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BINDING OF ADRENERGIC LIGANDS ([^{3}H]CLONIDINE AND [^{3}H]WB-4101) TO MULTIPLE SITES IN HUMAN BRAIN

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Abstract—In order to identify alpha-adrenoceptors in post-mortem human brain and to detect the possible existence of multiple types of binding sites for adrenergic [^{3}H]ligands, we studied the binding of [^{3}H]clonidine and [^{3}H]WB-4101 to human brain cerebral cortex, hippocampus, hypothalamus and striatum. Frontal cortex revealed two binding sites for [^{3}H]clonidine (with K_D values of approximately 1 and 8 nM), as indicated by the biphasic Scatchard plot, the biphasic pattern of dissociation kinetics, and the biphasic inhibition by phentolamine on the binding of [^{3}H]clonidine; the high-affinity site was heat-labile. Two high-affinity binding sites for [^{3}H]WB-4101 were also detected in the human frontal cortex (with K_D values of about 0.09 and 1.5 nM), as revealed by a biphasic pattern of dissociation. A third site with low affinity binding for [^{3}H]WB-4101 was detected by the biphasic inhibition by phentolamine (as well as by WB-4101 and prazosin) on the binding of [^{3}H]WB-4101. The three other brain regions revealed very similar patterns exhibited by the frontal cortex, except that the density of the [^{3}H]clonidine sites (of either high or low affinity) was highest in the hypothalamus, whereas the density of [^{3}H]WB-4101 sites was highest in the hippocampus.

Although adrenergic receptors have been studied extensively in rat brain by means of various radioreceptor assays [1–3], there is little information on the properties of such adrenergic binding sites in post-mortem human brain. In particular, it has been found that the rat frontal cortex and cerebellum contain multiple binding sites for [^{3}H]clonidine and [^{3}H]WB-4101, based on biphasic dissociation kinetics, low Hill slopes, and biphasic Scatchard plots [4–6]. For example, the rat frontal cortex contained two binding sites for [^{3}H]clonidine (with K_D values of about 0.2 and 7.1 nM), as well as two sites for[^{3}H]WB-4101 (with K_D values around 0.01 and 1.8 nM), and it was only possible to resolve the two sites in each case by detecting the biphasic pattern of [^{3}H]ligand desorption [6]. In the case of the rat cerebellar cortex, however, multiple sites for [^{3}H]clonidine could readily be detected by Scatchard analysis and competition curves. This present study was done to determine whether such evidence for multiple binding sites could also be obtained in post-mortem human brain, and a preliminary communication on this work has been reported [7].

MATERIALS AND METHODS

[^{3}H]*Ligands*

[^{3}H]Clonidine hydrochloride (2-[2,6-dichlorophenyl-(4-^{3}H)-amino]-2-imidazoline hydrochloride) (22.2 Ci/mmole) and [^{3}H]WB-4101 (23 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA) and Amersham, (Arlington Heights, IL), respectively, and were stored in ethanol at −20°.

Tissue preparation

Post-mortem neurologically normal brains were from individuals 22- to 91-years-old. The interval between death and freezing of the brains was 3–25 hr. The causes of death were car accidents, pneumonia, myocardial infarction, bronchogenic carcinoma or exposure. Brain areas were dissected from frozen slices (non-frontal cerebral cortex was defined as all cerebral cortex other than frontal cortex), and homogenized in 50 ml of ice-cold Tris–HCl buffer (pH 7.7 at 25°) using a Brinkmann Polytron PT-10 (10 sec; setting 7). The homogenates were centrifuged four times at 40,000 *g* for 15 min at 4°. The final pellets were resuspended in buffer and frozen. Before use the samples were thawed, centrifuged an additional two times, and rehomogenized with a Brinkmann polytron at a setting of 7 for 10 sec. Prolonged freezing of the homogenate reduced the specific binding of [^{3}H]clonidine; therefore, frozen homogenates were discarded if not used within 2 weeks.

Radioreceptor assays

Both [^{3}H]clonidine and [^{3}H]WB-4101 radioreceptor assays were done in glass test tubes (12 × 75 mm) in which the following aliquots were placed (using Eppendorf Brinkmann pipettes with polypropylene tips): 0.2 ml [^{3}H]ligand (final concentrations: [^{3}H]clonidine, 2 nM; [^{3}H]WB-4101, 0.25 nM); 0.2 ml brain homogenate (always added last and containing 20 mg original wet weight of tissue); 0.2 ml Tris–HCl buffer (pH 7.5 at 25°) or 0.2 ml of drug. Triplicate samples were incubated at 25° for 30 min and then filtered under vacuum through Whatman GF/B filters (24 mm diameter) with a 10-ml wash of ice-cold buffer. The specific binding of [^{3}H]clonidine or [^{3}H]WB-4101 was defined as the amount bound in the absence of 10 μM clonidine or 100 μM WB-4101

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(or 100 μM prazosin), respectively, minus the amount bound in the presence of these drugs.

The filters were placed directly in liquid scintillation vials, 8 ml Aquasol (New England Nuclear Corp.) was added, and the samples were assayed for ^{3}H (35–40 percent efficiency) after storage at 4° for at least 6 hr to allow temperature equilibration and to permit the glass fiber filters to become uniformly translucent.

Dissociation kinetic experiments

Dissociation of specifically bound [^{3}H]clonidine was evaluated by the method of U'Prichard *et al.* [4]. The suspensions were incubated to equilibrium with [^{3}H]clonidine (2 nM). Clonidine (10 μM) or phentolamine (100 μM) was then added and the amount bound was measured at varying time intervals thereafter. The dissociation of specifically bound [^{3}H]WB-4101 was done in the same way except that 0.25 nM [^{3}H]WB-4101 was incubated to equilibrium and 100 μM WB-4101 or 100 μM prazosin was then added (see Refs. 8 and 9 for additional details).

Drugs

Norepinephrine and yohimbine were obtained from the Sigma Chemical Co. (St. Louis, MO), epinephrine from the Sterling-Winthrop Research Institute (Rensselaer, NY) LSD from Sandoz Pharmaceuticals (East Hanover, NJ), clonidine from C. H. Boehringer Sohn Ingelheim (Ingelheim, West Germany), WB-4101 from Ward Blenkinsop & Co. Ltd., (Wembley, U.K.) and prazosin from Pfizer Co. Ltd. (Montreal, Canada).

RESULTS

Properties of [^{3}H]*clonidine binding*

Competition-type experiments. Competition of clonidine with specific [^{3}H]clonidine binding (Fig. 1, top) was monophasic but with a shallow slope. The Hill coefficient of this competition curve was 0.57, possibly suggesting multiple sites for [^{3}H]clonidine. Epinephrine and norepinephrine showed monophasic competition curves, with a plateau occurring at 70 percent of total binding. This indicated that both epinephrine and norepinephrine competed for only one of the [^{3}H]clonidine sites, since their maximal displacements occurred at 30 percent of the maximal displacement of clonidine itself. Phentolamine, on the other hand, showed a biphasic competition curve, with a plateau level occurring at 65 percent of total binding. Thus, phentolamine was

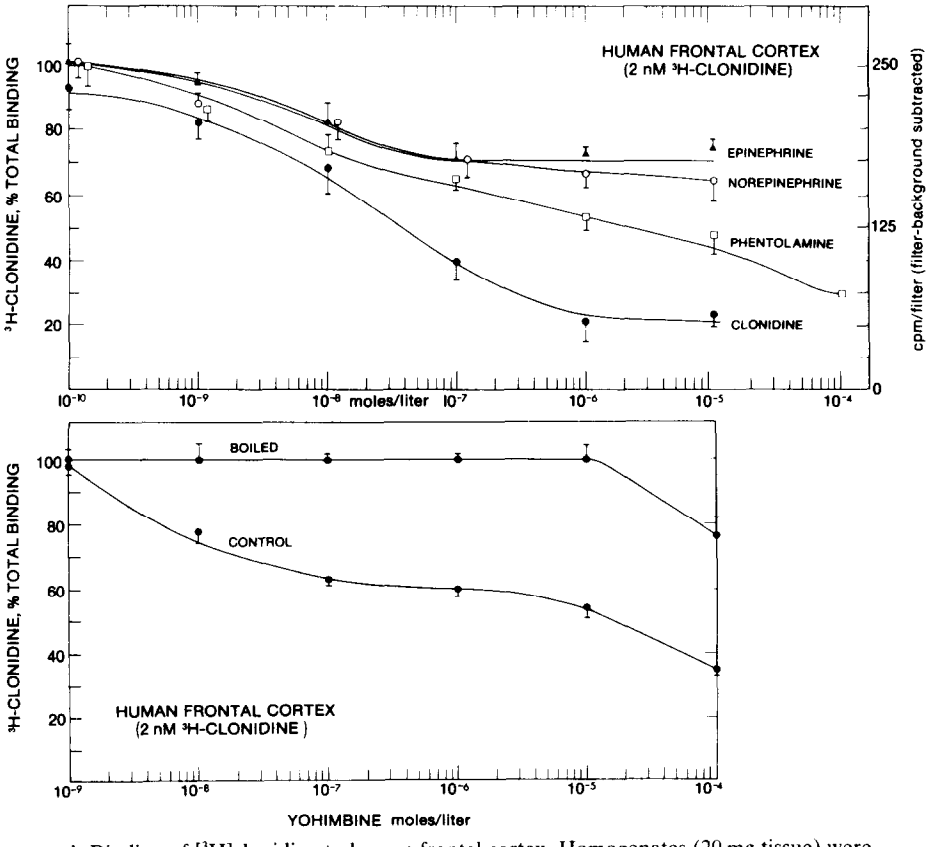

Fig. 1. Top panel: Binding of [^{3}H]clonidine to human frontal cortex. Homogenates (20 mg tissue) were incubated with 2 nM [^{3}H]clonidine for 30 min at 25° in the presence of various concentrations of unlabelled drugs. Values are expressed as percentage of total binding minus the filter background. Experiments were repeated two to four times in six separate human brains with triplicate determinations. Bottom panel: Competition of yohimbine against [^{3}H]clonidine binding (2 nM) in boiled and normal tissue. Tissue was boiled for 1 hr, allowed to cool to room temperature, and the competition experiment was begun. Total binding was approximately 250 cpm/filter for both boiled and normal tissue.

Table 1. IC_{50} Values for various drugs against [^{3}H]clonidine (2 nM) and [^{3}H]WB-4101 (0.25 nM) binding in human frontal cortex

	[^{3}H]WB-4101 IC_{50} (nM)	[^{3}H]Clonidine IC_{50} (nM)
Prazosin	1.5 7,500	> 1,000
Phentolamine	7.5 >10,000	2.8 >10,000
WB-4101	9.8 38,000	50
Phenoxybenzamine	50	
LSD*	60 >10,000	25
Epinephrine	660	6.6
Yohimbine	2,000	7.5 >10,000
Clonidine	3,600	13
Norepinephrine	4,500	5.6

* LSD = lysergic acid diethylamide.

able to resolve at least two clonidine sites.

The rank order of potencies of a series of catecholamine agonists and antagonists in inhibiting the binding of the high-affinity [^{3}H]clonidine site (Table 1) was phentolamine > norepinephrine > epinephrine > yohimbine > clonidine > LSD > WB-4101 > prazosin. These IC_{50} values were similar to those found in the rat (correlation coefficient = 0.88).

Effect of heat on binding sites for [^{3}H]*clonidine.* Boiling the tissue and then competing yohimbine for [^{3}H]clonidine binding (Fig. 1, bottom) revealed that the high-affinity component found in the normal tissue had disappeared in the boiled tissue. (It is difficult to tell whether the low-affinity component had disappeared or not.) This demonstrated that at least the high-affinity component was heat-labile.

Association kinetics of [^{3}H]*clonidine.* Association of [^{3}H]clonidine with human frontal cortex (Fig. 2, top) showed that specific binding reached plateau values by 20 min. Half-maximal level of specific binding was attained at about 1.5 min. The observed rate constant (k_{ob}) of association (Fig. 2, top, inset) was 0.22 min^{-1}.

Desorption kinetics of [^{3}H]*clonidine.* Dissociation of [^{3}H]clonidine from its binding sites after equilibration with the membranes (Fig. 2, bottom) yielded biphasic curves with offset exponential decay constants of 0.46 min^{-1} and 0.06 min^{-1}. The half-lives for the fast and slow phases of dissociation were about 1.0 min and 11 min respectively. Similar results were obtained when using 100 μM phentolamine as the dissociating agent. The equation for the second-order rate constant of association is:

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

Thus, the k_1 value, ascertained from the k_{ob} and slow phase k_{-1} values, was 0.08 nM^{-1} min^{-1}. Using this k_1 value, dissociation constants (K_D) calculated from the ratio k_{-1}/k_1 for the rapidly and slowly dissociating components of [^{3}H]clonidine specific binding were 5.7 and 0.75 nM respectively.

Once again this suggested the existence of two high-affinity [^{3}H]clonidine binding sites in the human frontal cortex. The previous analyses were also carried out in hippocampus and hypothalamus with similar results.

Saturation experiments with [^{3}H]*clonidine.* Scatchard analysis of [^{3}H]clonidine binding revealed biphasic curves in four out of six brains with the largest amount of high- and low-affinity binding in the hypothalamus, followed by frontal cortex, hippocampus and parieto-occipital cortex (Table 2).

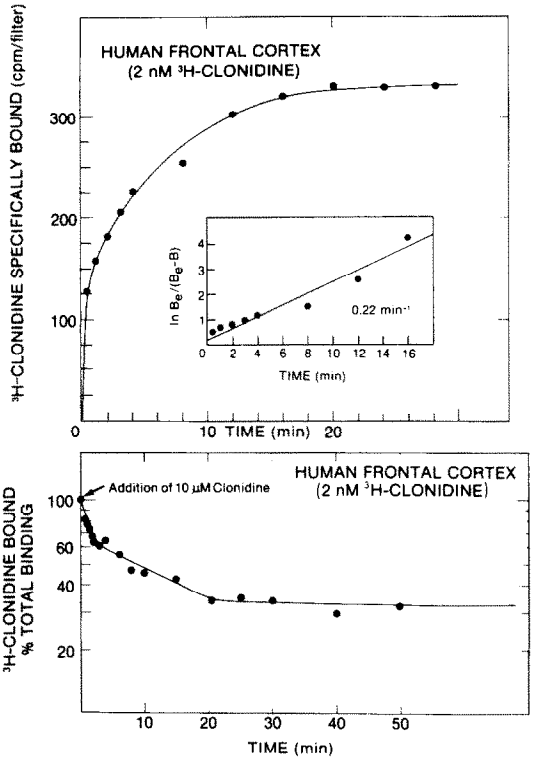

Fig. 2. Top panel: Association of specific [^{3}H]clonidine binding to human frontal cortex. [^{3}H]clonidine binding to the homogenates at 25° was measured at various intervals following addition of 2 nM [^{3}H]clonidine. Specific binding at each point was defined as the difference between binding obtained in the absence and presence of 10 μM clonidine. Points shown are from a single experiment replicated in three separate human brains. Inset: Pseudo first-order kinetic plot of initial [^{3}H]clonidine specific binding. The slope is equal to k_{ob}, the observed rate constant of the pseudo first-order reaction. Bottom panel: Dissociation of [^{3}H]clonidine. The dissociation was measured at 25° following incubation with 2 nM [^{3}H]clonidine to equilibrium (30 min). At time zero, 10 μM clonidine was added to the incubation mixtures and the reactions were terminated by filtration at various times. Points are shown as percent total binding (minus filter background). The plateau level values represent the amount of non-specific binding in the frontal cortex (25–30 percent total binding) which is similar to the amount of non-specific binding seen in Fig. 1 (20 percent total binding). Points shown are from a single experiment which was replicated in three separate human brains. The results were then computer-fitted. The observations were described well by the expression % Bound = $A_1e^{-B_1t}$ + $A_2e^{-B_2t}$ + A − A_1 − A_2, where t is time in min and A_1, A_2, B_1, and B_2 are parameters. This analysis yielded two components with dissociation rate constants of 0.46 min^{-1} and 0.06 min^{-1} for the rapidly and slowly dissociating components respectively.

Table 2. Scatchard analysis of [^{3}H]clonidine and [^{3}H]WB-4101 binding in different areas of the human brain*

Human brain region	[^{3}H]Clonidine: High-affinity site† K_D (nM)	[^{3}H]Clonidine: High-affinity site† B_{max} (fmoles/mg protein)	[^{3}H]Clonidine: Low-affinity site K_D (nM)	[^{3}H]Clonidine: Low-affinity site B_{max} (fmoles/mg protein)
Hypothalamus	1.9	51	12.3	158
Frontal cortex	0.98	32	8.4	118
Hippocampus	0.77	26	8.3	86
Cerebral cortex	0.5	10	4.6	59

Human brain region	[^{3}H]WB-4101 K_D (nM)	[^{3}H]WB-4101 B_{max} (fmoles/mg protein)
Hippocampus	3.5	992
Hypothalamus	3.0	875
Caudate nucleus	2.0	325
Cerebral cortex	1.2	267
Frontal cortex	1.0	169

* For each area N = 6 (individual human brains).
† As noted in text, there may be two high affinity sites.

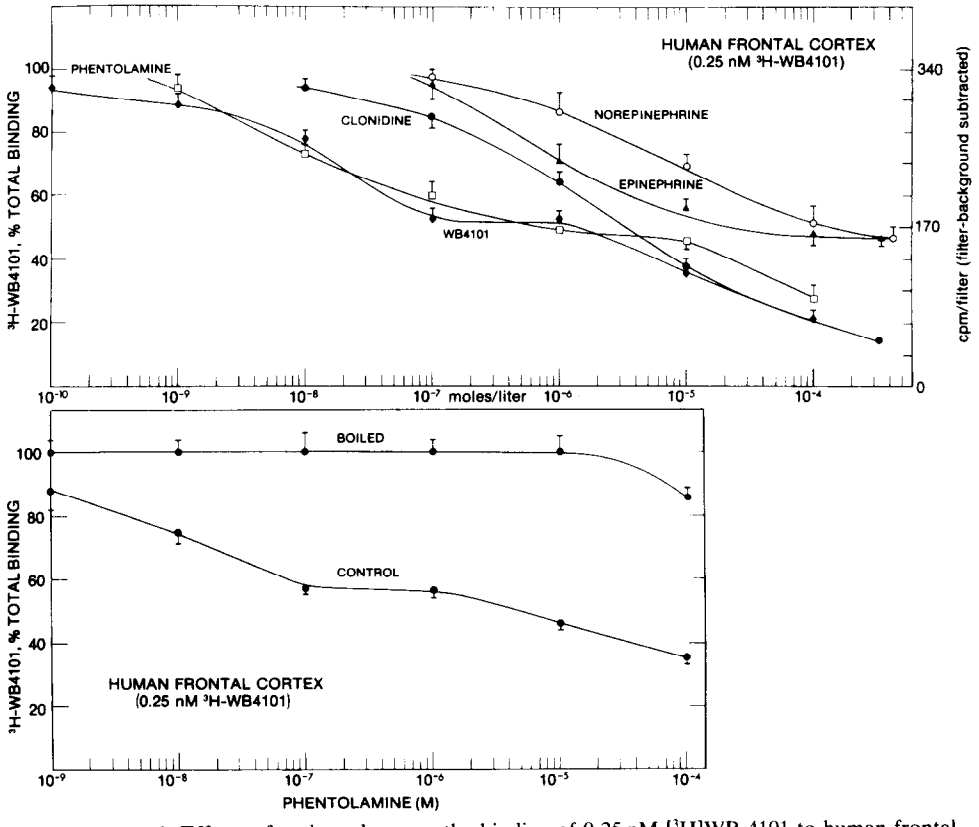

Fig. 3. Top panel: Effects of various drugs on the binding of 0.25 nM [^{3}H]WB-4101 to human frontal cortex particulate. Values are expressed as percentage of total binding minus filter background. Experiments were replicated two to four times in six separate human brains with triplicate determinations. Bottom panel: Competition of phentolamine against [^{3}H]WB-4101 binding (0.25 nM) in boiled and normal tissue. Tissue was boiled for 1 hr, allowed to cool to room temperature, and the competition experiments were begun. Total binding (minus filter background) was approximately 400 cpm/filter in boiled tissue and approximately 340 cpm/filter in normal tissue.

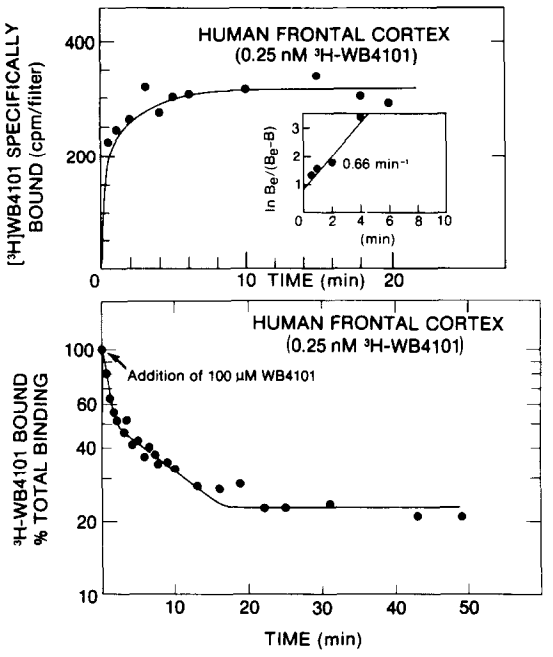

Fig. 4. Top panel: Association of specific [³H]WB-4101 binding to human frontal cortex. The binding of [³H]WB-4101 to the homogenates (20 mg tissue) at 25° was measured at various intervals following addition of 0.25 nM [³H]WB-4101. Specific binding at each point was defined as the difference between binding obtained in the absence and the presence of 100 µM WB-4101 (or 100 µM prazosin). Points shown are from a single experiment replicated in three separate human brains. Inset: Pseudo first-order kinetic plot of initial [³H]WB-4101 specific binding. The slope is equal to k_{ob}. Bottom panel: Dissociation of [³H]WB-4101. Conditions for the dissociation of [³H]WB-4101 binding were the same as in Fig. 2 (bottom), except that 0.25 nM [³H]WB-4101 was incubated with the membranes for 30 min and 100 µM WB-4101 was added at time zero. The results were then computer-fitted. The observations were described well by the expression % Bound = $(100 - A_1 - A_2)e^{-B_1 t} + A_1 e^{-B_1 t} + A_2$. According to this model there existed two components, with dissociation rate constants of 2.9 min^{-1} and 0.175 min^{-1} for the fast and slow dissociating components respectively.

The approximate K_D values of the high- and low-affinity components, as estimated by Scatchard analysis, were 1.0 and 8.4 nM respectively (Table 2). These K_D values were closely similar to the estimated high- and low-affinity components of binding observed in dissociation kinetic experiments.

Properties of [³H]*WB*-4101 *binding*

Competition-type experiments. Competition curves of various drugs against specific [³H]WB-4101 binding (Fig. 3, top) were biphasic for phentolamine, WB-4101 and prazosin, suggesting two binding sites. Epinephrine and norepinephrine competition curves were monophasic, with maximal displacement occurring at about 45 percent of total binding. This indicated that epinephrine and norepinephrine only competed at the high-affinity WB-4101 site. Clonidine, on the other hand, showed a monophasic but shallow competition curve with a Hill coefficient of 0.58, indicating closer affinities for these two sites.

The relative potencies of various drugs in competing for the high-affinity [³H]WB-4101 binding sites were prazosin > phentolamine > WB-4101 > phenoxybenzamine > LSD > epinephrine > yohimbine > clonidine > norepinephrine. These IC_{50} values were similar to those found in the rat (correlation coefficient = 0.84). Boiling the tissue and then competing phentolamine for [³H]WB-4101 binding (Fig. 3, bottom) revealed that both components found in the normal tissue had disappeared in the boiled tissue. This demonstrated that both components were heat-labile.

Association kinetics of [³H]*WB*-4101. Association of [³H]WB-4101 to human frontal cortex (Fig. 4, top) showed that, by about 8 min, specific binding reached equilibrium. Half-maximal levels of specific binding were attained at about 30 sec. The observed rate constant (k_{ob}) of association (Fig. 4, top, inset) was 0.66 min^{-1}.

Desorption kinetics of [³H]*WB*-4101. Dissociation of [³H]WB-4101 from its binding sites after equilibration with the membranes (Fig. 4, bottom) yielded biphasic curves with offset exponential decay constants of 2.9 min^{-1} and 0.175 min^{-1}. The half-lives for the fast and slow phases of dissociation were about 40 sec and 5 min respectively. Similar results were obtained when using 100 µM prazosin as the dissociating agent. Thus, the k_1 value for [³H]WB-4101 binding was 1.9 $nM^{-1}\,min^{-1}$. Using this k_1 value, the dissociation constants (K_D) of the rapidly and slowly dissociating components of [³H]WB-4101 specific binding were 1.5 and 0.09 nM respectively.

The previous analyses were carried out in hippocampus and hypothalamus with similar results.

Saturation of binding sites for [³H]*WB*-4101. Scatchard analysis of the high-affinity [³H]WB-4101 binding component (as seen by competition curves) revealed monophasic curves in five out of six brains with the largest amount of binding in the hippocampus and hypothalamus, followed by caudate nucleus, cerebral cortex, and frontal cortex (Table 2). Scatchard analysis corroborated the existence of only one of the high-affinity [³H]WB-4101 sites (with an approximate K_D value of 2.0 nM). One explanation could be that technically one cannot detect a very high affinity site using Scatchard analysis since the actual cpm per filter are very small. Furthermore, a binding site of extremely low affinity for the

Table 3. Comparison of the K_D values of [³H]clonidine and [³H]WB-4101 as determined by dissociation kinetic experiments in rat and human frontal cortex

		Frontal cortex	
	Affinity type	Rat K_D (nM)	Human K_D (nM)
[³H]Clonidine	High	0.2	0.75
	Low	7.1	5.7
[³H]WB-4101	High	0.01	0.09
	Low	1.8	1.5

[^{3}H]ligand would also be very difficult to detect using this technique, since at higher [^{3}H]ligand concentrations the signal/noise ratio becomes small so that the actual specific binding is overshadowed by the non-specific binding.

DISCUSSION

The present study has demonstrated the existence of multiple sites for [^{3}H]clonidine and [^{3}H]WB-4101 binding to the human frontal cortex. Comparison of the characteristics of [^{3}H]clonidine and [^{3}H]WB-4101 binding in human and rat showed that in both species there were multiple binding sites for each ligand. Table 3 compares the K_D value for the [^{3}H]clonidine and [^{3}H]WB-4101 binding sites (as estimated from dissociation kinetic experiments) in rat and human. It seems that in both species there were two high-affinity [^{3}H]clonidine and [^{3}H]WB-4101 binding sites with similar K_D values from one species to the other. [^{3}H]WB-4101 binding also displayed a third very low affinity site in human frontal cortex, detected only by competition curves.

Comparison of the pharmacological profiles of these multiple binding sites in rat and human revealed an interesting difference. In rat all drugs competed at the two high-affinity [^{3}H]clonidine binding sites with similar affinity for the two sites [4, 6]. This was seen by parallel, monophasic competition curves of these drugs against 2 nM [^{3}H]clonidine binding with similar maximal displacements. In the human, on the other hand, epinephrine and norepinephrine only competed against one of these [^{3}H]clonidine binding sites, while phentolamine and yohimbine were able to distinguish between the two sites (as seen by their biphasic competition curves). Recently, U'Prichard [10] suggested that there existed at least two states of the alpha-2-receptor, one with a high and low affinity for alpha agonists, and another with equal affinity for alpha antagonists; GTP shifted the affinity of the agonists from high to low. Thus, the biphasic dissociation of [^{3}H]clonidine could indicate the existence of these two states in the rat frontal cortex. However, in the human frontal cortex the biphasic dissociation of [^{3}H]clonidine does not seem to correspond to two states of the same receptor, since norepinephrine and epinephrine only displace one of these sites, while phentolamine and yohimbine, alpha-2 antagonists, displace both sites with very different affinities for each site. These data suggest two different clonidine binding sites of which the high-affinity clonidine site corresponds to an alpha-2 receptor.

[^{3}H]WB-4101 as well showed multiple binding sites in human frontal cortex. Once again, the neurotransmitters, epinephrine and norepinephrine, bound only to the high-affinity WB-4101 binding sites. These sites displayed an alpha-1 pharmacological profile. This finding agrees with previous studies in the rat brain [6, 11] in which a high- and a low-affinity site were demonstrated for [^{3}H]WB-4101 (Table 3), both of which were displaceable by both alpha agonists and antagonists. It was suggested by Davis *et al.* [12] that WB-4101 may bind to membrane receptor sites, mediating an alpha-adrenergic accumulation of cyclic AMP in rat cerebral cortex. This explanation may also apply to the human high-affinity alpha-1 sites. There is, as well, a third very low-affinity [^{3}H]WB-4101 binding site. This site does not bind norepinephrine or epinephrine (as seen in their competition curves). The [^{3}H]WB-4101 low-affinity site is different from the low-affinity [^{3}H]clonidine site in that the two sites display different pharmacological profiles. For example, LSD and WB-4101 did not display a low affinity for a [^{3}H]clonidine site, while they did display a low affinity for a [^{3}H]WB-4101 site (Table 1). Thus, the third site found in the human is different from any of the [^{3}H]clonidine sites or those sites found in the rat.

In the future, the regulation and resolution of each binding site of both clonidine and WB-4101 in the human brain will have to be studied.

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