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Multiple Apparent *Alpha*-Noradrenergic Receptor Binding Sites in Rat Brain: Effect of 6-Hydroxydopamine

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SUMMARY

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[^{3}H]Clonidine (26.7 Ci/mmole) binds with high affinity to sites on rat brain membranes with properties of *alpha*-noradrenergic receptors. [^{3}H]Clonidine binding shows a biphasic pattern in kinetic and saturation experiments. Fifty percent of the specific binding of 0.4 nM [^{3}H]clonidine is dissociated rapidly in 2 min by excess norepinephrine or clonidine, and the remaining 50% dissociates at a 10-fold slower rate. When slowly-dissociating [^{3}H]clonidine binding is examined in isolation, saturation curves show a single population of high-affinity sites with a K_D of 0.5 nM and B_{max} of 1.3-1.6 pmoles/g tissue in cerebral cortex. Rapidly-dissociating [^{3}H]clonidine binding, estimated from the difference between total binding and high-affinity binding, involves a single population of lower affinity sites with a K_D of 3.0 nM and B_{max} of 9-10 pmoles/g cerebral cortex tissue. *Alpha* agonists are in general more potent at the high affinity [^{3}H]clonidine site, while *alpha* antagonists are more potent at the low affinity site. α-Methylnorepinephrine is less potent than norepinephrine at the high affinity site, but more potent at the low affinity site. Neither [^{3}H]-clonidine binding site resembles the *alpha* receptor site labeled by [^{3}H]WB-4101. The distribution of high and low affinity [^{3}H]clonidine binding throughout rat central nervous system is different. High affinity binding levels vary 14 fold between lowest values in corpus striatum and cerebellum, and highest values in cerebral cortex. Low affinity binding varies less regionally, with highest levels in hypothalamus. 6-Hydroxydopamine treatment doubles the number of high affinity [^{3}H]clonidine sites in the cortex, but does not alter the number of low affinity sites. 6-Hydroxydopamine increases high affinity binding much more than low affinity binding throughout the brain. 6-Hydroxydopamine also increases by 50% the number of [^{3}H]WB-4101 and [^{3}H]epinephrine *alpha* receptor sites in the cerebral cortex.

INTRODUCTION

A variety of pharmacological evidence indicates the existence of more than one type of *alpha*-noradrenergic receptor. Besides classical postsynaptic *alpha*-receptors, noradrenergic nerve terminals appear to possess autoreceptors at which *alpha*-noradrenergic agonists inhibit release of norepinephrine and *alpha*-antagonists block these effects (1, 2). Clonidine is

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thought to be one of the most potent α-agonists at these autoreceptors (3, 4). However, in initial studies of [^{3}H]clonidine binding to putative *alpha*-noradrenergic sites we found only one population of saturable binding sites and observed no decrease in binding when noradrenergic neurons were destroyed by intraventricular injections of 6-hydroxydopamine (5). Recently we have obtained [^{3}H]clonidine of much higher specific activity and now report evidence for discrete high and low affinity binding sites, with specific properties resembling *alpha*-receptors, which have different responses to 6-hydroxydopamine treatment.

METHODS

Methods were generally similar to previous CNS *alpha*-receptor binding studies (5, 6). Different fresh brain regions from adult male Sprague-Dawley rats were homogenized in 20 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.7 at 25°, with a Brinkmann Polytron PT-10. After two centrifugations at 50,000 × *g* for 10 min with intermediate rehomogenization, the final pellets were resuspended in 49 volumes ([^{3}H]clonidine and [^{3}H]WB-4101 assays) or 22 volumes ([^{3}H]epinephrine assays) of cold 50 mM Tris-HCl buffer, pH 7.7 at 25°.

Binding assays. [^{3}H]Clonidine, 26.7 Ci/mmole, [^{3}H]WB-4101, 25 Ci/mmole and (±)-[^{3}H]epinephrine, 15 Ci/mmole, were obtained from New England Nuclear, at a stated purity of greater than 95%. For standard [^{3}H]clonidine binding assays, incubation tubes received 20 μl [^{3}H]clonidine at a final concentration of 0.4 nM, 20 μl of various concentrations of unlabeled drugs, 0.94 ml of 50 mM Tris-HCl buffer, pH 7.7 at 25°, and 1.0 ml of tissue suspension. After a 30 min incubation at 25°, the contents were rapidly filtered under vacuum over Whatman GF/B filters with 3 × 5 ml rinses of cold buffer. Filters were counted by liquid scintillation spectrometry in 10 ml Formula 947 (New England Nuclear) at 37.5% efficiency.

Specific [^{3}H]clonidine binding was defined as the excess over blanks containing 10 μM (—)-norepinephrine. At 0.4 nM [^{3}H]clonidine concentrations, in assays containing 20 mg original wet weight of rat cortex tissue, total binding was 1000 cpm and nonspecific binding was 80–100 cpm. To isolate high- and low-affinity components of binding, membranes were incubated with [^{3}H]clonidine for 30 min at 25°, following which 200 μl of a 10 μM solution of unlabeled clonidine was added to the incubation to dissociate [^{3}H]clonidine from low-affinity binding sites. The contents of the assay tubes were filtered 2 min after addition of unlabeled clonidine. The residual specific binding left after 2 min dissociation represented binding of [^{3}H]clonidine to high-affinity, slowly-dissociating sites. Parallel samples were assayed without short-term dissociation to determine overall specific binding. The difference between specific binding determined normally, and specific binding left after 2 min dissociation with unlabeled clonidine, represented binding of [^{3}H]clonidine to low-affinity, rapidly dissociating sites. In typical experiments using 0.4 nM [^{3}H]clonidine, overall specific binding was 900 cpm, of which the residual high-affinity component comprised 400 cpm, and the low-affinity component 500 cpm. In drug inhibition studies, IC_{50} values at the high-affinity site were determined by measuring residual binding following dissociation with unlabeled clonidine, in the presence of a range of drug concentrations. IC_{50} values at the low-affinity site were determined from the difference between binding determined normally, and residual binding after dissociation, in the presence of a range of drug concentrations.

[^{3}H]WB-4101 and [^{3}H]epinephrine binding to rat brain α-receptors was determined as described previously (5–7). Unless otherwise indicated, all binding assays were run in triplicate.

6-Hydroxydopamine treatments. Adult male Sprague-Dawley rats were injected in each lateral ventricle with 6-hydroxydopamine, 250 μg base, in 20 μl of 0.9% NaCl containing 0.1% ascorbic acid. The two injections were given 24 hr apart, and control animals received equivalent injections of vehicle. The animals were killed 4–5 weeks after treatment, the cortex dissected from the rest of the brain, and the cortex and the rest of the brain homogenized in 7 volumes

of 0.32 M sucrose. Brains from 6-hydroxydopamine-treated rats that did not show the expected abolition of twitching reflex following decapitation were discarded (8). [^{3}H]Norepinephrine uptake in synaptosomes prepared from control and 6-hydroxydopamine-treated tissues was determined by a modification of the method of Coyle and Snyder (9), using 0.1 μM (−)[^{3}H]norepinephrine (New England Nuclear, 27 Ci/mmole) and 10 μM desmethylimipramine as a blank to define uptake into noradrenergic terminals. Uptake assays were conducted for 5 min at 37°, and terminated by centrifugation. Uptake into 6-hydroxydopamine treated synaptosome preparations was 19 ± 5% and 6 ± 1% of paired control levels in cerebral cortex and the rest of the brain respectively.

Control and 6-hydroxydopamine homogenates in which [^{3}H]norepinephrine uptake was assayed, were prepared for *alpha*-receptor binding experiments by addition of a large volume of 50 mM Tris-HCl to lyse the homogenates, and two subsequent centrifugations and resuspensions as described above for standard assays. In 6-hydroxydopamine experiments, the binding assay contained 10 mg instead of the standard 20 mg tissue. The protein concentrations of the binding assay tissue suspensions were determined by the method of Lowry (10), using bovine albumin as a standard.

Drugs. Phenylethylamine enantiomers were generously donated by Sterling-Winthrop; clonidine, tramazoline and St-600, by Boehringer Ingelheim; dihydroergotamine, by Sandoz; WB-4101 by Ward Blenkinsop; prazosin, by Ely Lilly; and indoramin, by Wyeth. p-Aminoclonidine and 2,5-dichlorophenylimino-2-imidazolidine were provided by our colleague, Dr. B. Rouot. Other drugs and compounds were obtained from the pharmaceutical company of origin or commercial sources.

RESULTS

Kinetics of [^{3}H]clonidine binding. Specific [^{3}H]clonidine binding at 25° was reversible (Fig. 1). By 30 min, specific binding to cerebral cortex membranes reached plateau values which were 8 times the levels of nonspecific binding measured in the presence of 10 μM (−)-norepinephrine. Half maximal levels of specific binding occurred at about 30 min. On a semilogarithmic plot the pseudo-first order kinetics of association of specific binding were linear (Fig. 1A). From three different experiments, the observed rate constant (k_{ob}) of association was 0.13 ± 0.01 min^{-1} (mean ± standard error).

Dissociation of specifically bound [^{3}H]-clonidine was evaluated by incubating membranes to equilibrium with [^{3}H]clonidine, whereupon 10 μM (−)-norepinephrine was added and residual binding estimated at varying time intervals (Fig. 1B). When plotted on a semilogarithmic scale, dissociation of [^{3}H]clonidine was biphasic. The half lives for the fast and slow phases of dissociation were about 1 min and 13 min respectively. From three separate experiments, the rate constants for dissociation (k_{-1}) for the fast and slow phases were 0.68 ± 0.15 min^{-1} and 0.063 ± 0.008 min^{-1} respectively (mean ± standard error). When 1.0 μM clonidine instead of 10 μM (−)-norepinephrine was used to dissociate [^{3}H]-clonidine specific binding, dissociation remained biphasic with k_{-1} values of 0.69 min^{-1} and 0.064 min^{-1} for the fast and slow phases, similar to values obtained with 10 μM (−)-norepinephrine. Similar results were obtained using 10 μM and 100 μM clonidine. In other experiments in which dissociation was determined following 100-fold dilution of the medium, the same dissociation pattern was observed. The equation for the second order rate constant of association

$$k_1 = \frac{(k_{ob} - k_{-1})}{[^3\text{H}]\text{clonidine}}$$

which is the best estimate of k_1 (11), could not be used with accuracy for the observed association and fast dissociation phases. However, a k_1 value could be ascertained from the k_{ob} and slow phase k_{-1} values, which from three experiments was 0.16 ± 0.045 nM^{-1} min^{-1} (mean ± standard error). Using this k_1 value, dissociation constants (K_D) subsequently calculated from the ratio k_{-1}/k_1 for the rapidly and slowly dissociating components of [^{3}H]clonidine specific

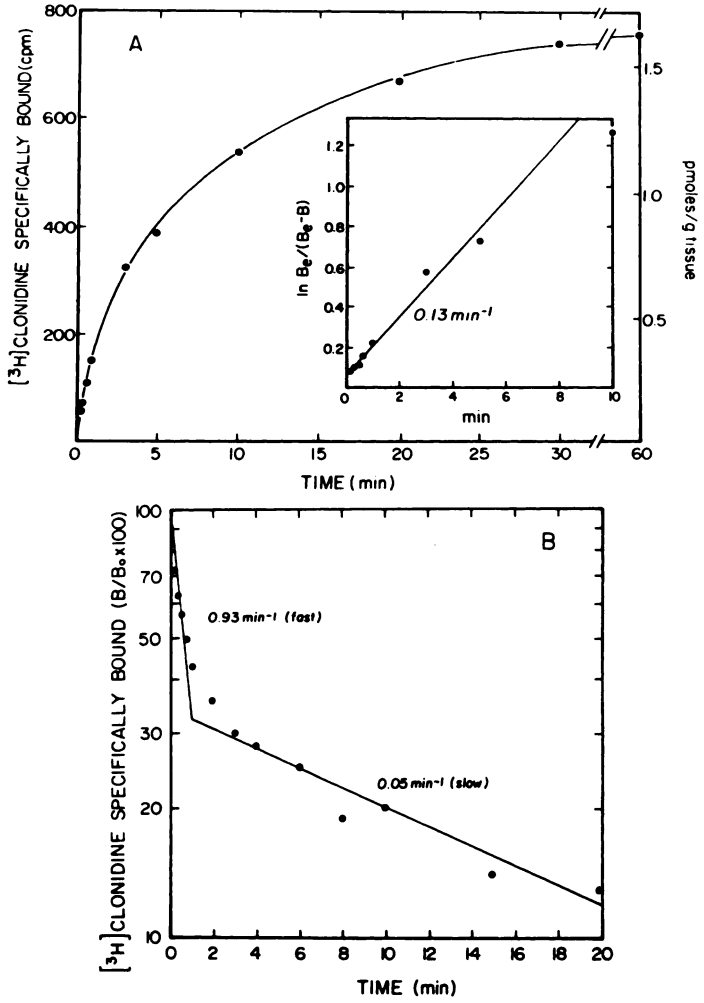

FIG. 1. *Time course of association and dissociation of [^{3}H]clonidine specific binding*

(Panel A) Association of [^{3}H]clonidine specific binding to rat cortex homogenates (20 mg, original tissue weight) at 25° was measured at various intervals following addition of 0.4 nM [^{3}H]clonidine. Specific binding at each point was defined as the difference between binding obtained in the absence and presence of 10 μM (—)-norepinephrine. Points shown are for a single experiment performed in triplicate, which was replicated three times. *Inset*, pseudo-first order kinetic plot of initial [^{3}H]clonidine specific binding. The slope is equal to k_{ob}, the observed rate constant for the pseudo-first order reaction. (Panel B) Dissociation of specifically bound [^{3}H]clonidine was measured at 25° following incubation with 0.4 nM [^{3}H]clonidine to equilibrium (30 min). At time zero, 10 μM (—)-norepinephrine was added to the incubation mixtures and the reactions terminated by filtration at various times. Nonspecific binding, determined in parallel samples, containing either 10 μM (—)-norepinephrine or 1.0 μM clonidine (which reduce [^{3}H]clonidine binding to the same extent), did not change during dissociation. Points shown are from a single experiment, performed in triplicate, which was replicated three times.

binding were 4.2 ± 1.7 nM and 0.39 ± 0.14 nM respectively.

Saturation of [^{3}H]clonidine binding. Specific [^{3}H]clonidine binding to rat cerebral cortex membranes was saturable with maximal binding apparent at about 9.0 nM and half maximal binding occurring at about 2–3 nM. Scatchard analysis of these data indicated two discrete components of binding. The approximate K_D values for the high and low affinity components were 0.4 nM and 2.6 nM respectively (Fig. 2). The

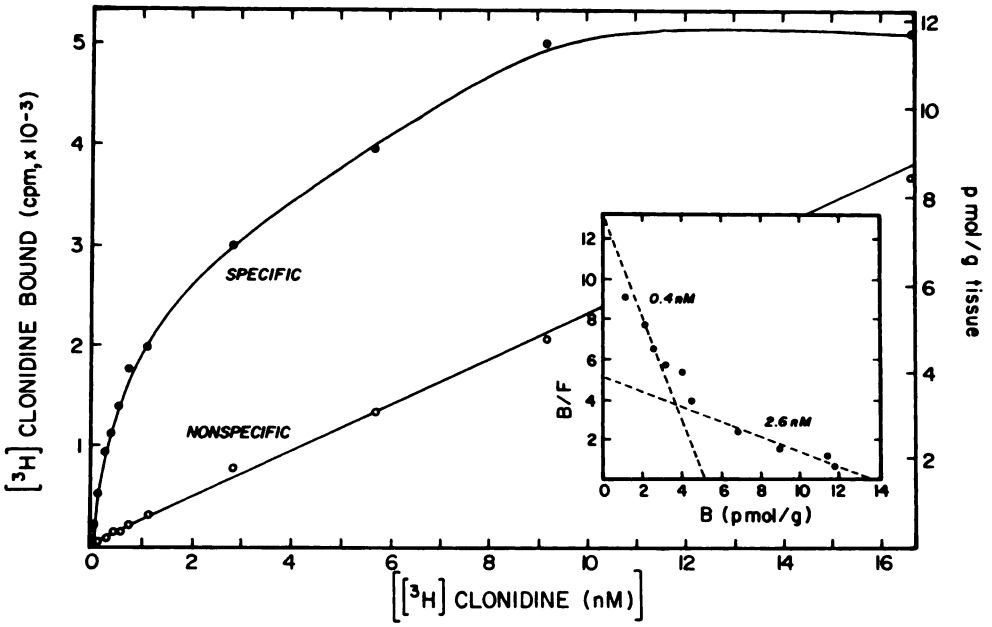

FIG. 2. *[^{3}H]Clonidine binding as a function of increasing concentrations of [^{3}H]clonidine*

Rat cortex homogenates (20 mg tissue) were incubated for 30 min at 25° with various concentrations of [^{3}H]clonidine. Nonspecific binding was determined in the presence of 10 μM (—)-norepinephrine. Points shown are from a single experiment, performed in triplicate, which was replicated 13 times. *Inset*, Scatchard plot of saturation data, with approximate K_D values.

apparently biphasic nature of [^{3}H]clonidine binding was observed in most, but not all experiments on cerebral cortex membranes. Linear regression analysis of all saturation points from 13 experiments gave a mean [^{3}H]clonidine K_D value of 2.0 ± 0.3 nM (mean ± standard error). It was noticed that in 12-week-old rats there was an approximate 30% decrease in binding sites compared to 7 week old rats.

To quantify the high and low affinity components of binding separately, we assumed that the lower affinity binding would dissociate more rapidly than the higher affinity binding. The dissociation data (Fig. 1) from repeated experiments suggested that at 2 min after onset of dissociation with norepinephrine or clonidine, the rapid phase of dissociation is essentially complete, and less than 10% of [^{3}H]clonidine is lost from the slowly dissociating phase. Accordingly, we conducted some incubations to equilibrium for 30 min and immediately terminated the reaction, while in parallel incubations we added, at equilibrium, either 10 μM (−)-norepinephrine or 1 μM clonidine and continued incubation for 2 min at which time residual binding was measured. We assumed that residual binding after this treatment would tend to reflect high affinity components of [^{3}H]clonidine binding. When residual binding of increasing concentrations of [^{3}H]clonidine was determined in this way and analyzed by Scatchard plots, the presumptive high affinity binding displayed only one component, whether the dissociation had been performed with norepinephrine or clonidine. This binding had a K_D value of about 0.4 nM determined from linear regression analysis, closely similar to the estimated high affinity component of binding observed in equilibrium experiments. The maximal number of binding sites (B_{max}) was about 1.5–1.8 pmol/g (Fig. 3A).

We determined the low affinity component of binding by subtracting the apparent high affinity binding from total equilibrium binding values. Scatchard analysis of the presumptive low affinity sites revealed K_D values of 2.3–2.6 nM, similar to the K_D for the low affinity component of binding ob-

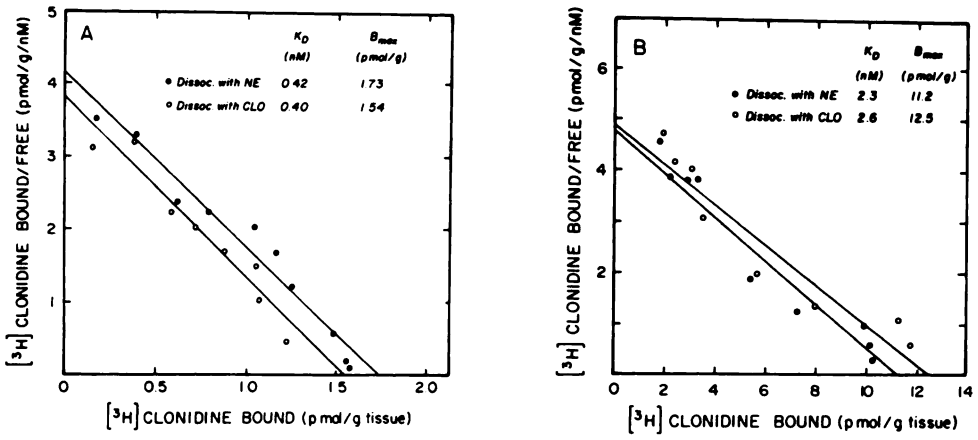

FIG. 3. *Quantification of high and low affinity binding sites*

(Panel A) Scatchard plots of high-affinity [^{3}H]clonidine binding. Rat cortex homogenates (20 mg tissue) were incubated for 30 min at 25° with various concentrations of [^{3}H]clonidine, and then for a further 2 min with 10 μM (—)-norepinephrine (NE, ●) or 1.0 μM clonidine (CLO, ○) before filtration, and residual specific binding was determined. Points shown are from the same experiment as in Fig. 2, where total equilibrium specific binding is shown, and are means of triplicate determinations. The experiment was replicated 10 times. (B) Scatchard plots of low affinity [^{3}H]clonidine binding. Points shown are the *difference* between total equilibrium specific binding at each [^{3}H]clonidine concentration (data in Fig. 2) and residual specific binding after dissociation with 10 μM (—)-norepinephrine or 1.0 μM clonidine for 2 min (data in Fig. 3A).

served in equilibrium experiments. The B_{max} value for the apparent low affinity sites was 11–12 pmol/g (Fig. 3B). The mean ± standard error K_D and B_{max} values from ten saturation experiments were 0.50 ± 0.05 nM and 1.6 ± 0.1 pmoles/g tissue for high affinity [^{3}H]clonidine binding, and 3.0 ± 0.6 nM and 9.2 ± 0.9 pmoles/g tissue for low affinity [^{3}H]clonidine binding. The K_D values for high and low affinity [^{3}H]clonidine binding obtained from saturation experiments closely resembled the dissociation constants (K_D) derived above from kinetic experiments (Fig. 1). The sum of the B_{max} values for the calculated high and low affinity components resembles the B_{max} obtained in equilibrium experiments of about 13 pmole/g, and the B_{max} value obtained in earlier experiments using low specific activity [^{3}H]clonidine (5). The K_D value of 5.8 nM for [^{3}H]clonidine binding reported earlier (5) suggests that in those experiments only low affinity [^{3}H]clonidine binding had been observed.

Drug specificity of the two [^{3}H]clonidine binding sites. To examine pharmacological differences between the two populations of [^{3}H]clonidine binding sites, we evaluated the influence of a variety of *alpha* receptor-specific drugs (Fig. 4, Table 1). In these experiments, drug influences on high affinity binding were estimated by conducting initial incubations in the presence of the drug and at 30 min adding 1 μM clonidine, continuing the incubation for a further 2 min, and estimating residual binding. Low affinity binding was estimated by subtracting high affinity from total equilibrium binding.

Both low and high affinity sites displayed profiles characteristic of *alpha*-noradrenergic receptors. The pharmacological profiles of both sites were very similar to the sites labeled previously in rat and bovine central nervous system by low specific activity [^{3}H]clonidine (5, 12) and [^{3}H]epinephrine and [^{3}H]norepinephrine (6, 7). The slopes for displacement curves of agonists and antagonists were parallel for both high and low affinity sites (Fig. 4), with log-logit slopes equal to 1.0. The major difference between the two sites was the greater potency of agonists in competing for high affinity than low affinity [^{3}H]clonidine binding sites. However, there were differences among the agonists in their relative potencies at high and low affinity sites. Thus, (−)-alpha-methylnorepinephrine

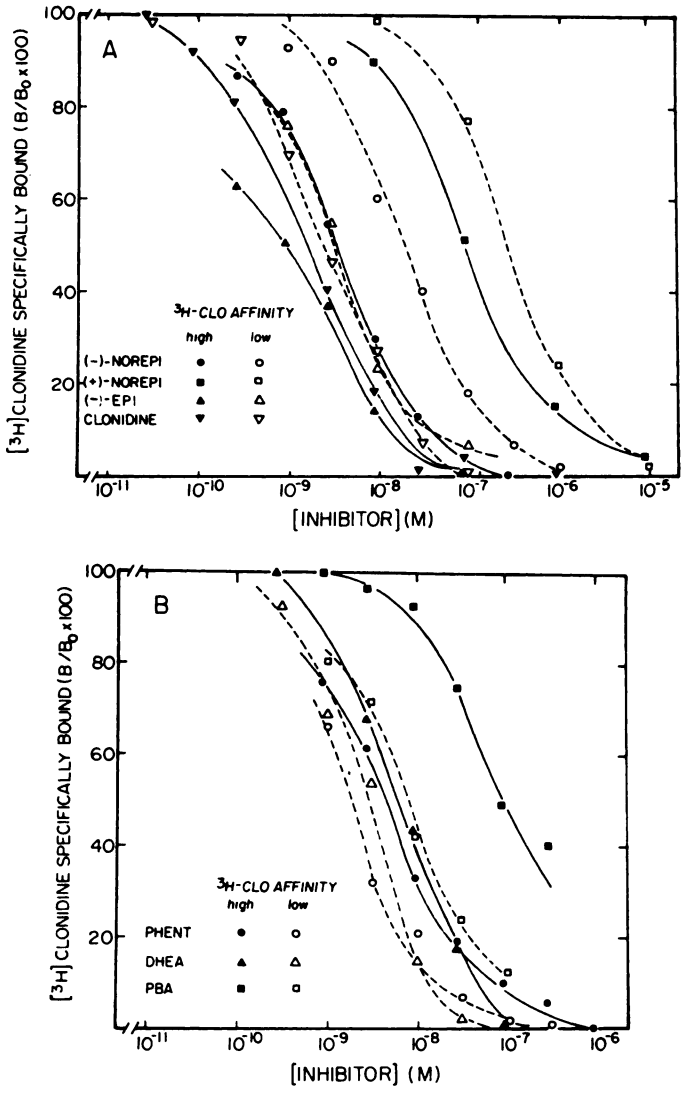

FIG. 4. *Inhibition of high affinity and low affinity specific [^{3}H]clonidine binding by agonists and antagonists*

Rat cortex homogenates (20 mg tissue) were incubated with 0.4 nM [^{3}H]clonidine for 30 min at 25° in the presence of various concentrations of unlabeled drugs, and high and low affinity [^{3}H]clonidine binding was subsequently isolated as described in Fig. 3 legend and METHODS. Values are specific binding expressed as percent of high affinity or low affinity specific binding in the absence of inhibitors (B_0), and are from a typical experiment, performed in duplicate. (Panel A) Agonists. (Panel B) Antagonists.

had approximately the same affinity at the two sites, while (−)-norepinephrine itself was 4.5 times more potent at the high than low affinity sites. Clonidine displayed the greatest discrepancy between the two sites, being about seven times more potent at high than low affinity sites (Table 1). The apparent K_i values for clonidine inhibiting high and low affinity binding were closely similar to the K_D values of [^{3}H]clonidine at the two sites determined from saturation experiments.

Relative effects of antagonists on the high and low affinity [^{3}H]clonidine sites differed from those of agonists. Except for piperoxan and tolazoline, which also act as

TABLE 1

Inhibition by Alpha Receptor Agents of High and Low Affinity [^{3}H]Clonidine Binding to Rat Cerebral Cortex Membranes

Drug inhibition of high affinity and low affinity binding of 0.4 nM [^{3}H]clonidine, using 6–9 concentrations of each unlabeled drug, was determined as described in METHODS. IC_{50} values were determined graphically by log probit analysis, and apparent K_i values calculated from the equation $K_i = IC_{50}/(1 + [[^3H]clonidine]/K_D)$. The K_D values used were 0.50 nM for high affinity binding and 3.0 nM for low affinity binding. Values are means ± standard errors of three or four experiments, with binding at each drug concentration assayed in duplicate in each experiment. Values without standard errors are from one experiment only. 2,5-Dichlorophenylimino-2-imidazolidine is an isomer of clonidine.

Drug	K_i at high affinity site (A)	K_i at low affinity site (B)	B:A
	nM	nM	
Agonists			
Phenylethylamines			
(—)-Epinephrine	0.83 ± 0.19	2.9 ± 0.2	3.5
(—)-Norepinephrine	2.5 ± 0.27	15.0 ± 4.0	6.0
(—)-α-Methylnorepinephrine	2.8 ± 0.27	3.9 ± 0.6	1.4
(—)-Phenylephrine	50.0 ± 4.0	160.0 ± 34.0	3.2
(+)-Norepinephrine	57.0	351.0	
Imidazolines			
p-Aminoclonidine	0.27	0.73	2.7
Naphazoline	0.58 ± 0.01	2.6 ± 0.1	4.5
Oxymetazoline	0.60 ± 0.03	1.6 ± 0.7	2.7
Clonidine	0.62 ± 0.08	4.3 ± 1.4	6.9
Tramazoline	1.2 ± 0.3	2.6 ± 0.2	2.2
2,5 Dichlorophenylimino-2-imidazolidine	2.3 ± 0.2	5.9 ± 0.6	2.6
St 600	2.6 ± 0.4	4.2 ± 0.3	1.6
Antagonists			
Phentolamine	2.5 ± 0.5	1.9 ± 0.7	0.76
Dihydroergotamine	3.7 ± 0.1	2.5 ± 0.2	0.68
Piperoxan	29 ± 1	36 ± 9	1.2
Phenoxybenzamine	44 ± 9	8.8 ± 1.2	0.20
Tolazoline	56 ± 13	110 ± 10	2.0
Yohimbine	73 ± 7	40 ± 5	0.55
WB-4101	106 ± 4	92 ± 5	0.87
Chlorpromazine	760	520	0.68
Thioridazine	1400	870	0.62
Droperidol	3100	1200	0.39
Prazosin	6400 ± 2700	2600 ± 1300	0.41
Indoramin	9000 ± 2000	26,000 ± 100	2.9

a partial agonist on alpha-receptors (13), all of the antagonists were more potent at low affinity than high affinity sites. There was variation among these drugs, ranging from phenoxybenzamine, which was five times more potent at low affinity than high affinity sites, to WB-4101, which was almost as potent at high affinity as at low affinity sites. It is possible that irreversible effects of phenoxybenzamine could influence differential actions of the two sites. Some antagonists, such as phentolamine and dihydroergotamine at both sites, and phenoxybenzamine at the low affinity site, were about as potent as inhibitors of [^{3}H]clonidine as they are of [^{3}H]WB-4101 *alpha*-receptor binding (5). Certain other antagonists, however, such as neuroleptic agents, WB-4101, indoramin and prazosin are markedly more potent at the [^{3}H]WB-4101 sites (5, 14, 15) than at either [^{3}H]clonidine site (Table 1). Thus neither the high- nor the low-affinity [^{3}H]clonidine site appears to correspond to the [^{3}H]WB-4101 alpha-receptor site. The low affinity of prazosin suggests that both [^{3}H]clonidine binding

sites resemble pharmacologically the presynaptic *alpha* receptor, or *alpha*$_2$ receptor (15, 16), whereas [^{3}H]WB-4101 labels the *alpha*$_1$ or postsynaptic *alpha* receptor.

Regional variations in high and low affinity [^{3}H]clonidine binding sites. The high and low affinity specific [^{3}H]clonidine binding sites showed differences in their regional distribution throughout rat brain (Table 2). A much more marked regional variation was apparent for the high affinity site, which showed a 14 fold difference between highest levels in the frontal cerebral cortex and lowest values in the corpus striatum. Outside of the cerebral cortex, highest levels of binding were observed in the hippocampus, thalamus, hypothalamus and the colliculi, whose values were about half those of the cerebral cortex. The next highest regions were the pons and the medulla oblongata, with levels of 22–30% of those of the frontal cerebral cortex. High affinity clonidine binding was only 11–13% of cerebral cortex values in the cerebellum and a "midbrain area" comprising tissue anterior to the pons, posterior to the thalamus, ventral to the colliculi, and containing the substantia nigra and nucleus ruber.

By contrast, much smaller regional variations were observed in low affinity binding. Lowest levels in the pons were about one fifth of highest levels, which occurred in the hypothalamus. Hypothalamus, anterior thalamus, cerebral cortex and colliculi had relatively similar levels of binding, while corpus striatum, midbrain area and hippocampus had 50–70% of values in the frontal cerebral cortex. The cerebellum, pons and medulla oblongata had similar levels of binding, about 30% of frontal cortex levels.

TABLE 2

Regional Distribution of High Affinity and Low Affinity [^{3}H]Clonidine Binding in Rat Brain

High and low affinity specific binding of 0.4 nM [^{3}H]clonidine was determined as described in METHODS. Values are expressed as the mean ± standard error of three determinations of [^{3}H]clonidine bound per mg protein. Each determination was performed in triplicate on pooled tissue from two brains, and each assay contained 8–20 mg tissue, or 0.35–1.0 mg protein. Protein assays were conducted for every tissue. The area of the midbrain region is defined in the text. Values in parentheses are binding in each region expressed as percent of frontal cortex binding.

Region	[^{3}H]Clonidine specific binding	
	High affinity	Low affinity
	(*fmoles/mg protein*)	(*fmoles/mg protein*)
Frontal cortex	16.9 ± 1.3 (100)	39.4 ± 1.2 (100)
Occipital cortex	15.2 ± 1.6 (90)	35.1 ± 1.3 (89)
Parietal cortex	16.2 ± 2.5 (96)	39.3 ± 4.4 (100)
Corpus striatum	1.2 ± 0.7 (7)	20.2 ± 3.6 (51)
Hippocampus	8.4 ± 1.6 (50)	27.6 ± 1.8 (70)
Anterior thalamus	9.7 ± 4.4 (57)	39.4 ± 9.9 (100)
Posterior thalamus	6.8 ± 0.4 (40)	26.2 ± 2.9 (67)
Hypothalamus	8.0 ± 1.0 (47)	50.9 ± 2.2 (129)
Colliculi	9.4 ± 2.7 (56)	37.8 ± 3.2 (96)
Midbrain	2.2 ± 1.5 (13)	25.4 ± 2.6 (65)
Cerebellum	1.8 ± 0.7 (11)	14.0 ± 1.3 (36)
Pons	3.8 ± 0.4 (22)	10.6 ± 1.7 (27)
Medulla	5.3 ± 0.4 (31)	14.6 ± 1.7 (37)

Influences of 6-hydroxydopamine on the two [^{3}H]clonidine binding sites. If one of the [^{3}H]clonidine binding sites involves presynaptic autoreceptors on noradrenergic terminals (1), then its binding should diminish following destruction of noradrenergic neurons with 6-hydroxydopamine. Accordingly, we administered 6-hydroxydopamine intraventricularly under conditions which produce almost total depletion of norepinephrine uptake capability and endogenous norepinephrine (17) (see METHODS). Four to five weeks following 6-hydroxydopamine treatment, we evaluated high and low affinity specific [^{3}H]clonidine binding in numerous brain regions (Fig. 5, Table 3). Saturation experiments were performed in the cerebral cortex, and Scatchard analysis indicated that 6-hydroxydopamine treatment doubled the number of high affinity binding sites with no apparent change in the K_D values (Fig. 5A). Both control and 6-hydroxydopamine-treated animals displayed a single population of high affinity sites. By contrast, after 6-hydroxydopamine treatment there was no significant change in the numbers of low affinity binding sites but there was an apparent two-fold increase in the affinity of [^{3}H]clonidine at that site. In these experiments, one

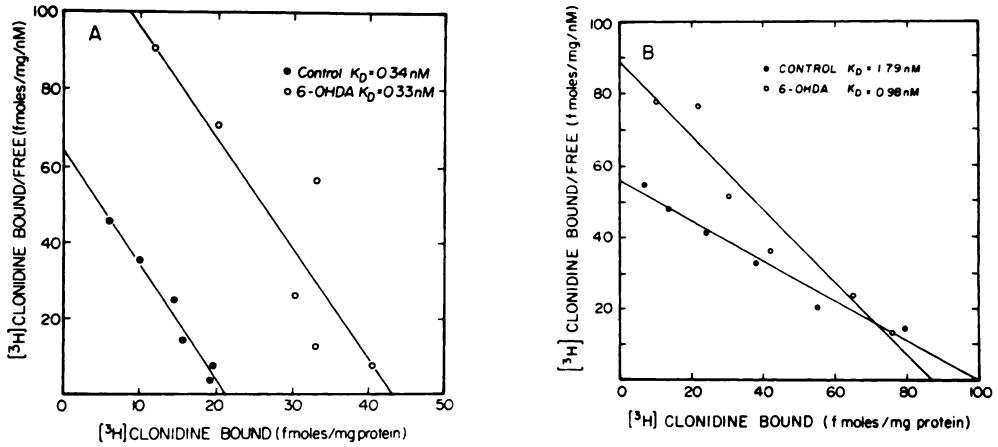

FIG. 5. *Scatchard plots of saturation of [^{3}H]clonidine specific binding in the cerebral cortex of control and 6-hydroxydopamine treated rats*

High affinity and low affinity [^{3}H]clonidine binding was isolated as described in METHODS. Values represent mean binding from triplicate determinations, at each [^{3}H]clonidine concentration, on six control (●) and six 6-hydroxydopamine-treated (6-OHDA) (○) rats. Lines of best fit were obtained by linear regression analysis. (A) High affinity binding. (B) Low affinity binding.

TABLE 3

Effect of 6-Hydroxydopamine on High Affinity and Low Affinity [^{3}H]Clonidine Binding in Different Rat Brain Regions

High and low affinity specific binding of 0.4 nM [^{3}H]clonidine in 6-hydroxydopamine-treated rats was determined as described in legend to Table 2. 6-Hydroxydopamine treated tissues were paired in each experiment with the control tissues whose values are given in Table 2. Values are expressed as the mean ± standard error of three determinations, each determination being performed on pooled tissue from two brains. Statistical comparisons were done using Student's *t*-test.

Region	[^{3}H]Clonidine specific binding			
	High affinity		Low affinity	
	(*fmoles/mg protein*)	(*% control*)	(*fmoles/mg protein*)	(*% control*)
Frontal Cortex	29.6 ± 1.3**	175	51.8 ± 0.5**	132
Occipital Cortex	28.7 ± 1.5***	189	56.2 ± 6.2**	160
Parietal Cortex	23.1 ± 2.0*	143	49.8 ± 3.7	127
Corpus Striatum	5.0 ± 1.1*	417	28.3 ± 0.7**	140
Hippocampus	15.9 ± 1.7*	189	40.3 ± 3.6*	146
Anterior Thalamus	10.6 ± 3.2	109	42.1 ± 1.8	107
Posterior Thalamus	10.2 ± 0.6**	150	38.8 ± 6.1	148
Hypothalamus	14.7 ± 1.2**	184	62.6 ± 7.8	123
Colliculi	17.4 ± 0.5*	185	45.4 ± 0.6	120
Midbrain	5.4 ± 2.8	246	37.4 ± 7.0	147
Cerebellum	6.4 ± 0.2**	356	13.1 ± 1.6	94
Pons	3.7 ± 0.9	97	14.2 ± 2.7	134
Medulla	5.5 ± 2.1	104	23.5 ± 2.5*	161

* Significantly different from control value, $p < 0.05$
** Significantly different from control value, $p < 0.01$
*** Significantly different from control value, $p < 0.001$

pair of control and 6-hydroxydopamine cortices were assayed in each experiment, and high and low affinity saturation constants calculated for each individual cortex. From six such experiments, the mean B_{max} values for the high affinity site were 22 ± 3 fmoles/mg protein for control and 41 ± 7 fmoles/mg protein for 6-hydroxydopamine ($p <$

0.01, Student's paired t-test). For the low affinity site, the values were 102 ± 14 fmoles/mg protein for control and 83 ± 19 fmoles/mg protein for 6-hydroxydopamine ($p > 0.2$).

The influence of 6-hydroxydopamine on binding of a single concentration of [^{3}H]-clonidine varied markedly among various brain regions (Table 3). The most marked augmentations occurred in the cerebellum and corpus striatum, where high affinity binding was increased 3.5–4 fold from very low control levels. The next highest increase was in the midbrain area, where binding was enhanced 2.5 fold. These increases occurred in the three regions with the lowest control levels of high-affinity [^{3}H]clonidine binding, and although statistically significant in the case of the corpus striatum and the cerebellum, may not be functionally significant. The posterior thalamus, hypothalamus, colliculi, hippocampus and areas of the cerebral cortex showed 50–100% increases in binding. Binding was not changed by 6-hydroxydopamine treatment in the anterior thalamus, pons and medulla oblongata.

Changes in low affinity [^{3}H]clonidine binding were less marked after 6-hydroxydopamine treatment. Statistically significant alterations were apparent only in the medulla oblongata, hippocampus, corpus striatum and frontal and occipital cerebral cortex, where augmentations of 30–60% were noted. Except for the cerebral cortex, binding in all areas was assayed only at a single concentration of [^{3}H]clonidine, so that it is not clear whether changes were due to alterations in affinity or numbers of sites.

To determine whether 6-hydroxydopamine-induced changes in [^{3}H]clonidine binding to *alpha* receptors would be demonstrable with other ligands, we assayed [^{3}H]WB-4101 and [^{3}H]epinephrine binding associated with *alpha* receptors in animals four weeks following 6-hydroxydopamine treatment (Table 4, Fig. 6). For both [^{3}H]WB-4101 and [^{3}H]epinephrine, the numbers of binding sites in the cerebral cortex increased by about 50%, while in the rest of the brain a 23–36% increase in binding sites occurred (Table 4). Scatchard plots remained monophasic, and no changes in K_D values were obtained after 6-hydroxydopamine treatment (Fig. 6).

TABLE 4

Effect of 6-Hydroxydopamine on [^{3}H]WB-4101 and [^{3}H]Epinephrine Binding to Alpha Receptors in Rat Brain

Specific binding to membranes from cerebral cortex or the rest of the brain at seven concentrations of [^{3}H]WB-4101 (0.05–5.0 nM) or [^{3}H]epinephrine (1.5–65.0 nM) was measured in individual control rats and 6-hydroxydopamine-treated (6-OHDA) rats (see METHODS). [^{3}H]WB-4101 and [^{3}H]epinephrine binding, in duplicate assays at each ligand concentration, were determined in the same rat brain, and in each experiment, one control rat was paired with one 6-OHDA rat. Saturation data from each brain for each ligand was evaluated by Scatchard plot, and K_D and B_{max} values for individual brains were derived by linear regression analysis. Values are mean ± standard error of 5 determinations on separate brains. Statistical comparisons were performed using Student's t-test.

Region	K_D (nM)	B_{max} (*fmoles/mg protein*)	ΔB_{max} (%)
[^{3}H]WB-4101			
Control cortex	0.14 ± 0.01	84 ± 9	
6-OHDA cortex	0.16 ± 0.01	123 ± 8*	+49
Control rest of brain	0.16 ± 0.02	61 ± 4	
6-OHDA rest of brain	0.16 ± 0.02	75 ± 9	+23
[^{3}H]Epinephrine			
Control cortex	13.4 ± 1.1	163 ± 12	
6-OHDA cortex	14.7 ± 1.5	240 ± 18*	+49
Control rest of brain	18.4 ± 3.1	100 ± 10	
6-OHDA rest of brain	20.6 ± 5.6	133 ± 15	+36

* Significantly different from control value, $p < 0.01$.

DISCUSSION

Several parameters examined suggest that the biphasic nature of [^{3}H]clonidine binding, observed in kinetic and saturation experiments, reflects the existence of two distinct sites resembling *alpha*-receptors at which [^{3}H]clonidine has differential affinity. The experimental procedure used to isolate high- and low-affinity [^{3}H]clonidine relies on the reasonable assumption that high-affinity binding is identical with the

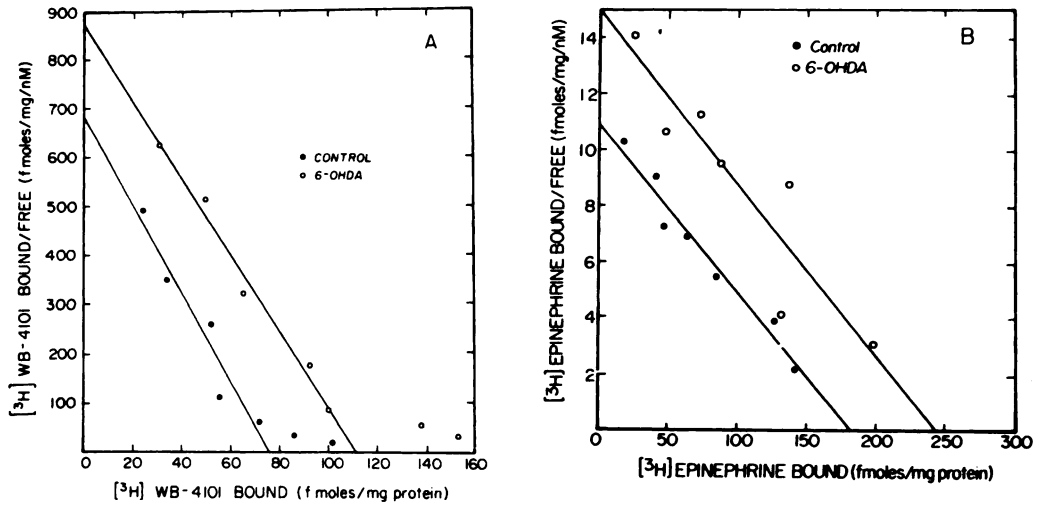

FIG. 6. *Scatchard plots of saturation of [^{3}H]WB-4101 and [^{3}H]epinephrine α-receptor binding in the cerebral cortex of control and 6-hydroxydopamine-treated (6-OHDA) rats*

Values represent mean binding from duplicate determinations, at each [^{3}H]ligand concentration, on 5 control (●) and 5 6-OHDA (○) rats. Lines of best fit were obtained by linear regression analysis on the first five points ([^{3}H]WB-4101) and on all points ([^{3}H]epinephrine). (A) [^{3}H]WB-4101. (B) [^{3}H]epinephrine.

slowly dissociating component, and low-affinity binding with the rapidly dissociating component (Figs. 1, 2). This procedure does not afford complete separation of the two phases of binding. The two-component pattern in a Scatchard plot is also consistent with negative cooperativity. However the persistence of similar dissociation rates in experiments using several dilution and excess norepinephrine or clonidine concentrations argues against negative cooperativity.

Substrate specificities of the two sites differ considerably. In general, agonists are more potent at the high affinity and antagonists more potent at low affinity sites. However, there are differences within each group. Compared to norepinephrine, *alpha*-methylnorepinephrine is more active at low affinity than at high affinity sites. Relative to other antagonists, phenoxybenzamine also appears relatively more active at low affinity than high affinity sites. Interestingly, *alpha*-methylnorepinephrine is thought to be a relatively selective drug for influencing central and peripheral *alpha*-noradrenergic autoreceptors (18). Neither the high affinity nor the low affinity [^{3}H]-clonidine site has the substrate specificity observed with the antagonist [^{3}H]WB-4101. In competing for [^{3}H]WB-4101 binding certain antagonists tend to be much more potent, and all agonists much weaker, than at either of the [^{3}H]clonidine binding sites. The differential affinities of prazosin at the [^{3}H]WB-4101 site compared to the [^{3}H]-clonidine binding sites suggest that [^{3}H]-clonidine labels sites pharmacologically similar to presynaptic receptors, whereas [^{3}H]WB-4101 binding has the characteristics of *alpha*$_1$ postsynaptic receptors.

The affinities of agonists and antagonists at the low-affinity [^{3}H]clonidine binding site resemble their affinities in inhibiting [^{3}H]epinephrine binding in the rat cortex, although the affinities at the [^{3}H]epinephrine site are somewhat lower (19). Thus (±)-[^{3}H]epinephrine has a K_D value of 13 nM (Table 4) and (−)-epinephrine has a K_i value of 5.2 nM at the [^{3}H]epinephrine site (19), as compared with 2.9 nM at the low-affinity [^{3}H]clonidine site (Table 1). (±)-[^{3}H]Epinephrine apparently labels one site in the cortex, which may correspond to the low-affinity [^{3}H]clonidine site. Binding of (±)-[^{3}H]epinephrine to the low-capacity, high affinity site is not readily detectable at present.

The regional distribution of binding also differentiates the high and low affinity [^{3}H]clonidine sites. The low affinity sites

are more uniformly distributed, resembling results obtained with [^{3}H]clonidine of lower specific activity (5). The corpus striatum, which contains negligible levels of endogenous norepinephrine (20), has substantial amounts of low affinity [^{3}H]clonidine binding, but the lowest levels in the brain of high affinity [^{3}H]clonidine binding. The cerebellum has fairly low levels of endogenous norepinephrine (19) and the major noradrenergic input to the cerebellum involves β-receptor effects at the Purkinje cells (21). The cerebellum displays very low levels of high affinity [^{3}H]clonidine sites. Thus, the regional distribution of high affinity [^{3}H]clonidine binding aligns better with regional variations in noradrenergic innervation and apparent *alpha*-receptor-mediated neuronal influences.

6-Hydroxydopamine treatment also markedly differentiates high and low affinity clonidine binding sites. The high affinity sites in almost all regions, but especially in the cerebellum and corpus striatum where control high affinity binding is very low, display much greater augmentations in binding following 6-hydroxydopamine than those apparent for any low affinity sites. The observation that, in almost all regions of the brain, neither high affinity nor low affinity [^{3}H]clonidine binding was decreased by 6-hydroxydopamine treatment suggests that neither site is associated with presynaptic autoreceptors on noradrenergic terminals. The four to five week period between treatment and assay of 6-hydroxydopamine tissue probably is sufficient time for phagocytosis and removal of presynaptic membrane components following the initial creation of a chemical lesion.

It is possible that the increases in numbers of *alpha*-noradrenergic binding sites following 6-hydroxydopamine treatment are associated with physiological supersensitivity to norepinephrine. These findings are an elaboration of initial findings in 6-hydroxydopamine-treated rats (5) and are similar to results in which norepinephrine depletion by reserpine treatment also elicited an augmentation in numbers of *alpha*-noradrenergic receptor binding sites (19). While we observed increases in *alpha*-receptor binding of the antagonist [^{3}H]WB-4101 after 6-hydroxydopamine (Skolnick *et al.*) failed to observe changes in [^{3}H]WB-4101 binding following 6-hydroxydopamine (8). These authors, however, reported an increase in *alpha*-adrenergic mediated stimulation of cyclic AMP production in cortical slices following 6-hydroxydopamine. Differences between our results and those of Skolnick *et al.* may relate to the lower dose of 6-hydroxydopamine, a shorter interval between treatment and binding assay, and the higher concentrations of [^{3}H]WB-4101 used by these authors, compared with our own investigation.

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