenoceptor repopulation in the cerebral cortex yielded estimates for the appearance (r) and disappearance (k) rate constants of 12.9 ± 1.4 fmol/mg of protein/day and 0.18 ± 0.03 day^{-1}, respectively, and a half-life ($t_{1/2}$) for the receptor of 3.9 ± 0.7 days (Fig. 2B; Table 1). The estimated value for the limit of the recovery function (eventual steady state density of α_2-adrenoceptors after complete recovery) was slightly lower (r/k = 72 ± 7 fmol/mg of protein; $\Delta = -24\%$; $p < 0.05$) than the density of α_2-adrenoceptors obtained in control rats (B_{max} = 95 ± 4 fmol/mg of protein) (Fig. 2A). The density of α_2-adrenoceptors at time zero after EEDQ (R_0) was assumed to be not different from zero, because the analysis of receptor recovery according to eq. 3 did not improve the goodness of fit ($F[1,28] = 1.24$; $p = 0.27$). Similarly, fitting the experimental data to a biexponential model (eq. 4) did not increase significantly the goodness of the analysis ($F[2,27] = 0.61$; $p = 0.55$), which suggested the absence of subpopulations or subtypes of cortical α_2-adrenoceptors with different turnover parameters.

Similar quantitative evaluations of receptor repopulation in different brain regions showed the existence of regional differences in the turnover parameters of the α_2-adrenoceptor. As in the cortex, EEDQ (4 mg/kg, intraperitoneally) treatment also resulted in marked reductions (87–96% at 6 hr) in the specific binding of [^{3}H]clonidine to neural membranes from the hippocampus, brainstem, hypothalamus, and striatum, which were followed by time-dependent recoveries towards control values (Fig. 3). The simultaneous analysis of experimental data obtained for the different brain regions according to a sole monoexponential function (same r and k values) resulted in a significant increase ($F[8,105] = 5.1$; $p < 0.001$) in the sum of squares, compared with the analysis without constraints (independent r and k values for each region), which suggested regional differences in turnover functions. Thus, simultaneous analyses of the data with and without sets of constraints indicated the existence of marked regional differences for the parameters r ($F[4,105] = 3.7$; $p < 0.01$), k ($F[4,105] = 3.2$; $p < 0.05$), and r/k ($F[4,105] = 4.0$; $p < 0.01$).

The results of the quantitative evaluations of the process of α_2-adrenoceptor repopulation in the rat brain are summarized in Table 1. α_2-Adrenoceptors in the cortex and hippocampus showed similar turnover parameters, which were at variance with the faster turnover rate of the receptor in the hypothalamus, striatum, and to a lesser extent the brainstem (Table 1). In the hypothalamus, as in the cortex, but not in other brain regions the recovery of α_2-adrenoceptors after EEDQ treatment was estimated to be incomplete, because the corresponding r/k value was lower ($\Delta = -30\%$; $p < 0.05$) than the respective receptor density before receptor inactivation (compare r/k values in Table 1 with B_{max} values in Table 2). In all brain regions studied, recovery of α_2-adrenoceptors showed a monoexponential distribution with R_0 values not different from zero. Moreover, the fitting of the experimental data to a biexponential function (eq. 4) did not result in increases of the goodness of fit (i.e., absence of subpopulations or subtypes of α_2-adrenoceptors with different turnover parameters).

Effect of long term treatment with desipramine on α_2-adrenoceptor turnover in the rat cerebral cortex and other brain regions. Analyses of saturation curves for [^{3}H]clonidine binding to cortical membranes from saline and desi-

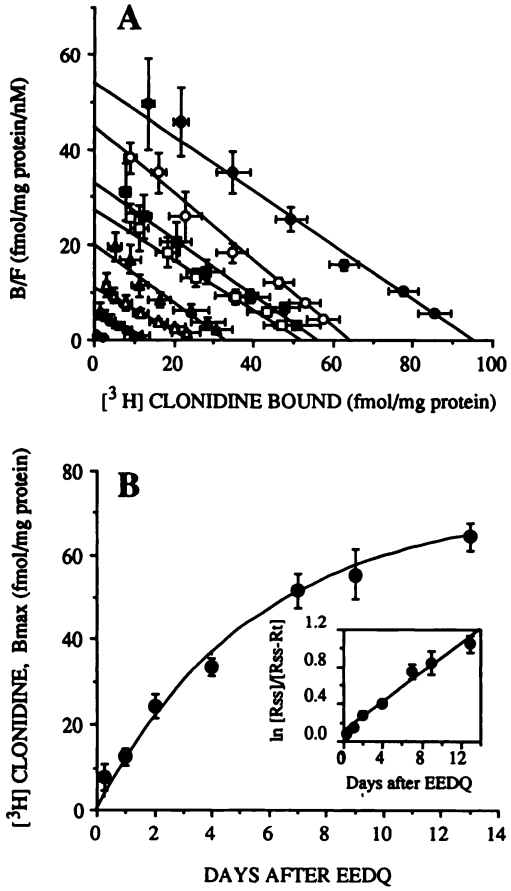

Fig. 2. Recovery of α_2-adrenoceptor density in the rat cerebral cortex after EEDQ-induced receptor inactivation. Data shown are means ± standard errors derived from three to five experiments carried out using pooled tissues from two animals. A, Mean Scatchard plots for [^{3}H] clonidine (0.25–16 nM) binding to cortical membranes of rats killed at 0.25 (◇), 1 (◆), 2 (△), 4 (▲), 7 (□), 9 (■), or 13 (○) days after the administration of EEDQ (4 mg/kg, intraperitoneally). ●, Vehicle-treated controls (B_{max} = 95 ± 4 fmol/mg of protein; K_d = 2.0 ± 0.7 nM). Range for K_d values was 1.6 ± 0.3 to 2.5 ± 0.7 nM. ANOVA for K_d values gave $F[6,27] = 0.26$ and $p = 0.95$. B, Recovery of mean B_{max} values for [^{3}H] clonidine binding after EEDQ treatment as a function of time. The B_{max} values were determined, for each time, from complete saturation experiments (see above) using the nonlinear regression program LIGAND. *Solid line*, computer-assisted curve fitting of experimental data to the monoexponential model described by eq. 1. *Inset*, semilogarithmic plot of the time course for the recovery of [^{3}H]clonidine binding sites according to the equation (25): $\ln R_{ss}/(R_{ss} - R_t) = kt$, where R_{ss} and R_t are the density of α_2-adrenoceptors in control 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). The coefficient of correlation is $r = 0.97$. See Table 1 for turnover parameters.

pramine-treated rats (3 mg/kg, intraperitoneally, every 12 hr for 7, 14, 21, or 35 days) revealed, as expected (11), the existence of a time-dependent modulation of α_2-adrenoceptor density induced by the antidepressant drug (ANOVA for B_{max} values, $F[4,16] = 15.4$; $p < 0.001$) (Fig. 4). Thus, desipramine for 7–14 days clearly induced significant decreases in α_2-adrenoceptor density (control, B_{max} = 102 ± 12 fmol/mg of protein, six experiments; desipramine for 7–14 days, B_{max} = 50 ± 3 and 72 ± 5 fmol/mg of protein, four experiments; $p < 0.001$), whereas in spite of continued treatment for 21–35 days receptor density was regulated to base-line values (B_{max} = 98 ± 6 and 96 ± 15 fmol/mg of protein, four experiments, respectively). Long term treatment with desipramine did not alter the binding

TABLE 1

Parameters of α_2-adrenoceptor turnover in different brain regions of the rat

Rats were injected with a single dose of the irreversible antagonist EEDQ (4 mg/kg, intraperitoneally) and killed after different periods of time to assess the recovery of the specific binding of [^{3}H]clonidine to neural 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 Figs. 2B and 3 (see Experimental Procedures). Reappearance of receptors was analyzed by nonlinear analysis according to the equation: $R_t = (r/k)(1 - e^{-kt})$, where r is the appearance (synthesis) rate constant, k is the disappearance (degradation) rate constant of the receptor, and the ratio r/k is the density of receptors at steady state after irreversible inactivation. $t_{½}$ represents the apparent half-life of the receptor and was calculated by the equation: $t_{½} = \ln 2/k$. Turnover parameters are expressed as the best fit values (mean ± standard error) calculated by the matrix inversion method, using the nonlinear regression program GraFit (26). Analysis of experimental data for the different brain regions with a sole monoexponential function (same r and k values) resulted in a significant decrease of goodness of fit, compared with a model without constraints (five different r and k values) ($F[8,105] = 5.1$; $p < 0.001$). Statistical comparisons between brain regions were made by comparing the goodness of fit of simultaneous analyses with and without a set of constraints by means of an F test.

Brain region	Turnover parameters			
	k	$t_{½}$	r	r/k
	day^{-1}	*day*	*fmol/mg of protein/day*	*fmol/mg of protein*
Cortex	0.18 ± 0.03	3.9 ± 0.7	12.9 ± 1.4	72.2 ± 6.5
Hippocampus	0.15 ± 0.03	4.3 ± 0.8	13.0 ± 1.3	87.9 ± 9.1
Brainstem	0.27 ± 0.07	2.6 ± 0.7	17.8 ± 3.3	65.6 ± 4.8
Hypothalamus	0.33 ± 0.08[a]	2.1 ± 0.5[a]	23.2 ± 4.0[b]	70.3 ± 5.6
Striatum	0.33 ± 0.05[a]	2.1 ± 0.3[a]	17.9 ± 2.0[a]	53.1 ± 3.1[b]

[a] $p < 0.05$, compared with cortex or hippocampus values (F test).
[b] $p < 0.01$.

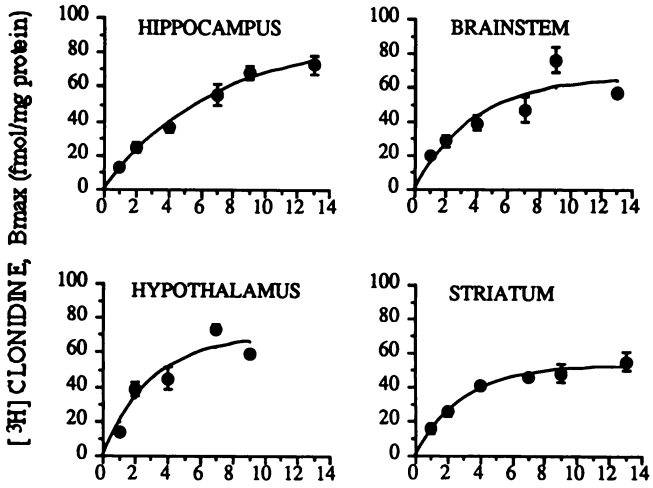

Fig. 3. Recovery of α_2-adrenoceptor density (B_{max} for [^{3}H]clonidine binding) in the hippocampus, brainstem, hypothalamus, and striatum after EEDQ-induced receptor inactivation. Rats were killed 0.25, 1, 2, 4, 7, 9, or 13 days after the administration of EEDQ (4 mg/kg, intraperitoneally). The B_{max} at each time interval was determined from the specific binding of a saturating concentration (16 nM) of [^{3}H]clonidine, as described in Experimental Procedures. Receptor densities (B_{max}) in vehicle-treated controls are shown in Table 2. Data shown are means ± standard errors derived from three to five experiments performed in triplicate using pooled tissues from two animals. *Solid lines*, computer-assisted curve fitting of experimental data to the monoexponential model described by eq. 1. See Table 1 for regional differences in α_2-adrenoceptor turnover parameters.

TABLE 2

Effect of long term treatment with desipramine on the specific binding of [^{3}H] clonidine to rat brain membranes

Rats were treated with desipramine (DMI) (3 mg/kg, intraperitoneally, every 12 hr) for 7, 14, or 21 days and were killed 36 hr after the last dose. The K_d and B_{max} values for [^{3}H]clonidine (0.25–16 nM) binding were determined from complete saturation experiments using the nonlinear regression program LIGAND. Each value represents the mean ± standard error derived from four experiments carried out using pooled tissues from six animals.

Treatment	Hippocampus		Brainstem		Hypothalamus		Striatum	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
	nM	fmol/mg of protein	nM	fmol/mg of protein	nM	fmol/mg of protein	nM	fmol/mg of protein
Control	3.8 ± 0.3	91 ± 6	4.2 ± 0.9	71 ± 11	5.8 ± 0.9	105 ± 11	5.2 ± 1.0	57 ± 6
DMI, 7 days	3.2 ± 0.3	51 ± 6[a]	7.2 ± 1.4	37 ± 6[b]	5.9 ± 0.5	54 ± 5[a]	3.1 ± 0.7	35 ± 2[a]
DMI, 14 days	3.9 ± 0.4	79 ± 4	5.2 ± 0.7	58 ± 12	5.6 ± 0.9	95 ± 7	4.6 ± 0.3	56 ± 6
DMI, 21 days	3.6 ± 0.3	92 ± 4	3.2 ± 0.1	72 ± 7	4.2 ± 0.6	91 ± 11	3.9 ± 0.3	57 ± 6

[a] $p < 0.01$, compared with the corresponding control value (ANOVA followed by Scheffé's test).
[b] $p < 0.05$.

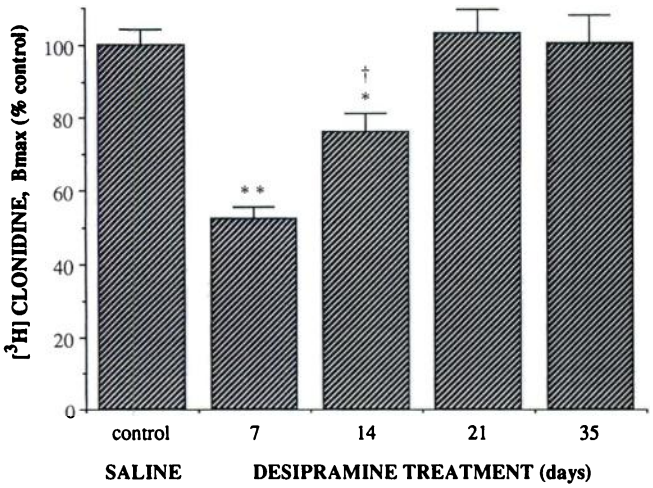

Fig. 4. Development of α_2-adrenoceptor down-regulation induced by desipramine in the rat cerebral cortex. Rats were treated with desipramine (3 mg/kg, intraperitoneally, every 12 hr) for 7, 14, 21, or 35 days and were killed 36 hr after the last dose. The B_{max} values for [^{3}H]clonidine (0.25–16 nM) binding were determined from complete saturation experiments using the nonlinear regression program LIGAND. *Bars*, means ± standard errors derived from four experiments carried out using pooled tissues from six animals, as percentage of values for saline-treated rats (B_{max} = 102 ± 11 fmol/mg of protein; K_d = 2.9 ± 0.6 nM). Range for K_d values (desipramine-treated rats) was 1.9 ± 0.1 to 2.6 ± 0.9 nM. ANOVA for K_d values (all groups) gave $F[4,16] = 0.61$ and $p = 0.66$. *, $p < 0.05$; **, $p < 0.01$, compared with the control value; †, $p < 0.05$, compared with desipramine for 7 days (ANOVA followed by Scheffé's test).

affinity of [^{3}H]clonidine for the α_2-adrenoceptor (Fig. 4). Similar results were obtained in other brain regions (hippocampus, brainstem, hypothalamus, and striatum), although the development of α_2-adrenoceptor down-regulation induced by desipramine was less pronounced (at 7 days of treatment receptor densities were decreased by 39–49%, at 14 days they were decreased by only 10–18%, and at 21 days they were regulated to base-line values) (Table 2).

Because desipramine modulated the steady state expression of brain α_2-adrenoceptors with a pattern of receptor down-regulation followed by a return to control steady state density, such a pattern might be expected to be the consequence of altered rates of receptor appearance (synthesis) and/or disappearance (degradation). Therefore, α_2-adrenoceptor turnover was assessed both during desipramine-induced receptor down-regulation (7–14 days) and during the phase of treatment without apparent receptor down-regulation (21–35 days).

Similarly to control rats, EEDQ (4 mg/kg, intraperitoneally) treatment in desipramine-treated rats (3 mg/kg, intraperitoneally, every 12 hr for 7 or 21 days) resulted in almost complete reductions (>90% at 6 hr) in α_2-adrenoceptor density in the cerebral cortex (Fig. 5). During both phases of desipramine treatment (7–14 and 21–35 days) the initial reductions of α_2-adrenoceptors were followed by rapid and time-dependent recoveries in receptor density towards control values (Fig. 5). The binding affinity of [^{3}H]clonidine for the α_2-adrenoceptor was unaltered during recovery from EEDQ-induced receptor inactivation (ANOVA for K_d) values: phase 7–14 days, $F[3,12] = 1.2$; $p = 0.34$; phase 21–35 days, $F[5,18] = 2.6$; $p = 0.06$). Also during desipramine treatment, recovery of α_2-adrenoceptors showed a monoexponential distribution with R_0 values not different from zero. Moreover, the fitting of the experimental data to a biexponential function (eq. 4) did not result in an increase of the goodness of fit (see above).

The simultaneous analysis of recovery functions in control rats and in desipramine-treated rats for 7–14 and 21–35 days revealed the existence of a marked modulation by the antidepressant of cortical α_2-adrenoceptor turnover function ($F[2,68] = 7.15$; $p < 0.001$) (Fig. 5). The results of these quantitative evaluations are summarized in Table 3. Desipramine significantly increased the disappearance (degradation) rate constant of the receptor in the cerebral cortex either during the phase of receptor down-regulation (phase 7–14 days, Δk = 122%; $p < 0.05$) or during the phase of treatment, without apparent receptor down-regulation (phase 21–35 days, Δk = 161%; $p < 0.01$), leading to significant reductions in the half-life of the receptor (control, $t_{1/2}$ = 3.9 days; desipramine, $t_{1/2}$ = 1.7 and 1.5 days). Moreover, the appearance (synthesis) rate constant was also increased by desipramine (phase 7–14 days, Δr = 68%; $p < 0.05$; phase 21–35 days, Δr = 129%; $p < 0.001$).

Similar results were obtained during the phase of receptor down-regulation (7–14 days) induced by desipramine in the hippocampus (k = 0.30 day^{-1}, $t_{1/2}$ = 2.3 days, r = 19.6 fmol/mg of protein/day), brainstem (k = 0.66 day^{-1}, $t_{1/2}$ = 1.1 days, r = 30.7 fmol/mg of protein/day), and hypothalamus (k = 0.65 day^{-1}, $t_{1/2}$ = 1.1 days, r = 42.5 fmol/mg of protein/day) but not in the striatum (k = 0.30 day^{-1}, $t_{1/2}$ = 2.4 days, r = 15.2 fmol/mg of protein/day) (means of quadruplicate determinations from a single experiment using pooled tissues from four rats; compare data with control values in Table 1). Therefore, in noradrenergic brain regions desipramine clearly increased α_2-adrenoceptor degradation (Δk = 97–144%) and shortened the half-life of the receptor, and it tended to increase the rate of synthesis (Δr = 51–83%).

Discussion

The irreversible blockade/inactivation of receptors and subsequent evaluation of their reappearance has been the approach

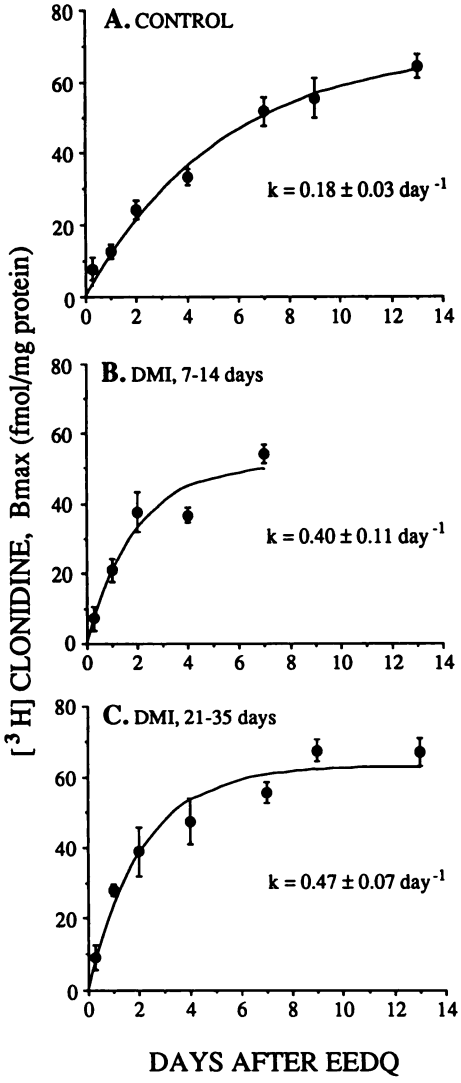

Fig. 5. Recovery of α_2-adrenoceptor density in the rat cerebral cortex (B_{max} for [^{3}H]clonidine binding) after EEDQ-induced receptor inactivation in control rats (B_{max} = 95 ± 4 fmol/mg of protein; K_d = 2.0 ± 0.7 nM) (A) and during the different phases of desipramine (*DMI*) treatment, 7–14 days (B) (induction of receptor down-regulation) and 21–35 days (C) (absence of apparent receptor down-regulation) (see Fig. 4). Desipramine-treated rats were injected with EEDQ (4 mg/kg, intraperitoneally) at 7 and 21 days in the corresponding experimental groups and treatment with desipramine was continued until 36 hr before killing. The B_{max} values were determined from complete saturation experiments for [^{3}H]clonidine (0.25–16 nM) using the nonlinear regression program LIGAND. Data shown are means ± standard errors derived from three to five experiments carried out using pooled tissues from two animals. *Solid lines*, computer-assisted curve fitting of experimental data to the monoexponential model described by eq. 1. See Table 3 for desipramine-induced changes in α_2-adrenoceptor turnover parameters. Note that desipramine markedly increased the disappearance (degradation) rate constant (k) of the receptor.

most widely used to study the metabolism of adrenoceptors *in vivo* and *in vitro* (16). In the present *in vivo* study the peptide-coupling agent EEDQ was selected as the irreversible antagonist for brain α_2-adrenoceptors. Although benextramine is considered to be the most specific irreversible ligand for this receptor, the drug does not cross the blood-brain barrier (20). Also, the use of phenoxybenzamine is limited by its higher affinity for α_1- than for α_2-adrenoceptors (30, 31) and by its relatively limited capacity to inactivate α_2-adrenoceptors *in vivo* (30, 32). In contrast, EEDQ appears to be more selective for α_2-adrenoceptors (33) and is able to almost completely inactivate these receptors *in vivo* (19). However, EEDQ can also block, at higher doses, dopaminergic (33–36), serotonergic (33), and cholinergic receptors (37). Meller *et al.* (33, 34) have reported the following order of sensitivity to and maximal degree of inactivation by EEDQ (6 mg/kg, subcutaneously, 24 hr) for rat brain neurotransmitter receptors: α_2 (95%) > α_1 (80%) > $D_2 \simeq D_1$ (70%) > 5-$HT_2 \simeq$ 5-HT_1 (60%) > β (25%) > muscarinic (10%) > opioid and γ-aminobutyric acid (<10%). Higher doses of EEDQ (up to 20 mg/kg) are needed to elicit substantial irreversible inactivation of most neurotransmitter receptors other than α-adrenoceptors. Therefore, the dose of EEDQ (4 mg/kg) used in the present study appears to be rather selective for α-adrenoceptors, especially of the α_2-adrenoceptor subtype. However, the ability of EEDQ to interact also with α_1-adrenoceptors and 5-HT receptors and the possibility that altered cross-talk between receptors could influence the quantitation of α_2-adrenoceptor turnover cannot be dismissed, although this possibility appears unlikely (38, 39).

In agreement with a previous study (40), EEDQ treatment induced marked decreases in α_2-adrenoceptor density in brain regions of the rat. Also, the progressive recovery of receptor density towards control values was observed without changes in receptor affinity for the radioligand as described in different tissues after the use of various irreversible receptor blockers (19, 20, 41, 42), which indicated that newly expressed brain α_2-adrenoceptors are similar to those previously inactivated. Brain α_2-adrenoceptors are heterogeneous in nature and the existence of at least two receptor subtypes (α_{2A} and α_{2B}) has been demonstrated (43). In the rat brain, the various regions appear to possess mixed populations of α_{2A}- and α_{2B}-adrenoceptors (Ref. 44; present study), although the precise distribution of the receptor subtypes has yet to be determined. The present results from the EEDQ protection experiments with oxymetazoline (selective α_{2A} agonist), ARC 239 (selective α_{2B} antagonist), and clonidine (mixed $\alpha_{2A/B}$ agonist) (43, 45, 46) clearly demonstrate that the peptide-coupling agent EEDQ does not discriminate between α_2-adrenoceptor subtypes. Therefore, the identification of more specific tools (subtype-selective alkylating and radioligand agents) will be required if accurate determination of the turnover of α_{2A}- and α_{2B}-adrenoceptors is to be achieved.

The overall results of this study demonstrate that regional differences exist in the turnover rates of brain α_2-adrenoceptors and, more relevantly, that desipramine-induced down-regulation of these receptors is associated with accelerated receptor turnover in the cerebral cortex and other brain regions.

The quantitative evaluations of receptor repopulation in the cerebral cortex, hippocampus, hypothalamus, striatum, and brainstem revealed significant regional differences in α_2-adrenoceptor turnover rates. The fastest receptor turnover rates were found in the hypothalamus, striatum, and brainstem, whereas the cerebral cortex and the hippocampus showed slower turnover rates. These regional variations do not appear to be related to the different proportions of α_{2A}- and α_{2B}-adrenoceptors in a particular brain region. Thus, the cortex and hypothalamus probably possess similar proportions of both receptor subtypes but differ markedly in α_2-adrenoceptor turnover rates; conversely, the cortex and brainstem possess different proportions of receptor subtypes but have similar receptor turnover parameters (see Fig. 1 and Table 1). It is noteworthy

TABLE 3

Effect of long term treatment with desipramine on α_2-adrenoceptor turnover parameters in the rat cerebral cortex

Receptor turnover parameters were calculated from data shown in Fig. 5 (see Experimental Procedures). Receptor turnover was assessed during desipramine (DMI) (3 mg/kg, intraperitoneally every 12 hr)-induced receptor down-regulation (phase 7–14 days) and during continued treatment without apparent receptor down-regulation (phase 21–35 days) (see Fig. 4). Other details are as for Table 1. Turnover parameters are expressed as the best fit values (mean ± standard error) calculated by the matrix inversion method, using the nonlinear regression program GraFit (26). Analysis of experimental data for the different periods of treatment with a sole monoexponential function (same r and k values) resulted in a significant decrease of goodness of fit, compared with a model without constraints (three different r and k values). ($F[2,68] = 7.15$; $p < 0.001$). Statistical comparisons were made by comparing the goodness of fit of simultaneous analyses with and without a set of constraints by means of an F test.

Phase of treatment	Turnover parameters			
	k	$t_{½}$	r	r/k
	day^{-1}	day	fmol/mg of protein/day	fmol/mg of protein
Control	0.18 ± 0.03	3.9 ± 0.7	12.9 ± 1.4	72.2 ± 6.5
DMI, 7–14 days	0.40 ± 0.11[a]	1.7 ± 0.3[a]	21.7 ± 4.1[a]	54.0 ± 6.1
DMI, 21–35 days	0.47 ± 0.07[b]	1.5 ± 0.3[b]	29.5 ± 3.6[c]	63.0 ± 2.7

[a] $p < 0.05$, compared with the corresponding control (F test).
[b] $p < 0.01$.
[c] $p < 0.001$.

that similar values for the turnover of this inhibitory α_2-adrenoceptor, for which there is evidence of a presynaptic location in the brain (47, 48), were obtained in the cortex and hippocampus, two brain regions that show similarities with respect to cytoarchitectural characteristics and innervation (49). The finding of a slightly faster turnover rate of α_2-adrenoceptors in the rat brainstem, compared with the cortex, is consistent with previous results in the rabbit (18). Recently, regional differences in the turnover of D_1 dopamine receptors also have been described in the rat brain (50).

The repopulation parameters of central α_2-adrenoceptors obtained here are in agreement with those previously reported (18, 19) and indicate that the turnover of these receptors in the central nervous system is very slow, compared with that in peripheral tissues such as the spleen (17), kidney, and adipose tissue (20). An interesting aspect of the recovery of α_2-adrenoceptors in the rat brain after EEDQ treatment is that the estimated limit for this process (r/k parameter) in some regions (cortex and hypothalamus) was significantly lower than the steady state density before blockade. This fact has also been reported for D_2 dopamine (51) and 5-HT_2 (52) receptors in senescent but not in mature rats. However, it is difficult to evaluate the real frequency of this finding, because most turnover studies have been analyzed using the linear transformation described by Mauger *et al.* (25), which is based on the assumption, not always statistically checked, that the limit of the recovery function is similar to the steady state receptor density before blockade. In fact, as shown in Fig. 2 (see legend), a semilogarithmic plot of transformed data that do not tend to receptor steady state resulted in a good correlation coefficient, which could suggest that the recovery process tends towards the density of receptors present before blockade. The observed lower limit (r/k) of the recovery function in the cortex and hypothalamus might be explained by the existence of a subpopulation of receptors subject to very slow metabolism or even not subject to turnover (51). Preliminary results from this laboratory[2] indicate that the aforementioned discrepancy also might be associated with the period of time allowed for receptor recovery after EEDQ (13 days in the present study) and/or with the use of agonist radioligands ([^{3}H]clonidine in the present study) that label preferentially the high affinity state of the α_2-adrenoceptor.

As expected (11), chronic treatment with desipramine resulted in a time-dependent modulation of brain α_2-adrenoceptors, with a marked initial reduction in receptor density followed by a progressive return to base-line values. It appeared, therefore, relevant to know whether the modulation of receptor density during receptor down-regulation was due to a modulation of receptor metabolism. Thus, the kinetics of recovery of brain α_2-adrenoceptors after EEDQ treatment revealed marked differences between the processes that regulate receptor density in control and desipramine-treated rats, which in turn can explain the time-dependent modulation of receptor density induced by the antidepressant drug (presence and absence of receptor down-regulation).

Desipramine treatment (phases 7–14 days and 21–35 days) resulted in marked increases in both the receptor appearance (synthesis) and disappearance (degradation) rate constants. Of these two changes, only the increase in the disappearance rate could explain the initial down-regulation of brain α_2-adrenoceptor density induced by desipramine, indicating that this accelerated degradation is the first change in the metabolism of the receptor induced by the antidepressant drug. Similarly to the reported increase in the turnover of α_1-adrenoceptors (53), β-adrenoceptors (54), and insulin receptors (55) as a consequence of exposure to an agonist, one can suggest that sustained stimulation of α_2-adrenoceptors by endogenous norepinephrine, after inhibition of neuronal uptake by desipramine (13, 15), increases the disappearance rate (degradation), leading to an initial reduction in receptor number. Conversely, the decrease in D_2 dopamine receptor turnover in rats treated with haloperidol (56) would agree with the concept of a relationship between occupancy of a receptor by an agonist and its disappearance rate. In this context, the observed increase in the appearance (synthesis) rate induced by desipramine treatment could be viewed as a later compensatory mechanism that would lead to restoration of brain α_2-adrenoceptor density. The relatively larger increase in receptor appearance observed during the phase of 21–35 days of desipramine treatment, compared with the phase of 7–14 days, is in good agreement with this hypothesis. The induction of such an adaptive increase in receptor synthesis tending to overcome the initial down-regulation of brain α_2-adrenoceptors would depend on the antidepressant drug, the dose, the duration of treatment, and even the brain region used and could explain the wide variability of results obtained in the different studies (11) that have evaluated the effects of antidepressant treatments on brain α_2-adrenoceptors.

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