Biochemical Pharmacology, Vol. 42, No. 5, pp. 1099–1105, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

REGULATION OF 5-HT$_2$ RECEPTORS IN RAT CORTEX

STUDIES WITH A PUTATIVE SELECTIVE AGONIST AND AN ANTAGONIST*

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(*Received* 23 *September* 1987; *accepted* 17 *April* 1991)

Abstract—The phenylisopropylamine derivative 1-(2,5-dimethoxy-4-iodo-phenyl)-2-aminopropane (DOI) has been suggested recently as a selective serotonin$_2$ (5-HT$_2$) receptor agonist. Because of the potential importance of such a tool for investigations of 5-HT$_2$ receptor regulation, receptor binding studies were performed in rats after acute and chronic treatment with DOI, the selective 5-HT$_2$ antagonist ketanserin, or vehicle. Single injections of 5 or 10 mg/kg DOI reduced the B_{max} of cortical sites labeled with [^{3}H]1-(2,5-dimethoxy-4-bromo-phenyl)-2-aminopropane and [^{3}H]ketanserin (9–32 or 32–46%, respectively). Chronic daily treatment with DOI (3–9 mg/kg) further down-regulated 5-HT$_2$ sites in cortex identified with either [^{3}H]ketanserin (−60%) or with [^{3}H]DOB (−75%), without altering K_d values or affecting 5-HT$_1$ sites. *In vitro* addition to the [^{3}H]ketanserin or [^{3}H]DOB binding assay of 10 nM to 1 μM DOI resulted in competitive inhibition, suggesting that down-regulation found *in vivo* was not secondary to residual drug. Chronic treatment with ketanserin (10 mg/kg) also down-regulated both [^{3}H]ketanserin (−38%) and [^{3}H]DOB (−58%) sites in cortex without changes in 5-HT$_1$ sites. In naive cortex, competition experiments revealed a K_i (nM) for (±)-DOI of 1.7 ± 0.02 at sites labeled by [^{3}H]DOB, and a K_H and K_L of 4.8 ± 1.5 and 53 ± 2 nM at sites labeled by [^{3}H]ketanserin. These data indicate that in chronic treatment, DOI, like ketanserin, is highly selective for 5-HT$_2$ vs 5-HT$_1$ sites at behaviorally useful doses. However, a representative putative 5-HT$_2$ selective agonist and antagonist have similar effects on 5-HT$_2$ receptors labeled by agonist or antagonist radioligands.

Glennon and co-workers have proposed that the apparent low affinity of classical serotonin (5-HT) agonists for the 5-HT$_2$ site is due, in part, to the use of 5-HT antagonists as labeling radioligands [1]. They identified several phenylisopropylamine derivatives as potential 5-HT$_2$ agonists, including 1-(2,5-dimethoxy-4-substituted phenyl)-2-amino-propanes, such as DOB (bromo-substitution) and DOI (iodo-substitution) [2, 3]. [^{3}H]DOB has been used to label a binding site on the 5-HT$_2$ receptor for which 5-HT agonists show greater affinity than antagonists [4, 5], in contrast to the 5-HT$_2$ site labeled with [^{3}H]ketanserin [6, 7]. It is currently unclear whether the agonist- and antagonist-labeled 5-HT$_2$ receptors represent a high (5-HT$_{2H}$) and low (5-HT$_{2L}$) affinity state of the same site [2] or two separate receptors (5-HT$_{2A}$ and 5-HT$_{2B}$ [8], respectively. It has been shown recently that cells transfected with a cDNA encoding the 5-HT$_2$ receptor express a protein that can bind both [^{3}H] ketanserin and [^{3}H]DOB [9, 10].

Under these circumstances, studies of 5-HT$_2$ receptor regulation are complex. Previous studies have been limited by lack of a highly specific 5-HT$_2$ agonist. It has been widely reported that chronic treatment with 5-HT$_2$ antagonists down-regulates antagonist-labeled 5-HT$_2$ receptors [11–16], but this difference from 5-HT$_1$ receptor regulation remains unexplained. The new 5-HT$_2$ ligands may be a valuable tool. Therefore, in this study, the effects of acute and chronic treatment with the 5-HT$_2$ agonist DOI and the antagonist ketanserin were compared in the rat. The *R*(−)isomer is more potent than its racemate [3], but DOI is commercially available only as a racemic mixture.

MATERIALS AND METHODS

Chemicals. Drugs were prepared immediately before use. For binding assays, (±)-1-(2,5-dimethoxy-4-iodo-phenyl)-2-aminopropane HCl (DOI; Research Biochemicals, Inc., Natick, MA), cinanserin HCl (E. R. Squibb & Sons, Inc., Princeton, NJ), 5-hydroxytryptamine creatinine sulfate (5-HT; Sigma, St. Louis, MO), and methysergide maleate (Sandoz-Ltd., Basel, Switzerland) were sonicated in assay buffer. For injections, DOI was dissolved in 0.9% saline, and ketanserin (Janssen Pharmaceutica, Beerse, Belgium) was sonicated in propylene glycol. Radioligands were obtained from New England Nuclear (Boston, MA): [^{3}H](−)DOB (sp. act. 20.8 to 22.1 Ci/mmol), [^{3}H]ketanserin (61.8 to 76.5 Ci/mmol), and [^{3}H]5-HT (28.2 Ci/mmol). Other chemicals were obtained from Sigma.

* These data were presented in abstract form at the 1988 Society for Neuroscience Meeting in Toronto, Canada.

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Animals. Male Sprague–Dawley rats (Charles River, Wilmington, MA), 200–250 g, were housed three to a cage with free access to food and water under constant temperature (23°) and a 12-hr light/12-hr dark cycle.

Single drug injections. Naive rats were injected intraperitoneally (i.p.) with a single dose of DOI (0.5, 5, 10 mg/kg) or saline. DOI doses were selected to begin at the threshold of behavioral effects [17, 18].

Chronic drug treatments. Naive rats were injected with one drug only at doses which have behavioral effects [18–20]. DOI or saline was injected i.p. once daily for 21 consecutive days between 8:00 and 9:00 a.m. at an initial dose of 3 mg/kg. An increasing agonist dose schedule was followed to induce tolerance and imitate clinical dose-ranging studies. Each week, the dose was increased by 3 mg/kg. Other rats received a fixed 10 mg/kg dose of ketanserin or vehicle i.p. for 21 days.

Tissue preparation. Rat brains were rapidly removed after decapitation 24 hr after the last DOI injection and 48 hr after the last ketanserin injection, a larger interval to allow for drug clearance. Frontal cortex was separated rapidly from rest-of-cortex on a glass petri dish on ice and both were frozen immediately on dry ice prior to placement in a −80° freezer. For each experiment, tissues from drug- and vehicle-treated rats were prepared at the same time.

Receptor binding assays. Assays for each binding site shared many features. Tissue was homogenized (Brinkmann Polytron setting 6 × 10 sec) in 40 vol. of buffer (50 mM Tris–HCl, pH 7.4, at 37°). The homogenate was preincubated for 15 min at 37° before centrifugation in a Sorvall centrifuge at 49,000 *g* for 20 min at 2–4°, and the pellet was resuspended in the appropriate assay buffer described below. The number of isotope concentrations was selected to allow both drug- and vehicle-treated tissues for incubation and filtration under the same conditions (48 tubes/experiment). In preliminary studies, values obtained from six isotope concentrations did not differ significantly from values obtained from twelve concentrations. The reaction was terminated by rapid filtration using a Brandel Harvester M-48R and No. 32 Schleicher & Schuell (Keene, NH) glass fiber filters. Filters were washed twice (5 mL each) with ice-cold Tris buffer and counted in 3a70 (Research Products International, Chicago, IL) by liquid scintillation spectroscopy (counting efficiency of 40% for chronic studies; 58% for all other studies).

In the 5-HT_1 assay [21], the assay buffer was Tris–HCl with 0.1% ascorbic acid, 4 mM $CaCl_2$, and 10 μM pargyline. One-hundred microliters of [^{3}H]5-HT (20 to 0.5 nM) was added last to tubes containing 100 μL of cold 5-HT (10 μM) or blank and 800 μL of tissue homogenate. Assay tubes were incubated for 10 min at 37°. Total isotope bound was 1–4% with approximately 60–85% specific binding at high-to-low isotope concentrations, respectively.

The agonist-labeled 5-HT_2 site was measured under two different assay conditions. Using the method of Titeler *et al.* [4], the Tris assay buffer contained 0.5 M Na_2EDTA and 10 mM $MgCl_2$. Fifty microliters [^{3}H]DOB (10 to 0.3 nM) was added last to tubes containing 400 μL of tissue homogenate and 50 μL of cinanserin (10 μL) or blank (buffer). The samples were incubated at 37° for 10 min; total bound was 0.5–3% with 39–60% specific binding. The commercially available [^{3}H]DOB has a much lower specific activity than that available to Titeler *et al.* [4]. Under these assay conditions, it yielded similar B_{max} values but higher K_d values, whether 1 μM ketanserin or cinanserin was used to define nonspecific binding. For competition experiments, the dpm values of [^{3}H]DOB specifically bound were too low (201 ± 20 dpm at 0.5 nM) to study concentrations of radioligand below 1 nM.

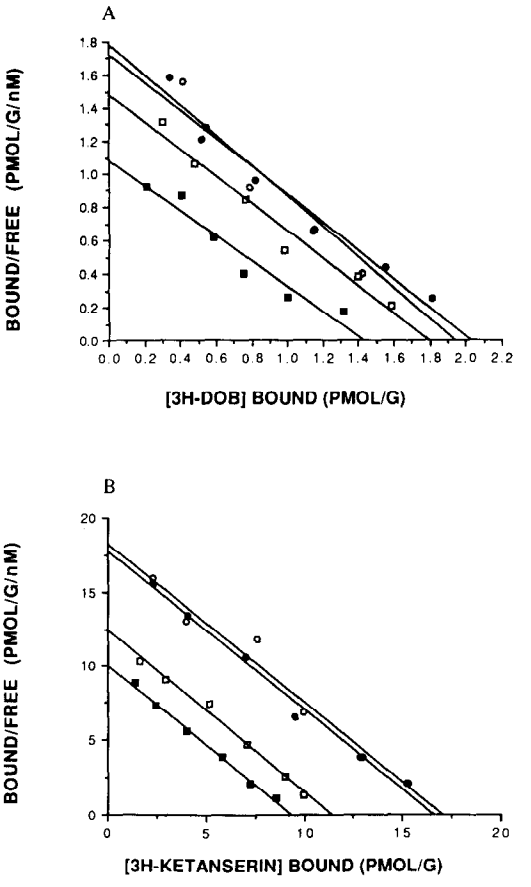

Fig. 1. Effect on binding sites in frontal cortex labeled by (A) [^{3}H]DOB and (B) [^{3}H]ketanserin of a single i.p. injection of various doses of DOI 24 hr earlier. Data are means ± SEM of 3–5 experiments performed in duplicate and presented in Table 1. DOI doses (mg/kg): 0 (○), 0.5 (●), 5 (□), and 10 (■).

The agonist-labeled 5-HT_2 site was also measured using the modification of Pierce and Peroutka [8]. In this assay, the assay buffer also contained 0.1% ascorbic acid. The assay volume was 1 mL, nonspecific binding was defined with 100 nM 5-HT, the incubation lasted for 30 min at 25°, and a lower range of [^{3}H]DOB concentrations was used (5 to 0.02 nM). Total bound was 1.5–7% with 59–77% specific binding.

The antagonist-labeled 5-HT_2 site was measured in the assay buffer of Titeler *et al.* [4, 22] or in

Table 1. Dose effect of a single injection of DOI on cortical binding sites labeled by [^{3}H]ketanserin or [^{3}H]DOB

DOI dose (mg/kg)	B_{max} (pmol/g)	K_d (nM)	n_H
[^{3}H]Ketanserin			
0 (N = 4)	17.1 ± 0.7	1.0 ± 0.1	1.0 ± 0.0
0.5 (N = 4)	16.8 ± 1.0	1.0 ± 0.1	1.1 ± 0.0
5 (N = 4)	11.6 ± 0.5*	1.0 ± 0.1	1.0 ± 0.0
10 (N = 4)	9.3 ± 0.3*	1.0 ± 0.1	1.0 ± 0.0
[^{3}H]DOB[1]			
0 (N = 4)	2.0 ± 0.1	1.3 ± 0.2	1.1 ± 0.0
0.5 (N = 3)	2.2 ± 0.3	1.3 ± 0.2	1.0 ± 0.0
5 (N = 5)	1.9 ± 0.2	1.3 ± 0.2	1.0 ± 0.1
10 (N = 5)	1.4 ± 0.1*	1.3 ± 0.1	1.0 ± 0.0
[^{3}H]DOB[2]			
0 (N = 3)	1.6 ± 0.1	0.6 ± 0.1	1.1 ± 0.0
0.5 (N = 3)	1.4 ± 0.1	0.4 ± 0.1	1.0 ± 0.0
5 (N = 3)	1.1 ± 0.2	0.4 ± 0.1	1.1 ± 0.0

Data are means ± SEM obtained by Scatchard and Hill plot analysis of 3–5 separate experiments performed in duplicate. The assay conditions for [^{3}H]ketanserin and [^{3}H]DOB[1] binding were similar, including the same assay buffer (10 mM $MgCl_2$, 0.5 mM Na_2EDTA, 50 mM Tris–HCl, pH 7.4, at 37°), tissue vol. (40), tissue type (frontal cortex from different rats of the same group), and assay vol. (500 μL). The assay conditions for [^{3}H]DOB[2] binding were different as described in Materials and Methods, including the drug used to define nonspecific binding (5-HT). Mean correlation coefficients were ≥0.91 for [^{3}H]DOB.

* $P < 0.05$ (PDIFF).

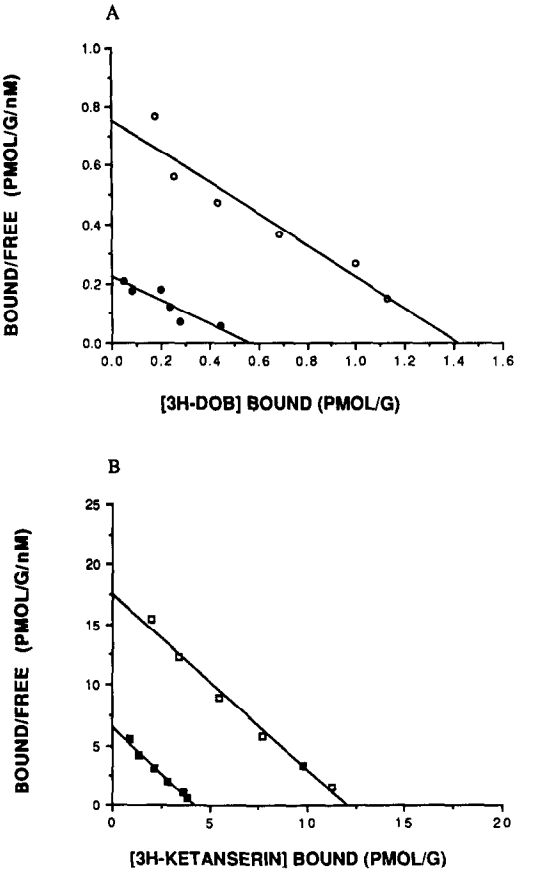

Fig. 2. Saturation studies of (A) [^{3}H]DOB and (B) [^{3}H]-ketanserin binding in cortex of rats treated for 3 consecutive weeks with DOI (●, ■) in increasing dosage (3–9 mg/kg) or with saline (○, □). Cinanserin was used in [^{3}H]DOB assays to define nonspecific binding under the assay conditions described in Materials and Methods. Data are means of 4–10 different experiments performed in duplicate and presented in Table 2.

Tris–HCl, pH 7.4, at 37° [7, 8]. Fifty microliters [^{3}H]ketanserin (10 to 0.1 nM), 50 μL methysergide (10 μM) and 400 μL homogenate were incubated at 37° for 20 min. Total isotope bound was 1–4% with 65–90% specific binding. In competition studies, a single isotope concentration of 0.5 nM was used instead.

Data analysis. For each assay, the difference in radioligand bound in the absence and presence of displacer was defined as specific binding. Saturation and competition experiments were analyzed using EBDA [23] to obtain B_{max}, K_d, Hill coefficients (n_H), and IC_{50} values. EBDA is a software program (Biosoft, Milltown, NJ) for the IBM PC that fits competition data to the Hill equation using nonlinear regression. Values of K_i (the dissociation constant) were derived from IC_{50} values (drug concentration inhibiting 50% of radioligand specific binding) of the equation [24]: $K_i = IC_{50}/[1 + ([I]/K_d)]$, where I is the concentration of radioligand. The data were analyzed statistically by using one- or two-way analysis of variance (ANOVA) in the Statistical Analysis System (SAS) [25]. Independent variables included drug treatment, drug dose, and binding site. For significant main effects ($P < 0.05$), differences between groups were then compared using *t*-tests in PDIFF [25]. Competition and Scatchard experiments were plotted on Sigma Plot (Jandel Scientific, Corte Madera, CA) and Cricket Graph (Malvern, PA), respectively.

RESULTS

Acute drug studies. There was a significant dose effect of DOI on 5-HT$_2$ receptor binding ($P < 0.0001$, ANOVA). A single injection of 5 or 10 mg/kg of DOI (Fig. 1) down-regulated binding sites labeled by [^{3}H]DOB [−9% (NS) and −32% ($P < 0.05$), respectively] or [^{3}H]ketanserin (−32% and −46%, respectively) ($P < 0.05$). A dose of 0.5 mg/kg DOI had no significant effect on B_{max} compared to saline. There were no significant changes in K_d or n_H at any dose of DOI studied for either 5-HT$_2$ site (Table 1). The results were replicated in two different batches of animals and under a variety of assay conditions.

Chronic drug studies. In cortex of rats treated chronically with DOI (Fig. 2), B_{max} values of sites identified with either [^{3}H]ketanserin (−60%) or [^{3}H]DOB (−75%) were reduced significantly without affecting K_d, compared to saline-treated rats ($P < 0.0001$, ANOVA). Different assay conditions yielded similar results (Table 2). [^{3}H]5-HT-labeled sites were unaltered: B_{max} (pmol/g) was 14.2 ± 0.5

Table 2. Effect of chronic treatment with DOI or ketanserin on cortical binding sites labeled by [^{3}H]ketanserin or [^{3}H]DOB

		B_{max} (pmol/g)	K_d (nM)	n_H
		DOI study		
[^{3}H]Ketanserin				
Saline	(N = 4)	12.5 ± 0.4	0.70 ± 0.1	1.1 ± 0.04
DOI	(N = 10)	4.4 ± 0.3*	0.71 ± 0.1	0.95 ± 0.01
[^{3}H]DOB†				
Saline	(N = 7)	1.5 ± 0.2	2.1 ± 0.3	0.94 ± 0.02
DOI	(N = 4)	0.52 ± 0.1*	2.0 ± 0.3	0.83 ± 0.10
[^{3}H]DOB‡				
Saline	(N = 4)	3.0 ± 0.2	0.92 ± 0.1	1.0 ± 0.02
DOI	(N = 3)	0.64 ± 0.1*	1.1 ± 0.2	0.93 ± 0.05
		Ketanserin study		
[^{3}H]Ketanserin				
Vehicle	(N = 5)	13.9 ± 0.8	0.65 ± 0.04	1.0 ± 0.02
Ketanserin	(N = 3)	8.6 ± 1.0*	1.5 ± 0.2*	0.99 ± 0.02
[^{3}H]DOB†				
Vehicle	(N = 4)	1.3 ± 0.2	1.8 ± 0.3	0.87 ± 0.13
Ketanserin	(N = 4)	0.56 ± 0.1*	1.4 ± 0.2	0.83 ± 0.16

Rats received a daily i.p. injection of saline or DOI (3 mg/kg/day for the first week, 6 mg/kg/day the second week, and 9 mg/kg/day the last week). Other rats were injected i.p. with 10 mg/kg ketanserin or vehicle. Within treatment groups, the same rats were used for [^{3}H]DOB and [^{3}H]ketanserin binding but rest-of-cortex and frontal cortex were used in the assays, respectively. Different assay buffers were used, as described in Materials and Methods.

* $P < 0.05$ (PDIFF).

† Cinanserin as displacer.

‡ 5-HT as displacer.

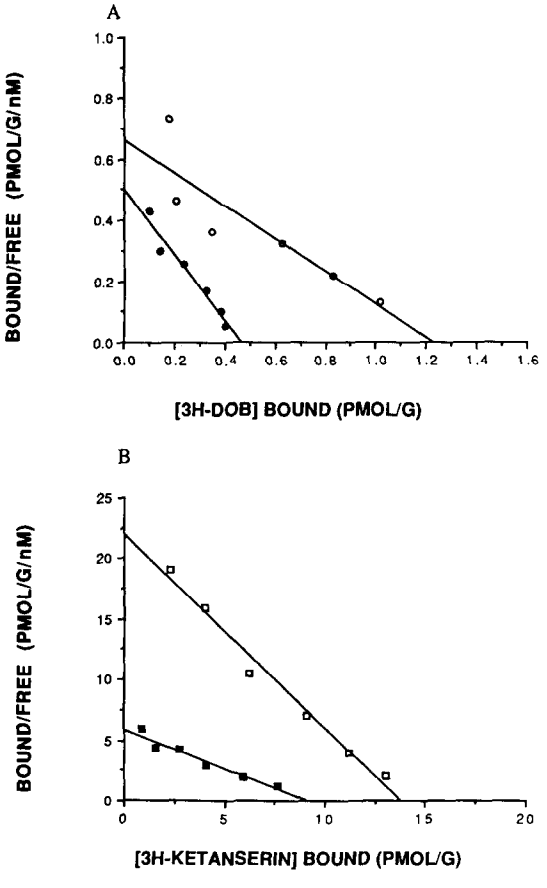

Fig. 3. Saturation studies of (A) [^{3}H]DOB and (B) [^{3}H]-ketanserin binding in cortex of rats treated for 3 consecutive weeks with 10 mg/kg ketanserin (●, ■) or with vehicle (○, □). Specific binding of radioligands was determined, as described in Materials and Methods. Data are means of 3–5 experiments performed in duplicate and presented in Table 2.

(DOI) and 14.0 ± 0.3 (saline); K_d (nM) was 1.3 ± 0.2 (DOI) and 1.5 ± 0.3 (saline).

In cortex of rats treated chronically with ketanserin (Fig. 3), the B_{max} of sites identified with either [^{3}H]ketanserin (−38%) or [^{3}H]DOB (−58%) was reduced significantly ($P < 0.05$). 5-HT_1 sites were not altered significantly: B_{max} (pmol/g) values of [^{3}H]5-HT sites were 13.5 ± 0.5 (ketanserin) and 14.6 ± 0.3 (vehicle), with corresponding K_d (nM) values of 1.5 ± 0.2 (ketanserin) and 1.6 ± 0.5 (vehicle).

Competition studies. In vitro, when DOI was allowed to compete with either [^{3}H]DOB or [^{3}H]-ketanserin in Scatchard experiments (Fig. 4), K_d values increased in a dose-dependent manner with little effect on B_{max}, suggesting competitive inhibition. In the presence of 10–100 μM DOI, the competition was complete.

Competition studies in naive cortex with DOI or 5-HT (Fig. 5) revealed data best fit by a one-site model for [^{3}H]DOB and by a two-site model for [^{3}H]ketanserin ($P < 0.002$ for 5-HT, $P < 0.0001$ for DOI, ANOVA).

In cortex of rats treated chronically with saline or DOI, competition studies of 5-HT at [^{3}H]ketanserin-

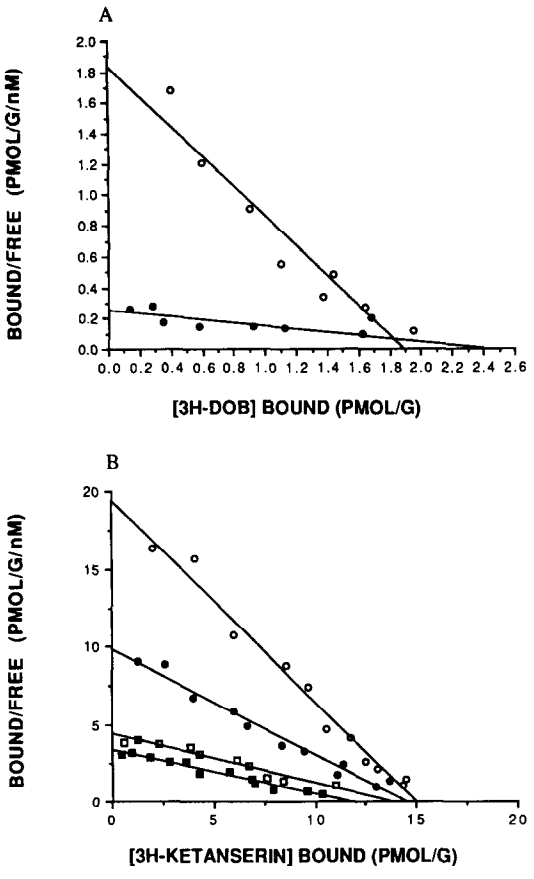

Fig. 4. Influence of DOI addition on *in vitro* (A) [^{3}H]DOB and (B) [^{3}H]ketanserin specific binding in naive rat cortex. The concentrations of DOI were 0 (○), 10 nM (●), 100 nM (□), and 1 μM (■). Data are the results of single experiments performed in duplicate using 10–12 isotope concentrations of 20 to 0.3 nM for [^{3}H]DOB and 30 to 0.3 nM for [^{3}H]ketanserin. In panel A, K_d values were 1.1 and 9.7 nM. In panel B, the K_d values were 0.77, 1.5, 3.1, and 3.4 nM in order from doses 0 to 1 μM.

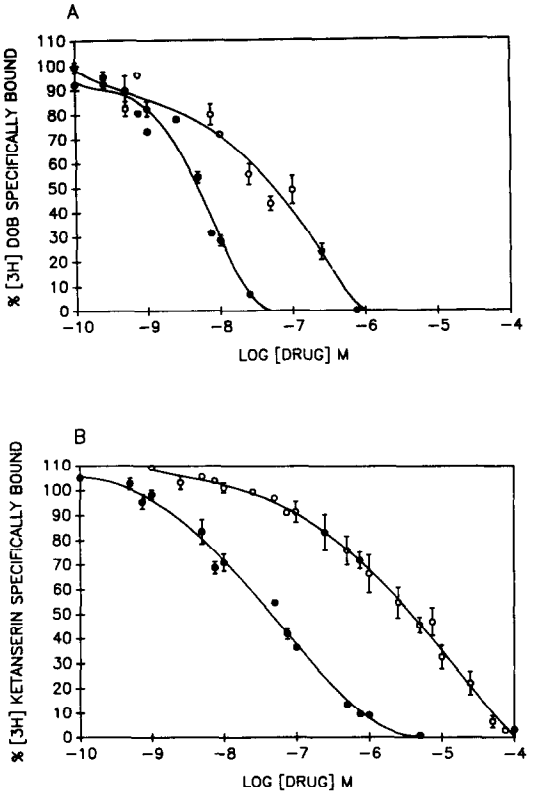

Fig. 5. Competition studies for cortical 5-HT$_2$ binding sites. (±)DOI (●) and 5-HT (○) competed for binding sites labeled by (A) 1.5 nM [^{3}H]DOB or (B) 0.5 nM [^{3}H]-ketanserin using 21 concentrations of competing drug and 10 μM cinanserin or 10 μM methysergide to define nonspecific binding in A and B, respectively. Data are means ± SEM of 3–4 separate experiments performed in duplicate. Error bars not shown fall within symbols. K_i values (nM) were 1.7 ± 0.02 for DOI and 17 ± 2 for 5-HT at sites labeled by [^{3}H]DOB. Data for drug competition with [^{3}H]ketanserin were best fit by a two-site model. For DOI, K_H was 4.8 ± 1.5 and K_L was 53 ± 2 nM. For 5-HT, K_H was 254 ± 57 and K_L was 3178 ± 408 nM.

labeled sites revealed a significant difference between groups only for K_H ($P < 0.002$). K_H was 23 ± 5 nM for saline treatment and 1.5 ± 0.5 for DOI treatment. K_L values were 744 ± 60 and 498 ± 175 nM for the respective groups.

DISCUSSION

These data provide evidence for the concept that 5-HT$_2$ receptors exhibit unexpected regulation. It has been shown previously that selective 5-HT$_2$ antagonists down-regulate 5-HT$_2$ sites [14], whereas other data suggest that 5-HT$_1$ antagonists [15] or at least nonselective 5-HT antagonists [26] up-regulate 5-HT$_1$ sites. Here a putative selective 5-HT$_2$ agonist and an antagonist, with 40-fold differences in affinity for their respective sites, down-regulated both the agonist- and antagonist-labeled 5-HT$_2$ receptor. This finding is unexplained. Behavioral paradigms, such as shaking behavior, support the designation of DOI as a 5-HT$_2$ agonist [1, 18, 20] and ketanserin as a 5-HT$_2$ antagonist [8, 19]. *In vitro*, the DOI cogener 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM) stimulates phosphoinositide hydrolysis as a partial 5-HT$_2$ agonist [27]. The Scatchard studies with *in vitro* addition of DOI presented here show that residual presence of DOI would have resulted in a competitive pattern affecting K_d rather than B_{max}, which did not occur. The issue of residual drug presence in brain to explain the down-regulation of 5-HT$_2$ sites by antagonists also has been addressed with negative findings [16].

These data do not resolve the issue of multiple 5-HT$_2$ sites versus affinity states. The similar response of putative 5-HT$_2$ states or sites to treatment with 5-HT$_2$ agonists and antagonists may suggest a single 5-HT$_2$ site with two affinity states or interconvertible states of the same receptor molecule. A decrease in the density of one of the affinity states did not result in a reciprocal increase in the density of the second

state; however, selective reduction of one state could serve instead to "pull" the other state in its direction. In view of the greater affinity of DOI for the agonist-labeled site, one might have expected that low doses of DOI would down-regulate only the agonist-labeled site, whereas high doses should down-regulate both sites. This response was not observed, perhaps due to the fact that DOI has high affinity for both agonist- and antagonist-labeled 5-HT_2 receptors. A variety of approaches will be necessary to resolve these issues.

Three laboratories simultaneously reported that DOI administration altered 5-HT_2 binding sites [28–30]. Buckholtz *et al.* [29] found that single high (7 mg/kg) but not low (1 mg/kg) doses of DOI (DOB or LSD) down-regulated [^{3}H]ketanserin-labeled 5-HT_2 sites in rat cortex (−26%), whereas both doses caused down-regulation after 7 days of treatment. Himeno *et al.* [30] reported that chronically administered 1 mg/kg DOI reduced the B_{max} of rat cortical 5-HT_2 sites labeled by [^{3}H]-ketanserin but not [^{125}I]DOI, which instead showed a higher K_d. The present data [28] extend those findings by showing that the 5-HT_2 antagonist ketanserin had effects similar to those of putative agonist DOI on 5-HT_2 receptors measured by either [^{3}H]DOB or [^{3}H]ketanserin, that chronic treatment increased the down-regulation induced by single injections, and that these effects are not explained by residual DOI in brain. Unlike McKenna *et al.* [31], a significant change in K_d of sites labeled by the agonist-radioligand at low DOI doses was not found, but there is agreement that the B_{max} was not affected. It is unclear if this difference reflects the various radioligands used or the presence of residual drugs in their study.

There have been few previous studies of other putative 5-HT_2 agonists for comparison. Quipazine, a 5-HT_2 agonist, is less selective than DOI in chronic treatment since it down-regulates 5-HT_1 sites [11]. This difference may relate to the 10-fold lower IC_{50} of quipazine than DOI at the 5-HT_1 site [2, 3]. Phencyclidine, another purported putative 5-HT_2 agonist, but with activity at other neurotransmitter receptors, also has been reported to down-regulate (−30%) the antagonist-labeled 5-HT_2 receptor [32]. Chronic studies of other putative selective 5-HT drugs to confirm these results are in progress in this laboratory.

Acknowledgements—This research was supported by NIH Grant 1-KO8-NS-1158 (CIDA) and RR00645, the Myoclonus Research Fund, the United Cerebral Palsy Research and Education Foundation (R381-88), the William Randolph Hearst Foundation, and the Children's Research Institute. The author thanks Ann Zaragoza for excellent word processing, Robert Pluchino for assistance with animal injections and computer data entry, and the pharmaceutical companies cited for their drug donations.

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