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# Identity of [$^3$H]-Dihydroalprenolol Binding Sites and β-Adrenergic Receptors Coupled with Adenylate Cyclase in the Central Nervous System: Pharmacological Properties, Distribution and Adaptive Responsiveness

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SUMMARY

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The binding of (−)-[$^3$H]-dihydroalprenolol and β-adrenergic-sensitive adenylate cyclase were measured in particulate fractions prepared from cat and rat brain. [$^3$H]-Dihydroalprenolol interacted with a single class of rat cortical sites, having an affinity of 7 ± 0.5 nM (n = 12) and a concentration of 169 ± 8 fmole/mg protein (n = 12). [$^3$H]-Dihydroalprenolol inhibited competitively the (−)-isoproterenol-sensitive adenylate cyclase with an apparent $K_I$ of 10 nM. The $K_D$app of [$^3$H]-dihydroalprenolol and the total number of specific binding sites were identical whether or not the determinations were made under conditions of adenylate cyclase assay. The apparent affinities of β-adrenergic agonists and antagonists for adenylate cyclase stimulation or inhibition were highly correlated with their apparent affinities for [$^3$H]-dihydroalprenolol binding sites, whether determined under adenylate cyclase incubation conditions ($r$ = 0.98) or not ($r$ = 0.95). Both processes were stereospecific for agonists and antagonists and showed the characteristics of a β-adrenergic receptor. Salbutamol, a $\beta_2$-adrenergic agonist in peripheral tissues, appeared to be an antagonist of the $\beta_1$-adrenergic receptor coupled to an adenylate cyclase in the cerebral cortex. The topographical distribution of [$^3$H]-dihydroalprenolol binding sites in rat frontal cerebral cortex was parallel to that of (−)-isoproterenol sensitive adenylate cyclase but not to that of dopamine sensitive adenylate cyclase. Similarly, the topographical distribution of [$^3$H]-dihydroalprenolol binding sites in different areas of cat brain was highly correlated with that of (−)-isoproterenol sensitive adenylate cyclase ($r$ = 0.93), but not with endogenous norepinephrine content. Intraventricular administration of 6-hydroxydopamine to five-day old cats resulted in an increase both in [$^3$H]-dihydroalprenolol binding sites and in adenylate cyclase stimulation by (−)-isoproterenol. The augmentation in binding sites increased with time after the lesion, whereas the increase observed in (−)-isoproterenol-sensitive adenylate cyclase activity did not. Chronic treatment of rats with reserpine produced a 50% increase in [$^3$H]-dihydroalprenolol binding sites and 43% increase in (−)-isoproterenol-sensitive adenylate cyclase. Chronic propranolol treatment also resulted in a significant increase in the concentration of [$^3$H]-dihydroalprenolol

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binding sites (31%), which was more pronounced than that observed in the (−)-isoproterenol-sensitive adenylate cyclase (17%). Chronic treatment with either chlorpromazine or phenoxybenzamine had no effect on either process. The affinity of [$^3$H]-dihydroalprenolol for its binding sites or of (−)-isoproterenol for adenylate cyclase stimulation was not affected by any of the treatments. Thus, the similarities between the pharmacological characteristics, the topographical distribution and the homeostatic regulation of the binding sites for [$^3$H]-dihydroalprenolol and of the β-adrenergic receptor coupled with an adenylate cyclase leads to the conclusion that these two components are identical in the central nervous system.

## INTRODUCTION

Two experimental approaches have been used to study receptors for neurotransmitters in the central nervous system. These are the binding of radiolabeled agonists or antagonists, and the measurement of the accumulation of cyclic AMP[2] resulting from the interaction of certain neurotransmitters, such as dopamine, serotonin and norepinephrine with their receptors (reviewed in [1]). From the present state of research, it is as yet unclear whether the receptors detected by binding studies are identical with those coupled to an adenylate cyclase.

Most of the studies on β-adrenergic stimulation of cyclic AMP production in the central nervous system have been performed using brain slices. However, this experimental system is not amenable to a precise study of the β-adrenergic receptors involved. This is due to the presence of complicating factors, such as presynaptic uptake and metabolism (2, 3), phosphodiesterase activity (2) and the possible alteration by β-adrenergic agonists of the local concentration of other agents affecting cyclic AMP accumulation, such as $Ca^{2+}$ or adenosine (4). Recently we have reported that in cerebral cortex homogenates it is possible to measure with accuracy the stimulation of an adenylate cyclase coupled to a β-adrenergic receptor (5). Thus in this report, we have attempted to compare the pharmacological properties and topographical distribution of the β-adrenergic sensitive adenylate cyclase with the binding sites detected using [$^3$H]-DHA. This radiolabeled β-adrenergic antagonist has previously been used by Alexander *et al.* (6) and Bylund and Snyder (7) to investigate cerebral β-adrenergic receptors.

Modification of the activity of central noradrenergic pathways by the destruction of noradrenergic innervation or by pharmacological treatment has been shown to result in an alteration in the NE or (−)-isoproterenol-induced accumulation of cyclic AMP in brain slices (2, 8, 9). Recently, Sporn *et al.* (10, 11) have reported that the intraventricular administration of 6-OHDA to rats resulted in 30–50% increase in the number of $^{125}$I-labeled iodohydroxybenzylpindolol binding sites in the cerebral cortex. A concomitant but proportionally greater increase (80%) in norepinephrine or isoproterenol-induced cyclic AMP accumulation was found in brain slices from the same area. Similarly after isoproterenol and 6-OHDA treatment, respectively, Nahorski (12) and Skolnick *et al.* (13) found changes in responsiveness of the cyclic AMP generating system to NE in brain slices, which were of greater magnitude than alterations in labeled β-adrenergic antagonist binding. It is, however, difficult to analyze the mechanisms responsible for any modification in responsiveness of adenylate cyclase measured in brain slice preparations, as in addition to alterations in receptor number, there are likely to be changes in the other factors mentioned above (2, 3, 4).

In this report we have examined the effect of various treatments chronically altering cerebral noradrenergic activity, in parallel on the binding of [$^3$H]-DHA and on (−)-isoproterenol-sensitive adenylate cy-

[2] The abbreviations used are: cyclic AMP, adenosine cyclic 3′,5′ monophosphate and [$^3$H]-DHA, (−) [$^3$H]-dihydroalprenolol. $K_D$app refers to the apparent dissociation constant of either agonists or antagonists for the [$^3$H]-DHA binding sites; $K_A$app refers to the apparent activation constant for agonists and $K_I$app to the apparent inhibition constant for antagonists of the β-adrenergic sensitive adenylate cyclase.

clase. Both activities have been measured in particulate fractions of cerebral cortex, in order that they be more directly comparable. The treatments investigated include intraventricular 6-OHDA administration to 5-day old cats and chronic administration to adult rats of reserpine or propranolol, a β-adrenergic antagonist.

## MATERIALS AND METHODS

*[$^3$H]-Dihydroalprenolol binding.* Particulate fractions were prepared by homogenizing fresh tissues (0.1 g wet weight/ml) in Tris-maleate 2 mM, pH 7.2; EGTA 2 mM; sucrose 300 mM, using a teflon-glass homogenizer. The homogenate was diluted 6-fold with hypotonic medium (Tris-HCl, 50 mM, pH 8) and centrifuged at 47,000 × *g* for 30 minutes. The pellet was washed four times using the same hypotonic medium.

The final pellet was resuspended usually in 10 volumes (of initial fresh weight) of Tris-HCl (50 mM, pH 8; containing ascorbic acid 0.1% and pargylline $10^{-6}$ M) and filtered through a silk screen. Aliquots (usually 90 μl) of this particulate fraction were incubated for 10 minutes at 35° with [$^3$H]-DHA and other drugs to a total volume of 100 μl (protein concentration: 2–3 mg/ml). Nonspecific binding was determined in the presence of (−)-alprenolol ($10^{-6}$ M).

When [$^3$H]-DHA binding was determined under adenylate cyclase incubation conditions, the particulate fraction was finally resuspended in Tris-maleate (50 mM, pH 7.2), and incubated as above in a total volume of 100 μl in the presence of ATP (0.5 mM), $MgSO_4$ (1 mM), creatine kinase (0.2 mg/ml), creatine phosphate (20 mM), theophylline (10 mM), EGTA (0.5 mM) and sucrose (75 mM) final concentrations.

At the end of the incubation period the samples were diluted with 1 ml ice-cold Tris-HCl, 50 mM, pH 8, filtered through a glass fiber filter (G F/B, Whatman) and washed with 5 × 4 ml of the same medium. The filters were dried and their retained radioactivity determined by liquid scintillation counting. For the determination of the topographical distribution of [$^3$H]-DHA binding in rat frontal cerebral cortex, the forebrain was frozen at −7°, cut in slices (500 μm thickness), and the different areas were dissected with a microscalpel; the particulate fractions were then prepared as described for fresh tissues.

For studies of the pharmacological characteristics of the β-adrenergic receptors in rat cerebral cortex, the whole cerebral cortex was used, dissected free from the internal capsule and other surrounding structures.

Under the conditions used, the specific binding of [$^3$H]-DHA (7 nM) showed at $t_{1/2}$ of 1 minute; and remained at equilibrium during between 5 and 45 minutes of incubation at 35°. Binding of 7 nM [$^3$H]-DHA was proportional to tissue concentration up to 0.5 mg protein/tube.

*Adenylate cyclase assay.* Homogenate preparation: for experiments investigating the β-adrenergic sensitive adenylate cyclase in rat cerebral cortex, the molecular layer or other regions of the cortex were dissected at −7° as previously described (5), while in experiments using cat cerebral tissue it was dissected at 4° and used immediately. Homogenates were prepared as for binding studies, and used without washing.

Adenylate cyclase activity was measured by the conversion of [α-$^{32}$P]ATP into $^{32}$P-cyclic AMP. The final medium (40 μl) contained 25 mM Tris-maleate pH 7.2; 0.5 mM nonlabeled ATP; 1 mM $MgSO_4$; 0.2 mg/ml creatine kinase; 20 mM creatine phosphate; 10 mM theophylline; 0.5 mM EGTA; 75 mM sucrose; 1.5 μCi [α-$^{32}$P]ATP and 0.001 μCi [$^3$H]-cyclic AMP. Fluphenazine ($10^{-5}$ M) was included in the incubation medium for β-adrenergic-sensitive adenylate cyclase, to avoid possible effects of certain β-adrenergic agonists on any dopamine-sensitive adenylate cyclase present. The reaction was initiated by the addition of 10 μl of homogenates and was allowed to proceed for 5 to 10 minutes at 35° (protein concentration: 1.3–2.0 mg/ml). It was stopped by the addition of 100 μl of a solution containing 5 mM ATP, 5 mM cyclic AMP, 50 mM Tris HCl (pH 7.4) and 1% sodium lauryl sulfate.

The purification of the $^{32}$P cyclic AMP was performed according to Salomon *et al.* (14). Adenylate cyclase activities were expressed in pmoles of cyclic AMP produced/min/mg protein. The production of

$^{32}$P cyclic AMP from [$\alpha$-$^{32}$P]ATP was linear for at least 12 min (5). The range of variation between triplicate determinations was always less than 10%.

*Determination of equilibrium constants for binding and adenylate cyclase activation and inhibition.* The $K_D$app values for unlabeled agonists and antagonists were calculated from the $IC_{50}$ determined from displacement experiments as previously described (15). The $K_A$app was the agonist concentration giving 50% of maximal activation of adenylate cyclase. The $K_I$app was calculated from the antagonist concentration giving 50% inhibition of (−)-isoproterenol-activated adenylate cyclase (15).

*Endogenous norepinephrine determinations.* The dissected tissues were immediately homogenized in 8 volumes of an ethanol:water solution (74:16 v/v) containing 0.05% ascorbic acid and 0.05% EDTA. After storage for 12 h at −30°, the homogenates were centrifuged, and the monoamines in the supernatant were then selectively absorbed on an Amberlite CG 50 column (0.4 cm diameter, 2.5 cm long). NE was eluted together with the other monoamines in 2 ml of 0.3 M $HClO_4$. NE was then estimated in a 0.5 ml aliquot according to the method of Laverty and Taylor (16).

*Drug treatments.* a) Reserpine (dissolved in acetic acid, diluted with 0.9% NaCl and neutralized with NaOH) or its vehicle was administered i.p. to rats at a dose of 2.5 mg/kg on the first day, followed by 0.5 mg/kg on three subsequent days. During this period rats were fed a diet of mashed biscuits in milk. They were killed 48 hr after the last injection.

b) (±)-Propranolol (10 mg/kg in 0.9% NaCl) or its vehicle was administered i.p. at 8 hr intervals for seven days. The rats were killed 8 hr after the final injection.

c) Chlorpromazine (5, 10 or 20 mg/kg), phenoxybenzamine (10 mg/kg) or their vehicle (0.9% NaCl) were administered i.p. daily for seven days. The rats were killed 48 hr after the final injection.

*6-Hydroxydopamine administration.* 6-OHDA (1 mg free base in 100 µl of 1% ascorbic acid in 0.9% NaCl) was injected bilaterally into the frontal horns of the two lateral ventricles of 5 day-old cats by means of two cannulae (0.8 mm external diameter) previously implanted and fixed to the skull with dental cement, as described in detail elsewhere (17). Sham animals were injected intraventricularly with 1% ascorbic acid in 0.9% NaCl.

*Analysis of the effects of drug treatments.* The comparison between the adenylate cyclase activities of control and treated animals was performed by variance analysis. The affinities ($K_D$app) of $\beta$-adrenergic receptors for [$^3$H]-DHA and the concentration of receptor sites were determined by regression analysis of Scatchard plots for control and treated animals. The standard deviation of $K_D$ was determined from the error on the slope of the regression line. The significance of differences between $K_D$ values for treated and control animals were determined using Student's two-tailed *t*-test. The total number of [$^3$H]DHA binding sites was always determined by Scatchard plot analysis.

## MATERIALS

*Animals.* Male Charles River rats of Sprague Dawley strain (200–300 g) and male or female cats were kept in a controlled environment (24°, alternative cycles of 12 hr light and 12 hr darkness, food and water *ad libitum*) for at least one week before use.

*Chemicals.* Chemicals and drugs were purchased or donated (*) as follows: ATP (disodium salt), (±)-propranolol, (−)-isoproterenol, (−)-epinephrine and (−)-NE (Sigma); cyclic AMP, creatine kinase and creatine phosphate (Boehringer, Mannheim), dopamine (DA, HCl salt, Calbiochem), chlorpromazine* (Rhône Poulenc); (+)-propranolol*, (−)-propranolol* and practolol* (ICI), (−)-alprenolol* and phentolamine* (Ciba Geigy); phenoxybenzamine* (SKF), protokylol* (Lakeside); salbutamol* (Allen and Hanbury's); 6-OHDA (HBr salt; Regis Chemicals); (+)-LSD* (Sandoz), [$^3$H]-cyclic AMP (ammonium salt; 25 Ci/mmole), [$\alpha$-$^{32}$P]-ATP (sodium salt; 10–20 Ci/mmole) and (−)-[$^3$H]dihydroalprenolol (32.6 Ci/mmole) were obtained from NEN.

## RESULTS

*Comparison between the pharmacological properties of the [$^3$H]-DHA binding sites and the β-adrenergic receptors involved in adenylate cyclase stimulation in rat cerebral cortex.* [$^3$H]-DHA interacted with a single category of binding sites (see Scatchard plots in the various figures) having an affinity of 7 ± 0.5 nM (n = 12). The concentration of receptors in the cerebral cortex was 169 ± 8 fmole/mg protein (n = 12). This radiolabeled ligand also competitively inhibited the (−)-isoproterenol sensitive adenylate cyclase, with an apparent inhibition constant ($K_I$app) of 10 nM (Fig. 1). The effects of including ATP (0.5 mM) in the incubation medium was investigated on the concentration and affinity of cortical [$^3$H]-DHA binding sites. In the same experiment, the $K_D$app was 6.4 in control conditions and in the presence of ATP. The number of [$^3$H]-DHA binding sites determined by Scatchard plot was not changed, being 138 fmole/mg protein under both conditions. The $K_D$app and number of [$^3$H]-DHA binding sites determined in the same medium as that used for the adenylate cyclase experiments were 6.7 and 148 fmole/mg protein, respectively.

This similarity between the affinity of [$^3$H]-DHA for its binding sites whether or not measured under adenylate cyclase conditions and for the β-adrenergic receptor coupled to an adenylate cyclase was generally true for several β-adrenergic agonists and antagonists (Table 1, Fig. 2). The [$^3$H]-DHA binding sites and β-adrenergic stimulation of the adenylate cyclase were stereospecific for β-adrenergic agonists and antagonists; the (−)-stereoisomers being between 25 and 200 times more potent than the (+)-stereoisomers (Fig. 2). Practolol, a specific $\beta_1$-adrenergic antagonist (18) both displaced [$^3$H]-DHA from its binding sites and inhibited (−)-isoproterenol-induced activation of adenylate cyclase. Interestingly, salbutamol, previously defined as a specific agonist of $\beta_2$-adrenergic receptors in peripheral tissues (19), was found to interact with [$^3$H]-DHA binding sites; however, it behaved as an antagonist of the (−)-isoproterenol stimulated adenylate cyclase, while having no effect on basal adenylate cyclase activity. Two α-adrenergic antagonists, phentolamine and phenoxybenzamine, were inactive on both systems, while chlorpromazine, a drug with dopaminergic antagonist properties, showed a very low affinity both for [$^3$H]-DHA binding sites and for antagonism of (−)-isoproterenol sensitive adenylate cyclase (Table 1).

*Distribution of [$^3$H]-DHA binding sites, and of β-adrenergic and dopamine-sensitive adenylate cyclases in rat frontal cerebral cortex (Fig. 3).* Of the four regions investigated, the molecular layer (I) of the

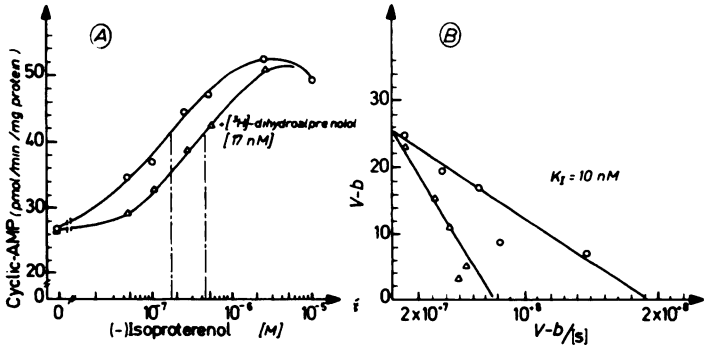


FIG. 1. *Determination of the apparent inhibition constant of [$^3$H]-DHA for (−)-isoproterenol sensitive adenylate cyclase*

A—Dose response curve for adenylate cyclase activation by (−)-isoproterenol in the absence (○) and presence (△) of 17 nM [$^3$H]-DHA. B—Eadie plot of the dose-response curves. In the presence of [$^3$H]-DHA the slope is equal to $K_A$ (1 + $I/K_I$app) where $I$ = [$^3$H]-DHA concentration. b = basal adenylate cyclase activity; v = adenylate cyclase activity in the presence of a concentration [S] of (−)-isoproterenol.

TABLE 1

*Comparison of apparent $K_D$ for displacement of binding of [$^3$H]-DHA and apparent $K_A$ for activation or $K_I$ for inhibition of (−)isoproterenol sensitive adenylate cyclase of various adrenergic agonists and antagonists in rat cerebral cortex*

Displacement of [$^3$H]-DHA by agonists and antagonists was performed (a) Under standard conditions of binding assay as described in the METHODS section; and (b) under the adenylate cyclase incubation conditions (Tris maleate 25 mM pH 7.2; 0.5 mM ATP; 1 mM $MgSO_4$; 0.2 mg/ml creatine kinase; 20 mM creatine phosphate; 10 mM theophylline; 0.5 mM EGTA; 75 mM sucrose).

| | Binding $K_D$app | | Adenylate cyclase $K_A$app or $K_I$app |
|---|---|---|---|
| | Standard conditions | In presence of adenylate cyclase incubation medium | |
| | *M* | *M* | *M* |
| *Agonists* | | | |
| (−)-isoproterenol | $2.6 \pm 0.9 \times 10^{-7}$ | $2.6 \times 10^{-7}$ | $3.6 \pm 0.8 \times 10^{-7}$ |
| Protokylol | $7.3 \pm 1.3 \times 10^{-7}$ | $8.6 \times 10^{-7}$ | $1.0 \times 10^{-6}$ |
| (−) Norepinephrine | $3.9 \pm 0.1 \times 10^{-6}$ | $3.8 \times 10^{-6}$ | $4.8 \pm 0.8 \times 10^{-6}$ |
| (−) Epinephrine | $6.9 \pm 1.8 \times 10^{-6}$ | $4.0 \times 10^{-6}$ | $5.7 \pm 0.7 \times 10^{-6}$ |
| (+) Isoproterenol | $7.6 \pm 0.6 \times 10^{-6}$ | $7.2 \times 10^{-6}$ | $1.2 \pm 1.0 \times 10^{-5}$ |
| *Antagonists* | | | |
| (−)-Propranolol | $5.5 \pm 2.0 \times 10^{-9}$ | $8.8 \times 10^{-9}$ | $3.2 \pm 1.4 \times 10^{-9}$ |
| (−)-[$^3$H]-DHA | $7.0 \pm 0.5 \times 10^{-9}$ | $6.7 \times 10^{-9}$ | $10.0 \times 10^{-9}$ |
| (−)-Alprenolol | $1.8 \pm 0.3 \times 10^{-8}$ | $2.5 \times 10^{-8}$ | $7.6 \times 10^{-9}$ |
| (+)-LSD | $2.3 \pm 0.4 \times 10^{-7}$ | $1.6 \times 10^{-7}$ | $3.7 \pm 1.6 \times 10^{-7}$ |
| (+)-Propranolol | $1.1 \pm 0.4 \times 10^{-6}$ | $9.5 \times 10^{-7}$ | $2.7 \times 10^{-7}$ |
| Practolol | $1.4 \pm 0.2 \times 10^{-6}$ | $1.6 \times 10^{-6}$ | $7.7 \times 10^{-7}$ |
| Salbutamol | $6.7 \pm 2.2 \times 10^{-6}$ | $6.7 \times 10^{-6}$ | $8.0 \times 10^{-6}$ |
| Phentolamine | N[a] | — | N[a] |
| Phenoxybenzamine | N[a] | — | N[a] |
| Chlorpromazine | $1.6 \times 10^{-4}$ | — | $5.0 \times 10^{-6}$ |
| | $r = 0.96$ | | $r = 0.98$ |
| | $r = 0.95$ | | |

[a]N = no detectable activity. Correlations were performed by linear regression analysis. The $K_D$app and $K_I$app for chlorpromazine were not included in the regression analysis owing to the uncertainty that chloropromazine has a specific effect on the β-adrenergic receptor. The $K_D$app and the $K_I$app values given are either the means of two independently determined values differing by less than 10% or the mean ± SEM of between 3 and 12 independently determined values.

frontal cerebral cortex showed the highest content of [$^3$H]-DHA binding sites and of β-adrenergic sensitive adenylate cyclase, while not having any DA-sensitive adenylate cyclase activity. This association between [$^3$H]-DHA binding sites and β-adrenergic sensitive adenylate cyclase was also found in the three other areas dissected, the activities being lower in the interior than in the exterior cortical regions. An inverse distribution was found for DA-sensitive adenylate cyclase.

*Distribution of [$^3$H]-DHA binding sites, β-adrenergic sensitive adenylate cyclase and endogenous norepinephrine in cat brain (Table 2).* The distribution of both [$^3$H]-DHA binding sites and β-adrenergic sensitive adenylate cyclase showed wide variations between the different regions of adult cat brain investigated. Both activities were greatest in cerebellum, although this structure, of all those examined, contained the lowest concentration of endogenous NE. Furthermore, the hypothalamus, with the highest NE content, showed the lowest activity of β-adrenergic sensitive adenylate cyclase. This distribution of [$^3$H]-DHA binding sites in the eight brain areas examined was highly correlated ($r = 0.92$) with that of β-adrenergic sensitive adenyl-

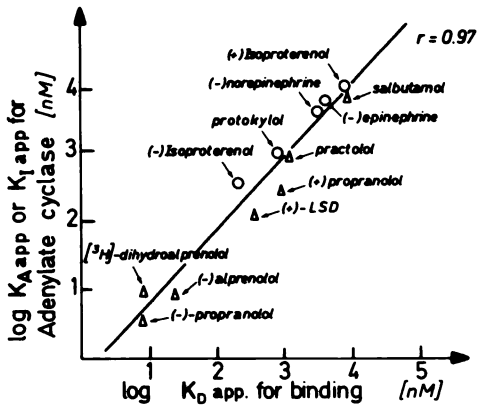


FIG. 2. *Correlation of $K_D$app for [$^3$H]-DHA binding with either $K_A$app or $K_I$app for adenylate cyclase activity*

The line of correlation was determined by regression analysis of the log values. $r$ = regression coefficient. (○) = agonists, (△) = antagonists. The binding was performed under conditions of adenylate cyclase assay.

ate cyclase, but not with endogenous levels of NE ($r$ = 0.49).

*Effect of 6-hydroxydopamine-induced destruction of cortical NE innervation on both [$^3$H]-DHA binding sites and β-adrenergic sensitive adenylate cyclase.* The effect of the intraventricular administration of 6-OHDA to 5-day old rats was examined two, 11, 55 and 65 days later. An 80% reduction in cortical NE levels was observed three days after the lesion as reported in detail elsewhere (20). The treatment resulted in a significant and time-dependent increase in the number of [$^3$H]-DHA binding sites (by 12.5, 32, 65 and 71%, respectively, two, 11, 55 and 65 days after lesion, Table 3). The increase in the number of [$^3$H]-DHA binding sites was highly correlated with time elapsing after lesion ($r$ = 0.98). It is clear that degeneration of presynaptic innervation also induced an increase in maximal β-adrenergic stimulation of the adenylate cyclase; this however appeared temporally more rapid than the increase in the number of [$^3$H]-DHA binding sites and was not correlated with time after lesion ($r$ = 0.38). No changes were detected in basal adenylate cyclase activity, or in the affinity of [$^3$H]-DHA for its binding sites or of (−)-isoproterenol for the activation of adenylate cyclase (Table 3).

*The effect of chronic drug treatments on both [$^3$H]-DHA binding sites and β-adrenergic-sensitive adenylate cyclase in rat cerebral cortex.* Following chronic reserpine treatment there was a 50 ± 11% increase in specific [$^3$H]-DHA binding sites (187 ± 3 and 280 ± 23 fmole/mg protein in control [n = 4] and treated [n = 4] animals, respectively; $p < 0.01$), which was not accompanied by any difference either in the number of non-specific binding sites or in the $K_D$ of [$^3$H]-DHA for binding (Fig. 4). This treatment also produced an increase in the maximal stimulation of adenylate cyclase by (−)-isoproterenol (by 44%) while the $K_A$ was not altered (Fig. 5). Chronic

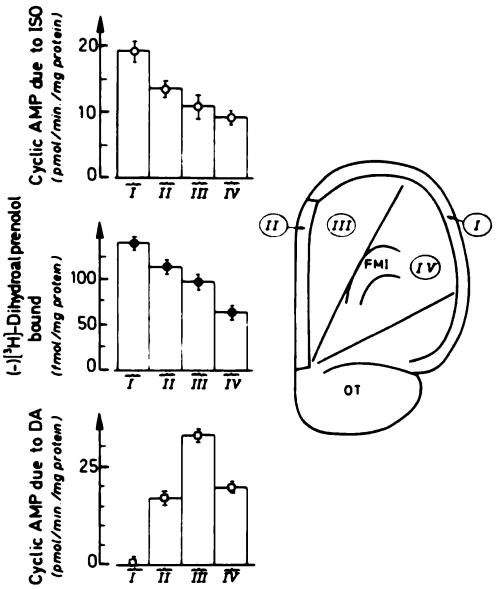


FIG. 3. *Comparison between the topographical distribution of [$^3$H]-DHA binding sites, and β-adrenergic and DA-sensitive adenylate cyclase in the frontal cerebral cortex*

The frontal cortex was cut into transverse slices of 500 μm thickness (from 12,300 to 10,300 μm according to König and Klippel (46)). Each slice (depicted RH-side of figure) was dissected into four main areas which were pooled. These areas were: I—the outer molecular layer of the cerebral cortex; II—the medial molecular layer; III—medial internal cortical region; IV—lateral internal cortical region. Isoproterenol (ISO), dopamine (DA) and [$^3$H]-DHA concentrations were $10^{-5}$ M, $10^{-4}$ M and $2 \times 10^{-8}$ M, respectively. The basal adenylate cyclase activities of areas I, II, III and IV were 14.6, 14.3, 13.0 and 10.7 pmol/min/mg protein. FMI = forceps minor, OT = olfactory tubercule.

TABLE 2

*Comparison of (−)-isoproterenol stimulation of adenylate cyclase in homogenates, specific [$^3$H]-DHA binding sites in particulate fractions and endogenous NE content in regions of adult cat brain*

The (−)-isoproterenol concentration used was $5 \times 10^{-5}$ M. The number of specific [$^3$H]-DHA binding sites was estimated by performing the binding in the presence of 8 nM [$^3$H]-DHA (n = 6). In separate experiments the $K_D$ value for each region was determined by Scatchard analysis; and the total concentration of specific [$^3$H]-DHA binding sites was calculated using these values.

| Brain regions | Basal cyclic AMP level | Cyclic AMP due to (−)-isoproterenol | $K_D$ for [$^3$H]-DHA binding | Total [$^3$H]-DHA binding sites n = 6 | Endogenous norepinephrine n = 3–5 |
|---|---|---|---|---|---|
| | *pmol/min/mg protein* n = 4 | | *nM* | *fmol/mg protein* | *μg/g fresh weight* |
| Cerebellum | 3.5 ± 0.2 | 8.5 ± 0.6 | 3.2 | 126 ± 14 | 0.16 ± 0.03 |
| Temporal cortex | 9.4 ± 0.1 | 3.6 ± 0.2 | 5.8 | 85 ± 25 | 0.18 ± 0.01 |
| Hippocampus | 9.1 ± 0.1 | 3.3 ± 0.4 | 6.1 | 39 ± 7 | 0.21 ± 0.03 |
| Striatum | 19.9 ± 0.5 | 2.2 ± 0.2 | 2.4 | 42 ± 4 | 0.30 ± 0.05 |
| Tuberculum olfactorium | 14.5 ± 0.1 | 1.8 ± 0.5 | 2.1 | 16 ± 5 | 0.57 ± 0.07 |
| Lateral brainstem | 4.4 ± 0.5 | 0.69 ± 0.05 | 4.9 | 16 ± 9 | 0.43 ± 0.01 |
| Raphé | 5.2 ± 0.1 | 0.52 ± 0.09 | 4.0 | 27 ± 12 | 0.63 ± 0.03 |
| Hypothalamus | 13.8 ± 0.2 | 0.3 ± 0.2 | * | 28 ± 6* | 2.54 ± 0.22 |
| | | | $r = 0.92$ | $r = 0.36$ | |
| | | | $r = 0.49$ | | |

* Because of high non-specific binding of [$^3$H]-DHA in the hypothalamus (70–90% of total binding) the $K_D$ was not determinable in this region. The value given for the number of binding sites is that determined using the mean $K_D$ from the other regions (4 nM).

propranolol treatment also resulted in a 32 ± 4% increase in the number of [$^3$H]-DHA binding sites (202 ± 9 [n = 3] and 266 ± 20 [n = 3] fmole/mg protein in control and treated animals, respectively [$p < 0.05$]; Fig. 6) and in the maximal (−)-isoproterenol stimulation of adenylate cyclase (17%, Table 4). The apparent affinities of [$^3$H]-DHA for the binding sites or (−)-isoproterenol for the β-adrenergic-sensitive adenylate cyclase were not modified by chronic propranolol treatment indicating that propranolol did not rest in the cortical tissue in sufficient amount to affect the results. Basal adenylate cyclase activity was also not altered by either treatment.

Chronic treatment with phenoxybenzamine or chlorpromazine had no effect on the concentration of [$^3$H]-DHA binding sites or on the activity of the β-adrenergic sensitive adenylate cyclase (data not shown).

## DISCUSSION

There are several indications that certain receptor populations detected by binding studies are distinct from those detected by adenylate cyclase studies. For example, the affinities of DA and serotonin for their binding sites have been found to be up to 100-fold higher than for adenylate cyclase stimulation (for reviews see 1, 21). In addition, differences have been observed: 1) in the topographical distribution and ontogenic development of serotonin binding sites and serotonin-sensitive adenylate cyclase (22) and 2) in the subcellular distribution of DA-sensitive adenylate cyclase and its binding sites (23). Furthermore, in certain studies, the specificity and topographical distribution of neurotransmitter receptors have been found to vary depending on whether the radioligand used for their detection is an agonist or antagonist (24). For this reason it was important to determine whether it was possible to identify [$^3$H]-DHA binding sites in the brain with those implicated in the activation of a β-adrenergic sensitive adenylate cyclase.

The order of potencies of agonists and antagonists for [$^3$H]-DHA binding sites in cerebral cortex indicates that the receptor is of the $\beta_1$ type, according to its definition in peripheral tissues (25, 26). This is in agreement with the results of Bylund and Snyder (7) and Alexander *et al.* (6). In the

TABLE 3

*The effect of 6-OHDA treatment of 5 day-old cats on the concentration and characteristics of (−)-isoproterenol sensitive adenylate cyclase and [³H]-DHA binding sites on the cerebral cortex*

The stimulation of adenylate cyclase by (−)-isoproterenol and the binding of [³H]-DHA was determined on the same region of temporal cortex at different lengths of time after the administration of 6-OHDA or its vehicle to 5 day-old cats. For the determination of adenylate cyclase activity, the values given are the means of the values measured in triplicate or quadruplicate for the number (N) of animals investigated in each group.

| Days after lesion | | N | Mean cortical NE | $K_A$app for (−)-iso. | Adenylate cyclase activity | | ³H-DHA binding | | Effect of lesion (% control) on | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Basal | +(−)-iso. ($5 \times 10^{-5}$ M) | $K_A$app | No. sites | Cyclic AMP due to iso. | [³H]-DHA sites |
| | | | μg/g | μM | pmol/min/mg protein | | nM | fmol/mg protein | | |
| 2 | Control | 2 | 0.064 | — | 8.0 | 10.8 | 3.6 | 32 | 106 | 12, 5 |
| | Lesioned | 2 | 0.009 | — | 7.24[a] | 13.0[b] | 3.6 | 36 | | |
| 11 | Control | 2 | 0.077 | 0.4 | 16.3 | 25.1 | 2.2 | 60 | 60 | 32 |
| | Lesioned | 2 | 0.006 | 0.4 | 16.0[a] | 29.9[c] | 2.2 | 79 | | |
| 55 | Control | 2 | 0.124 | 0.37 | 43.2 | 68.0 | 3.1 | 127 | 94 | 65 |
| | Lesioned | 1 | 0.013 | 0.5 | 41.6[a] | 89.6[d] | 4.0 | 219 | | |
| 65 | Control | 1 | 0.150 | 0.4 | 29.2 | 48.4 | 4.0 | 130 | 80 | 71 |
| | Lesioned | 1 | 0.012 | 0.4 | 29.0[a] | 63.6[e] | 4.0 | 223 | | |

Statistical comparison was performed by variance analysis: between 6-OHDA lesioned and control groups:

[a] NS when compared to control basal level.

[b] DF = 1,14; F = 6.1; $p < 0.05$.

[c] DF = 1,10; F = 22.3; $p < 0.01$.

[d] DF = 1,8; F = 40.9; $p < 0.01$.

[e] DF = 1,6; F = 146; $p < 0.01$ when compared to respective control stimulated level. Regression analysis between days after 6-OHDA lesion and either % increase in cyclic AMP production due to isoproterenol or % increase in [³H]-DHA binding sites gave coefficients of correlation $r = 0.38$ and $r = 0.98$, respectively.

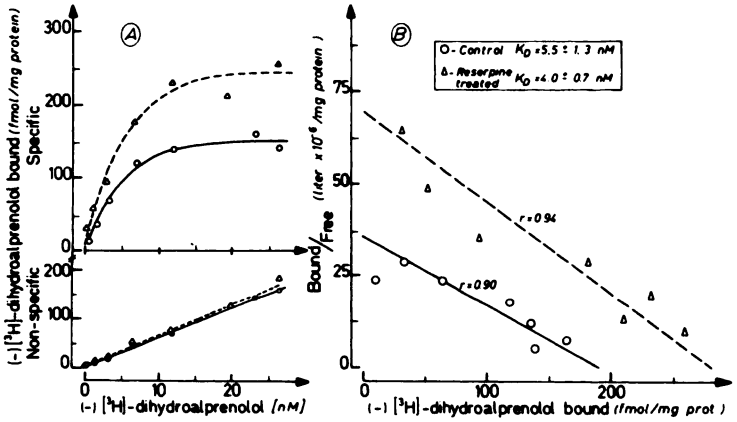


FIG. 4. *Equilibrium binding of [³H]-DHA to cortical particulate fractions of control and chronic reserpine-treated animals, and their Scatchard analysis*

Note that the non-specific binding was not altered by reserpine treatment. The concentration of [³H]-DHA binding sites were 191 and 281 fmol/mg protein in the cortices of control and reserpine-treated animals, respectively. The number of cortical [³H]-DHA binding sites from four experiments was 187 ± 3 and 280 ± 23 fmol/mg protein for control and reserpine-treated animals respectively ($p < 0.01$).

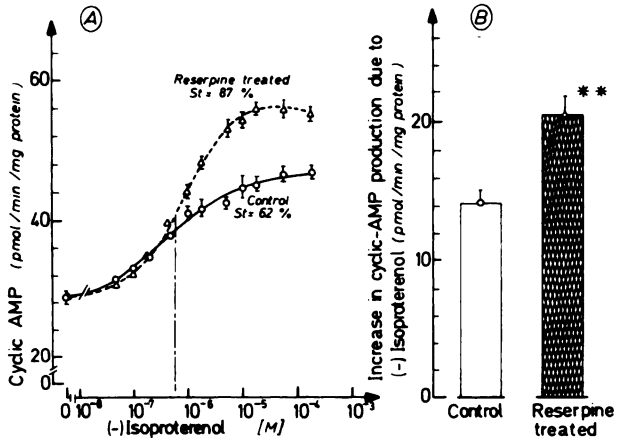


FIG. 5. *Stimulation of β-adrenergic sensitive adenylate cyclase in cortical homogenates of control* (○) *and reserpine-treated animals* (△)

A—Dose response curves for (−)-isoproterenol activation of the adenylate cyclase. Values are the mean ± SEM of triplicate determinations. B—Comparison between maximal activation of the adenylate cyclase by (−)-isoproterenol ($5 \times 10^{-5}$ M) in control and reserpine-treated animals. Values are the means ± SEM of triplicate determinations on three pairs of control and reserpine-treated animals. The statistical comparison was performed by variance analysis between the two groups, DF = 1.16; F = 13.32, $p < 0.01$.**

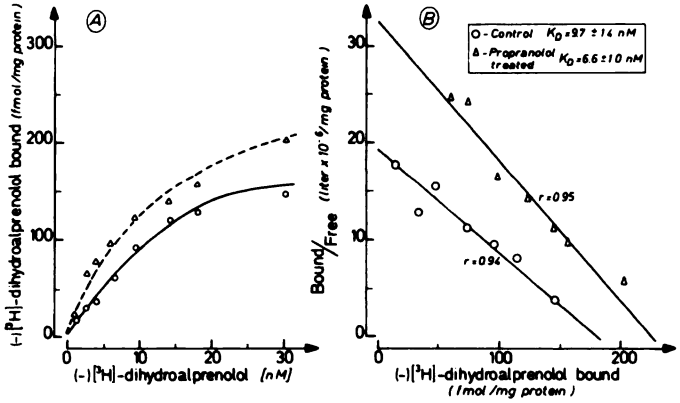


FIG. 6. *Equilibrium binding of [³H]-DHA to cortical particulate fractions of control and chronic propranolol-treated animals, and their Scatchard analysis*

The concentration of [³H]-DHA binding sites were 229 and 185 fmol/mg protein in propranolol-treated and control animals respectively. From three experiments the concentration of [³H]-DHA binding sites was 202 ± 9.5 and 266 ± 20 fmol/mg protein for control and propranolol-treated animals, respectively ($p < 0.05$).

presence of fluphenazine ($10^{-5}$ M), to block both any DA-sensitive adenylate cyclase present (5) and also α-adrenergic receptors 24), the β-adrenergic-sensitive adenylate yclase in homogenates of the cerebral cor- ex also appeared to involve a $\beta_1$-adrenergic eceptor. The pharmacological specificity f the β-adrenergic receptor associated oth with [³H]-DHA binding and with an denylate cyclase was confirmed by their nteraction with practolol, a specific $\beta_1$-adrenergic antagonist (18). In addition, the specific $\beta_2$-adrenergic agonist salbutamol (19), although displacing [³H]-DHA from its binding sites, did not stimulate adenylate cyclase activity in cerebral cortex but rather acted as an antagonist of (−)-isoproterenol stimulated adenylate cyclase, as it has previously been shown by Horn and Phillipson (27). This result highlights the necessity of using a functional test in conjunction with binding studies in order to

TABLE 4

*The stimulation of β-adrenergic sensitive adenylate cyclase in cortical homogenates of control and chronic propranolol-treated rats*

The concentration of (−)-isoproterenol used was $5 \times 10^{-5}$ M. Values are the mean ± SEM of duplicate or triplicate determinations in three pairs of control and propranolol treated-animals. $K_A$app for (−)-isoproterenol stimulation of adenylate cyclase was $6 \times 10^{-7}$ M in both control and propranolol-treated animals.

| | Treatment | Cyclic AMP | Variance analysis |
|---|---|---|---|
| | | *pmol/min/mg protein* | |
| Basal | Control | 19.4 ± 0.8 | DF: 1,12; F = 0.025, NS |
| | Propranolol | 19.6 ± 0.4 | |
| Increase in cyclic AMP production due to (−)-isoproterenol | Control | 9.2 ± 0.4 | DF: 1,12; F = 7.44, $p < 0.05$ |
| | Propranolol | 10.8 ± 0.4 | |

define unambiguously the pharmacological profile of a drug. (+)-LSD was also found to displace [$^3$H]-DHA binding and to block (−)-isoproterenol sensitive adenylate cyclase, as has recently been investigated in more detail (28).

The apparent affinities of β-adrenergic agonists and antagonists for the displacement of [$^3$H]-DHA binding ($K_D$app) were highly correlated with their apparent affinities for stimulation of adenylate cyclase ($K_A$app) or inhibition of its stimulation by (−)-isoproterenol ($K_I$app), a finding which held true for the radioligand [$^3$H]-DHA itself.

This result was obtained whether or not the determinations of $K_D$app were made under adenylate cyclase incubation conditions (Table 1, Fig. 2). Furthermore, the line of correlation only slightly differed from the line of identity (Fig. 2). Similar findings have been reported in C6 glioma cells (29) and frog erythrocytes (30) when binding was measured in simple media without added nucleotides, and also in turkey erythrocytes in presence of Gpp(NH)p (31). However, a three-fold difference has been found between the affinity of several agonists for [$^3$H]-DHA binding sites and β-adrenergic sensitive adenylate cyclase, when the two are measured under identical conditions in membranes of C6 glioma cells (15). Nucleotide triphosphates (e.g., GTP, Gpp(NH)p, ITP and ATP) have been found to affect the $K_D$app for agonists without modifying that for antagonists (15, 32, 33). The absence of any effect of ATP on $K_D$app values in the present system is not surprising since this nucleotide was much less potent than GTP and has been reported to be without effect in several systems at concentrations (0.5–1.0 mM) used for adenylate cyclase measurements (15, 32). Similarly, in the present system the total number of specific [$^3$H]-DHA binding sites was not modified by their measurement in adenylate cyclase incubation conditions.

The parallel topographical distribution of [$^3$H]-DHA binding sites and (−)-isoproterenol sensitive adenylate cyclase in different areas of cat brain (Fig. 2) is also a good indication of their identity. Furthermore, the endogenous NE content of these regions showed a very poor correlation with either the concentration of [$^3$H]-DHA binding sites or (−)-isoproterenol sensitive adenylate cyclase activity. A similar poor correlation between [$^3$H]-DHA binding and endogenous NE content has recently been found in rat and monkey brain (7). However, the involvement of α-adrenergic receptors post-synaptic to noradrenergic terminals in regions with dense noradrenergic innervation may account for these discrepancies. Indeed, the hypothalamus, which had the highest concentration of endogenous NE, showed a very low capacity for [$^3$H]-DHA binding and negligible (−)-isoproterenol-sensitive adenylate cyclase activity (Table 2), whereas it has been shown to have one of the highest cerebral concentrations of α-adrenergic receptors (24).

In contrast, the parallel fine distribution of [$^3$H]-DHA binding sites and (−)-isoproterenol-sensitive adenylate cyclase in rat frontal cerebral cortex was comparable to

that of noradrenergic terminals found by previous histochemical and biochemical studies (34, 35, 36). Their highest concentrations were found in the molecular layer of the neocortex in contrast to the highest concentration of DA-sensitive adenylate cyclase which was found in the internal prefrontal cortex (Fig. 3).

The nature of the cells on which β-adrenergic receptors are found in the central nervous system remains to be clarified. Glioma cell lines, and possibly glial cells themselves, exhibit β-adrenergic receptors coupled to an adenylate cyclase (29, 37). β-adrenergic receptors have been visualized on neuronal cells, in particular in cerebellar Purkinje cells, using a fluorescently labelled β-adrenergic antagonist (38) and these cells are hyperpolarized by iontophoretic administration of β-adrenergic agonist or dibutyryl cyclic AMP (39) suggesting the presence of a β-adrenergic receptor coupled to an adenylate cyclase. The marked non-uniform distribution of β-adrenergic receptors in the central nervous system found in the present study possibly indicates that the concentration of these receptors situated on glial cells represents a small percentage of the total, or alternatively that there is a heterogeneity in the distribution of properties of glial cells. It is also possible that a certain percentage of β-adrenergic receptors are situated in the walls of cerebral blood vessels.

The conclusion drawn from the present pharmacological and topographical studies is that [$^3$H]-DHA labels the β-adrenergic receptor implicated in adenylate cyclase stimulation. It was thus of great interest to investigate the changes in these two parameters after modifications of noradrenergic neurotransmission, either by the induction of denervation supersensitivity by use of 6-OHDA to destroy noradrenergic neurons, or the induction of decentralization supersensitivity using reserpine to deplete cerebral NE levels, or propranolol to block β-adrenergic receptors. The destruction of noradrenergic terminals in cat cerebral cortex by the intraventricular administration of 6-OHDA, five days after birth, resulted in an enhancement between two and 65 days later in the concentration of β-adrenergic receptors measured both by [$^3$H]-DHA binding and by the stimulation of adenylate cyclase by (−)-isoproterenol compared to controls of the same age. Although no increase in basal adenylate cyclase activity was found after 6-OHDA lesions, this cannot be taken as evidence that the number of catalytic units of this enzyme remained unchanged, since those which are specifically coupled to a β-adrenergic receptor may represent a small percentage of the total. Indeed, in a more homogeneous system such as the pineal gland, constant exposure of the animal to light increases the concentration of both β-adrenergic receptors and adenylate cyclase catalytic units (40). After 6-OHDA treatment, the percentage augmentation of the number of [$^3$H]-DHA binding sites was highly correlated with the length of time elapsing after the lesion ($r = 0.98$), whereas supersensitivity of the isoproterenol-sensitive adenylate cyclase did not show such a correlation ($r = 0.38$). This is in contrast to the results of Harden *et al.* (41), who found a parallel increase of cortical β-adrenergic receptors and of isoproterenol-induced cyclic AMP accumulation in tissue slices after administration of 6-OHDA to rats a few hours after birth. At the time of the lesion in the present experiments (five days) the cortical NE level is at least 25% of adult levels[3]; whereas in the experiments of Harden *et al.* (41) the noradrenergic innervation of the cortex is very low at the time of 6-OHDA administration.

Since 6-OHDA treatment permanently destroyed preexisting presynaptic cortical noradrenergic elements as evidenced by the rapid and prolonged fall in NE levels in this region (20), one hypothesis for the different evolution of [$^3$H]-DHA binding and β-adrenergic sensitive adenylate cyclase in the present experiments could be that there exist presynaptic β-adrenergic receptors (42) not coupled to an adenylate cyclase, which disappear with a time course similar to that of the fall in NE, which is more rapid than the increase of postsynaptic β-adrenergic receptors coupled with an adenylate cyclase. Similarly, Sporn *et al.* (10,

[3] Michel Hamon, unpublished observations.

11) found a greater augmentation of [$^{125}$I]-iodohydroxybenzylpindolol binding sites than of (−)-isoproterenol or NE induced accumulation of cyclic AMP in cortical slices after 6-OHDA lesions of adult rats. However, because of the uncertainty of the cellular localization of β-adrenergic receptors in central nervous system it is impossible to determine whether the hypersensitivity observed is situated on glial cells or on neurons postsynaptic to noradrenergic terminals. Furthermore, even if unlikely, it cannot be ruled out that after 6-OHDA lesions the increase in β-adrenergic receptors is not due to a glial proliferation.

There is some controversy concerning whether chronic reserpine treatment induces hypersensitivity of a (−)-isoproterenol or NE-sensitive adenylate cyclase in brain slices (3, 9, 12). In the present study, this treatment resulted in a similar increase in the number of [$^3$H]-DHA binding sites (50 ± 11%) and β-adrenergic sensitive adenylate cyclase (44%). The pattern of the increase in these two parameters contrasts with the effect of 6-OHDA treatment in the adult rat (10, 11), and may be accounted for by the fact that presynaptic terminals possibly containing β-adrenergic receptors are not destroyed by reserpine. It also contrasts with the results of Nahorski (12) in which a single dose of reserpine induced an increased responsiveness of isoproterenol-induced cyclic AMP accumulation in chick brain slices, whereas it had no effect on the number of [$^3$H]-DHA binding sites. The difference could be due to the single treatment with reserpine used in these experiments, compared to the four days' treatment which we have used.

In the present study, chronic propranolol treatment also produced an augmentation of the number of cortical β-adrenergic receptors (32 ± 4%) and β-adrenergic-sensitive adenylate cyclase (17%). Its effect was weaker than that of reserpine, possibly because propranolol is a competitive β-adrenergic antagonist with a short duration of action. Thus the treatment schedule used may not have achieved a complete and permanent blockade of these receptors. Chronic propranolol administration has recently been found to produce an augmentation in the number of specific [$^3$H]-DHA binding sites in rat heart (43) and of the maximal response of the cyclic AMP-generating system to NE in mouse limbic forebrain slices (9). In contrast, chronic phenoxybenzamine or chlorpromazine administration was not found to produce any alteration of the number of β-adrenergic receptors or in the stimulation of adenylate cyclase by isoproterenol, although both treatments have previously been found to modify NE-induced accumulation of cyclic AMP in brain slices (9, 44). It is possible that the effects of these agents on NE-stimulated adenylate cyclase in brain slices could be indirect and due to alterations in sensitivity of α-adrenergic receptors.

The supersensitivity of cortical β-adrenergic receptors after chronic propranolol treatment found in the present study, or of striatal dopaminergic receptors after haloperidol treatment (45), indicates that in the central nervous system the homeostatic control of the concentration of neurotransmitters receptors is exercised as a function of receptor occupancy by the agonist and does not depend on alterations in the release of other presynaptic regulatory factors.

These studies on the pharmacological properties, topographical distribution and adaptive regulation of [$^3$H]-DHA binding sites and β-adrenergic sensitive adenylate cyclase lead to the conclusion that in the central nervous system the β-adrenergic receptors coupled to an adenylate cyclase are those identified by [$^3$H]-DHA binding.

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