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CHARACTERISTICS OF [^{3}H](+)-AMPHETAMINE BINDING SITES IN THE RAT CENTRAL NERVOUS SYSTEM

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Summary

Recent studies in our laboratory have demonstrated the presence of specific binding sites for [^{3}H](+)-amphetamine in crude membrane preparations derived from rat brain. In this report we have further characterized the specific binding of [^{3}H](+)-amphetamine in various subcellular fractions of rat brain and demonstrate a greater than five-fold enrichment in the crude synaptosomal (P_2) fraction compared to a crude membrane preparation. Specific [^{3}H](+)-amphetamine binding in crude synaptosomal membranes is saturable and stereospecific with an apparent dissociation constant, K_d, of 2.8 ± 0.5 μM and an estimated maximum number of binding sites, B_{max}, of 60.4 ± 8.4 pmoles/mg protein derived by Scatchard or Klotz analysis of binding data using filtration assays. Centrifugation assays yield a similar K_d though the apparent B_{max} is higher. In addition specific [^{3}H](+)-amphetamine binding is: rapidly reversible, temperature sensitive, labile to preincubation in Tris buffer, inhibited by sodium ions and unevenly distributed in various brain regions. Specific [^{3}H](+)-amphetamine binding sites are found almost exclusively in the rat central nervous system (the brainstem, hypothalamus, and striatum exhibiting relatively high levels of binding), whereas peripheral tissues such as liver, kidney and heart have very low to undetectable levels of specific binding.

Amphetamine and related phenylethylamine derivatives possess pronounced pharmacologic and behavioral actions which include central stimulant, anorexic, vasoconstrictor, and hyperthermic effects (1,2). Biochemical studies have demonstrated potent effects of amphetamine on the neuronal release, reuptake, and metabolism of biogenic amines (1). However, it is still unclear which of these biochemical effects are responsible for the multiple pharmacologic actions of this class of drugs. Binding studies using radiolabeled psychotropic drugs have proven valuable in delineating the membrane sites mediating the actions of many of these agents. The availability of [^{3}H](+)-amphetamine has made similar binding studies using this ligand feasible. In a recent report from our laboratory, saturable and stereospecific binding site(s) for specific [^{3}H](+)-amphetamine were demonstrated in the rat hypothalamus and the relative affinities of a series of phenylethylamine-derivatives for this site were highly correlated with their potencies as anorexic agents in animals (3). In our

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initial studies describing [^{3}H](+)-amphetamine binding to rat hypothalamic membranes we observed non-linear saturation isotherms and Scatchard plots suggesting the presence of multiple binding sites or negative cooperativity. In subsequent studies, significant interassay differences in maximum binding capacity (B_{max}) were also observed. These differences were found to be dependent upon the time and storage conditions following preparation of the membranes. We now report a more detailed characterization of [^{3}H](+)-amphetamine binding in rat brain including the effects of preincubation time and temperature, subcellular and regional distribution, effects of ions, and a computer-generated analysis of the binding kinetics.

Materials and Methods

Tissue Preparation: Male Osborne-Mendel rats (120-150 g) were housed under diurnal lighting conditions (12:12) with free access to food and water. Rats were killed by decapitation. Their brains were removed quickly, dissected on ice, and immediately placed in ice-cold 0.32 M sucrose. Dissection of brain areas was performed as described by Glowinski and Iverson (4). Brain tissue was pooled and disrupted in 40 volumes (weight to volume) of ice cold 0.32 M sucrose with a teflon glass homogenizer. The homogenate was centrifuged at 1000 g for 10 minutes and the resulting supernatant was centrifuged at 27,000 g for 20 minutes. This pellet was then gently resuspended in ice-cold 0.32 M sucrose by vortexing and centrifuged at 27,000 g for an additional 20 minutes. The final pellet was resuspended in 40 volumes (25 mg/ml) of ice-cold 50 mM Tris-HCl (pH 7.4) and used immediately unless otherwise indicated. In studies on the subcellular distribution of [^{3}H](+)-amphetamine binding the sucrose gradient fractionation technique of Whittaker et al. (5) was used.

Binding Assay: The binding of [^{3}H](+)-amphetamine sulfate (Spec. Act. 15-19 Ci/mmole: New England Nuclear, Boston, MA.) was carried out on ice (0-4° C) for 30 minutes unless otherwise specified. A standard incubation contained 50 µl of buffer or unlabeled (+)-amphetamine sulfate (100 µM), 50 µl of [^{3}H](+)-amphetamine (10 nM to 20 µM) and 200 µl of membrane preparation in 12 x 75 mm plastic tubes. When the concentration of protein from brainstem or hypothalamic membranes was varied from 5 to 250 µg, specific binding increased in a linear fashion ($r = 0.99$). For routine binding assays 100 to 200 µg of membrane protein was used. All incubations were initiated by the addition of the membrane preparation. In preliminary experiments we observed that other buffers (e.g. 20 mM potassium phosphate, 50 mM Hepes, or 20 mM sodium phosphate pH 7.4) reduced specific binding in some cases by as much as 80%. The addition of reducing agents such as 0.5% ascorbate or 5mM dithiothreitol also reduced binding by 50% and 65%, respectively. Thus all assays were carried out in 50 mM Tris HCl (pH 7.4). Incubations were quickly filtered through glass fiber filters (Whatman GF/B) and the retained radioactivity was measured in a liquid scintillation counter (Beckman LS 9000). The binding assay was modified for experiments where bound [^{3}H](+)-amphetamine was separated from free ligand using centrifugation. In these experiments a typical incubation contained 150 to 200 µl of buffer, 50 µl of buffer or unlabelled (+)-amphetamine sulfate (100 µM), 50 µl of [^{3}H](+)-amphetamine sulfate, and 250 µl of the membrane preparation in 12 X 75 mm polypropylene tubes. After incubation at 0-4°C for 30 minutes, the samples were centrifuged at 27,000 g for 15 minutes. The resulting supernatants were decanted and the pellets gently rinsed twice with 5 ml of ice-cold buffer. Afterward 1 ml of NCS tissue solubilizer (Amersham, Arlington Hts., IL) was added to the pellets. The tubes were vortexed until all tissue was solubilized. Following solubilization 1 ml scintillation fluid (Ready-Solv™, Beckman Instruments, Fullerton, CA) was added and the tubes

decanted into counting vials containing an additional 10 ml of scintillation fluid. All tubes were rinsed with a final 1 ml of scintillation fluid prior to counting the radioactivity.

Specific binding was defined as the difference between the binding observed in the presence and absence of nonradioactive (+)-amphetamine sulfate (100 μM). The apparent K_d and B_{max} were determined using both linear regression analysis as well as a nonlinear least squares curve-fitting computer program, "Ligand", developed by Munson and Robard (6). Additionally, the binding data was transformed by the method of Klotz (7) and the apparent K_d and B_{max} estimated using a computer program for simultaneous curve fitting which employs a four parameter logistic equation ("All fit") (8). IC_{50} values were converted to K_i values using the common derivation of the Cheng-Prusoff equation (9).

Results

In our two earlier reports on [^{3}H](+)-amphetamine binding (3,10), a crude membrane fraction was used in all binding assays. Preliminary experiments on the subcellular distribution of specific [^{3}H](+)-amphetamine binding in brain revealed a significant enrichment in the synaptosomal fraction and consequently we examined the binding to crude synaptosomal membranes (P_2) for use in routine binding studies. Using the crude synaptosomal/mitochondrial (P_2) fraction specific [^{3}H](+)-amphetamine binding increased by two-fold compared to the crude membrane preparation (Table 1). Additionally, the percent specific binding increased from 47% to 70%. When the P_2 fraction was subjected to an additional isotonic wash in 40 vol. of 0.32 M sucrose, [^{3}H](+)-amphetamine binding was increased three-fold and the percent specific binding increased to 80% (Table 1). Consequently, a crude synaptosomal (P_2) fraction which had been washed once in 0.32 M sucrose was employed in all binding studies. The term "membrane preparation" used in this report refers to the crude synaptosomal/mitochondrial (P_2) fraction.

Since reanalysis of the binding kinetics in our original report (3) suggested that the pharmacologically-relevant binding of [^{3}H](+)-amphetamine to the brain membranes had an apparent K_d of approximately 1 μM (11), the kinetics of specific binding, as well as, the effects of multiple filter washings on specific binding were examined. Fig. 1 demonstrates the effects of "washing" the hypothalamic membranes following filtration of the incubation mixture over Whatman GF/B glass fiber filters. Specific [^{3}H](+)-amphetamine binding in the absence of any washing was only 30% of the total binding, but was approximately 4- to 5-fold greater than that observed after multiple washings. Both the absolute amount and percent specific [^{3}H](+)-amphetamine binding plateaued after one or more washings indicating that a rapidly dissociable binding component of [^{3}H](+)-amphetamine binding is lost during the routine filtration assay. Using a centrifugation assay, we were able to measure more completely the total amount of [^{3}H](+)-amphetamine binding. Nevertheless, the lack of any further loss of specific binding during multiple washes suggested that the filtration assay could be used to measure the less rapidly dissociable component of the total specific binding. Furthermore, the percent specific binding increased from 30% to almost 80% following two or more washes and the variability between replicate assays was far less with the filtration than centrifugation assays. Consequently, in the present experiments all binding assays were terminated by rapid vacuum filtration followed by two washes (5 ml) with ice-cold buffer (3 sec).

Experiments on the kinetics of specific [^{3}H](+)-amphetamine binding to crude synaptosomal membranes revealed both rapid association and dissociation rates at 0-4°C (Fig. 2). Specific binding increased rapidly and reached a

TABLE I

Membrane Preparation	Specific Binding (% total)	Specific Bound (fmoles/mg)
P_1	47 ± 4	277 ± 31
P_2 + Tris-HCl wash	71 ± 2	572 ± 18
P_2 + sucrose wash	79 ± 2	1,483 ± 36

[^{3}H](+)-Amphetamine binding in various hypothalamic membrane preparations. For the P_1 preparation, hypothalamic tissue was homogenized in 40 volumes of ice-cold 50 mM Tris-HCl (pH 7.4) with a Brinkman Polytron (setting 7, 30 sec) and the resulting homogenate was centrifuged at 27,000 g for 20 minutes. This pellet was resuspended in a similar volume of Tris-HCl buffer and recentrifuged at 27,000 g for 20 minutes twice more before being assayed as described in the text. P_2 + Tris-HCl wash indicates that the isotonic wash in 0.32 M sucrose was replaced by resuspending the P_2 pellet in 40 volumes of 50 mM Tris-HCl (pH 7.4) and centrifuging this resuspension at 27,000 g for 20 minutes. Mean ± SEM for 8 separate binding determinations at 100 nM of ligand. Values are from a typical experiment repeated 3 times.

maximum at approximately 1 min. Half-maximal binding occurred at approximately 20 sec. However, these experiments revealed a complex association rate. The initial maximal binding declined by 34% over the next 6 minutes until a new equilibrium was reached which remained stable for at least 40 min (Fig. 2). An association rate constant could not be calculated due to the complex association

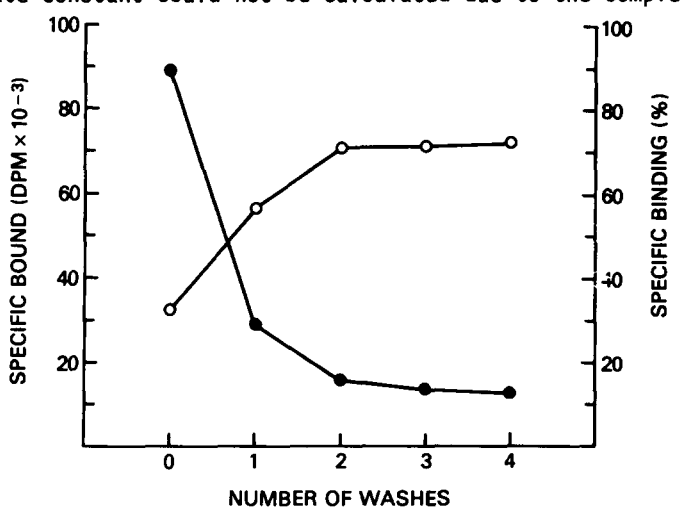

FIG. 1

Effect of number of washes on [^{3}H](+)-amphetamine binding in crude hypothalamic synaptosomes expressed as total specifically bound dpm's (●-●) and percent specific binding (O-O). Each data point represents 3 separate determinations using 100 nM of [^{3}H](+)-amphetamine ligand. Values are from a typical experiment repeated 5 times.

kinetics. The reversibility of specific [^{3}H](+)-amphetamine binding was readily demonstrated in dissociation experiments (Fig. 2). When plotted on a semilogarithmic scale, the dissociation of specifically bound [^{3}H](+)-amphetamine was monophasic with a half-life ($t_{1/2}$) of 43 sec. The mean dissociation rate constant, k_{-1}, calculated from 8 separate experiments was 1.73 ± 0.24 min^{-1}.

In our earlier reports (3,10) [^{3}H](+)-amphetamine binding was carried out in the presence of NaCl, since previous work on the binding of various antidepressants and specific uptake blockers revealed maximal binding in the presence of sodium. The effect of NaCl on [^{3}H](+)-amphetamine binding to brainstem membranes is depicted in Fig. 3. Inhibition of specific binding occurred at the lowest concentration of NaCl examined (5 mM). As the concentration of NaCl was increased over a physiologic range from 5 to 200 mM, the inhibition of binding increased to 59.0 ± 2.5% (n = 3). Scatchard analysis revealed that the apparent B_{max} was reduced in the presence of sodium while the apparent K_d was not changed (12). Since specific [^{3}H](+)-amphetamine binding was reduced by sodium, subsequent characterization was done in the absence of sodium.

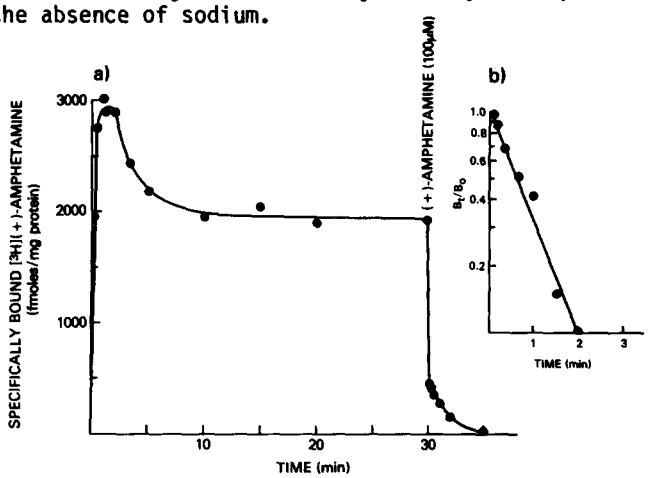

FIG. 2

Association and dissociation of [^{3}H](+)-amphetamine (100 nM) to hypothalamic synaptosomes (P_2). The complex association pattern (a) was demonstrated in 5 separate experiments. The rate of dissociation of bound [^{3}H](+)-amphetamine (b) was determined by adding 100 μM of unlabeled (+)-amphetamine to incubation mixtures and then terminating the reaction by rapid vacuum filtration at various times ranging from 5 sec to 5 min. Points shown represent the means of 4 separate determinations. The dissociation rate constantin this experiment, k_{-1}, determined by linear regression analysis was 1.20 min^{-1}.

The preincubation time following tissue preparation in 50 mM Tris buffer and subsequent assay at 4° C (Fig. 4) was another important variable in the measurement of [^{3}H](+)-amphetamine binding. In a representative experiment specific binding of [^{3}H](+)-amphetamine to the brainstem P_2 fraction decreased by approximately 40% during a two hour preincubation at 0-4°C. There was a gradual diminution in the relative amount of binding over the next 3 hrs. The percent specific binding also decreased to below 70%. Twenty-four hours after tissue preparation specific binding of [^{3}H](+)-amphetamine was 9% of the initial one hour point (data not shown). Thus, all binding assays were performed within two hours after sacrifice.

Specific [^{3}H](+)-amphetamine binding in Tris-HCl buffer was also sensitive to preincubation temperature (Fig. 5). Specific binding was decreased by approximately 50% when the membrane preparation was preincubated at 25°C for 30 min and subsequently assayed at 0-4°C. A dramatic reduction in binding (95%) was also observed during a 15 min preincubation at 37°C.

Fig. 6 represents the binding of [^{3}H](+)-amphetamine to rat hypothalamic membranes (P_2) as a function of increasing radioligand concentration. Specific binding saturated at a ligand concentration of approximately 10 µM whereas nonspecific binding increased linearly with [^{3}H](+)-amphetamine concentrations up to 22 µM, the highest concentration studied (data not shown). At 100 and 200 nM of [^{3}H](+)-amphetamine, concentrations routinely used in the standard binding assay, the specific binding represented greater than 70% of the total binding. Scatchard analysis of the saturation isotherm (Fig. 6) using the curve fitting computer program "Ligand", revealed a linear plot indicative of a single population of binding sites. The binding data could not be fitted to a two-site model though our previous study in crude hypothalamic membranes (assayed in the presence of NaCl) revealed a biphasic Scatchard plot (3). The apparent K_d for [^{3}H](+)-amphetamine was 2.8 ± 0.5µM and the estimated B_{max} was 60.4 ± 8.4 pmoles/mg protein. In four separate experiments the mean K_d calculated using the "Ligand" program was 3.2 ± 0.2 µM and the mean B_{max} was 66.7 ± 5.4 pmoles/mg protein. The saturation binding data was also analyzed by the method of Klotz (7). Specific binding plateaued above 10 µM of [^{3}H](+)-amphetamine and this method yielded an apparent B_{max} of 69.5 ± 1.0 pmoles/mg protein (Fig. 6). The apparent K_d estimated by this method was 2.10 ± 0.08 µM, in good agreement with that derived from Scatchard analysis.

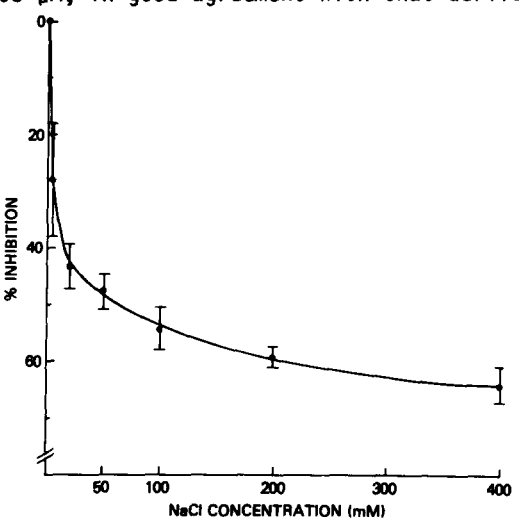

FIG. 3

Effect of NaCl on [^{3}H](+)-amphetamine binding (100-200 nM) in hypothalamic P_2. Mean ± SEM for 3 separate experiments.

Specific binding of [^{3}H](+)-amphetamine to hypothalamic P_2 membranes was also measured using a centrifugation assay. Using this method of separating bound drug from free ligand, binding again was found to be saturable (data not shown). Scatchard analysis of the binding data revealed an apparent K_d of 2.7 ± 0.8 µM, similar to the apparent K_d derived from the filtration binding assay. However, the estimated B_{max} using the centrifugation method (180 ± 44 pmoles/mg) was three-fold greater. In three separate experiments the mean K_d was 3.2 ± 0.5 µM and the B_{max} was 144 ± 15 pmoles/mg protein.

Subcellular distribution of [^{3}H](+)-amphetamine binding sites is depicated in Fig. 7. The highest density of binding sites was found in the synaptosomal fraction. The amount of specific binding present in the mitochondrial, nuclear, and microsomal fractions was only 20% of that observed in the synaptosomal fraction. Thus, the majority of specific [^{3}H](+)-amphetamine binding appears to be localized to synaptosomal membranes.

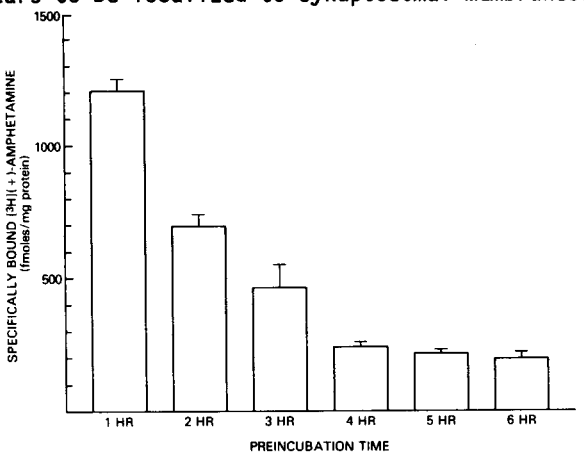

FIG. 4

Effect of preincubation time at 4°C on specific [^{3}H](+)-amphetamine binding in brainstem P_2. Mean ± SEM for 3 separate determinations using 300 nM of [^{3}H](+)-amphetamine.

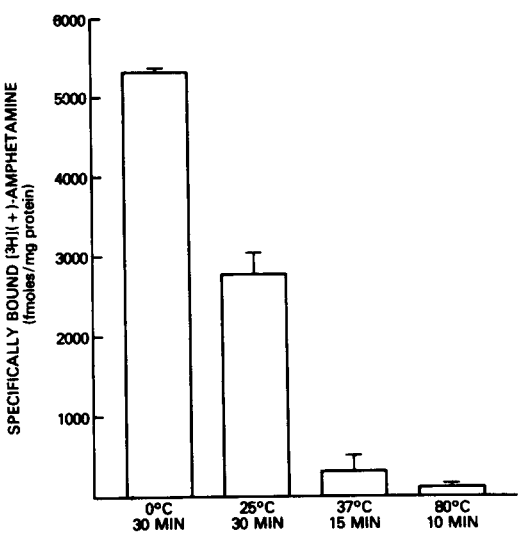

FIG. 5

Effect of preincubation temperature on [^{3}H](+)-amphetamine binding (300 nM). After brainstem membrane preparations (P_2) were preincubated at various temperatures for different periods of time, the amount of specific [^{3}H](+)-amphetamine binding was measured as described in the text. Values represent the Mean ± SEM for 3 separate determinations.

The regional distribution of [^{3}H](+)-amphetamine binding sites in the brain was heterogeneous (Fig. 8). The brainstem had the highest density of specific binding sites followed by the hypothalamus and striatum. The brain area with the least number of binding sites was the cerebellum, and the density in this area was similar to the density in the pituitary. Specific [^{3}H](+)-amphetamine binding appeared to be highly localized to the central nervous system since peripheral tissues such as liver, kidney, and heart had very low to undetectable levels of specific binding.

In a previous report we observed the stereospecific displacement of [^{3}H](+)-amphetamine binding in the crude membrane preparations employed (3). Using the crude synaptosomal preparation we again observed stereospecificity in the displacement of [^{3}H](+)-amphetamine binding by (+)- and (-)-amphetamine. The (+)-enantiomer of amphetamine (K_i = 1.9 μM) was at least three-fold more potent in displacing specific binding than the pharmacologically less active (-)-amphetamine (K_i = 5.9 μM). Additionally, the K_i for (+)-amphetamine corresponded well with the apparent K_d derived from Scatchard analysis.

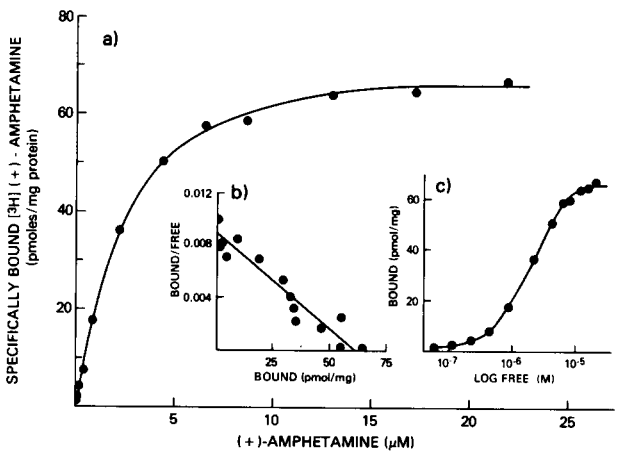

FIG. 6

Saturation isotherm (a), Scatchard plot (b), and analysis by the method of Klotz (7)(c) of [^{3}H](+)-amphetamine binding to crude hypothalamic synaptosomes (P_2). Scatchard analysis using the "Ligand" program (b) revealed a statistically good fit for only 1 site (sum of squares 1481, d.f. 21, mean squre 71, and a nonsignificant runs test, $p > 0.05$) Binding data transformed by the method of Klotz (c) was curve fitted using "All fit". Each point represents the mean of 3 separate determinations.

Discussion

In this report we have further characterized the saturable and stereospecific binding of [^{3}H](+)-amphetamine to crude synaptosomal (P_2) membrane preparations of rat brain. [^{3}H](+)-Amphetamine binding is highly localized to synaptosomal membranes, temperature sensitive, labile to preincubation in Tris buffer, unevenly distributed in various brain regions, and inhibited by sodium. In a previous report we described similar binding sites for [^{3}H](+)-amphetamine in crude membrane fractions of rat brain and here

demonstrate a greater than five-fold enrichment in the crude synaptosomal fraction. Since the P_2 fraction is enriched in synaptosomes, this finding is consistent with the subcellular distribution of specific [^{3}H]-(+)amphetamine binding measured using sucrose gradient centrifugation of the crude synaptosomal fraction reported here.

Reanalysis of the binding kinetics in our original report (3) using the nonlinear least squares curve fitting program ("Ligand") developed by Munson and Rodbard (6) gave a biphasic Scatchard plot with an apparent K_d for the low affinity site of 1.31 μM (11). In the present series of experiments we observed only a single class of binding sites in crude synaptosomal membranes with an apparent K_d similar to that of the "low affinity" site previously observed in the crude membrane preparation. Furthermore, in this report the estimation of the apparent K_d and B_{max} values by either the method of Scatchard or Klotz yielded very similar results. Although our present data suggest only a single class of noninteracting binding sites for [^{3}H](+)-amphetamine in crude synaptosomal membranes (Fig. 6 and ref. 12), we cannot entirely rule out the presence of multiple binding sites and (or) the presence of negative cooperativity in the crude membrane preparations originally employed. It is clear however that in the crude synaptosomal fraction of rat brain [^{3}H](+)-amphetamine binds with a dissociation constant (K_d) in the low micromolar range (1-3 μM). The number or density of binding sites is markedly dependent on a number of variables but is far greater than that previously observed in crude membrane preparations measured in the presence of sodium.

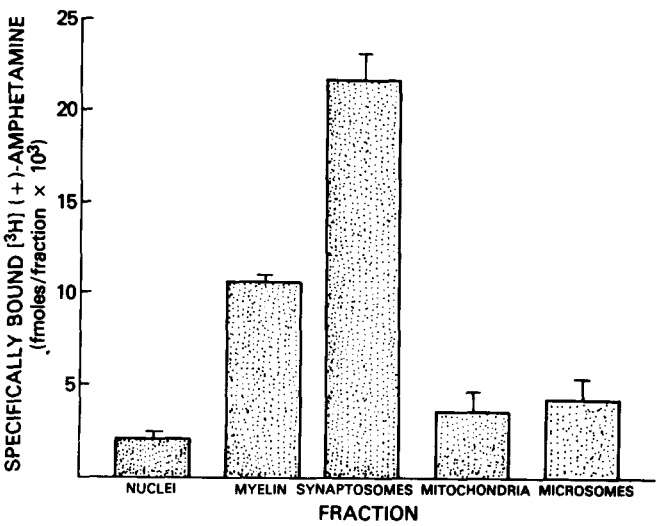

FIG. 7

Subcellular distribution of [^{3}H](+)-amphetamine binding in hypothalamic fractions. Subcellular fractions were prepared according to the method of Whittaker et al. (5) from 250 mg of rat hypothalamic tissue. Individual fractions were resuspended in a known volume of 50 mM Tris-HCl buffer (pH 7.4) and assayed as described in the text. Data is expressed as fmoles of specific [^{3}H](+)-amphetamine binding per total individual fraction which was calculated by multiplying the specific binding per incubation volume by the volume of the individual fraction. When the data was expressed as fmoles per mg of protein, similar results were obtained. Mean ± SEM for 3 separate determinations.

Because of the relatively low affinity and high capacity of the [^{3}H](+)-amphetamine binding site, the rapid filtration method was compared to the centrifugation method for separating bound from free ligand. The apparent K_d using the centrifugation method, was similar to the apparent K_d for the rapid filtration method. However, the estimated B_{max} was greater using

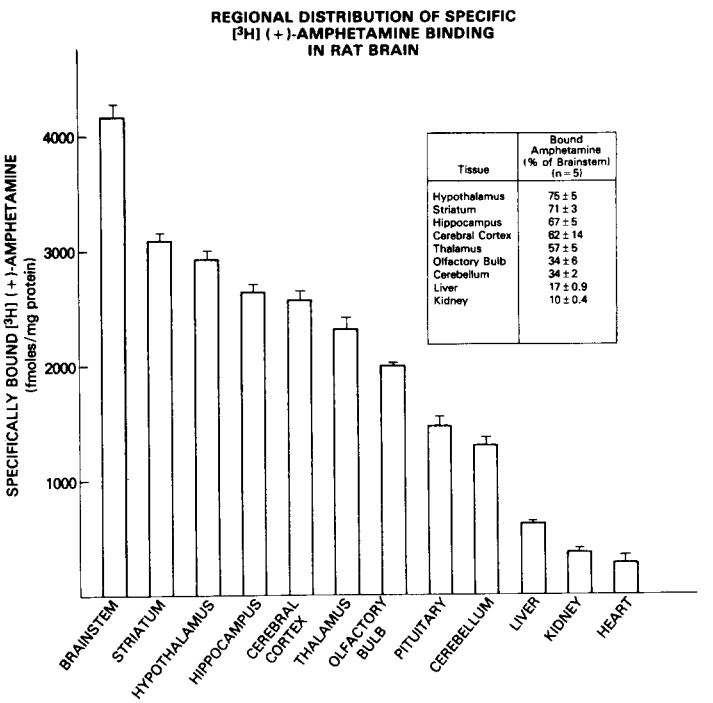

FIG. 8

Regional distribution of [^{3}H](+)-amphetamine binding. Mean ± SEM for 3 separate determinations at 100 nM of ligand from a representative experiment. Inset: Regional distribution (Mean ± SEM) normalized to amount of specific binding in brainstem crude synaptosomal membranes from 5 separate experiments.

the centrifugation method, although, the percent specific binding was considerably higher for the filtration method. Nevertheless, with a $t_{1/2}$ of 40-50 seconds for the dissociation of [^{3}H](+)-amphetamine at 4°C, the 3 second washing period in our filtration assay was sufficiently rapid to enable us to reliably measure specific binding. Additionally, multiple washes of the glass fiber filters following filtration of the assay did not result in any additional loss of saturable binding during the assay (Fig. 2). The discrepancy between the filtration and centrifugation methods may be due to the presence of a rapidly dissociable site, suggested by the wash experiment (Fig. 2) which would not be measured in the filtration assay. Thus, for routine characterization and structure activity studies we have found the filtration assay by virtue of its greater efficiency and ratio of specific to nonspecific binding (> 75%) to be preferable.

The exact identity and pharmacological significance of the [^{3}H](+)-amphetamine binding site is still not known. Since [^{3}H](+)-amphetamine binding is optimal in sodium-free media, this site is probably not associated with a neurotransmitter uptake site like the previously reported high affinity [^{3}H]-imipramine or [^{3}H]-desipramine binding sites (13). Furthermore, no loss of specific [^{3}H](+)-amphetamine binding is seen after 6-hydroxydopamine or 5,7 dihydroxytryptamine lesions (in preparation) indicating that these sites are not structurally associated with adrenergic or serotonergic terminals. The very high density of binding sites in synaptosomal membranes suggest that these sites may be associated with an ion channel or some large membrane constituent (e.g. enzyme) rather than a classical neurotransmitter receptor site.

Other central nervous system drug and neurotransmitter recognition sites exhibit apparent binding affinities in the micromolar range. For example, relatively low affinity binding sites have been described for GABA, phencyclidine, cocaine, and the benzodiazepines (14-16). Since the brain levels of amphetamine following pharmacologically active doses are in the range of 2 to 50 μM (17) and thus correspond closely to the apparent K_d for the [^{3}H](+)-amphetamine binding sites in rat brain, such a "low affinity" site may be pharmacologically relevant.

Whether the [^{3}H](+)-amphetamine binding sites reported here mediate the central action(s) of amphetamine is also not certain. However, the regional distribution of [^{3}H](+)-amphetamine binding in rat brain indicates that the density of binding sites is highest in the brainstem, hypothalamus, and striatum. We have previously shown that the relative affinities of a series of phenylethylamine derivatives for [^{3}H](+)-amphetamine binding sites in crude hypothalamic membranes is highly correlated to their potencies as anorexic agents (3). This finding suggests that the [^{3}H](+)-amphetamine site in hypothalamus may mediate the anorexic activity of amphetamine and related drugs. Recent data from our laboratory on the changes in [^{3}H](+)-amphetamine binding in rat brain during food deprivation and feeding (Hauger et al., submitted) suggest that these binding sites may also be involved in the regulation of appetite. Based on structure activity studies the [^{3}H](+)-amphetamine binding sites in hypothalamic membranes do not seem to be associated with the locomotor stimulant properties of amphetamine. However, it remains to be determined whether the binding sites in other brain regions (e.g. brainstem and striatum) are related to the other well-known properties of amphetamine. For example, the [^{3}H]-amphetamine binding sites in brainstem may be involved in the motor stimulant actions of amphetamine and particularly amphetamine's effect on arousal and sleep. Studies on the structure-activity relationships of various phenylethylamine derivatives as to their relative potencies in inhibiting [^{3}H](+)-amphetamine binding in these brain regions are in progress.

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