0026-895X/94/061111-09$03.00/0

MOLECULAR PHARMACOLOGY, 46:1111-1119, 1994.

Comparative Recovery Kinetics of 5-Hydroxytryptamine 1A, 1B, and 2A Receptor Subtypes in Rat Cortex After Receptor Inactivation: Evidence for Differences in Receptor Production and Degradation

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Received June 7, 1994; Accepted September 16, 1994

SUMMARY

The present study investigates the comparative repopulation kinetics of 5-hydroxytryptamine (5-HT)$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{2A}$ receptors in rat cortex homogenates after irreversible receptor inactivation by *N*-ethoxycarbonyl-1,2-ethoxydihydroquinoline. Adult male rats were administered a single subcutaneous dose of vehicle (1:1 ethanol/water) or *N*-ethoxycarbonyl-1,2-ethoxydihydroquinoline (10 mg/kg), and the recovery of 5-HT receptor subtypes was measured at various times after injection (4–336 hr). Despite comparable control B_{max} values for 5-HT$_{1A}$ (84 ± 2 fmol/mg of protein) and 5-HT$_{1B}$ (94 ± 4 fmol/mg) subtypes, marked differences were noted in their 1) receptor production rates (r = 0.349 versus 0.235 fmol/mg of protein/hr), 2) receptor degradation rate constants (k = 0.0056 versus 0.0033 hr^{-1}), and 3) half-lives of receptor recovery (124.1 versus 212.5 hr). For 5-HT$_{2A}$ receptors, both r and k for agonist [(±)-1-(2,5-dimethoxy-4-[^{125}I]iodophenyl)-2-aminopropane]- or antagonist ([^{3}H]ketanserin)-labeled sites were markedly greater than the respective values for the 5-HT$_1$ subtypes. In addition, the significantly different B_{max} values for agonist- versus antagonist-labeled 5-HT$_{2A}$ receptors (79 ± 4 versus 206 ± 10 fmol/mg) were reflected exclusively as a 2.6-fold difference in receptor production rates, because degradation rate constants (k) were identical. Moreover, the stoichiometry of agonist-labeled to antagonist-labeled 5-HT$_{2A}$ receptors was not altered at any time point during recovery. These data indicate that 1) comparable receptor steady state B_{max} values for 5-HT receptor subtypes may be due to markedly different receptor kinetic parameters (r and k), 2) differences in r and k are greater between 5-HT receptor families (i.e., 5-HT$_1$ versus 5-HT$_2$) than among subtypes within a family (i.e., 5-HT$_{1A}$ versus 5-HT$_{1B}$), and, 3) despite marked changes in 5-HT$_{2A}$ receptor density, the percentage of receptors in the agonist-labeled, high affinity state is maintained.

Regulation of receptor density and/or sensitivity plays an important role in modulating neurotransmission in the central nervous system. Changes in receptor density occur during development, in the pathophysiology of psychiatric disorders, and in response to therapeutic interventions in these psychiatric disorders. However, the underlying kinetic mechanisms responsible for increases or decreases in receptor density have not often been elucidated.

For example, although the density of most 5-HT receptor subtypes is regulated during development, receptor density does not always seem to correspond to alterations in mRNA levels (1–5). In addition to the developmental regulation of receptor numbers, altered density and/or sensitivity of specific central nervous system 5-HT receptor subtypes have also been implicated in the pathophysiology of several psychiatric disorders, including aggressiveness, panic disorders, obsessive-compulsive disorders, anxiety, depression, and suicidal behavior (6). However, information regarding the mechanisms by which receptors are altered in these disorders is limited. Likewise, successful therapeutic approaches to these disorders have involved 5-HT receptor-selective drugs that themselves produce marked changes in receptor number and/or sensitivity, thus presumably restoring normal serotonergic neurotransmission. However, whereas treatment-induced influences on receptor density have been extensively documented, little is known about the mechanistic processes through which different classes of serotonergic drugs change the density of 5-HT receptor subtypes.

Classical receptor theory predicts receptor up-regulation in response to conditions that prevent receptors from being activated, such as denervation or chronic antagonist treatment. Conversely, receptor down-regulation often occurs in response

This study was supported in part by the Loyola University Potts Foundation and United States Public Health Service Grant DA07741.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); EEDQ, *N*-ethoxycarbonyl-1,2-ethoxydihydroquinoline; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; 8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin; ICYP, iodocyanopindolol; ANOVA, analysis of variance.

to conditions in which receptors are persistently activated, such as monoamine uptake blockade or chronic agonist exposure. However, 5-HT receptor density is frequently regulated in an anomalous fashion, with no consistent compensatory changes in response to agonists or antagonists (7, 8). Moreover, although changes in functional parameters seem to generally parallel changes in receptor density (9, 10), mRNA levels are not consistently altered (11–14).

Although most studies have measured changes in receptor density at steady state levels (i.e., B_{max}), receptor density actually reflects the net effect of the underlying turnover kinetics for a receptor population. That is, receptor density at any time (i.e., steady state receptor density, or $[B_{max}]_{ss}$) is not a static phenomenon but is a consequence of two processes, receptor production and receptor degradation (15). Changes in receptor steady state levels, therefore, can occur as a consequence of altered rates of receptor production, receptor degradation, or both. For example, the decreased density of 5-HT_{2A} receptors in aged Fisher 344 rats, compared with young adult rats, was shown to be the result of reductions in both receptor production and degradation rates (16). Some implications of the kinetic model of receptor regulation are that 1) changes in both receptor production and degradation processes may offset each other, producing no net change in receptor steady state levels, and 2) treatments that produce identical changes in receptor density may do so by differentially influencing either receptor production or degradation. However, whereas increases or decreases in receptor density have been reported after pharmacological activation or antagonism (17, 18), only a limited number of studies have attempted to examine the receptor kinetic mechanisms by which pharmacological agents induce receptor regulation (19–22). Therefore, examining the turnover kinetics of receptors may elucidate mechanisms of normal regulation as well as treatment-induced changes of 5-HT receptor subtypes that do not generally follow the classical model of receptor regulation.

Investigation of receptor turnover can be accomplished by monitoring receptor recovery after irreversible inactivation and using these data to determine the receptor production rate constant (r), the receptor degradation rate constant (k), and the half-life of receptor recovery ($t_{1/2}$). We have recently demonstrated that EEDQ can dose-dependently and irreversibly inactivate various 5-HT receptor subtypes and that a time-dependent receptor repopulation occurs (23). EEDQ is known to activate carboxyl groups at or near the ligand recognition site of the receptor. These activated carboxyl groups then cross-link with accessible free nucleophilic groups (e.g., free α-NH, -OH, or -SH), presumably present at the ligand recognition site, thus irreversibly inactivating the receptor (24). Because a single 10 mg/kg dose of EEDQ can produce >75% reduction in 5-HT receptor B_{max} (23), EEDQ serves as a useful tool to investigate the comparative basal turnover of various serotonin receptor subtypes.

The present study investigates the comparative turnover kinetics of 5-HT receptors, both within a family (5-HT_{1A} and 5-HT_{1B}) and between different families (i.e., 5-HT_1 versus 5-HT_2), in pharmacologically naive rats. In addition, we examine the turnover kinetic parameters for the same receptor (i.e., 5-HT_{2A}) labeled with an agonist or an antagonist. The present study provides data on the "basal" turnover of these 5-HT receptor subtypes, to establish similarities or differences in the kinetic parameters that dictate the steady state levels of these receptors. These data on the basal kinetic parameters for various 5-HT receptor subtypes may prove useful in subsequent studies investigating treatment-induced changes in densities of these 5-HT receptor subtypes.

Materials and Methods

Chemicals

EEDQ was obtained from Aldrich Chemical Co. (Milwaukee, WI). DOI was purchased from Research Biochemicals (Natick, MA). [^{3}H]8-OH-DPAT (162.9 Ci/mmol), [^{3}H]ketanserin (60.02 Ci/mmol), [^{125}I]ICYP (2200 Ci/mmol), and [^{125}I]DOI (2200 Ci/mmol) were all obtained from New England Nuclear (Boston, MA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Drug Administration

Adult, male, Sprague-Dawley rats (250–300 g; Zivic Miller, Zelienople, PA) were housed two/cage in a temperature-controlled (22–25°) environment with a 12-hr light-dark cycle. Food and water were available *ad libitum*. On the day of the experiment, rats were weighed and injected subcutaneously with either vehicle (ethanol/water, 1:1, v/v) or freshly dissolved EEDQ at a dose of 10 mg/kg of body weight. EEDQ-treated animals were sacrificed by decapitation, at various time points after injection (4, 24, 72, 120, 168, 240, and 336 hr). Vehicle-treated control rats were sacrificed at 4 and 336 hr after injection. Brains were collected in ice-cold 0.9% saline solution, and left and right cerebral cortices were quickly dissected out over ice. Tissue was inserted into cryotubes, frozen in liquid nitrogen, and stored at −70° until assayed.

Tissue Preparation

Cortical tissue was weighed, thawed, and homogenized (maximum setting, 10 sec) in a Tekmar tissumizer in 50 volumes of ice-cold 50 mM Tris·HCl buffer, pH 7.4 at room temperature. The homogenate was centrifuged (30,000 × g at 4°) for 15 min, and the pellet was resuspended in 50 volumes of the same buffer. After subsequent centrifugation and resuspension, the pellet was resuspended to a final concentration of 30 mg of original wet weight/ml, in 50 mM Tris·HCl buffer containing 10 mM $MgSO_4$ and 0.5 mM EDTA.

Radioligand Binding Assays

All assays were performed in 50 mM Tris buffer, pH 7.7 at 23°, containing 10 mM $MgSO_4$, 0.5 mM EDTA, 0.02% ascorbic acid, and 10 μM pargyline. Assays were initiated by the addition of tissue and were allowed to reach equilibrium before being rapidly filtered through Whatman GF/C filters in a Brandel cell harvester. Filters were washed with 15 ml of cold 50 mM Tris buffer. Filters containing tritiated ligands were counted by liquid scintillation counting, using a Beckman LS 7500 counter at an efficiency of 52%. Filters containing radioiodinated ligands were counted in a Micromedic 10/600 automatic γ counter at an efficiency of 82%. Proteins were determined by the Lowry method (25). All radioligands labeled a single homogeneous population of sites under the assay conditions used.

5-HT_{1A} receptors. 5-HT_{1A} receptors were labeled with increasing concentrations of [^{3}H]8-OH-DPAT (0.5–8.0 nM) in a 0.5-ml assay volume containing 3 mg of cortex homogenate/tube. Tubes were incubated with or without 10 μM 5-HT to define nonspecific binding. Assay mixtures were incubated for 60 min at room temperature before being rapidly filtered. Specific binding represented 74–93% of total binding over the concentration range used.

5-HT_{1B} receptors. [^{125}I]ICYP concentrations of 0.05–0.5 nM were used to label 5-HT_{1B} receptors. The final assay volume was 0.5 ml and contained 1.5 mg of cortical homogenate/tube. Isoproterenol (3 μM) was included in all assay tubes to prevent the binding of [^{125}I]ICYP to β-adrenergic receptors. Assay tubes were incubated for 30 min at room temperature before the mixtures were rapidly filtered as described. Nonspecific binding was defined by 10 μM 5-HT. Specific binding

ranged from 64 to 89% of total binding over the concentration range used.

5-HT$_{2A}$ receptors. 5-HT$_{2A}$ receptors were labeled with the antagonist [^{3}H]ketanserin (0.4–5.0 nM) in a 2.5-ml assay volume containing 3 mg of cortical homogenate and 30 nM prazosin (to preclude binding to α_1-adrenergic receptors) in each tube. Tissue was incubated for 30 min at 37°. Nonspecific binding was defined with 10 μM 5-HT. Specific binding ranged from 51 to 82% of total binding within the concentration range used. The density of 5-HT$_{2A}$ receptors was also measured with the agonist [^{125}I]DOI (0.1 nM) in the presence of increasing concentrations of unlabeled DOI (0.1–30 nM) and 1.5 mg of cortex tissue, in a final assay volume of 1.0 ml. Incubations were carried out for 90 min at room temperature. Nonspecific binding was defined by 10 μM 5-HT.

Data Analysis and Statistics

Radioligand binding data were fit to a rectangular hyperbolic equation by a nonlinear least-squares method, using GraphPAD InPlot (GraphPAD Software, San Diego, CA) to obtain K_d and B_{max} values. Receptor turnover kinetic parameters were determined by linear regression analysis of recovery data after conversion into logarithmic form. Data were analyzed by one-way ANOVA or two-way ANOVA followed by the Student-Newman-Keul test, using GraphPAD InStat or SigmaStat (Jandel Scientific, San Rafael, CA).

Results

Comparative recovery of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors. As shown in Table 1, a small (13%) but statistically significant ($p < 0.05$) difference was observed between the B_{max} values of 5-HT$_{1A}$ (83.7 ± 1.7 fmol/mg of protein) and 5-HT$_{1B}$ (94.3 ± 4.5 fmol/mg of protein) receptors in non-EEDQ-treated controls. As shown in Table 1, EEDQ (10 mg/kg) reduced the B_{max} of 5-HT$_{1A}$ receptors in cerebral cortex to 25% of non-EEDQ-treated controls at 4 hr after treatment. The B_{max} of 5-HT$_{1B}$ receptors was also reduced by EEDQ (10 mg/kg), to a comparable extent (23% of control), at 4 hr after treatment. After initial irreversible receptor inactivation, there was a time-dependent increase in the B_{max} of 5-HT$_{1A}$ receptors (Fig. 1A). However, at 336 hr (i.e., the last time point measured) the B_{max} (74.1 ± 1.7 fmol/mg of protein) of 5-HT$_{1A}$ receptors remained significantly lower (11% lower) than control values. Similarly, the B_{max} of 5-HT$_{1B}$ receptors increased in a time-dependent manner after irreversible receptor inactivation (Fig. 1A; Table 1). The recovery of 5-HT$_{1B}$ receptors appeared to require a longer time, because the percentage recovery at 336 hr for 5-HT$_{1B}$ receptors (74% of control) was significantly lower than that observed for 5-HT$_{1A}$ receptors (89% of control) (Fig. 1B; Table 1). As shown in Fig. 1B, when recovery data were plotted as a percentage of control B_{max} values, there was a significant difference ($p < 0.05$) in the overall repopulation times between 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor subtypes.

In addition to the marked reduction of the 5-HT$_{1A}$ receptor B_{max}, the affinity of the residual (i.e., EEDQ-insensitive) 5-HT$_{1A}$ receptors measured at 4 hr after treatment was significantly lower (control K_d = 0.95 ± 0.06 nM, residual receptor K_d = 7.81 ± 0.46 nM). The affinity of 5-HT$_{1A}$ receptors measured at 24 hr after treatment (K_d = 3.51 ± 0.46 nM) was also different from control values but was significantly higher than that observed at 4 hr. At subsequent time points, the K_d was not significantly different from control values. The lower affinity of the 5-HT$_{1A}$ receptors measured immediately after inactivation by EEDQ may represent the affinity of a subpopulation of EEDQ-insensitive 5-HT$_{1A}$ receptors, (i.e., those receptors uncoupled from the G protein or not fully inserted into the plasma membrane) or it may be due to an effect of EEDQ in altering the affinity of the remaining 5-HT$_{1A}$ receptor population. Because the measured K_d value represents the average affinity of all 5-HT$_{1A}$ receptors, the significant difference in affinity noted at 24 hr could be attributed to a greater contribution of the residual population of receptors to the total density of 5-HT$_{1A}$ receptors measured at this time point, when the amount of newly synthesized membrane-bound receptors is small. As the recovery of the higher affinity, membrane-bound (i.e., G protein-coupled), 5-HT$_{1A}$ receptors proceeds, the contribution of the lower affinity, residual component to the overall affinity becomes smaller and the affinity of the 5-HT$_{1A}$ receptors at subsequent time points is not significantly different from controls. In contrast, there were no significant differences in the affinity of 5-HT$_{1B}$ receptors (K_d = 85 pM) at any time point after EEDQ inactivation.

Calculation of receptor kinetic parameters. Recovery of various serotonin receptors and other monoamine receptors

TABLE 1

Time-dependent recovery of rat cortical 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors after irreversible receptor inactivation by EEDQ

Data represent the mean ± standard error of B_{max} values obtained from three to six rats at each time point. Data are tabulated both as time-dependent changes in actual receptor density and as percentages of respective control values. The actual densities of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors in control and EEDQ-treated rats were analyzed by two-way ANOVA, followed by the Student-Newman-Keul test. When recovery data were plotted as a percentage of respective controls, two-way ANOVA indicated a significant difference ($p < 0.05$) in the overall recovery of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors.

Treatment/recovery period	5-HT$_{1A}$ B_{max}		5-HT$_{1B}$ B_{max}	
hr	*fmol/mg of protein*	*% of control*	*fmol/mg of protein*	*% of control*
Control	83.7 ± 1.7	100.0 ± 2.0	94.3 ± 4.5[a]	100.0 ± 4.8
4	21.2 ± 5.1[b]	25.3 ± 6.1	22.1 ± 5.1[b]	23.4 ± 5.4
24	27.3 ± 1.7[b]	32.6 ± 2.0	24.2 ± 0.8[b]	25.7 ± 0.9
72	45.5 ± 1.8[b]	54.4 ± 2.1	33.9 ± 1.5[b]	35.9 ± 1.6[c]
120	55.7 ± 2.4[b]	68.9 ± 2.9	43.3 ± 1.3[b]	45.9 ± 1.4[c]
168	60.8 ± 0.4[b]	72.6 ± 0.5	47.4 ± 2.5[b]	50.8 ± 2.6[c]
240	69.6 ± 0.6[d]	83.1 ± 0.7	58.3 ± 4.5[b]	61.8 ± 4.8[c]
336	74.1 ± 1.7[e]	88.5 ± 2.0	69.4 ± 0.9[b]	73.6 ± 0.9[c]

[a] Significant difference ($p < 0.05$) between the control B_{max} values for 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors.
[b] Significant difference ($p < 0.001$) from respective control.
[c] Significant difference ($p < 0.01$) in the percentage recovery of 5-HT$_{1B}$ receptors from the corresponding value for 5-HT$_{1A}$ receptors at the same time point, as determined by the Student-Newman-Keul test.
[d] Significant difference ($p < 0.01$) from respective control.
[e] Significant difference ($p < 0.05$) from respective control.

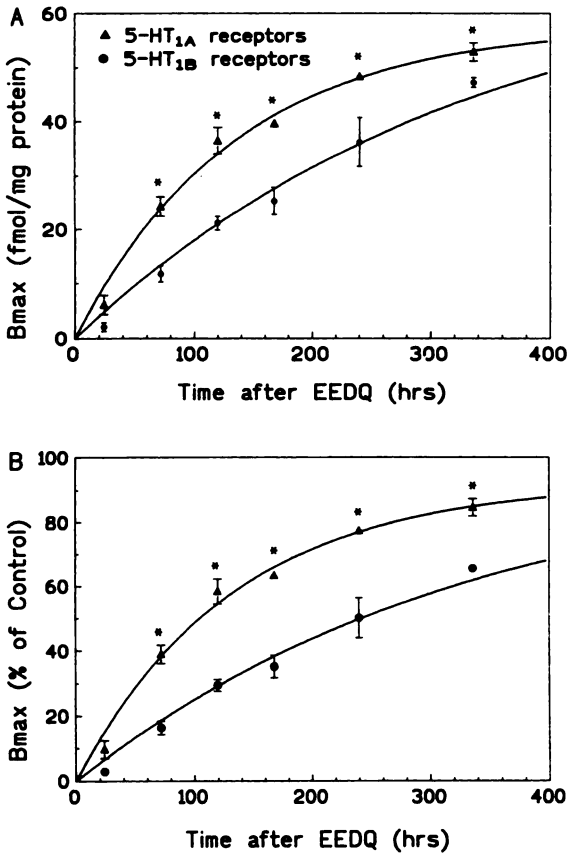

Fig. 1. A, Comparative recovery of 5-HT_{1A} and 5-HT_{1B} receptors after irreversible inactivation by EEDQ. Data represent the mean ± standard error of B_{max} values from three to six rats at each time point. Residual (EEDQ-insensitive) receptor densities for 5-HT_{1A} and 5-HT_{1B} subtypes (21.2 and 22.1 fmol/mg of protein for the 5-HT_{1A} and 5-HT_{1B} subtypes, respectively) were subtracted from B_{max} values for EEDQ-treated and nontreated controls. These experimentally determined data were iteratively fit to a monoexponential equation by computerized nonlinear regression. *, Significant difference (at least $p < 0.05$) between the receptor densities of the 5-HT_{1A} and 5-HT_{1B} subtypes, as indicated by two-way ANOVA followed by the Student-Newman-Keul test. B, Comparative recovery of 5-HT_{1A} and 5-HT_{1B} receptor density, expressed as a percentage of non-EEDQ-treated control $[B_{max}]_{ss}$. Data represent the mean ± standard error of three to six rats at each time point. Residual receptor values were subtracted from both $[B_{max}]_{ss}$ and $[B_{max}]_t$ at each time point, before the data were expressed as a percentage of control and fit to a monoexponential equation. *, Significant difference ($p < 0.05$) in the percentage recovery, as determined by two-way ANOVA followed by the *post hoc* Student-Newman-Keul test.

after EEDQ inactivation has been demonstrated to follow a monoexponential association model (16, 26–29). This model is based on two implicit assumptions, that 1) the receptor production rate follows zero-order kinetics (i.e., receptor production occurs at a constant rate), and 2) the receptor degradation raae follows first-order kinetics (i.e., the degradation rate depends on the concentration of receptors at any given time point).

Because receptor production is a zero-order process, the receptor production rate is equivalent to the receptor production rate constant. Therefore, the repopulation kinetics of a receptor after irreversible inactivation can be defined by the monoexponential association eq. 1.

$$[B_{max}]_t = (r/k)(1 - e^{-kt}) \quad (1)$$

In this equation, $[B_{max}]_t$ is the receptor concentration (femtomoles/milligram of protein) at time t, r is the receptor production rate constant (femtomoles/milligram of protein/hour), and k is the receptor degradation rate constant ($hour^{-1}$).

As time proceeds to infinity, receptor recovery becomes complete, and receptors achieve steady state levels ($[B_{max}]_{ss}$). At this time, as t approaches infinity, the term e^{-kt} approaches 0 and therefore eq. 1 may be restated as

$$[B_{max}]_t = [B_{max}]_{ss} = r/k \quad (2)$$

Substituting $[B_{max}]_{ss}$ for the term r/k and performing a logarithmic transformation of eq. 1 yields eq. 3.

$$\ln [B_{max}]_{ss}/([B_{max}]_{ss} - [B_{max}]_t) = kt \quad (3)$$

This is essentially the equation for a straight line ($y = mx + b$, where $b = 0$). In eq. 3, because we have experimentally determined B_{max} both in control animals (i.e., $[B_{max}]_{ss}$) and in EEDQ-treated animals at each time point t during recovery (i.e., $[B_{max}]_t$), we can obtain k, the receptor degradation rate constant, directly from the slope of the plot of $\ln [B_{max}]_{ss}/([B_{max}]_{ss} - [B_{max}]_t)$ versus time. Fig. 2 is the plot of this equation as applied to the recovery of 5-HT_{1A} and 5-HT_{1B} receptors. The close fit of our data to this equation (correlation coefficients of 0.990 and 0.996 for 5-HT_{1A} and 5-HT_{1B} receptors, respectively) validates the appropriateness of this model for determining the recovery kinetics of 5-HT_{1A} and 5-HT_{1B} receptors. In these calculations, the residual B_{max} values (i.e., the B_{max} values at 4 hr after EEDQ treatment) for both 5-HT receptor subtypes have been subtracted from B_{max} values determined at each of the individual recovery time points ($[B_{max}]_t$ values) as well as from control values in non-EEDQ-treated rats. This modification allows the values obtained to more accurately represent the turnover kinetics of only that portion of receptors actually undergoing recovery. A quantitative assessment of this difference in k can be seen in Fig. 2. The semilogarithmic representation of receptor recovery data reveals markedly different slopes for the 5-HT_{1A} receptors (0.0056 hr^{-1}) and the 5-HT_{1B} receptors (0.0033 hr^{-1}).

Implicit in eq. 3 is the fact that the time to reach any

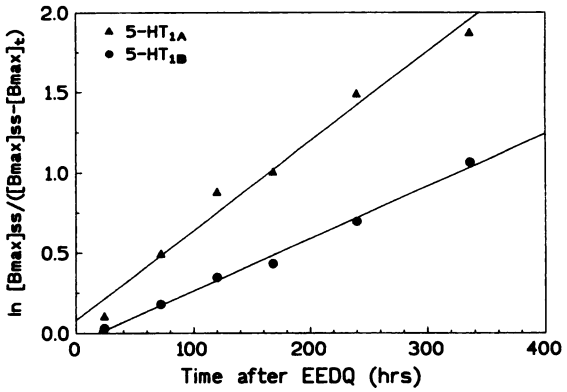

Fig. 2. Semilogarithmic plot of the time course of 5-HT_{1A} and 5-HT_{1B} receptor recovery in rat cerebral cortex. $[B_{max}]_{ss}$ is the steady state receptor density obtained from non-EEDQ-treated control rats. $[B_{max}]_t$ is the receptor density at various time points after EEDQ treatment. Levels of residual receptors left after EEDQ treatment were subtracted from both terms, $[B_{max}]_{ss}$ and $[B_{max}]_t$, used in this equation. Correlation coefficients for the 5-HT_{1A} and 5-HT_{1B} receptors were 0.996 and 0.992, respectively. Rate constants for receptor degradation (k) were 0.0056 and 0.0033 hr^{-1} for the 5-HT_{1A} and 5-HT_{1B} subtypes, respectively.

particular percentage of control $[B_{max}]_{ss}$ values is dependent only on k and not on r. Therefore, alterations in k would be expected to alter the time required to achieve a given percentage of the control $[B_{max}]_{ss}$. As shown in Fig. 1B and stated previously, when recovery of 5-HT_{1A} and 5-HT_{1B} was plotted as a percentage of maximal recoverable receptors (i.e., $[B_{max}]_{ss}$) there was a significant difference ($p < 0.05$) between these receptors in their repopulation times. Substitution of the respective values of k into eq. 2 for $[B_{max}]_t = [B_{max}]_{ss}/2$ yields receptor half-lives ($t_{½} = \ln 2/k = 0.693/k$) of 124.1 and 212.5 hr for the 5-HT_{1A} and 5-HT_{1B} receptors, respectively. Because the recovery time ($t_{½}$) was found to differ significantly ($p < 0.05$) between 5-HT_{1A} and 5-HT_{1B} receptors, the difference in the calculated degradation rate constants observed must also be significantly different. Therefore, because we have experimentally determined $[B_{max}]_{ss}$ and have calculated k, the receptor production rate (r) may be obtained directly from restating the equation $[B_{max}]_{ss} = r/k$ as $r = k[B_{max}]_{ss}$.

As mentioned previously, for all calculations of receptor recovery kinetic parameters (r and k), the value of $[B_{max}]_{ss}$ used is the $[B_{max}]_{ss}$ in non-EEDQ-treated control rats minus the residual number of receptors left 4 hr after EEDQ treatment, because this better approximates the kinetic parameters of only that portion of receptors undergoing recovery. As shown in Table 2, receptor production rates for 5-HT_{1A} and 5-HT_{1B} receptors were found to be different (0.349 and 0.235 fmol/mg of protein/hr, respectively). A summary of all kinetic parameters obtained for the 5-HT_{1A} and 5-HT_{1B} receptors is shown in Table 2.

Comparative recovery kinetics of agonist ([^{125}I]DOI)- and antagonist ([^{3}H]ketanserin)-labeled 5-HT_{2A} receptors. In non-EEDQ-treated control animals, [^{125}I]DOI labeled the high affinity, G protein-coupled state of 5-HT_{2A} receptors (79.1 ± 3.6 fmol/mg of protein), which comprised 38% of the total [^{3}H]ketanserin-labeled 5-HT_{2A} receptors (206.3 ± 10.4 fmol/mg of protein). As shown in Table 3, EEDQ did not differentially affect these sites, reducing the B_{max} values of agonist- and antagonist-labeled 5-HT_{2A} receptors to comparable extents (6.8 and 9.3% of control, respectively). After initial inactivation, there was a time-dependent increase in the B_{max} of the agonist ([^{125}I]DOI)-labeled receptors (Fig. 3A), and

TABLE 2

Summary of recovery kinetic parameters for cortical 5-HT_{1A} and 5-HT_{1B} receptors

$[B_{max}]_{ss}$ is the steady state receptor B_{max} value in non-EEDQ-treated control rats, r is the receptor production rate, k represents the receptor degradation rate, and $t_{½}$ is the half-life of receptor repopulation. For calculation of r, k, and $t_{½}$, values for residual receptors left after EEDQ treatment (21.2 and 22.1 fmol/mg of protein for the 5-HT_{1A} and 5-HT_{1B} subtypes, respectively) were subtracted from the B_{max} values for both control and EEDQ-treated animals, to ensure that the receptor kinetic parameters obtained (r, k, and $t_{½}$) represent only those receptors actually undergoing recovery.

Parameters	5-HT_{1A}	5-HT_{1B}
$[B_{max}]_{ss}$ (fmol/mg of protein)	83.7 ± 1.7	94.3 ± 4.5[a]
r (fmol/mg of protein/hr)	0.349	0.235
k (hr^{-1})	0.0056	0.0033[b]
$t_{½}$ (hr)	124.1	212.5[c]

[a] Statistically significant difference ($p < 0.05$) between the $[B_{max}]_{ss}$ values of 5-HT_{1A} and 5-HT_{1B} receptors in non-EEDQ-treated controls.

[b] Statistically significant difference in the receptor degradation rate constants (k) for these receptor subtypes. This difference is inferred because k is exclusively dependent on $t_{½}$ values [$t_{½} = (\ln 2)/k$], which were significantly different.

[c] Significantly different ($p < 0.05$) half-lives of recovery ($t_{½}$) between the 5-HT_{1A} and 5-HT_{1B} subtypes, as determined by two-way ANOVA of receptor B_{max} values expressed as percentage of control.

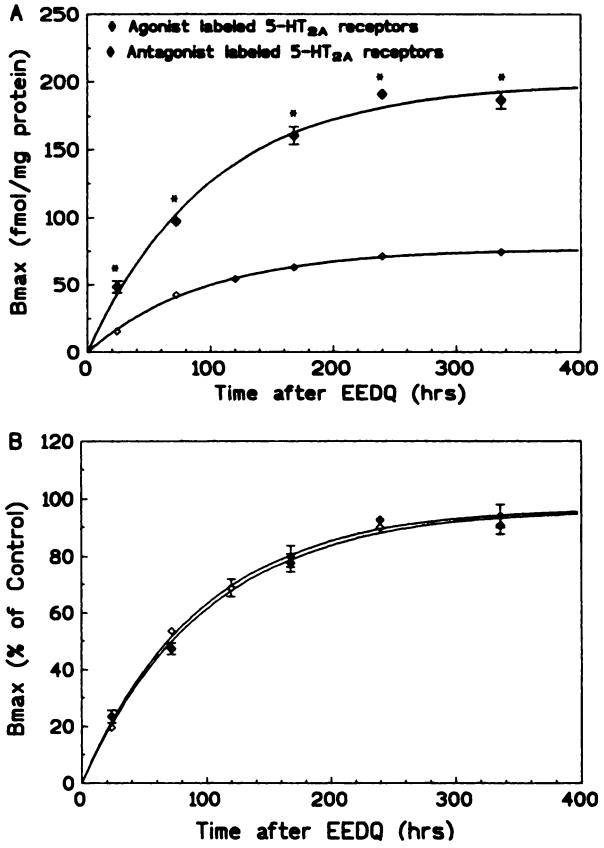

Fig. 3. A, Comparative recovery of agonist ([^{125}I]DOI)- and antagonist ([^{3}H]ketanserin)-labeled 5-HT_{2A} receptor density after irreversible receptor inactivation by EEDQ. Data represent the mean ± standard error of B_{max} values obtained from three to six rats at each time point, from which values for residual receptors (5.4 and 19.2 fmol/mg of protein for the agonist- and antagonist-labeled 5-HT_{2A} receptors, respectively) have been subtracted. These experimentally determined data were then iteratively fit to a monoexponential equation by computerized nonlinear regression. *, Significant difference (at least $p < 0.01$) between the receptor densities of agonist- and antagonist-labeled 5-HT_{2A} receptors, as determined by two-way ANOVA followed by the Student-Newman-Keul test. B, Comparative recovery of [^{125}I]DOI- and [^{3}H]ketanserin-labeled 5-HT_{2A} receptors expressed as a percentage of non-EEDQ-treated control $[B_{max}]_{ss}$. Data represent the mean ± standard error of three to six rats at each time point. Values for residual receptors were subtracted from both $[B_{max}]_{ss}$ and $[B_{max}]_t$ at each time point, before the data were expressed as a percentage of control and fit to a monoexponential equation. Two-way ANOVA indicated no significant difference in the overall recovery between the [^{125}I]DOI- and [^{3}H]ketanserin-labeled 5-HT_{2A} receptors.

at 336 hr the receptor B_{max} (79.7 ± 3.2 fmol/mg of protein) was equivalent to control values. Similarly, the B_{max} of [^{3}H]ketanserin-labeled 5-HT_{2A} receptors increased in a time-dependent manner (Fig. 3A), and at 336 hr the receptor B_{max} (205.9 ± 6.1 fmol/mg of protein) was not significantly different from control values (Table 3). The affinity of 5-HT_{2A} receptors labeled with either the agonist [^{125}I]DOI (0.39 ± 0.02 nM) or the antagonist [^{3}H]ketanserin (0.65 ± 0.06 nM) was not altered by EEDQ treatment or at any time point during recovery.

The recovery of 5-HT_{2A} receptors could also be defined by a monoexponential association equation (eq. 1). Receptor recovery data were fit to eq. 2 and the receptor degradation rate constants (k) were obtained from the slope of the plot of ln $[B_{max}]_{ss}/([B_{max}]_{ss} - [B_{max}]_t)$ versus time (Fig. 4). The degradation constant (k) was found to be nearly identical for [^{125}I]DOI-

TABLE 3

Time-dependent recovery of [^{125}I]DOI- and [^{3}H]ketanserin-labeled cortical 5-HT$_{2A}$ receptors after irreversible receptor inactivation by EEDQ

Data represent the mean ± standard error of B_{max} values obtained from three to six rats at each time point. Data are tabulated both as time-dependent changes in actual receptor density and as percentages of control values. The actual receptor densities for both agonist ([^{125}I]DOI)- and antagonist ([^{3}H]ketanserin)-labeled 5-HT$_{2A}$ receptors in control and EEDQ-treated rats were analyzed by two-way ANOVA, followed by the Student-Newman-Keul test. When recovery data were plotted as a percentage of respective controls, two-way ANOVA indicated no significant differences in overall receptor recovery between agonist- and antagonist-labeled 5-HT$_{2A}$ receptors.

Treatment/recovery period	[^{125}I]DOI B_{max}		[^{3}H]Ketanserin B_{max}	
hr	*fmol/mg of protein*	*% of control*	*fmol/mg of protein*	*% of control*
Control	79.1 ± 3.6	100.0 ± 4.5	206.3 ± 10.4[a]	100.0 ± 5.1
4	5.4 ± 1.6[b]	6.8 ± 2.0	19.2 ± 3.8[b]	9.3 ± 1.8
24	20.9 ± 0.6[b]	26.4 ± 0.8	67.6 ± 4.6[b]	32.8 ± 2.2
72	47.6 ± 1.4[b]	60.2 ± 1.8	116.6 ± 4.1[b]	56.5 ± 1.99
120	59.7 ± 2.4[c]	75.5 ± 3.0	121.2 ± 4.2[b]	58.7 ± 2.1
168	68.6 ± 2.9[d]	86.7 ± 3.7	179.5 ± 6.6[b]	87.0 ± 3.2
240	76.5 ± 0.2	96.7 ± 0.2	210.1 ± 1.8	101.8 ± 0.87
336	79.7 ± 3.2	100.8 ± 4.1	205.9 ± 6.1	99.8 ± 2.96

[a] Significantly different ($p < 0.001$) control B_{max} values for the [^{125}I]DOI- versus [^{3}H]ketanserin-labeled 5-HT$_{2A}$ receptors.
[b] Significant difference ($p < 0.001$) from respective control.
[c] Significant difference ($p < 0.01$) from respective control.
[d] Significant difference ($p < 0.05$) from respective control.

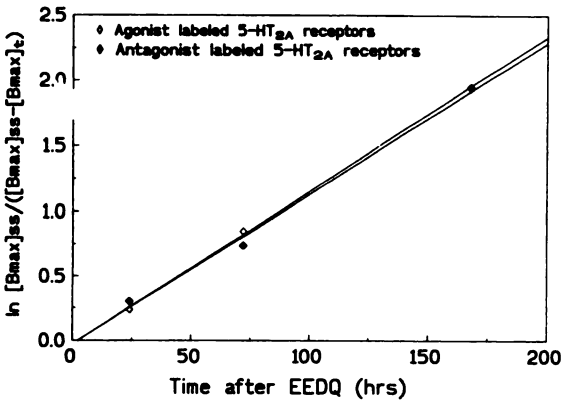

Fig. 4. Semilogarithmic plot of the time course of receptor recovery of [^{125}I]DOI- and [^{3}H]ketanserin-labeled 5-HT$_{2A}$ receptors in rat cerebral cortex. $[B_{max}]_{ss}$ is the steady state receptor density obtained from non-EEDQ-treated control rats. $[B_{max}]_t$ is the receptor density at various time points after EEDQ treatment. Values for residual receptors left after EEDQ treatment were subtracted from both terms, $[B_{max}]_{ss}$ and $[B_{max}]_t$, used in this equation. Correlation coefficients for the [^{125}I]DOI- and [^{3}H]ketanserin-labeled 5-HT$_{2A}$ receptors were 0.998 and 0.996, respectively. Rate constants for receptor degradation (k) were 0.0117 and 0.0118 hr^{-1} for the agonist- and antagonist-labeled 5-HT$_{2A}$ receptors, respectively.

and [^{3}H]ketanserin-labeled 5-HT$_{2A}$ receptors (k = 0.0117 and 0.0118 hr^{-1}, respectively). The high correlation coefficients obtained in the logarithmic-linear plots of receptor recovery for agonist-labeled (0.998) and antagonist-labeled (0.996) 5-HT$_{2A}$ receptors are consistent with the model (eq. 1) for 5-HT$_{2A}$ receptor repopulation after irreversible inactivation. As described for the calculation of the kinetic parameters for 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, the residual B_{max} values for the 5-HT$_{2A}$ receptors were subtracted from the recovery data, as well as from the control data points used for calculating the receptor kinetic parameters r and k.

The quantitative assessment of k for the agonist- and antagonist-labeled 5-HT$_{2A}$ sites indicates that k is identical in the two cases (Fig. 4; Table 4). As shown in Fig. 3B, where receptor recovery is plotted as a percentage of control, there was no significant difference in the time course of recovery between [^{125}I]DOI- and [^{3}H]ketanserin-labeled 5-HT$_{2A}$ receptors. Because the time required to reach any particular percentage of control B_{max} values is exclusively dependent on k [$t_{½} = (\ln 2)/k$], the half-lives of receptor repopulation for the agonist-labeled and antagonist-labeled 5-HT$_{2A}$ receptors were not significantly different ($t_{½}$ = 59.4 and 58.9 fmol/mg of protein/hr, respectively).

TABLE 4

Summary of recovery parameters for agonist ([^{125}I]DOI)- and antagonist ([^{3}H]ketanserin)-labeled 5-HT$_{2A}$ receptors in cortex

$[B_{max}]_{ss}$ is the steady state receptor B_{max} value in non-EEDQ-treated control rats, r is the receptor production rate, k represents the receptor degradation rate, and $t_{½}$ is the half-life of receptor repopulation. For calculation of r, k, and $t_{½}$, values for residual receptors left after EEDQ treatment (5.4 and 19.2 fmol/mg of protein for the agonist- and antagonist-labeled 5-HT$_{2A}$ receptors, respectively) were subtracted from the B_{max} values for both control and EEDQ-treated animals, to ensure that the receptor kinetic parameters obtained (r, k, and $t_{½}$) represent only those receptors actually undergoing recovery. Two-way ANOVA of receptor recovery expressed as percentage of control indicated that overall receptor recovery kinetics were not significantly different. This implies that $t_{½}$ and therefore k are not significantly different.

Parameters	5-HT$_{2A}$ ([^{125}I]DOI)	5-HT$_{2A}$ ([^{3}H]ketanserin)
$[B_{max}]_{ss}$ (fmol/mg of protein)	79.1 ± 3.6	206.3 ± 10.4[a]
r (fmol/mg of protein/hr)	0.859	2.202[b]
k (hr^{-1})	0.0117	0.0118
$t_{½}$ (hr)	59.4	58.9

[a] Significant difference ($p < 0.001$) between the $[B_{max}]_{ss}$ values of the agonist ([^{125}I]DOI)- and antagonist ([^{3}H]ketanserin)-labeled 5-HT$_{2A}$ receptors in non-EEDQ-treated controls.
[b] Inferred significant difference in receptor production rates (r), obtained from the equation $[B_{max}]_{ss} = r/k$.

The calculated receptor production rate for the agonist-labeled 5-HT$_{2A}$ receptors (r = 0.859 fmol/mg of protein/hr) was found to be markedly different from the receptor production rate for the antagonist-labeled 5-HT$_{2A}$ receptors (r = 2.202 fmol/mg of protein/hr). As indicated in Fig. 3A and Table 3, the B_{max} values for the agonist-labeled and antagonist-labeled 5-HT$_{2A}$ receptors are significantly different ($p < 0.001$). Therefore, because there was no significant difference between agonist- and antagonist-labeled 5-HT$_{2A}$ receptors in the half-life of recovery (and consequently in k), despite the $[B_{max}]_{ss}$ values being significantly different, the differences in receptor production rates for agonist- and antagonist-labeled 5-HT$_{2A}$ receptors must be significant (eq. 3). Indeed, the receptor production rate for the agonist-labeled sites, which was 38% of the respective value for the antagonist-labeled 5-HT$_{2A}$ receptors, reflected the proportion of receptor densities for the agonist- and antagonist-

labeled 5-HT_{2A} receptors in control animals and throughout recovery. Receptor turnover parameters for the agonist- and antagonist-labeled 5-HT_{2A} sites are summarized in Table 4.

Discussion

The present study investigated the comparative receptor kinetic parameters responsible for steady state control levels (i.e., $[B_{max}]_{ss}$) of 5-HT receptor subtypes, both within a family (e.g., 5-HT_{1A} and 5-HT_{1B}) and between families (e.g., 5-HT_1 versus 5-HT_2) in the same brain region (e.g., cerebral cortex) in rats. In addition, the turnover of 5-HT_{2A} receptors labeled by an agonist versus an antagonist radioligand was assessed. Because 5-HT_{1A} and 5-HT_{1B} receptor subtypes are constituents of the 5-HT_1 receptor family and have intronless genes, fairly high sequence homology, a common transduction mechanism (inhibition of adenylyl cyclase), and some similarity in their pharmacology (30), these receptor subtypes might be expected to be under similar transcriptional and post-translational regulatory controls and to exhibit similar receptor production rates. Given the high degree of amino acid sequence homology and similarities in tertiary structure (both are seven-transmembrane domain G protein-linked receptors), these subtypes might also be expected to exhibit comparable receptor degradation rates. In contrast, 5-HT_{2A} receptors, which have less homology with the 5-HT_1 class of receptors, would be expected to differ markedly from both the 5-HT_{1A} and 5-HT_{1B} subtypes in both receptor production and degradation rates.

However, the present study indicates that in rat cerebral cortex the 5-HT_{1A} and 5-HT_{1B} receptor subtypes exhibit markedly different repopulation kinetics. The 5-HT_{1B} receptor had a significantly longer half-life of recovery (212.5 hr) and consequently a significantly different degradation rate constant, compared with that of the 5-HT_{1A} receptor (124.1 hr). To achieve a comparable $[B_{max}]_{ss}$, given the lower receptor degradation rate, the 5-HT_{1B} subtype also exhibited a markedly lower receptor production rate (r) than did the 5-HT_{1A} receptor.

Although differential production rates might be attributed to these receptor subtypes being distinct gene products and therefore possibly subject to distinct regulatory processes with respect to receptor production, this does not completely explain why the degradation rates of these receptor subtypes are so markedly different. One explanation for differences in both receptor production and degradation could be that these receptor subtypes are differentially localized on cell types with different rates or distinct mechanisms of protein synthesis and metabolism *per se*. Alternatively, the differences may be due not only to the localization of the receptors on different cells but also to different inherent rates of receptor production and degradation for each subtype. Although there is no unequivocal evidence for differential receptor protein metabolism in different neuronal cells, there is evidence to suggest differential localization (with some overlap) of the 5-HT_{1A} and 5-HT_{1B} receptor subtypes in the cortex. Within various regions of the neocortex, cells expressing 5-HT_{1A} mRNA are found predominantly in deep cortical layers (layers IV–VI), and corresponds to the neuroanatomical distribution of [^{3}H]8-OH-DPAT-labeled 5-HT_{1A} receptors (31). For example, in the rat cingulate cortex, peak specific [^{3}H]8-OH-DPAT binding occurs in layer Vb, with only moderate binding in layers Va and VI and lowest binding in layers I-IV (32). In contrast, [^{125}I]ICYP-labeled 5-HT_{1B} receptor density is highest in layers Ia–Ic and markedly lower in the deeper layers. Moreover, evidence from pharmacological and autoradiographic studies suggests that 5-HT_{1B} receptors are largely presynaptic on axons and terminals (33, 34). In contrast, 5-HT_{1A} receptors in cortex are predominantly postsynaptic, and the majority are located on pyramidal cells in layers V and VI (35). These data suggest that the differential rates of receptor production and degradation for 5-HT_{1A} and 5-HT_{1B} receptors could be attributed to a greater extent to differences in the synthetic and catabolic mechanisms between serotonergic and non-serotonin-containing neurons than to differences in receptor-specific processes within the same cell.

Therefore, in comparing the subtypes of the 5-HT_1 family with 5-HT_{2A} receptors, it may be important to consider the differences in both the neuroanatomical localization and the biochemical characteristics of these receptors. The 5-HT_{2A} receptor has an overall low level of sequence homology with the 5-HT_{1A} receptor (36), as well as a distinct transduction mechanism (phosphoinositide hydrolysis). Also, with respect to the neuroanatomical localization, 5-HT_{2A} receptors exhibit maximal density in layers IIIb and IV (37, 38), as well as fairly high density in layer I, which partially overlaps with the distribution of 5-HT_{1B} receptors (high levels in layers Ia–Ic) but differs markedly from the distribution of 5-HT_{1A} receptors (lowest binding in layers I–IV). Moreover, in rat cortex 5-HT_{2A} receptors are predominantly postsynaptic and localized on non-serotonergic neurons (39). Based on the similarity in neuroanatomic distribution of 5-HT_{2A} and 5-HT_{1B} receptors, it might be expected that the receptor kinetic paramenters of the 5-HT_{2A} receptor would be more like those of the 5-HT_{1B} receptor than those of the 5-HT_{1A} receptor.

However, our data demonstrate that both receptor production and degradation rates for the 5-HT_{2A} receptor differ markedly from the respective kinetic parameters obtained for either the 5-HT_{1A} or 5-HT_{1B} subtype. The receptor degradation rate constant for the 5-HT_{2A} receptor labeled with either an agonist or an antagonist was greater than that for either the 5-HT_{1A} (2-fold) or the 5-HT_{1B} (3.6-fold) subtype, respectively. Likewise, the receptor production rates for either agonist- or antagonist-labeled 5-HT_{2A} receptors were markedly greater than those for the 5-HT_{1A} or 5-HT_{1B} subtypes, suggesting that these differences in kinetic parameters could not be attributed merely to differences in agonist or antagonist receptor recognition sites. In fact, our data from the agonist- and antagonist-labeled 5-HT_{2A} receptors suggest that the receptor production rate (r) obtained by using an agonist radioligand may actually underestimate the true production rate for a receptor population. Likewise, in the case of 5-HT_{1A} and 5-HT_{1B} receptors, which were labeled with an agonist and an antagonist, respectively, the difference in receptor production rates (r) between these subtypes may actually be greater than our results indicate. These data suggest that the differences in receptor turnover kinetics between the 5-HT_1 and 5-HT_2 families may be partly attributed to their distribution on different cell populations but are more likely due to the differential biochemical characteristics of these receptor subtypes.

Because the receptor production rate (r) is composed of the summed rates of transcription, translation, and post-translational processes, it is not possible, using the present experimental paradigm, to discern which one or more of these steps may be different. Likewise, the degradation rate is also a multistep process, and it is not possible to identify the specific

steps at which changes in rate may contribute to differences in the overall degradation rate constant. Nevertheless, receptor recovery after irreversible inactivation can provide valuable information regarding which component of the regulatory process (i.e., production or degradation) is responsible for maintaining and/or altering receptor steady state levels after specific experimental procedures. In this respect, this method may prove to be more useful than more direct approaches, such as measurement of mRNA levels. For example, mianserin (a 5-HT_{2A} antagonist) causes down-regulation of 5-HT_{2A} receptors, whereas SR46349B (also an antagonist) has been shown to cause up-regulation of 5-HT_{2A} receptors (13, 14). However, neither of these changes in 5-HT_{2A} receptor levels has been shown unequivocally to be related to changes in mRNA levels, suggesting that there may not be a change in receptor "production." However, because changes in mRNA levels may not necessarily result in increased protein synthesis and because changes in mRNA levels represent only one of the steps that may alter receptor production rates, the absence of alterations in mRNA levels cannot be interpreted as conclusive evidence that changes in the overall rate of receptor production have not occurred. Moreover, as the receptor turnover model (eq. 3) suggests, changes in $[B_{max}]_{ss}$ may also be due to changes in k, the receptor degradation rate constant. Therefore, in evaluating the mechanisms of treatment-induced changes in B_{max}, the receptor turnover approach could identify changes in the overall receptor production and degradation processes, whereas measuring mRNA would be less useful in this respect.

The present studies demonstrate that the agonist ([^{125}I]DOI) labeled 38% of the antagonist ([^{3}H]ketanserin)-labeled 5-HT_{2A} receptors, both at steady state and throughout the recovery time period. These data indicate that, despite marked alterations in receptor density (10–90% of control values), the ratio of agonist-labeled to antagonist-labeled sites, or the contribution of agonist-labeled sites to the antagonist-labeled 5-HT_{2A} receptor population, is maintained. Based on these data, one would predict that treatment-induced changes in receptor B_{max} values, which are typically <40%, would not cause changes in the stoichiometry of agonist- and antagonist-labeled sites unless the treatment directly affected the efficiency of receptor-G protein coupling.

Taken as a whole, the present data indicate that, within the same brain region, 1) comparable steady state densities of 5-HT receptor subtypes can be achieved due to markedly different underlying kinetic rates (r and k), 2) differences in receptor production rates (r) and receptor degradation rate constants (k) appear to be greater between different 5-HT receptor families (i.e., 5-HT_1 versus 5-HT_2) than among subtypes within a family (e.g., 5-HT_{1A} versus 5-HT_{1B}), 3) differences in the calculated receptor production rates (r) for agonist-labeled versus antagonist-labeled 5-HT_{2A} receptors reflect the differences in the $[B_{max}]_{ss}$ for the receptor labeled with either an agonist or antagonist radioligand, and, 4) despite marked alterations in 5-HT_{2A} receptor density, the stoichiometry between agonist- and antagonist-labeled receptors is maintained.

Acknowledgments

The authors thank David Guran for technical assistance and Theresa Cabrera for helpful suggestions.

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