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Beta Adrenergic Receptor Binding in Membrane Preparations from Mammalian Brain

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SUMMARY

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[^{3}H]Dihydroalprenolol ([^{3}H]DHA) binding sites in membrane preparations of rat and monkey brain appear to involve *beta* adrenergic receptors. [^{3}H]DHA binding is saturable, with high affinity and an apparent dissociation constant of about 1 nM. Determination of the dissociation constant by kinetic studies measuring rate constants for association and dissociation provided K_D values similar to those obtained in equilibrium experiments. [^{3}H]DHA binding is stereospecific for adrenergic agonists and antagonists. The relative potencies of numerous drugs in competing for [^{3}H]DHA binding sites parallel their pharmacological activity at *beta* receptors, and suggest that the receptors are of the $beta_1$ type. Subcellular fractionation studies show an enrichment of [^{3}H]DHA binding sites in "synaptic membrane" fractions. Regional distribution studies reveal fairly low densities of binding sites in the hypothalamus, which contains the highest norepinephrine concentration, suggesting that norepinephrine receptors in the hypothalamus are predominantly of the *alpha* variety. The relatively high levels of [^{3}H]DHA binding in the cerebral cortex and cerebellum suggest that synaptic actions of norepinephrine in these regions may involve *beta* receptors.

INTRODUCTION

Direct biochemical labeling of *beta* adrenergic receptor sites has been reported in red blood cell membrane preparations, using both antagonists (1-5) and the agonist isoproterenol (1), and in heart (6) and pineal gland (7) preparations, using the antagonist alprenolol.

In the brain, where norepinephrine is an important neurotransmitter, the nature of the adrenergic receptor sites is quite unclear (8). Because the physiological monitoring of neurotransmitter receptors is much more difficult in brain tissue than in peripheral nervous systems, major characteristics of adrenergic receptors in the brain, such as drug sensitivity, have not been ascertained. Although there is some neurophysiological evidence for *beta* receptors in the cerebellum (9, 10), most central adrenergic receptors have not been characterized in terms of *alpha* and *beta* types (11).

In the present investigation we report the labeling of *beta* adrenergic receptors in membrane preparations from monkey and rat brains, using the *beta* antagonist [^{3}H]dihydroalprenolol. The characteristics of [^{3}H]DHA[3] binding, including drug spec-

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[3] The abbreviation used is: DHA, dihydroalprenolol.

ificity, kinetics, detailed regional distribution, and subcellular localization, are described.

MATERIALS AND METHODS

Materials. (−)-Alprenolol HCl was tritiated for us at New England Nuclear Corporation by reduction with tritium gas, using a palladium catalyst. The resulting compound was purified in our laboratory, using Eastman Chromagram 13179 silica gel thin-layer chromatography sheets developed with acetone–benzene–acetic acid, 70:25:5 (4). The resulting compound, which had a specific activity of 30 Ci/mmole, was stable for at least 3 months when stored in absolute ethanol at −20°. Aliquots of this solution were diluted 200-fold in H_2O to give a stock solution, which was stored at −20° for not more than 4 days.

In the thin-layer chromatography system described above, [^{3}H]DHA and (−)-alprenolol have similar R_F values (0.3). However, if chromatography sheets treated with a saturated solution of $AgNO_3$ in methanol are developed with 1-butanol–acetic acid–water (25:4:10), [^{3}H]-DHA has an R_F of about 0.8 while that for (−)-alprenolol is about 0.4.[4] Using this chromatography system, we were unable to detect any (−)-[^{3}H]alprenolol in our [^{3}H]DHA preparation. This result helps to exclude the possibility of contamination with (−)-alprenolol, because tritium exchange reactions occurring during the tritiation procedure would be expected to result in general labeling of (−)-alprenolol with low specific activity.[4] Thus, if there were a significant amount of (−)-alprenolol remaining after reduction, it would have been detected in the $AgNO_3$ chromatography system.

Recently [^{3}H]DHA [with no apparent (−)-alprenolol contamination] has become routinely available from New England Nuclear. This [^{3}H]DHA (lot 874-102, 32.6 Ci/mmole) gave essentially the same results as our [^{3}H]DHA when compared in our binding assay system. As part of the characterization of [^{3}H]DHA at New England Nuclear, deuterium-labeled DHA was prepared by the procedure used for [^{3}H]DHA, and the structure was confirmed by nuclear magnetic resonance and mass spectroscopy. In these studies no contamination by (−)-alprenolol was detected.[4] Other preparations of [^{3}H]DHA have been shown to possess the same biological activity as (−)-alprenolol in activation of adenylate cyclase (4).

The following drugs were kindly donated by the indicated companies: (−)- and (+)-alprenolol HCl and (±)-dihydroalprenolol, Hässle; (−)- and (+)-isoproterenol bitartrate, (+)-norepinephrine bitartrate, (+)-epinephrine bitratrate, (±)-phenylephrine HCl, and (+)-phenylephrine bitartrate, Sterling-Winthrop; hydroxybenzylpindolol, Dr. D. Hauser, Sandoz; (±)-dichloroisoproterenol, Lilly; (−)- and (+)-practolol, Imperial Chemical; (−)- and (+)-propranolol, Ayerst; (±)-salbutamol, Allen and Hanbury; (±)-terbutaline sulfate and phentolamine, Ciba-Geigy; and (−)- and (+)-sotalol HCl and (+)-MJ-1998, Mead Johnson. (±)-Cc-34 benzoate, (±)-Cc-25 benzoate, and (±)-MJ-9184-1 were gifts from Dr. R. Lefkowitz. (−)-Norepinephrine bitartrate, (−)-epinephrine bitartrate, and Tris were obtained from Sigma Chemical Company, and (−)-[^{3}H]norepinephrine (27.7 Ci/mmole) and [^{14}C]tryptamine (55.2 Ci/mmole), from New England Nuclear Corporation. Other drugs and materials were obtained from the pharmaceutical company of origin or commercial sources.

[^{3}H]DHA binding assay. Male Sprague-Dawley rats (45–55 days old) were killed by decapitation, and the brains were rapidly removed and chilled in ice-cold 0.9% NaCl. The cerebral cortices were removed, homogenized using a Brinkmann Polytron (model PT-10 ST, setting 6, 20 sec) in 10–30 volumes of ice-cold 0.05 M Tris (pH 8.0 at 25°), and centrifuged at 49,000 × g for 15 min. The pellet was rehomogenized in the same buffer and centrifuged as before. The pellet was finally resuspended in 97 ml of the buffer per gram of original wet weight of tissue. If catecholamines were to be used as ligands, 0.1% ascorbic acid and 1 μM pargyline were added to prevent destruction of the catecholamines. Inhibition of [^{3}H]DHA binding by (−)-alprenolol was

[4] M. Randall, New England Nuclear Corporation, personal communication.

the same with or without these additions. In other studies of binding to brain membranes associated with opiate, glycine, γ-aminobutyric acid, serotonin, dopamine, and muscarinic cholinergic receptors, we have found that homogenization with a Polytron instrument produces more uniform dispersions of smaller membrane fragments and more reproducible results than conventional glass or Teflon pestles and homogenizers (12).

[^{3}H]DHA binding was routinely determined at 23° by adding to 0.97 ml of the above particulate suspension (representing 10 mg of tissue, or about 0.4 mg of protein) 10 μl of the appropriate concentration of drug followed by about 66,000 dpm of [^{3}H]DHA in 20 μl of water (final [^{3}H]DHA concentration, 1 nM). The protein concentration of 0.4 mg/10 mg of tissue was consistent in analyses of representative samples from all tissue preparations. After 15–20 min the reaction mixtures were filtered under reduced pressure through Whatman glass fibers (GF/B). The filters were rinsed four times with 4 ml of ice-cold Tris buffer (total filtering time was about 5 sec) and placed in plastic vials along with 12 ml of Hydromix (Yorktown Research). The vials were mechanically shaken for 2 hr and then stored at 4° for 6–24 hr, until the radioactivity was measured in a Packard 3385 scintillation spectrometer at 45% efficiency. All assays were conducted in triplicate.

The extent of nonspecifically bound [^{3}H]DHA was estimated from parallel assay tubes which contained a large excess (1 μM) of (−)-alprenolol. Specific binding, defined as the difference between total and nonspecific binding, was 70–80% of the total binding. To check the possibility that some of the specifically bound [^{3}H]DHA was removed by the rinsing procedure, the volume of buffer used for rinsing was varied from 4 to 60 ml. The amount of specifically bound [^{3}H]DHA was constant through this range, while both the total and nonspecific binding decreased markedly, resulting in an increase of the percentage of specific binding from 38% at 4 ml to 90% at 60 ml. The amount of [^{3}H]DHA bound by the filter in the absence of tissue was about 0.7% of the total radioactivity in the incubation tube (or about 10% of the total binding in the presence of tissue) and was the same in the presence and absence of 1 μM (−)-alprenolol. This filter binding was part of the nonspecific binding defined above and was subtracted as part of the "blank."

The stability of [^{3}H]DHA under our incubation conditions was examined at pH 6.8, 8.0, and 9.4. At each pH, 5 ml of a standard incubation mixture were centrifuged at 49,000 × *g* for 10 min, and the supernatant and pellet were each extracted with ethyl acetate. The extracts were reduced in volume under a stream of N_2 and analyzed by thin-layer chromatography in the acetone–benzene–acetic acid (70:25:5) system. The major peak in each case (80–95% of the total radioactivity) corresponded to unincubated [^{3}H]DHA.

The filter assay was used in preference to the microfuge (4) assay, because the percentage of specific binding is much higher. For example, in preliminary experiments using frog and turkey red blood cell membranes, the microfuge assay gave about 50% specific binding, whereas the filter assay gave more than 90%. Similarly, with brain tissue, about 20% specific binding was obtained using the microfuge assay, and 75% using the filter assay.

Subcellular fractionation. Various subcellular fractions of rat brain were obtained by differential and discontinuous sucrose gradient centrifugation. Cerebral cortices were homogenized in 10 volumes of ice-cold 0.32 M sucrose, using a glass homogenizer with a motor-driven Teflon pestle. After centrifugation at 1000 × *g* for 10 min, the crude nuclear pellet (P_1) was washed with an equal volume of 0.32 M sucrose. The crude mitochondrial pellet (P_2) was obtained by centrifuging the combined supernatant fractions at 17,500 × *g* for 20 min. The supernatant was centrifuged at 100,000 × *g* for 1 hr to obtain the crude microsomal pellet (P_3). Part of the P_2 pellet was suspended in 0.32 M sucrose and further fractionated on a 0.8–1.2 M sucrose two-step discontinuous gradient (13). Another part of P_2 was lysed by resuspension in water and centrifuged at 10,000 × *g* for

20 min. The resulting supernatant fluid was layered on discontinuous sucrose gradients (equal volumes of 0.4, 0.6, 0.8, 1.0, and 1.2 M sucrose), which were centrifuged at 61,000 × *g* for 120 min (14).

The pellet fractions (except P_3; see below) were suspended in 0.32 M sucrose. After removal of aliquots for the determination of norepinephrine uptake (15), all fractions were diluted 3–6-fold with Tris buffer and centrifuged at 49,000 × *g* for 15 min. The resulting pellets were suspended in Tris buffer with the Polytron and centrifuged as before. The crude microsomal pellet (P_3) was suspended in Tris buffer and centrifuged at 100,000 × *g* for 30 min. After resuspension with the Polytron, aliquots were removed to determine [^{3}H]DHA binding and monoamine oxidase activity (16).

Protein concentration was determined by the method of Lowry *et al.* (17), using bovine serum albumin as a standard.

RESULTS

Tissue linearity and pH optimum. Specific [^{3}H]DHA binding increases linearly with increasing tissue concentration over the range of 2.5–20 mg of original wet weight of tissue per milliliter (about 0.1–0.8 mg of membrane protein per milliliter). At higher tissue concentrations, the binding is less than linear. Assays were routinely conducted at 10 mg of tissue per milliliter, which was well within the linear range.

Specific [^{3}H]DHA binding displays a rather broad pH optimum, with no major variations between 6.8 and pH 9.4 (Fig. 1). However, there is a marked decrease in nonspecific binding of [^{3}H]DHA with increasing pH. Thus nonspecific binding falls about 3-fold between pH 6.8 and 8.0 and remains approximately constant at higher pH values. Routine assays were conducted at pH 8.0 in order to maximize the ratio of specific to nonspecific binding.

Saturation of [^{3}H]DHA binding. Specific binding of [^{3}H]DHA is saturable, with half-maximal binding at about 1 nM (Fig. 2A). By contrast, nonspecific binding increases linearly with increasing [^{3}H]DHA concentration. In routine binding assays

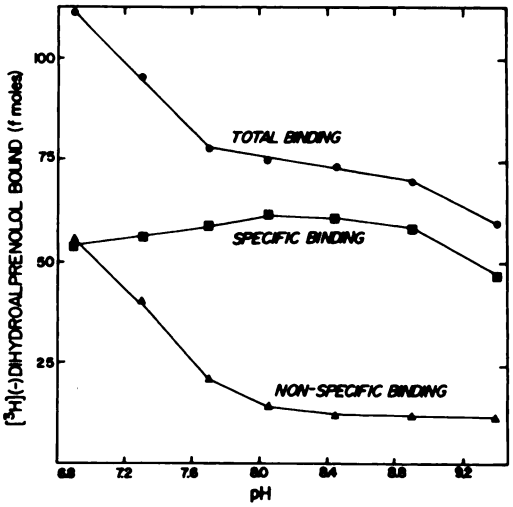

FIG. 1. *Specific [^{3}H]DHA binding as a function of pH*

The membrane fraction from rat cerebral cortex was prepared to a final concentration of 50 mg of original wet weight of tissue per milliliter and then diluted to a concentration of 10 mg/ml with 0.05 M Tris buffer of varying pH values. The pH of the suspension was estimated with a Radiometer pH meter. Aliquots (1.0 ml) were incubated with 1.0 nM [^{3}H]DHA with and without 1 μM (−)-alprenolol and assayed as described in MATERIALS AND METHODS. Specific binding is the difference between total binding [without (−)-alprenolol] and nonspecific binding [in the presence of 1 μM (−)-alprenolol]. Values are the means of triplicate incubations, which differed by less than 10%. The experiment was repeated three times.

with 1.0 nM [^{3}H]DHA, specific binding is 3 times nonspecific values. Scatchard analysis of this saturation curve indicates a single binding component with an apparent dissociation constant of about 1.3 nM (Fig. 2B). The mean ± standard error for six similar experiments is 1.1 ± 0.1 nM. The amount of [^{3}H]DHA bound at saturation provides an estimate of the number of specific binding sites. The mean ± standard error for the six experiments using rat cerebral cortex is 11 ± 1 pmoles/g of tissue, or about 0.3 pmole/g of protein.

To test for cooperativity, we plotted these data according to the Hill equation (Fig. 2C). A straight line is obtained with a Hill coefficient of 1.00 ± 0.02 (mean ± standard error for six experiments), suggesting the absence of positive or negative

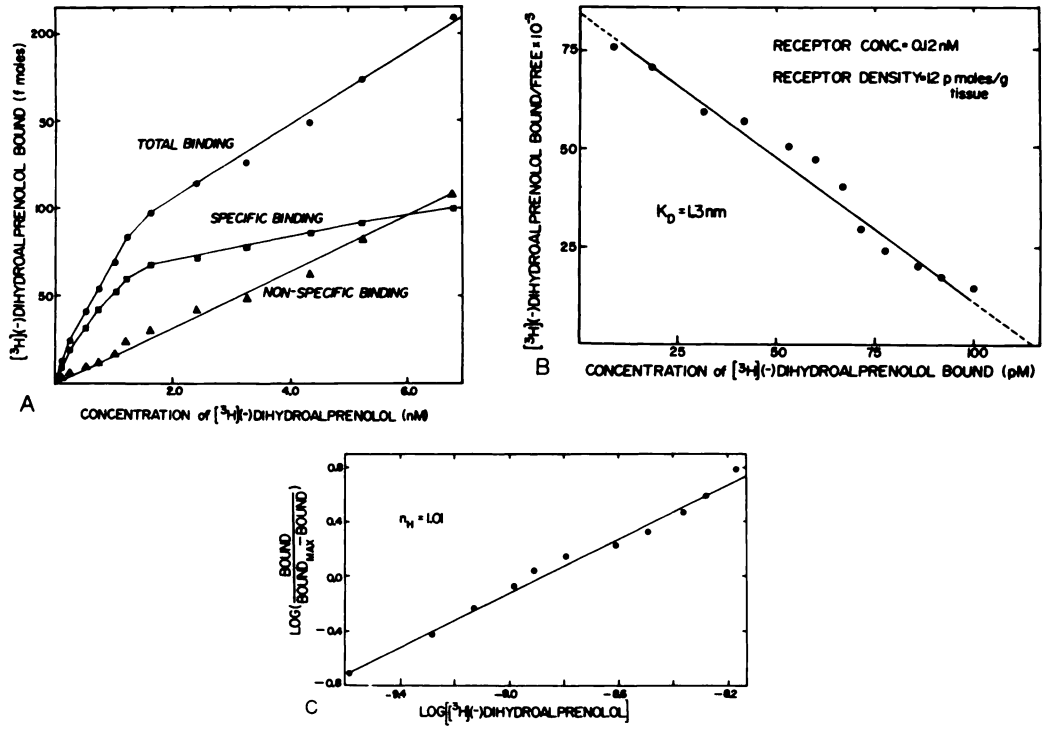

FIG. 2. *[³H]DHA binding as a function of increasing concentrations of [³H]DHA*

The membrane fraction from rat cerebral cortex (10 mg of tissue per milliliter) was assayed for [³H]DHA binding using increasing concentrations of [³H]DHA. A. A direct plot of the data, showing total binding, nonspecific binding [with the addition of (−)-alprenolol to 1 μM], and specific binding (the difference between total and nonspecific). B. Scatchard plot, which gives an apparent K_D of 1.3 nM and a receptor density of 12 pmoles/g of tissue. C. A Hill plot of the data, which gives a Hill coefficient (n_H) of 1.0.

cooperative effects within the concentration range studied. By contrast, in red blood cell membranes, Limbird *et al.* (18) found a Hill coefficient of 0.82 for (−)-alprenolol, using high tissue and (−)-alprenolol concentrations.

Kinetics of [³H]DHA binding. When studied at 23°, [³H]DHA associates with binding sites fairly rapidly (Fig. 3A). Binding attains equilibrium by about 7–10 min, with half-maximal binding at about 2 min. Using this data, the bimolecular rate constant is calculated to be 0.13 nM^{-1} min^{-1} at 23° (mean ± standard error for three experiments, 0.12 ± 0.03) from the equation $K_1 = [1/t(a - b)] \ln [b(a - x)/a(b - x)]$, where a is the initial concentration of [³H]DHA (1.0 nM), b is the receptor concentration (0.11 nM; see above), and x is the concentration of specifically bound [³H]DHA at time t.

The rate of dissociation of [³H]DHA was examined at three temperatures (Fig. 3B). At all three temperatures, 37°, 23°, and 0°, dissociation appears to be a first-order process, represented by a straight line when plotted on a semilogarithmic scale. The half-lives for dissociation at 37°, 23°, and 0° are about 3, 7, and 20 min, respectively. The rate constant k_2 for [³H]DHA dissociation from the receptor at 23° is calculated to be 0.10 ± 0.007 min^{-1} (mean ± standard error of 11 experiments, using all three procedures described below) from the equation $k_2 = (1/t) \ln (a/x)$, where a is the initial concentration of bound [³H]DHA and x is the concentration of bound [³H]DHA at time t. The dissociation constant for [³H]DHA-receptor interactions calculated from the ratio of dissociation rate constant to association rate constant (k_2/k_1), based on these direct kinetic data,

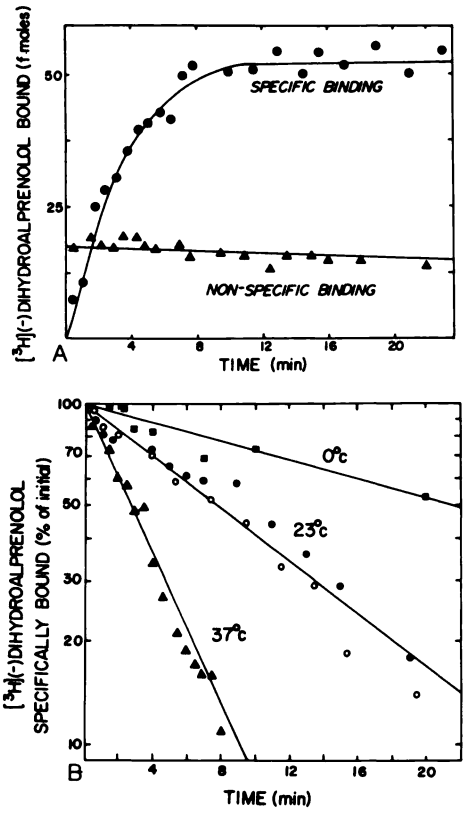

FIG. 3. *Kinetics of [³H]DHA binding*

A. Association of [³H]DHA and its receptor was measured under the standard assay conditions as a function of time from the addition of [³H]DHA (1.0 nM); the nonspecific binding [with 1 μM (−)-alprenolol added] was subtracted from the total binding [without (−)-alprenolol] to give specific binding. B. Dissociation of [³H]DHA from its receptor was measured at three temperatures: 0° (■), 23° (●, ○), and 37° (▲). At 0° and 37°, (−)-alprenolol (1 μM) was added at zero time to standard incubations which had been exposed to 1.0 nM [³H]DHA for 40 min and 15 min, respectively. At 23°, the incubation contained 0.3 g (original wet weight of tissue) of the standard membrane preparation and 3.4 pmoles of [³H]DHA in a total volume of 11 ml. After 30 min, 539 ml of buffer were added either with (○) or without (●) (−)-alprenolol (1 μM), and 10-ml aliquots were filtered according to the standard procedure. Nonspecific binding was estimated from equivalent incubations in the presence of 1 μM (−)-alprenolol and from the [³H]DHA remaining bound at times greater than five half-lives.

is 0.8 nM, which corresponds closely to the K_D estimated from equilibrium studies.

The measured rate of dissociation is the same whether excess (−)-alprenolol or dilution with a large volume of buffer, or both, is used to prevent the rebinding of dissociated [³H]DHA (Fig. 3B). The dissociation of [³H]DHA from frog red blood cell membranes at 10° has been reported to be more rapid with excess (−)-alprenolol than in experiments in which dissociation is facilitated only by dilution (18). It was suggested that these findings indicate negative cooperativity in the receptor interactions with (−)-alprenolol. Our experiments failed to reveal any such interactions in brain membranes.

Drug specificity. (−)-Alprenolol inhibits the binding of [³H]DHA 50% at 2 nM (Fig. 4), which corresponds to a K_i of about 1 nM (Table 1) and is similar to the K_D for [³H]DHA obtained from kinetic and equilibrium experiments. The IC_{50} value for the inhibition of [³H]DHA binding by (−)-DHA can be estimated to be 1.3 nM [assuming that it is one-half the IC_{50} for (±)-DHA], which is essentially the same as the IC_{50} for (−)-alprenolol. The accuracy of the K_i values as estimated from the data in Table 1, using the equation $K_i = IC_{50}/(1 + [[^3H]DHA]/K_D)$, is limited by the accuracy of both the IC_{50} value and the K_D value for [³H]DHA. Since both these values vary about 2-fold, the calculated K_i values could vary about 3-fold.

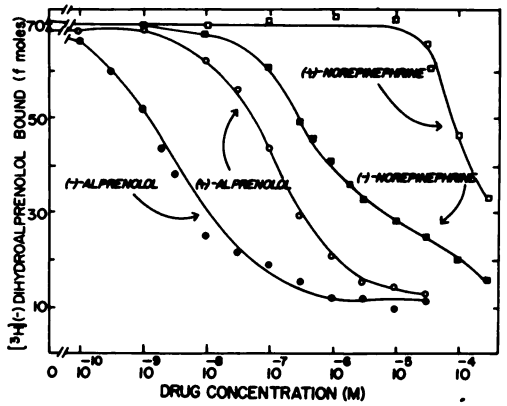

FIG. 4. *Inhibition of [³H]DHA binding by alprenolol and norepinephrine*

The inhibition of [³H]DHA binding was determined as described in MATERIALS AND METHODS. Concentrations which gave half-maximal inhibition [using 1 μM (−)-alprenolol as maximal inhibition] are: (−)-alprenolol, 2.5 nM; (+)-alprenolol, 0.16 μM; (−)-norepinephrine, 1 μM; (+)-norepinephrine, 180 μM.

TABLE 1

Inhibition of [³H]DHA binding

The IC_{50} for each drug was determined as described in Figs. 4 and 5. When catecholamines were studied, 0.1% ascorbic acid and 1 μM pargyline were included in the incubation. Values given are the means ± standard errors of two or more determinations, each done in triplicate. Since the concentration of [³H]DHA was 1.0 nM, the K_i value calculated for each drug is one-half the IC_{50} value, using the equation (19) $K_i = IC_{50}/(1 + [[^3H]DHA]/K_D)$. The following compounds had IC_{50} values greater than 100 μM: acetophenazine, apomorphine, ATP, (+)- and (−)-butaclamol, carbinoxamine, cyclic AMP, cyclizine, epinine, GTP, α- and β-flupenthixol, mesoridazine, methapyrilene, morphine, naloxone, octopamine, phenethylamine, (−)-*threo*- and (+)-*erythro*-phenyl-2-piperidylcarbinol, and tranylcypromine. The following did not inhibit binding at a concentration of 20 μg/ml (approximately 100 μM): acetylcholine, *N*-acetylglucosamine, α-alanine, Δ-aminolevulinic acid, 6- aminopenicillanic acid, anserine, antazoline, arginine, aspartate, atropine, benzylamine, butyrylcholine, caffeine, carnosine, carphenazine, catechol, chlorcyclizine, *N*-(2-chloroethyl)dibenzylamine, cycloleucine, cycrimine, dexbrompheniramine, dexchlorpheniramine, dianisidine, diazepam, diethylpropion, dopa, fluphenazine, γ-aminobutyric acid, glutamate, glutamine, 6-hydroxydopamine, 5-hydroxyindoleacetic acid, 4-hydroxy-3-methoxymandelic acid, hydroxyzine, iproniazid, leucine, α-methyldopa, methylphenidate, α-methyltyrosine, *p*-bromdylamine, perphenazine, phendimetrazine phenelzine, phenylalanine, (+)-*threo*-phenyl-2-piperidylcarbinol, physostigmine, procyclidine, promethazine, 2-*n*-propylphenol, parathiazine, scopolamine, serine, trimeprazine, tyramine, and valine.

Compound	IC_{50}	Compound	IC_{50}
	M		*M*
	Adrenergic antagonists		
(±)-Hydroxylbenzylpindolol	$5.0 \pm 1.0 \times 10^{-10}$	(−)-Practolol	$8.8 \pm 1.5 \times 10^{-7}$
(−)-Alprenolol	$2.3 \pm 0.2 \times 10^{-9}$	(−)-Sotalol	$1.9 \pm 0.5 \times 10^{-6}$
(−)-Propranolol	$2.1 \pm 0.4 \times 10^{-9}$	(±)-MJ-1998	$1.2 \pm 0.4 \times 10^{-5}$
(±)-Dihydroalprenolol	$2.6 \pm 0.7 \times 10^{-9}$	Ergotamine	$1.7 \pm 0.5 \times 10^{-5}$
(±)-Dichloroisoproterenol	$1.0 \pm 0.3 \times 10^{-7}$	(+)-Practolol	$3.3 \pm 0.8 \times 10^{-4}$
(+)-Alprenolol	$1.7 \pm 0.3 \times 10^{-7}$	(+)-Sotalol	$4.9 \pm 1.1 \times 10^{-4}$
(+)-Propranolol	$4.0 \pm 0.6 \times 10^{-7}$	Phentolamine	$>1 \times 10^{-4}$
		Phenoxybenzamine	$>1 \times 10^{-4}$
	Adrenergic agonists		
(−)-Isoproterenol	$4.6 \pm 0.9 \times 10^{-8}$	(−)-*erythro*-Phenyl-2-piperidyl-carbinol	$1.8 \pm 0.5 \times 10^{-5}$
(±)-Cc-25	$1.8 \pm 0.3 \times 10^{-7}$	(±)-Phenylephrine	$2.1 \pm 0.6 \times 10^{-5}$
(±)-Cc-34	$4.9 \pm 0.5 \times 10^{-7}$	(−)-Amphetamine	$4.2 \pm 1.8 \times 10^{-5}$
(±)-MJ-9184-1	$4.6 \pm 0.5 \times 10^{-7}$	(±)-Synephrine	$5.1 \pm 1.0 \times 10^{-5}$
(−)-Norepinephrine	$8.0 \pm 1.1 \times 10^{-7}$	(+)-Epinephrine	$8.0 \pm 1.4 \times 10^{-5}$
(−)-Epinephrine	$1.1 \pm 0.4 \times 10^{-6}$	(+)-Amphetamine	$8.2 \pm 3.8 \times 10^{-5}$
(+)-Isoproterenol	$2.8 \pm 0.7 \times 10^{-6}$	(+)-Norepinephrine	$1.2 \pm 0.3 \times 10^{-4}$
(±)-Salbutamol	$2.7 \pm 0.5 \times 10^{-6}$	Dopamine	$>1 \times 10^{-4}$
(±)-2,5-Dimethoxy-4-ethyl-amphetamine	$8.0 \pm 1.5 \times 10^{-6}$	(+)-Phenylephrine	$>1 \times 10^{-4}$
(−)-Ephedrine	$1.1 \pm 0.5 \times 10^{-5}$	(+)-Ephedrine	$>1 \times 10^{-4}$
(±)-Terbutaline	$2.7 \pm 0.4 \times 10^{-5}$	(+)- and (−)-Pseudoephedrine	$>1 \times 10^{-4}$
	Other		
trans-N-Methylphenylcyclopropylamine	$1.5 \pm 0.6 \times 10^{-5}$	*cis*-Thiothixene	$6.4 \pm 2.0 \times 10^{-5}$
trans-N,N-Dimethylphenylcyclopropylamine	$3.3 \pm 0.7 \times 10^{-5}$	Promazine	$6.6 \pm 1.7 \times 10^{-5}$
Nortriptyline	$3.7 \pm 0.5 \times 10^{-5}$	Protriptyline	$6.7 \pm 2.2 \times 10^{-5}$
Dimethindene	$4.1 \pm 1.0 \times 10^{-5}$	Imipramine	$8.0 \pm 2.8 \times 10^{-5}$
Haloperidol	$5.5 \pm 1.2 \times 10^{-5}$	Benztropine	$>1 \times 10^{-4}$
Bulbocapnine	$6.1 \pm 0.9 \times 10^{-5}$	Serotonin	$>1 \times 10^{-4}$
Chlorprothixene	$6.3 \pm 1.6 \times 10^{-5}$	Iprindole	$>1 \times 10^{-4}$
		trans-Thiothixene	$>1 \times 10^{-4}$

Binding of [³H]DHA is stereospecific, both for isomers of alprenolol and other adrenergic antagonists and for isomers of norepinephrine and other agonists (Fig. 4 and Table 1). (+)-Alprenolol is only about 1% as potent as (−)-alprenolol in inhibiting [³H]DHA binding. (−)-Norepinephrine has considerably less affinity for binding sites than (−)-alprenolol, with half-maximal inhibition at 0.8 μM. Inhibition by norepinephrine is also stereospecific, as (+)-norepinephrine has only about 1% of the affinity of (−)-norepinephrine for receptor sites. Log probit analysis indicates that the slopes for inhibition of [³H]DHA binding by alprenolol and other antagonists, and by norepinephrine and other agonists, are the same (Fig. 5). This differs from the lack of parallelism in the competition of agonist and antagonists for muscarinic cholinergic[5] (20) and postsynaptic

[5] H. I. Yamamura, K. J. Chang, and S. H. Snyder, manuscript in preparation.

serotonin (21) receptor binding, but resembles the patterns seen for agonists and antagonists at the opiate receptor (22). The most potent *beta* antagonist is hydroxybenzylpindolol, whose IC_{50} of 0.5 nM indicates an affinity about 4 times greater than that of (−)-alprenolol. Propranolol and alprenolol have essentially the same affinity for the receptor, as has been reported for red blood cell and pineal gland membranes (3, 7). Other antagonists are substantially less potent. Dichloroisoproterenol has an IC_{50} of 0.1 μM, and (−)-practolol, a selective $beta_1$ antagonist, inhibits binding half-maximally at 0.9 μM. The *beta* antagonists sotalol and MJ-1998 have IC_{50} values between 2 and 10 μM, while the *alpha* receptor antagonists ergotamine, phentolamine, and phenoxybenzamine are substantially weaker.

Of the catecholamines, (−)-isoproterenol is the most potent, with an IC_{50} of 50 nM. (−)-Isoproterenol has about 20 times greater affinity for [^{3}H]DHA binding sites than (−)-norepinephrine, whose affinity is similar to that of (−)-epinephrine. This order of potency is characteristic of a $beta_1$ type of receptor. The importance of the β-hydroxyl group is exemplified by the failure of dopamine to inhibit [^{3}H]DHA binding (IC_{50} > 100 μM). The catechol group is required for binding for simple phenylethylamines, sine phenylephrine, which differs from epinephrine only in the absence of the *p*-hydroxyl moiety, is 5% as potent as (−)-epinephrine. The synthetic *beta* agonists Cc-25, Cc-34, and MJ-9184-1, with IC_{50} values of 0.2–0.5 μM, are more potent than any *beta* agonists except for isoproterenol. Salbutamol and terbutaline, which are selective $beta_2$ agonists, are relatively weak, with IC_{50} values of 3 and 20 μM.

The extent of stereospecificity is similar for most catecholamines examined. The (−) isomers are about 100 times more potent than the (+) isomers for isoproterenol, norepinephrine, and epinephrine. Sympathomimetic amines possessing two asymmetrical centers evince considerable stereospecificity. Of the four isomers of ephedrine, only (−)-ephedrine shows activity, with an IC_{50} of 10 μM, while the IC_{50} values for the other isomers are greater than 100 μM. Similarly, (−)-*erythro*-phenyl-2-piperidylcarbinol possesses activity, while the other three isomers fail to inhibit [^{3}H]DHA binding.

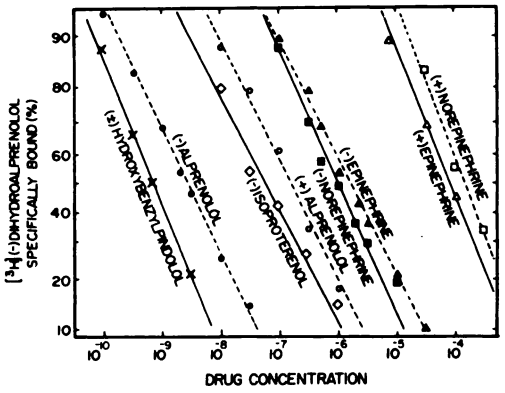

FIG. 5. *Log probit plots of agonist and antagonist inhibition of [^{3}H]DHA binding*

The inhibition of [^{3}H]DHA was determined as in Fig. 4. The ordinate is the percentage of maximal specific binding as determined in the absence of added drugs.

The psychedelic mescaline-amphetamine derivative 2,5-dimethoxy-4-ethylamphetamine (23) lacks a catechol or β-hydroxy grouping but does inhibit binding 50% at 8 μM. A large number of phenothiazines, tricyclic agents, antihistamines, and anticholinergic drugs are weak or inactive in competing for [^{3}H]DHA binding sites.

Regional variations of [^{3}H]DHA binding. To assess the extent to which norepinephrine actions in various brain regions involve *beta* receptors, we evaluated [^{3}H]DHA binding in various regions of rat and monkey brain (Table 2). Marked regional differences exist which fail to parallel regional variations in endogenous norepinephrine concentrations. Thus the highest density of binding sites occurs in the corpus striatum and cerebral cortex, both areas with very low endogenous norepinephrine concentrations. By contrast, the hypothalamus, which contains the highest endogenous norepinephrine levels of rat brain, has one of the smallest levels of [^{3}H]DHA binding sites. The fewest [^{3}H]DHA binding sites occur in the medulla-pons. The cerebellum, which has

TABLE 2

Regional distribution of (−)-[³H]dihydroalprenolol binding in rat and monkey brain

Three rhesus monkeys *(Macaca mulatta)* were injected intraperitoneally with 40 mg/kg of sodium pentobarbital and decapitated 1 hr later. The skull was opened with an autopsy saw, and the brain was transferred to ice-cold 0.9% NaCl. After dissection, the membrane fractions were prepared as described for rat tissue in MATERIALS AND METHODS. The concentrations of [³H]DHA used were 2.0 nM for monkey preparations and 1.5 nM for rat. Since these concentrations are not saturating, the values given are only proportional to the receptor density. However, as shown in Table 3, the K_i values for (−)-alprenolol and (−)-norepinephrine are similar in various brain regions, and thus the values presented in this table are relative receptor densities. Values are the means and standard errors of the number of determinations shown.

Region	[³H]DHA bound	Region	[³H]DHA bound
	pmoles/g tissue		*pmoles/g tissue*
	Rat		
Cerebral cortex	6.3 ± 0.4 (5)	Midbrain-thalamus	3.1 ± 0.2 (5)
Hippocampus	3.9 ± 0.2 (5)	Cerebellum	3.8 ± 0.2 (5)
Hypothalamus	2.8 ± 0.3 (5)	Medulla-pons	2.3 ± 0.3 (5)
Corpus striatum	7.0 ± 0.4 (5)		
	Monkey		
Olfactory bulb	0.4 (1)	Extrapyramidal areas	
Cerebrum		Head of caudate	3.0 ± 0.5 (3)
Frontal pole	4.2 ± 0.2 (3)	Body of caudate	2.4 ± 0.2 (3)
Occipital pole	4.3 ± 0.2 (3)	Tail of caudate	1.8 ± 0.4 (2)
Temporal pole	3.8 ± 0.4 (2)	Putamen	2.5 ± 0.2 (3)
Precentral gyrus	3.0 ± 0.2 (3)	Globus pallidus interior	2.5 ± 0.2 (2)
Postcentral gyrus	3.9 ± 0.3 (3)	Globus pallidus exterior	2.2 ± 0.7 (2)
Superior temporal gyrus	4.8 ± 0.3 (3)	Substantia nigra	2.1 ± 0.3 (3)
Inferior temporal gyrus	3.7 ± 0.2 (3)	Midbrain	
Medial temporal gyrus	4.1 ± 0.8 (3)	Ventral	1.8 ± 0.3 (3)
Cingulate gyrus	4.9 (1)	Superior colliculi	1.3 ± 0.2 (3)
Corpus callosum		Inferior colliculi	2.2 ± 0.1 (3)
Anterior	0.7 ± 0.1 (3)	Periaqueductal gray	2.0 ± 0.3 (3)
Posterior	2.4 ± 0.4 (2)	Cerebellum	
Splenium	1.4 (1)	Lateral cortex	2.3 ± 0.2 (3)
Limbic cortex		Dorsal lateral cortex	1.5 ± 0.2 (2)
Amygdala	2.2 ± 0.1 (3)	Inferior lateral cortex	1.0 ± 0.1 (2)
Hippocampus	3.5 ± 0.1 (3)	Deep nuclei	2.2 ± 0.9 (3)
Septum	1.4 ± 0.1 (2)	Lower brain stem	
Hypothalamus		Uvula	0.9 ± 0.2 (2)
Anterior	1.5 ± 0.3 (3)	Culmen	2.2 ± 0.6 (2)
Posterior	1.7 ± 0.2 (2)	Pyramidal tract	0.3 ± 0.2 (2)
Thalamus		Floor fourth ventricle	1.5 ± 0.3 (3)
Anterior medial	0.8 ± 0.1 (3)	Pons	2.8 ± 0.4 (3)
Posterior medial	2.1 ± 0.6 (3)	Lower medulla	1.8 ± 0.3 (3)
Pulvinar	0.9 ± 0.4 (3)	Upper medulla	1.9 ± 0.3 (3)

among the lowest norepinephrine concentrations in the brain, has intermediate levels of binding sites.

Regional variations in monkey brain are similar to those of the rat brain, with the highest binding in monkey brain occurring in the cerebral cortex. There does not appear to be any marked variation in the density of *beta* receptors among different parts of the cerebral cortex. Unlike the rat brain, the levels of binding in areas of the corpus striatum are not the highest but only intermediate. As in the rat, levels of [³H]DHA binding sites in the monkey hypothalamus are among the lowest in the brain. Moreover, as in the rat, the cerebellum of the monkey contains intermediate values of *beta* receptor binding. Among the lowest levels of [³H]DHA binding sites in monkey brain are regions which consist

primarily of white matter, such as the anterior corpus callosum and pyramidal tract.

The basic experiments exploring substrate specificity of [^{3}H]DHA binding sites used membranes from cerebral cortex of rat tissue. The validity of regional studies depends on the assumption that [^{3}H]DHA binds to the same receptor sites in different regions. Accordingly, we evaluated the relative affinities of (−)-alprenolol and norepinephrine for [^{3}H]DHA binding sites in the different regions of both monkey and rat brain (Table 3). The relative affinities are essentially the same as those observed for rat cerebral cortex.

Subcellular distribution of [^{3}H]DHA binding. Neurotransmitter receptors are generally assumed to involve postsynaptic membranes. However, high densities of *beta* adrenergic receptor-related adenylate cyclase activity have been demonstrated in glial cells in tissue culture (24). Accordingly, we determined the extent of [^{3}H]DHA binding to various subcellular fractions (Table 4). In the principal subcellular fractions obtained by differential centrifugation of whole homogenates of rat cerebral cortex, the greatest amount and specific activity of [^{3}H]DHA binding occur in the crude mitochondrial (P_2) fraction, which contains mitochondria as well as pinched-off nerve terminals or "synaptosomes." This fraction is also most enriched in the activity of monoamine oxidase, a marker for mitochondria, and in the high-affinity uptake of [^{3}H]norepinephrine, which presumably involves only nerve terminals.

When P_2 pellets are subfractionated on a discontinuous sucrose gradient designed to separate synaptosomes from mitochondria and myelin as well as other membrane fractions, the greatest percentage and specific activity of [^{3}H]DNA binding occur in the synaptosomal fraction. [^{3}H]Norepinephrine uptake is also most enriched in this fraction, while monoamine oxidase is equally distributed between the synaptosomal and mitochondrial fractions. Presumably mitochondria within synaptosomes possess substantial monoamine oxidase activity.

Hypotonic lysis of P_2 pellets destroys synaptosomes, liberating synaptic membranes into the supernatant fractions obtained after centrifuging the lysed pellet at 10,000 × *g* for 20 min. Under these conditions more than twice as much [^{3}H]DHA binding remains in the superna-

TABLE 3

Relative affinities of alprenolol and norepinephrine isomers for [^{3}H]DHA binding sites in rat and monkey brain regions

The IC_{50} values were determined by log probit analysis of 5–10 drug concentrations, each concentration tested in triplicate. The mean ± standard error is given if two or more determinations were made. The membrane fraction of rat and monkey tissue was prepared as described in Table 2. The fractions were assayed using 1.0 nM [^{3}H]DHA as described in Table 1.

Region	IC_{50}		
	(−)-Alprenolol	(−)-Norepinephrine	(+)-Norepinephrine
	nM	*μM*	*mM*
Rat			
Cerebral cortex	2.3 ± 0.2	0.8 ± 0.1	0.12 ± 0.03
Corpus striatum	3.0		
Cerebellum	1.5		
Hippocampus		1.2	0.10
Medulla-pons		1.6	
Monkey			
Frontal pole	2.3 ± 0.7	0.6 ± 0.1	0.11
Superior temporal gyrus	1.8	0.5 ± 0.1	0.07
Occipital pole	2.5	0.9	
Hippocampus	2.0		
Medulla	2.3		

TABLE 4

Subcellular distribution of specific (−)-[^{3}H]dihydroalprenolol binding in rat cerebral cortex

The specific binding of [^{3}H]DHA was determined in various subcellular fractions using the standard assay procedure. The data are presented in terms of specific binding per milligram of protein and as a percentage of the total binding activity recovered. Ninety-five per cent of the binding measured in the whole homogenate was recovered in $P_1 + P_2 + P_3$ pellets. Ninety-seven per cent of the binding in P_2 was recovered in the two lysed P_2 fractions. The data for monoamine oxidase activity (marker for mitochondria) and norepinephrine uptake (marker for synaptosomes) are also given as a percentage of the recovered activity. Values are the means ± standard errors of three separate experiments. The P_2 pellet was subfractionated on a two-step discontinuous sucrose gradient (equal volumes of 0.8 M and 1.2 M), which was centrifuged at 61,000 × *g* for 120 min (12). The 10,000 × *g* supernatant of the lysed P_2 was subfractionated (13) on a five-step discontinuous sucrose gradient (equal volumes of 0.4 M, 0.6 M, 0.8 M, 1.0 M, and 1.2 M). Further details are given in MATERIALS AND METHODS.

Fraction	[^{3}H]DHA bound	[^{3}H]DHA binding	Monoamine oxidase activity	Norepinephrine uptake
	fmoles/mg protein	*% total*	*% total*	*% total*
Whole homogenate	160 ± 7			
P_1 (crude nuclear)	82 ± 48	8 ± 2	3 ± 1	2 ± 1
P_2 (crude mitochondrial)	164 ± 25	53 ± 3	79 ± 2	91 ± 4
P_3 (crude microsomal)	142 ± 31	39 ± 2	18 ± 3	7 ± 3
Subfractionation of P_2				
0.32 M		0	0	
0.32–0.8 M (myelin, etc.)	45 ± 22	13 ± 7	2 ± 1	1 ± 1
0.8–1.2 M (synaptosomes)	155 ± 6	62 ± 4	51 ± 3	87 ± 2
0.12 M (mitochondria)	60 ± 2	25 ± 3	47 ± 2	12 ± 3
Lysed P_2, 10,000 × *g* supernatant	205 ± 9	78 ± 2	47 ± 1	
Lysed P_2, 10,000 × *g* pellet	91 ± 23	23 ± 2	53 ± 2	
Subfractionation of lysed P_2, 10,000 × *g* supernatant				
Lysate (no organized structure)		0	0	
0.0–0.4 M (synaptic vesicles)	118 ± 90	2 ± 1	1 ± 1	
0.4–0.6 M (microsomes, etc.)	283 ± 150	9 ± 5	2 ± 1	
0.6–0.8 M (synaptosome ghosts, membrane fragments)	195 ± 44	24 ± 3	5 ± 1	
0.8–1.0 M (same)	261 ± 71	19 ± 3	7 ± 1	
1.0–1.2 M (damaged synaptosomes)	146 ± 14	27 ± 5	60 ± 8	
1.2 M (small mitochondria, shrunken synaptosomes)	123 ± 34	19 ± 3	25 ± 7	

tant fractions than in the pellet, while monoamine oxidase activity is equally distributed between pellet and supernatant. Subfractionation of this 10,000 × *g* supernatant fluid with discontinuous sucrose density gradients separates fractions enriched in synaptic vesicles, microsomes, synaptic membranes, damaged synaptosomes, and free mitochondria (14). In such sucrose density gradients very little [^{3}H]DHA binding occurs in synaptic vesicle fractions, none can be detected in the supernatant fluid, and most binding is distributed in fractions enriched in synaptic membranes or damaged synaptosomes. By contrast, monoamine oxidase activity tends to be most concentrated in the more dense fractions containing free mitochondria and damaged synaptosomes.

DISCUSSION

The major finding of the present study is that [^{3}H]DHA appears to label *beta* adrenergic receptor sites in the brain. Binding is saturable, with high affinity and marked stereospecificity for *beta* adrenergic agonists and antagonists. The relative potencies of catecholamines and drugs in inhibiting [^{3}H]DHA binding closely parallel their relative pharmacological potencies and their relative affinities for *beta* receptor binding sites described by others

(2–7). There are some discrepancies, however, in the apparent absolute affinities of these drugs. The IC_{50} values determined by the procedure of Lefkowitz (3, 4, 6, 7) are higher than the IC_{50} values in the present study. A major difference between the two procedures is the 10–20-fold higher [^{3}H]DHA concentration and the 10–50-fold higher tissue concentrations used in the former procedure. The basis for higher IC_{50} value with increased concentrations of labeled ligand and receptor has been recently described (25). Our values are very similar to the K_D values reported by Atlas *et al.* (2) for [^{3}H]propranolol binding to turkey erythrocytes. We included an inhibitor of monoamine oxidase and ascorbic acid in the incubations to prevent degradation of catecholamines, whereas no such additions were employed by others (1–7).

In contrast to peripheral tissues, it is difficult to evaluate the nature of physiological catecholamine receptors in the brain. Not only are the properties of central adrenergic receptors in various brain regions not known, but it is unclear whether they can even be classified in terms of *alpha*, *beta*$_1$, and *beta*$_2$ types. Direct studies of receptor binding facilitate investigation of these questions. The binding sites in the brain labeled with the general *beta* antagonist [^{3}H]DHA have properties very similar to the *beta*$_1$ receptor in the periphery. *Beta*$_1$ receptors, which predominate in the heart, have similar affinities for epinephrine and norepinephrine, whereas *beta*$_2$ receptors, which are most concentrated in tissues such as bronchiolar smooth muscle, display considerably greater affinity for epinephrine than norepinephrine (26). The similarity in affinities of norepinephrine and epinephrine for [^{3}H]DHA binding sites in the cerebral cortex suggests that these receptors are predominantly *beta*$_1$. This conclusion is supported by the low affinities of salbutamol (27) and terbutaline (28), both of which are selective *beta*$_2$ agonists, and the moderate affinity of practolol, a selective *beta*$_1$ antagonist (29). Receptors in the various brain regions seem to be similar, as shown by the similar affinities for (−)-alprenolol and (−)-norepinephrine. In addition, the order of potency of agonists and antagonists on a norepinephrine-sensitive adenylate cyclase in rat limbic forebrain homogenates is indicative of a *beta*$_1$ receptor (30).

[^{3}H]DHA binding in the various subcellular fractions prepared by centrifugation techniques (Table 4) is greatest in those fractions containing higher concentrations of synaptic membranes and synaptosomes. This suggests that [^{3}H]DHA binds predominantly to synaptic receptor sites, although some binding to non-neuronal receptors (on glial cells, for instance) is not excluded.

Endogenous norepinephrine and the histofluorescent density of norepinephrine nerve terminals are greatest in the hypothalamus and parts of the limbic system. One of our most striking observations is the relatively low [^{3}H]DHA binding in the hypothalamus. This suggests that norepinephrine receptors in the hypothalamus may be predominantly of the *alpha* variety, which has been inferred from neurophysiological studies (8, 31). The corpus striatum, which contains the highest dopamine concentrations of the brain, but low levels of norepinephrine, has the highest density of [^{3}H]DHA binding sites of any region in rat brain and a relatively high level of binding in monkey brain. This suggests that norepinephrine receptors in the corpus striatum may be predominantly of the *beta* type (32). The cerebellum, with one of the lowest norepinephrine levels of the brain, displays moderately high [^{3}H]DHA binding. The inhibitory effects of norepinephrine on the firing of Purkinje cells of the cerebellum may involve *beta* receptors (9, 10). The observation that the majority of cerebellar Purkinje cells receive norepinephrine innervation (33) provides a rationale for the high density of receptor sites despite low levels of endogenous norepinephrine, assuming that cells receiving many terminals have receptor densities similar to those of cells with few terminals.

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