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Serotonin Depletion Unmasks Serotonergic Component of [^{3}H] Dihydroalprenolol Binding in Rat Brain

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SUMMARY

Selective lesions of serotonin neurons or inhibition of serotonin synthesis results in an increase in the number of [^{3}H]dihydroalprenolol binding sites in several areas of rat brain. Previously, this increase in binding sites was interpreted as an increase in β-adrenergic receptors. However, the lesion-induced increase in [^{3}H]dihydroalprenolol is not accompanied by an increase in isoproterenol- or norepinephrine-stimulated cyclic AMP production. The increased binding of [^{3}H]dihydroalprenolol is blocked by the addition of serotonin but not by the addition of norepinephrine or dopamine to the assays. Furthermore, the addition of metergoline, a serotonin antagonist, also blocked the increase in lesioned tissues. Thus, the lesion-induced increase in [^{3}H]dihydroalprenolol binding appears to represent an increase in serotonergic binding sites. Among drugs with some selectivity for serotonin-1 receptor subtypes, trifluoromethylphenylpiperazine and RU-24929 were as effective as serotonin in blocking lesion-induced increases in [^{3}H]dihydroalprenolol binding. However, 8-hydroxy-*N,N*-dipropyl-2-aminotetralin (8-OH-DPAT), spiperone, and mesulergine were either much less effective than serotonin or completely ineffective. Radioligand binding to serotonin-1A and serotonin-1B sites with [^{3}H]8-OH-DPAT and ^{125}I-cyanopindolol, respectively, after lesions of serotonin axons or depletion of serotonin was not increased, despite a marked increase in [^{3}H]dihydroalprenolol binding in the same tissues. When tissues from control rats or rats with serotonin lesions were preincubated at 37° for 10 min to remove endogenous serotonin bound to receptors, the binding of [^{3}H]dihydroalprenolol in controls was increased to the level seen in lesioned tissues. Thus, [^{3}H]dihydroalprenolol binds primarily to β-adrenergic receptors in control membranes that are not preincubated; however, either preincubation of control tissues or serotonin depletion unmasks a serotonin-1 receptor subtype to which [^{3}H]dihydroalprenolol binds in addition to the β-adrenergic receptor.

Selective lesions of 5-HT neurons with 5,7-DHT or PCA increase the number (B_{max}) of [^{3}H]DHA binding sites in rat cerebral cortex and hippocampus (1–3). However, this increase is restricted to [^{3}H]DHA binding sites with low affinity for isoproterenol, while the number of sites with high affinity for isoproterenol is unchanged (2–4). The isoproterenol-stimulated accumulation of cyclic AMP was reported to be increased by 5-HT lesions in one study (1) but unchanged in other studies (2, 5).

Recently, low concentrations of 5-HT *in vitro* were found to block the increases in [^{3}H]DHA binding sites induced by 5-HT lesions (5). This was interpreted as indicating that 5-HT regulates a population of β-adrenergic receptors with low affinity for isoproterenol, whereas norepinephrine regulates the population of β-adrenergic receptors with high affinity for isoproterenol (5).

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The main purpose of the studies reported here was to determine whether the increase in [^{3}H]DHA binding sites induced by 5-HT lesions represents β-adrenergic binding sites or 5-HT binding sites. Although [^{3}H]DHA is one of the most frequently used ligands for measuring β-adrenergic receptor binding sites in brain, its utility for this purpose relies on the way specific binding is defined. Typically, specific binding is defined as the difference between binding in the absence and presence of a high concentration (1–20 μM) of propranolol or alprenolol. This definition, in turn, relies heavily on the presumed selectivity of [^{3}H]DHA and propranolol or alprenolol for β-adrenergic receptor binding sites. However, the selectivity of these and other β-adrenergic antagonists for this purpose is questionable, because they have been found to bind to 5-HT sites as well as to β-adrenergic sites in brain (6–8). We provide evidence that both *in vivo* depletion of 5-HT and *in vitro* preincubation of control membranes increase [^{3}H]DHA binding in rat brain, but the increased binding sites appear to be a type of serotonergic binding site rather than a β-adrenergic receptor.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine or serotonin; PCA, *p*-chloroamphetamine; PCPA, *p*-chlorophenylalanine; [^{3}H]DHA, [^{3}H]dihydroalprenolol; 5,7-DHT, 5,7-dihydroxytryptamine; TFMPP, 1-(*m*-trifluoromethylphenyl)piperazine; 8-OH-DPAT, 8-hydroxy-*N,N*-dipropyl-2-aminotetralin; CYP, cyanopindolol.

Materials and Methods

Animals. Male Sprague-Dawley rats (200–250 g) (Zivic Miller) were housed in groups in a light- and temperature-controlled room and had free access to food and water.

Lesions. For intracranial lesions, rats were anesthetized with pentobarbital (45 mg/kg) and injected with desmethylimipramine (25 mg/kg; intraperitoneally). Forty-five minutes later they were positioned in a Kopf stereotaxic instrument and 5,7-DHT creatinine sulfate, in a dose equivalent to 7 μg of free base, dissolved in 3 μl of vehicle (0.9% NaCl containing 0.01% ascorbic acid) was injected into both the dorsal and median raphé nuclei over a 5-min period. The stereotaxic coordinates for the dorsal and median raphé nuclei were AP, −0.6; L, 0.0; and H, −3.1 and −5 (9). In control rats, the injection cannula was lowered and positioned 1 mm above the dorsal and median raphé nuclei but no neurotoxin was injected. Other rats received an intraperitoneal injection of PCA·HCl on day 1 (12 mg/kg) and day 3 (15 mg/kg). Control rats were injected with saline at identical intervals. The rats were allowed to recover for 7 to 8 weeks (5,7-DHT) or 4–5 weeks (PCA) before being killed by decapitation.

Chronic drug treatment. Another group of rats received intraperitoneal injections of either saline or PCPA-methyl ester·HCl, which depletes 5-HT by inhibiting tryptophan hydroxylase. The dosage of PCPA was the free base equivalent of 300 mg/kg on day 1 and 75 mg/kg on 3 alternate days/week for 5 weeks. The rats were killed by decapitation 48 hr after the last injection.

Receptor binding assays. Following decapitation, the brains were quickly dissected on ice-chilled plate, as previously described (10). Various brain areas were frozen on dry ice and stored at −60° until used for ligand binding assays.

Binding of [^{3}H]DHA (31.3 or 54.8 Ci/mmol; New England Nuclear) was measured according to the method of Bylund and Snyder (11). Tissues were suspended in buffer containing 50 mM Tris·HCl, 1 mM $MgCl_2$, and 57 μM ascorbic acid (pH 7.9 at 25°) and were homogenized using a Brinkmann Polytron (setting 6; 8 sec). The homogenates were diluted with buffer and centrifuged at 48,000 × *g* for 10 min. The pellets were homogenized in fresh buffer and washed a second time by centrifugation. The final pellet was then rehomogenized in fresh buffer. Aliquots of tissue homogenate containing approximately 350 μg of protein (7 mg of tissue, wet weight) were incubated with [^{3}H]DHA (4–6 nM) at 25° for 15 min, in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of either 10 μM DL-propranolol·HCl, 1 μM *l*-alprenolol-*d*-tartrate salt, or 10 or 100 μM (−)-isoproterenol·HCl. In some experiments, 5-HT, metergoline, mesulergine, 8-OH-DPAT, spiperone, TFMPP, RU-24969, dopamine, or norepinephrine was included in the incubation buffer. When 5-HT, dopamine, or norepinephrine was present, pargyline·HCl (5 μM) was added to the final tissue homogenization buffer and to the incubation buffer. Incubations were terminated by addition of 4 ml of cold buffer to each tube and rapid filtration under reduced pressure through Schleicher and Schuell glass fiber filters (No. 34). The filters were washed three times with 4-ml aliquots of cold buffer, transferred to vials to which scintillation fluid was added, and counted by liquid scintillation counting. Specific binding, defined as the difference between total binding and nonspecific binding, was 65–80% of total binding, depending on the concentration of [^{3}H]DHA.

Serotonin-1A binding sites were measured using [^{3}H]8-OH-DPAT (201 Ci/mmol; Amersham), according to the method of Peroutka (12). Tissues were suspended in 50 mM Tris·HCl (pH 7.7 at 25°) and homogenized with a Polytron. The homogenates were diluted with buffer and centrifuged at 48,000 × *g* for 10 min. The pellets were resuspended, preincubated at 37° for 10 min, diluted, and centrifuged. The supernatant was discarded, and the pellet was resuspended in Tris-HCl incubation buffer containing 10 μM pargyline, 4 mM $CaCl_2$, and 0.1% ascorbic acid. Aliquots of tissue homogenates containing approximately 250 μg of protein (5 mg of tissue, wet weight) were incubated in triplicate with [^{3}H]8-OH-DPAT at 25° for 30 min, in a final volume of 0.5 ml. Nonspecific binding was determined in the presence of 10 μM 5-HT. Incubations were terminated and radioactivity was counted as outlined above. Specific binding was 90–95% of total binding.

Serotonin-1B binding sites were measured using ^{125}I-CYP (2200 Ci/mmol; New England Nuclear) according to the method of Hoyer *et al.* (13), as modified by Offord *et al.* (14). Aliquots of tissue homogenates, containing 25 μg of protein (0.5 mg of tissue, wet weight), were prepared and preincubated as described above and added to triplicate test tubes containing incubation buffer which was composed of 10 mM Tris·HCl, 154 mM NaCl, and 10 μM pargyline (pH 7.7 at 25°, final incubation volume was 0.5 ml). Binding of ^{125}I-CYP (50–600 pM) was done in the presence of (−)-isoproterenol (3–25 μM, depending on the concentration of ^{125}I-CYP) to prevent binding of the radioligand to β-adrenergic binding sites. Nonspecific binding was measured in the presence of 10 μM 5-HT. Incubations were performed at 37° for 30 min and then terminated as outlined above. Radioactivity was measured in a Micromedic γ-counter. Specific binding was 35–65% of total binding.

Assay of cyclic AMP accumulation. The β-adrenergic receptor-mediated stimulation of cyclic AMP by isoproterenol was carried out in minces of fresh hippocampus. Following decapitation, brains were quickly removed and placed in ice-cold oxygenated (95% O_2/5% CO_2) Krebs-Ringer bicarbonate buffer (pH 7.4 at 25°). The buffer contained 118 mM NaCl, 5 mM KCl, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 24 mM $NaHCO_3$, 0.02 mM EDTA, 10 mM glucose, and 2.5 mM $CaCl_2$. The hippocampi were dissected on an ice-chilled plate and then cross-chopped into 250 × 250 μ minces with a McIlwain Tissue Chopper. The minces were placed in oxygenated Krebs-Ringer buffer (37°), gently dispersed with a Pasteur pipette, and preincubated for 10 min in a water bath. The buffer was drawn off and the slices were incubated for two additional 10-min periods with fresh oxygenated buffer containing 50 μM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase. The buffer was then aspirated and 30 μl of gravity-packed slices (approximately 10 mg of tissue, wet weight) were added to tubes containing oxygenated warm Krebs-Ringer buffer and 50 μM 3-isobutyl-1-methylxanthine. (−)-Isoproterenol or L-norepinephrine·HCl was dissolved in Krebs-Ringer buffer and 100 μl of either one of the agonists or vehicle were added to the tubes. The final incubation volume was 2 ml. The tubes were oxygenated, capped, and placed in a shaking water bath at 37°. After 10 min of incubation, 200 μl of 1 N HCl were added to each tube, the tissues were homogenized with a Polytron, and the tubes were then placed in a boiling water bath for 10 min. The homogenates then were chilled in ice water and vortexed, and a sample was removed for protein determination. The remaining homogenate was centrifuged for 10 min at 2000 × *g* and the supernatant was removed and frozen. The content of cyclic AMP in the supernatant was measured using a radioimmunoassay kit (New England Nuclear). The protein content of tissue homogenates in the radioligand binding assays and the assay for the accumulation of cyclic AMP was measured by the method of Lowry *et al.* (15), using bovine serum albumin as the standard.

Tissue content of monoamines. High performance liquid chromatography with electrochemical detection was used to determine the content of 5-HT and norepinephrine in the frontal cortex by the method of Mefford and Barchas (16) and Sperk (17).

Data analysis and statistics. Data from saturation experiments were analyzed by nonlinear regression analysis using the McPherson adaptation (Elsevier, Cambridge) of the LIGAND program (18). Binding to a single binding site was assumed unless a more complex, two-site model better explained the experimental data. A two-site model was selected if it caused a significant decrease in the sum of squares of the residuals about the regression line for the more complex model, as evidenced by an *F* test ($p < 0.01$). For the assays in which binding was measured in the absence or presence of 5-HT or other competing drugs, repeated measures analysis of variance (for between and within subject group comparisons) was used to test for statistically significant effects of the 5-HT depletion and/or *in vitro* assay condition (i.e., in the presence or absence of 5-HT). If there was a significant effect of the animal treatment or assay condition or if there was a significant

interaction between animal treatment and assay condition, appropriate *post hoc* testing between related means was performed with Student's *t* test for paired or unpaired data. A p value of less than 0.05 was considered statistically significant.

Drugs. Metergoline was generously donated by Farmitalia (Milan, Italy). RU-24969 was obtained from Roussel Uclaf (Romainville, France). Mesulergine and (±)-CYP were generous gifts of Sandoz Ltd. (Basel, Switzerland). L-Norepinephrine·HCl, 5,7-DHT creatinine sulfate, PCA·HCl, and PCPA methyl ester·HCl were purchased from Regis Chemical Co. (Morton Grove, IL). TFMPP, spiperone, and (±)-8-OH-DPAT·HBr were purchased from Research Biochemicals, Inc. (Natick, MA). 5-HT·HCl, dopamine·HCl, DL-propranolol·HCl, l-alprenolol D-tartrate salt, (−)-isoproterenol·HCl, pargyline·HCl, and 3-isobutyl-l-methylxanthine were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Effects of 5,7-DHT lesions. Injections of 5,7-DHT into the dorsal and median raphé nuclei reduced the concentration of 5-HT in the frontal cortex by more than 90% when compared with the concentration in tissues from control rats (Fig. 1A). The content of norepinephrine was not significantly different from control values in homogenates of the same tissues (Fig. 1B).

This selective lesion of 5-HT neurons resulted in a significant increase in specific binding of [^{3}H]DHA in cortex, regardless of whether nonspecific binding was determined with 10 μM isoproterenol, 10 μM propranolol, or 1 μM alprenolol (Fig. 2). However, the increase appeared to be greater when either of the antagonists was used to determine nonspecific binding (Fig. 2). The addition of 1 μM 5-HT in the binding assay did not significantly affect specific binding of [^{3}H]DHA in control tissues when nonspecific binding was determined with isoproterenol; however, when either propranolol or alprenolol was used to determine nonspecific binding, the addition of 5-HT reduced specific binding of [^{3}H]DHA in control tissues by 16–19% (Fig. 2). More important, the presence of 1 μM 5-HT in the binding assay completely blocked the increase in [^{3}H]DHA

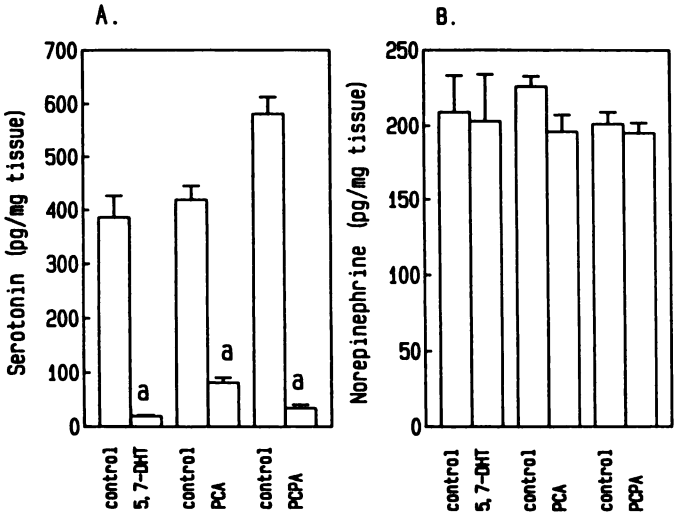


Fig. 1. Effect of long term treatment with 5,7-DHT, PCA, or PCPA on the content of 5-HT (A) and norepinephrine (B) in rat frontal cortex. See Materials and Methods for the duration of the treatments. 5-HT and norepinephrine were measured by high pressure liquid chromatography with electrochemical detection. Values represent the mean ± standard error of 9 to 12 rats/group. $^a p < 0.01$ versus control, based on Student's *t* test.

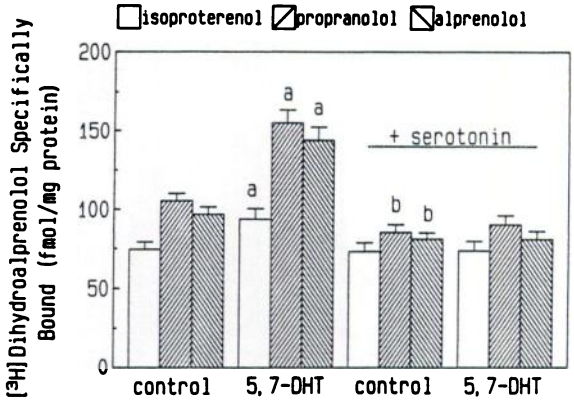


Fig. 2. Effect of lesions of the dorsal and median raphé nuclei with 5,7-DHT on the binding of [^{3}H]DHA (4.5 nM) in rat parietooccipital cortex. Rats were killed 7 to 8 weeks after the lesions were made. Nonspecific binding was defined with either 10 μM (−)-isoproterenol, 10 μM DL-propranolol, or 1 μM *l*-alprenolol and the assays were performed in the presence or absence of 1 μM 5-HT. Values represent the mean ± standard error of six rats/group. Repeated measures analysis of variance indicated a significant interaction between the treatment with 5,7-DHT and the presence of 5-HT in the assay ($p < 0.015$). $^a p < 0.05$ compared with control in the absence of 5-HT and compared with 5,7-DHT-lesioned rats in the presence of 5-HT. $^b p < 0.001$ compared with control in the absence of 5-HT.

TABLE 1

Effect of 5,7-DHT on the binding on [^{3}H]DHA in various brain regions

Rats were killed 7 weeks after the injection of 5,7-DHT into the median and dorsal raphé nuclei. The concentration of [^{3}H]DHA was 4–6 nM. Nonspecific binding was determined in the presence of 10 μM DL-propranolol. The values represent the mean ± standard error of five to eight rats/group.

	[^{3}H]DHA binding	
	Control	5,7-DHT
	fmol/mg of protein	
Cortex	83.1 ± 5.4	115.9 ± 6.4[a]
Hippocampus	94.8 ± 6.4	161.3 ± 3.9[a]
Thalamus	55.0 ± 3.4	81.4 ± 3.6[a]
Hypothalamus	66.2 ± 4.8	102.6 ± 5.5[a]
Cerebellum	45.9 ± 2.6	45.4 ± 3.5

$^a p < 0.01$ compared with control.

specific binding in cortex from lesioned rats, regardless of which compound was used to determine nonspecific binding (Fig. 2).

The 5,7-DHT lesion also markedly increased [^{3}H]DHA specific binding in the hippocampus, thalamus, and hypothalamus when 10 μM propranolol was used to determine nonspecific binding, but the binding in the cerebellum was unchanged by the lesion (Table 1). However, despite the marked increase in [^{3}H]DHA binding in the hippocampus, neither basal levels nor isoproterenol- or norepinephrine-stimulated accumulation of cyclic AMP was affected by the lesion (Fig. 3).

Effects of PCA lesions. Intraperitoneal injections of PCA reduced the concentration of 5-HT in the frontal cortex by more than 80% (Fig. 1A), without significantly affecting the concentration of norepinephrine (Fig. 1B).

This selective lesion of 5-HT neurons resulted in a significant increase in [^{3}H]DHA specific binding in the cortex when 10 μM propranolol was used to determine nonspecific binding (Fig. 4). Again, however, the addition of 5-HT to the assay blocked the PCA-induced increase in [^{3}H]DHA binding. In competition assays, 5-HT decreased binding of [^{3}H]DHA in lesioned tissues in a concentration-dependent manner, reducing the binding to

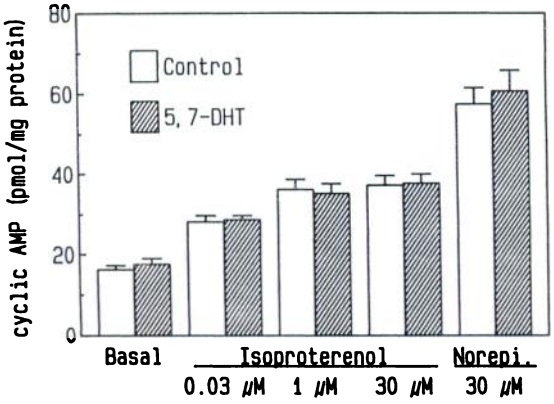


Fig. 3. Effect of lesions with 5,7-DHT on the net accumulation of cyclic AMP in response to isoproterenol or norepinephrine (*Norepi.*) in rat hippocampus. The cyclic AMP was measured by radioimmunoassay after a 10-min incubation of minces of fresh hippocampus in the absence (basal values) or presence of agonists. Values represent the mean ± standard error of eight rats/treatment group.

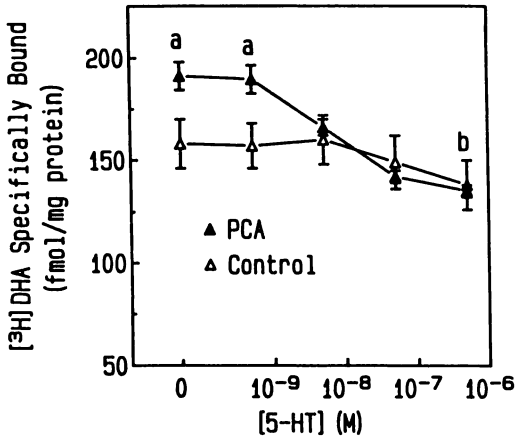


Fig. 4. Effect of lesions with PCA on the binding of [^{3}H]DHA in cortex in the absence (0 M) or presence of increasing concentrations of 5-HT. Rats were killed 4 weeks after the injection of PCA. The concentration of [^{3}H]DHA was 4.2 nM; nonspecific binding was measured in the presence of 10 μM propranolol. Values are fmol/mg of protein and represent the mean ± standard error of five rats/treatment group. The experiment was repeated once in other rats. Repeated measures analysis of variance indicated a significant interaction between the treatment with PCA and the presence of 5-HT in the assay ($p < 0.001$). [a]$p < 0.05$ compared with the respective control. [b]$p < 0.01$ for control in the presence of 500 nM 5-HT compared with control in the absence of 5-HT.

nonlesioned control levels at a concentration of approximately 5 nM (Fig. 4). Consistent with the results shown in Fig. 2, when propranolol was used to determine nonspecific binding of [^{3}H]DHA, higher concentrations of 5-HT slightly decreased specific binding in control cortex also (Fig. 4).

To determine the pharmacological specificity of 5-HT in blocking the lesion-induced increase in [^{3}H]DHA binding, serotonergic and nonserotonergic drugs were added to the assays. [^{3}H]DHA binding was significantly increased in the cortex from PCA-treated rats, and the addition of 500 nM 5-HT significantly decreased binding in control tissues and completely blocked the lesion-induced increase in [^{3}H]DHA binding (Table 2). Similarly, the addition of 500 nM metergoline, a 5-HT receptor antagonist, decreased binding in control tissues and completely blocked the lesion-induced increase in [^{3}H]DHA binding (Table 2).

In the hippocampus, [^{3}H]DHA binding was markedly increased by the PCA-induced lesion (Table 3). The addition of 100 nM 5-HT significantly decreased binding in control tissues and completely blocked the increase in binding in lesioned tissues (Table 3). In contrast to 5-HT, neither 100 nM norepinephrine nor 100 nM dopamine affected [^{3}H]DHA binding in control hippocampus, and neither blocked the increased binding in hippocampus from lesioned rats (Table 3). Even at concentrations of 1 μM, norepinephrine and dopamine appeared to only partially block the increased binding in lesioned rats (Table 3). As in the cortex, the 5-HT receptor antagonist metergoline decreased [^{3}H]DHA binding in hippocampus from control rats and completely blocked the increased binding in hippocampus from lesioned rats (Table 3).

These results indicated that, when propranolol is used to determine nonspecific binding of [^{3}H]DHA, a significant fraction of [^{3}H]DHA specific binding in rat brain is to a 5-HT binding site and that this fraction is increased by lesions of 5-HT axons. To try to determine the subtype of 5-HT binding site to which [^{3}H]DHA binds, assays were carried out in the presence of drugs with some selectivity for 5-HT-1A, 5-HT-1B, or 5-HT-1C binding sites. In the cortex, 5-HT slightly decreased binding in control tissues and completely blocked the PCA lesion-induced increase in binding (Fig. 5). Neither 8-OH-DPAT, a 5-HT-1A-selective drug, nor mesulergine, a 5-HT-1C- and 5-HT-2-selective drug, blocked the lesion-induced increase in [^{3}H]DHA binding (Fig. 5; Table 4). In contrast to 8-

TABLE 2

Effects of PCA on the binding of [^{3}H]DHA in cortex

Rats were killed 4 weeks after injections of PCA. The concentration of [^{3}H]DHA was 4 nM and nonspecific binding was determined with 10 μM propranolol. The values represent the mean ± standard error of four to six rats/group.

Addition	[^{3}H]DHA binding	
	Control	PCA
	fmol/mg of protein	
None	108.0 ± 2.9	137.2 ± 5.5[a]
5-HT (500 nM)	90.6 ± 2.3[b]	92.6 ± 2.7
Metergoline (500 nM)	92.4 ± 4.6[b]	92.4 ± 1.6

[a] $p < 0.002$ compared with control with no addition.
[b] $p < 0.02$ compared with control with no addition.

TABLE 3

Effect of lesions with PCA on the binding of [^{3}H]DHA in hippocampus in the absence or presence of 5-HT, norepinephrine, or dopamine

Rats were killed 4 weeks after injections of PCA. The concentration of [^{3}H]DHA was 4.0–4.7 nM; nonspecific binding was measured in the presence of 10 μM propranolol. Values represent the mean ± standard error of five or six rats/group. Repeated measures analysis of variance indicated a significant interaction between the treatment with PCA and the presence of 5-HT, metergoline, or dopamine in the assay ($p < 0.005$).

Addition	[^{3}H]DHA binding	
	Control	PCA
	fmol/mg of protein	
None	77.0 ± 6.5	123.3 ± 4.3[a]
5-HT (100 nM)	47.7 ± 3.1[b]	50.4 ± 2.7
None	116.2 ± 10.6	166.9 ± 11.1[a]
Norepinephrine (100 nM)	118.8 ± 12.2	170.1 ± 11.1[c]
Norepinephrine (1 μM)	91.1 ± 9.6	135.4 ± 9.2[c]
Dopamine (100 nM)	120.5 ± 12.0	160.9 ± 11.8[c]
Dopamine (1 μM)	115.4 ± 9.9	134.8 ± 7.3[c]
None	87.1 ± 4.2	139.5 ± 6.1[a]
Metergoline (500 nM)	47.2 ± 1.4[b]	41.8 ± 1.9

[a] $p < 0.05$ versus the respective control with no addition.
[b] $p < 0.01$ versus the respective control with no addition.
[c] $p < 0.05$ versus the respective control.

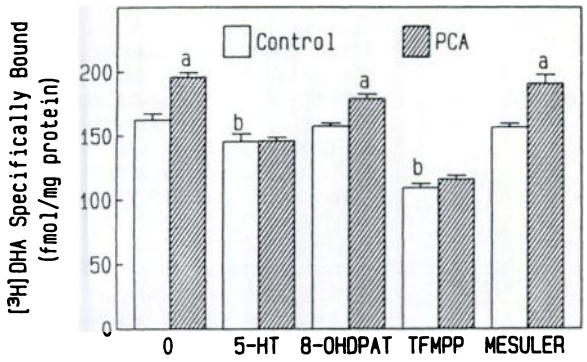


Fig. 5. Effect of lesions with PCA on the binding of [^{3}H]DHA in cortex in the absence (0) or presence of serotonergic compounds. Rats were killed 4 weeks after the injections of PCA. The concentration of [^{3}H]DHA was 4.1 nM; nonspecific binding was measured in the presence of 10 μM propranolol. The concentration of 5-HT, 8-OH-DPAT, and mesulergine (*MESULER*) was 100 nM; the concentration of TFMPP was 200 nM. Values are fmol/mg of protein and represent the mean ± standard error of four or five rats/group. The experiment was repeated once in other rats. Repeated measures analysis of variance indicated a significant interaction between the treatment with PCA and the presence of 5-HT or TFMPP in the assay ($p < 0.001$). $^a p < 0.002$ compared with the respective control. $^b p < 0.01$ compared with control in the absence of added drug.

TABLE 4

Effects of lesions with PCA on the binding of [^{3}H]DHA in cortex in the absence or presence of serotonergic drugs

The concentration of [^{3}H]DHA was 4 nM; nonspecific binding was measured in the presence of 10 μM propranolol. Values represent the mean ± standard error of four or five rats/treatment group. Repeated measures analysis of variance indicated no significant interaction between treatment with PCA and the presence of spiperone or 8-OH-DPAT; a significant interaction was observed between treatment with PCA and the presence of RU-24969 or CYP in the assay ($p < 0.002$).

Addition	[^{3}H]DHA binding	
	Control	PCA
	fmol/mg of protein	
None	138.0 ± 8.0	173.1 ± 9.1[a]
Spiperone (60 nM)	127.6 ± 3.5[b]	157.6 ± 8.2[c]
Spiperone (600 nM)	109.3 ± 4.3[b]	134.9 ± 12.6[c]
8-OH-DPAT (100 nM)	125.6 ± 5.0[b]	146.4 ± 8.2[c]
8-OH-DPAT (300 nM)	122.6 ± 4.2[b]	145.4 ± 6.8[c]
8-OH-DPAT (1 μM)	119.2 ± 2.5[b]	140.6 ± 8.5[c]
None	166.0 ± 7.2	201.0 ± 4.7[a]
RU-24969 (1 nM)	132.2 ± 7.5[b]	156.2 ± 3.2[c]
RU-24969 (3 nM)	126.2 ± 5.8[b]	135.6 ± 5.1
RU-24969 (10 nM)	125.0 ± 2.6[b]	126.6 ± 5.9
None	87.2 ± 4.2	115.6 ± 2.3[a]
CYP (100 nM)	20.5 ± 2.3[b]	18.1 ± 1.8

[a] $p < 0.02$ versus control with no addition.
[b] $p < 0.05$ versus the respective control with no addition.
[c] $p < 0.05$ versus the respective control.

OH-DPAT, TFMPP significantly decreased [^{3}H]DHA binding in control cortex and completely blocked the increase in binding in lesioned tissue (Fig. 5). Spiperone, which blocks 5-HT-1A and 5-HT-2 binding sites, also was unable to block the increase in binding (Table 4). At higher concentrations, 8-OH-DPAT and spiperone significantly reduced binding in tissue from control and PCA-treated rats, but the lesion-induced increase in binding persisted (Table 4). CYP, a potent β-adrenergic and 5-HT-1A and 5-HT-1B antagonist, blocked the lesion-induced increase in binding and greatly reduced binding in control and PCA-treated rats (Table 4). RU-24969, equipotent at 5-HT-1A and 5-HT-1B sites, potently blocked the lesion-induced increase in [^{3}H]DHA binding in cortex, with an IC_{50} of about 1 nM (Table 4); RU-24969 concentrations of 1 nM or higher also decreased binding in control tissues (Table 4). Similar results with RU-24969 were obtained when nonspecific binding was determined with 10 μM isoproterenol (data not shown).

In the hippocampus, 100 nM 5-HT significantly decreased [^{3}H]DHA binding in control tissues and completely blocked the PCA lesion-induced increase (Fig. 6). Although 100 nM 8-OH-DPAT also significantly decreased [^{3}H]DHA binding in control hippocampus, it did not completely block the lesion-induced increase in binding (Fig. 6). But again in contrast to 8-OH-DPAT, 100 nM TFMPP significantly decreased binding of [^{3}H]DHA in control tissues and nearly completely blocked the increased binding in lesioned tissues (Fig. 6).

Competition experiments were done to measure the potency of TFMPP in blocking the lesion-induced increase in [^{3}H]DHA binding. In experiments in which the concentration of TFMPP ranged from 1 nM to 20 μM, it blocked the increase in [^{3}H]DHA binding in lesioned cortex and hippocampus with an IC_{50} of approximately 3 nM (Fig. 7); at higher concentrations, it also decreased binding in control tissues (Fig. 7).

To directly determine whether binding to a 5-HT receptor subtype was increased by lesions to 5-HT neurons, binding of [^{3}H]8-OH-DPAT and ^{125}I-CYP to 5-HT-1A and 5-HT-1B sites, respectively, was measured in hippocampus from control and PCA-treated rats. [^{3}H]DHA binding was also measured in the same rats. The lesion induced a 37% increase in [^{3}H]DHA binding, but neither [^{3}H]8-OH-DPAT nor ^{125}I-CYP binding was increased (Table 5). Similarly, [^{3}H]5-HT binding in the hippocampus was not significantly affected by the lesion with PCA (data not presented).

The assays for 5-HT-1A and 5-HT-1B receptor binding include a 10-min preincubation at 37° to remove endogenous 5-HT bound to receptors (15, 16). To determine whether endogenous 5-HT was affecting [^{3}H]DHA binding, homogenized membranes from control and PCA-treated rats were divided and either preincubated at 37° for 10 min or left in an ice bath. Tissues were then washed by centrifugation as described in Materials and Methods. [^{3}H]DHA binding in cortical and hippocampal membranes that were not preincubated was signifi-

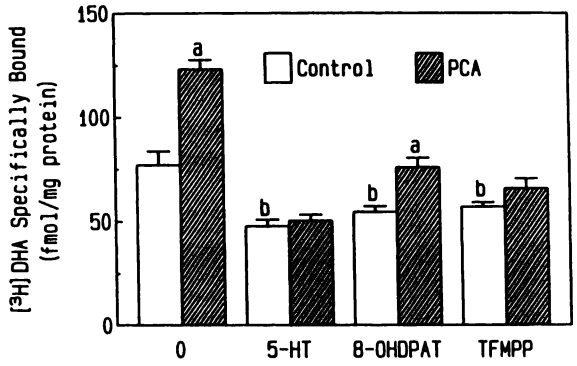
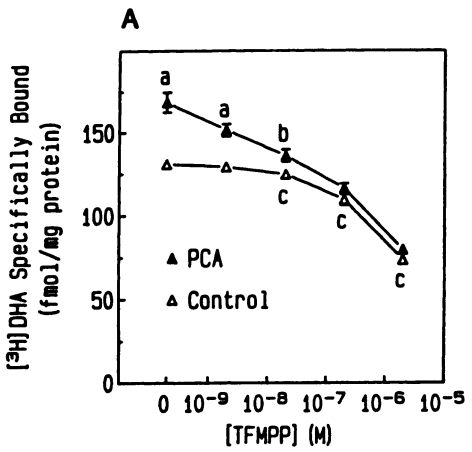


Fig. 6. Effects of lesions with PCA on the binding of [^{3}H]DHA in hippocampus in the absence (0) or presence of serotonergic compounds. Rats were killed 4 weeks after the injections of PCA. The concentration of [^{3}H]DHA was 4.0 nM; nonspecific binding was measured in the presence of 10 μM propranolol. The concentration of 5-HT, 8-OH-DPAT, and TFMPP was 100 nM. Values are fmol/mg of protein and represent the mean ± standard error of five rats/group. Repeated measures analysis of variance indicated a significant interaction between the treatment with PCA and the presence of 5-HT or TFMPP in the assay ($p < 0.001$). $^a p <$ 0.002 compared with the respective control. $^b p < 0.01$ compared with control in the absence of added drug.



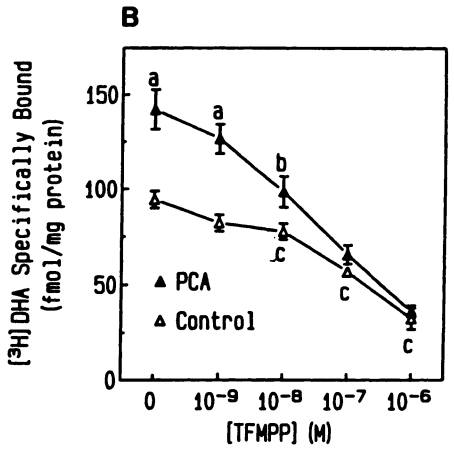


Fig. 7. Effect of lesions with PCA on the binding of [^{3}H]DHA in cortex (A) and hippocampus (B) in the absence (0 M) or presence of increasing concentrations of TFMPP. Rats were killed 4 weeks after the injections of PCA. The concentration of [^{3}H]DHA was 4 nM; nonspecific binding was measured in the presence of 10 μM propranolol. Values are fmol/mg of protein and represent the mean ± standard error of five rats/treatment group. The standard error was smaller than the size of the symbols for some of the data points. Repeated measures analysis of variance indicated a significant interaction between the treatment with PCA and the presence of TFMPP in the assay ($p < 0.001$). $^a p < 0.005$ compared with the respective control. $^b p < 0.05$ compared with the respective control. $^c p < 0.05$ compared with control in the absence of TFMPP.

TABLE 5

Effect of lesions with PCA on the binding of [^{3}H]DHA and on the binding of [^{3}H]8-OH-DPAT or ^{125}I-CYP to 5-HT-1A or 5-HT-1B binding sites, respectively, in rat hippocampus

Nonspecific binding was determined in the presence of 10 μM propranolol in the [^{3}H]DHA assay and 10 μM 5-HT in the [^{3}H]8-OH-DPAT and ^{125}I-CYP assays. Values represent the mean ± standard error of six rats in the [^{3}H]DHA assay and eight or nine rats in the other assays. The value listed for ^{125}I-CYP binding is the B_{max}; the K_d values for control and PCA-treated groups, respectively, were 100.4 ± 7.4 pM and 103.4 ± 10.3 pM. Hippocampi used in the [^{3}H]DHA assay were contralateral to those used in the other assays.

	Binding	
	Control	PCA
[^{3}H]DHA (4.3 nM)	69.7 ± 6.3	95.4 ± 6.9^a
[^{3}H]8-OH-DPAT		
0.75 nM	97.0 ± 14.1	114.0 ± 7.9
5.0 nM	210.2 ± 14.1	221.7 ± 10.5
^{125}I-CYP	117.8 ± 6.8	123.2 ± 8.2

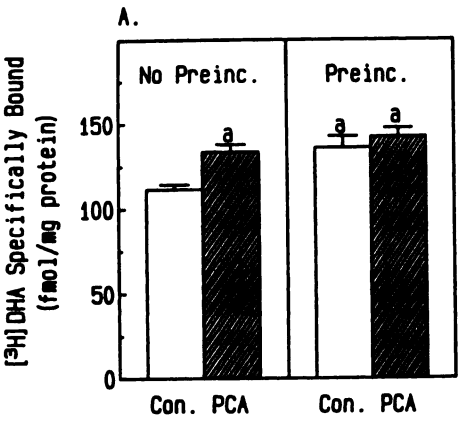
$^a p < 0.015$ compared with control.

cantly increased in PCA-treated rats compared with controls (Fig. 8). However, when tissues from control and PCA-treated rats were preincubated at 37° to remove endogenous 5-HT bound to receptors, the binding of [^{3}H]DHA in controls was increased to the level seen in PCA-treated rats (Fig. 8).

To determine whether any part of the increased [^{3}H]DHA binding in lesioned rats represented β-adrenergic receptors coupled to cyclic AMP production, isoproterenol-stimulated accumulation of cyclic AMP was measured in hippocampal slices from control and PCA-lesioned rats. In contrast to a previous report from our laboratory (1) and despite a large and consistent increase in [^{3}H]DHA specific binding, PCA lesions did not increase either isoproterenol- or norepinephrine-stimulated accumulation of cyclic AMP in hippocampus from lesioned rats (Fig. 9).

Effects of depletion of 5-HT with PCPA. Repeated injections of PCPA for 5 weeks depleted the 5-HT content in the cortex by more than 90% (Fig. 1A) without affecting the content of norepinephrine (Fig. 1B). As was found in 5,7-DHT-lesioned rats, this long term depletion of 5-HT produced by inhibition of synthesis resulted in a significant increase in specific binding of [^{3}H]DHA in the cortex, regardless of whether nonspecific binding was determined with 10 μM isoproterenol, 10 μM propranolol, or 1 μM alprenolol (Fig. 10). In control tissues, the addition of 1 μM 5-HT slightly but significantly decreased [^{3}H]DHA specific binding, regardless of which method was used to determine nonspecific binding; more important, it completely blocked the increase in [^{3}H]DHA binding (Fig. 10).

In the hippocampus, [^{3}H]DHA specific binding was defined using 100 μM isoproterenol (Fig. 11). Although the amount of specific binding was lower when isoproterenol was used to determine nonspecific binding, compared with when propranolol was used (e.g., compare values in Fig. 11 with the values in Table 3), the PCPA-induced depletion of 5-HT still resulted in a nearly 90% increase in specific binding of [^{3}H]DHA (Fig. 11). As in the cortex, the addition of 1 μM 5-HT completely blocked



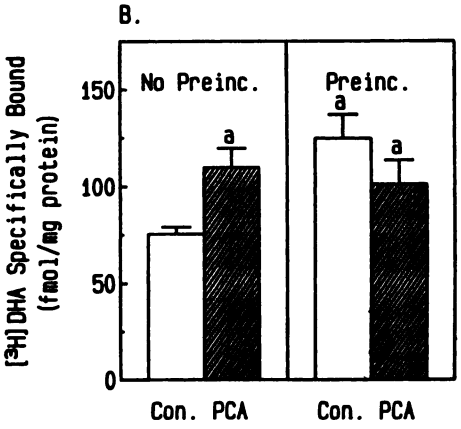


Fig. 8. Effect of preincubation on the binding of [^{3}H]DHA in cortex (A) and hippocampus (B) of control (*Con*) or PCA-treated rats. Membrane homogenates from each animal were either preincubated at 37° for 10 min or kept in ice-water. The concentration of [^{3}H]DHA was 4 nM; nonspecific binding was measured in the presence of 10 μM propranolol. Values are fmol/mg of protein and represent the mean ± standard error of 10 or 11 and 4 to 6 rats/treatment group in the cortex and hippocampus, respectively. $^a p < 0.02$ versus control, no preincubation.

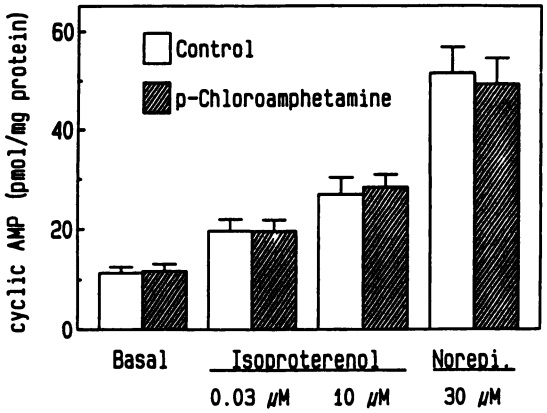


Fig. 9. Effect of lesions with PCA on the net accumulation of cyclic AMP in response to isoproterenol or norepinephrine (*Norepi.*) in rat hippocampus. Rats received two intraperitoneal injections of PCA and were killed 5 weeks later. The cyclic AMP was measured by radioimmunoassay after a 10-min incubation of minces of fresh hippocampus in the absence (basal values) or presence of the agonists. Values represent the mean ± standard error of six control and eight PCA-treated rats.

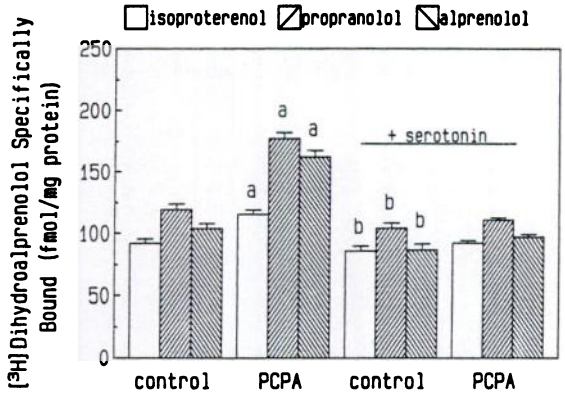


Fig. 10. Effect of repeated treatments with PCPA on the binding of [^{3}H]DHA (4.4 nM) in rat frontal cortex. Rats received intraperitoneal injections of PCPA 3 days/week for 5 weeks and were killed 2 days after the last injection. Nonspecific binding was determined with either 10 μM (−)-isoproterenol, 10 μM DL-propranolol, or 1 μM *l*-alprenolol and the assays were performed in the presence or absence of 1 μM 5-HT. Values represent the mean ± standard error of six rats/group. Repeated measures analysis of variance indicated a significant interaction between the treatment with PCPA and the presence of 5-HT in the assay ($p < 0.005$). $^a p < 0.01$ compared with control in the absence of 5-HT and compared with PCPA-treated rats in the presence of 5-HT. $^b p < 0.05$ compared with control in the absence of 5-HT.

the PCPA-induced increase in [^{3}H]DHA binding; in fact, in the presence of 5-HT, binding of [^{3}H]DHA in PCPA-treated rats appeared to be slightly lower than in controls (Fig. 11).

Finally, as in rats lesioned with either 5,7-DHT or PCA, neither the basal level nor the isoproterenol- or norepinephrine-stimulated accumulation of cyclic AMP in the hippocampus was affected by treatment with PCPA (data not shown).

Discussion

In this study, we found that [^{3}H]DHA binding is increased to a similar extent by either 5-HT neuron lesions or chronic inhibition of 5-HT synthesis with PCPA, indicating that it is the depletion of 5-HT per se and not the lesion of the 5-HT axon that is critical for the increase in binding. Although in a previous study (1) we reported that lesions of 5-HT neurons

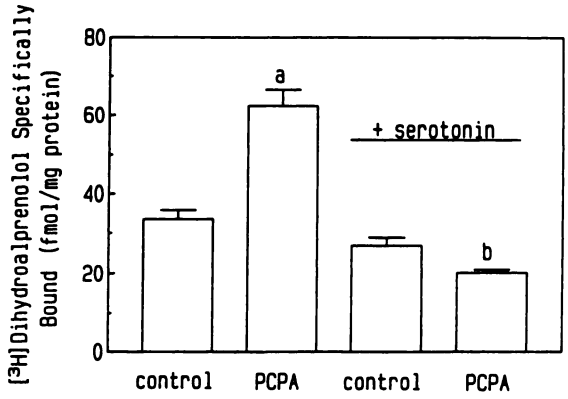


Fig. 11. Effect of repeated treatments with PCPA for 5 weeks on the binding of [^{3}H]DHA (4 nM) in rat hippocampus. Nonspecific binding was determined with 100 μM (−)-isoproterenol and the assays were performed in the presence or absence of 1 μM 5-HT. Values represent the mean ± standard error of six rats/group. Repeated measures analysis of variance indicated a significant interaction between the treatment with PCPA and the presence of 5-HT in the assay ($p < 0.001$). $^a p < 0.001$ compared with respective control. $^b p < 0.05$ compared with respective control.

with PCA increased isoproterenol-stimulated accumulation of cyclic AMP as well as the number of [^{3}H]DHA binding sites in rat hippocampus, in the presence study we were unable to replicate the finding of increased cyclic AMP accumulation after 5-HT neuron lesions with PCA, despite a marked increase in binding. We cannot explain these divergent results, but the present study also found that neither 5,7-DHT lesions nor depletion of 5-HT with PCPA increases cyclic AMP accumulation mediated by β-adrenergic receptors, despite a marked increase in [^{3}H]DHA binding in both cases. Furthermore, neither of these treatments increases norepinephrine-stimulated accumulation of cyclic AMP. Therefore, we conclude that, although both 5-HT neuron lesions and inhibition of 5-HT synthesis markedly increase [^{3}H]DHA binding sites, neither treatment increases the β-adrenergic receptor-mediated response of cyclic AMP.

Previous studies (2–5) have shown that the competition of isoproterenol for [^{3}H]DHA binding sites in rat brain fits a two-site model, with isoproterenol having nanomolar affinity for one site and micromolar affinity for the other. Lesions of 5-HT axons increase the number of [^{3}H]DHA binding sites that have low affinity for isoproterenol (2–5). Recently, Gillespie *et al.* (5) found that addition of low concentrations of 5-HT *in vitro* blocks the increase in [^{3}H]DHA binding induced by 5-HT lesions. These results were interpreted as indicating that β-adrenergic receptors exist in two conformations, one with high affinity and one with low affinity for β-adrenergic agonists (2, 3, 5), and, further, that the receptor conformation with low affinity for β-adrenergic agonists is regulated by 5-HT (5). This reinforced the concept of a possible link between 5-HT and norepinephrine transmission in brain, in which 5-HT modulates β-adrenergic receptors (1, 2, 5, 19, 20).

In the present study, we found that, in both the cortex and the hippocampus, the addition of 100 nM 5-HT to the assays completely blocks the increased binding. In contrast, at concentrations up to 1 μM, neither norepinephrine nor dopamine blocks the increased binding. Thus, the [^{3}H]DHA binding site that is increased by 5-HT depletion has some selectivity for 5-HT among agonists. However, the 5-HT antagonist metergoline also blocks the lesion-induced increase in binding. This strongly

indicates that the site increased by 5-HT depletion is a serotonergic binding site rather than a β-adrenergic receptor regulated by 5-HT. This would also explain why isoproterenol-stimulated cyclic AMP accumulation is not increased by 5-HT depletion despite the marked increase in [^{3}H]DHA binding.

Studies of the possible type of 5-HT binding site labeled by [^{3}H]DHA focused on 5-HT-1 subtypes because in competition studies, 5-HT blocked the increased binding of [^{3}H]DHA with an IC_{50} of approximately 3 nM (Fig. 4), which is characteristic of its affinity at 5-HT-1 subtypes. Furthermore, the 5-HT-2 subtype of receptor is not increased by depletion of 5-HT (c.f., Ref. 21 and references therein). Investigation of which 5-HT-1 subtype is labeled by [^{3}H]DHA in the brain of 5-HT-depleted rats found that in the cortex neither the 5-HT-1A-selective drug 8-OH-DPAT, the 5-HT-1A- and 5-HT-2-selective drug spiperone, nor the 5-HT-1C- and 5-HT-2-selective drug mesulergine significantly affected the binding of [^{3}H]DHA in lesioned rats. In contrast, TFMPP, RU-24969, and CYP completely blocked the increased binding. RU-24969 is equipotent at both the 5-HT-1A and 5-HT-1B sites; CYP is equipotent at the 5-HT-1A and 5-HT-1B sites (22) and is also a potent β-adrenergic antagonist. TFMPP shows very little selectivity for 5-HT binding subtypes (22). Thus, in the cortex, only compounds with relatively high affinity for the 5-HT-1B binding site, regardless of their affinities for the other subtypes, blocked the lesion-induced increase in [^{3}H]DHA binding. In the hippocampus also, TFMPP completely blocked the increased binding in lesioned rats, whereas the binding was still increased in the presence of 8-OH-DPAT, although at the high concentration of 8-OH-DPAT used (100 nM) the increase appeared to be diminished. Thus, although it is possible that in the hippocampus a component of the increased binding of [^{3}H]DHA represents 5-HT-1A sites, the predominant binding site labeled by [^{3}H]DHA in the cortex and probably also in the hippocampus of 5-HT-depleted rats appears to be the 5-HT-1B subtype.

Binding to the 5-HT-1A and 5-HT-1B binding sites was measured directly with [^{3}H]8-OH-DPAT and ^{125}I-CYP, respectively, to determine whether [^{3}H]DHA was binding to a 5-HT-1 receptor in PCA-treated rats. However, measurements of these sites failed to find any increase in binding in the hippocampus from PCA-treated rats, despite marked increases in [^{3}H]DHA binding in the contralateral hippocampus in each case. This absence of an increase in ^{125}I-CYP binding in the hippocampus after 5-HT depletion is consistent with a recent report by Offord *et al.* (14) that lesions of 5-HT neurons with 5,7-DHT do not increase ^{125}I-CYP binding in the rat cortex. Similarly, the absence of an increase in [^{3}H]8-OH-DPAT binding in this study is consistent with reports that lesions of 5-HT neurons do not increase 5-HT-1A sites in rat brain (23, 24).

The most probable explanation for the increases in [^{3}H]DHA binding sites comes from experiments in which the tissues were preincubated before assay. Preincubation removes endogenous 5-HT from the tissues and results in an increase in [^{3}H]DHA binding in control tissues to the levels seen in tissues from lesioned rats. This preincubation does not further increase binding in PCA-lesioned rats because most (greater than 80%) of the 5-HT has been depleted from the tissue *in vivo* by the lesion. This strongly suggests that endogenous 5-HT in non-preincubated control tissues masks all or most of the 5-HT-1 receptors. Thus, removal of endogenous 5-HT *in vitro* by preincubation or *in vivo* by depletion exposes a 5-HT-1 receptor to which [^{3}H]DHA binds. The binding of [^{3}H]8-OH-DPAT and ^{125}I-CYP to 5-HT-1A and 5-HT-1B receptors, respectively, is not affected by the lesion because these assays routinely include a preincubation step to remove endogenous 5-HT.

It is important to note that the increase in [^{3}H]DHA binding after 5-HT depletion is found regardless of whether nonspecific binding is determined with 10 μM propranolol, 1 μM alprenolol, or 10 μM isoproterenol, although in the cortex the increase appears to be largest when either of the antagonists is used. This indicates that even when isoproterenol is used to determine nonspecific binding, [^{3}H]DHA specific binding includes a component of serotonergic sites. The extent of this component depends on the brain area (e.g., hippocampus greater than cortex), the state of the 5-HT system, and the method of preparing membranes (preincubation versus no preincubation). In particular, experimental manipulations may markedly increase the extent of the serotonergic component of [^{3}H]DHA binding, as illustrated by these studies in 5-HT-depleted rats. Thus, [^{3}H]DHA binds predominantly to β-adrenergic receptors in control membranes that are not preincubated; however, either preincubation of control tissues to remove endogenous 5-HT or prior *in vivo* depletion of 5-HT unmasks a 5-HT-1 receptor subtype to which [^{3}H]DHA binds in addition to the β-adrenergic receptor.

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