# Characterization of Brain Interactions With Methylenedioxyamphetamine and Methylenedioxymethamphetamine

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## INTRODUCTION

Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA), like other amphetamine analogs, affect multiple *in vitro* monoamine parameters. These effects include stimulation of [$^3$H]serotonin and [$^3$H]dopamine release (Johnson et al. 1986) and inhibition of serotonin, dopamine, and norepinephrine uptake (Steele et al. 1987). In addition, recent radioligand binding studies have demonstrated interactions of MDA and MDMA at a variety of established postsynaptic brain recognition sites, including serotonergic, adrenergic, and cholinergic receptors (Battaglia et al. 1988).

Several studies have identified specific sites of interaction in brain that may mediate the actions of amphetamine and its substituted analogs. Binding sites for [$^3$H]amphetamine (Hauger et al. 1984) and [$^3$H]fenfluramine (Garattini et al. 1987) have been identified in discrete areas of rat brain. These sites have similar characteristics in that both have high binding capacities (amphetamine, $B_{max}$=60 pmol/mg protein; fenfluramine, $B_{max}$=63 pmol/g tissue), and binding to both sites is inhibited by sodium ions. To date, two groups have studied interactions of [$^3$H]MDMA with rat brain. Gehlert et al. (1985) reported high affinity specific binding ($K_d$=99 nM, $B_{max}$=31 fmol/mg protein) of [$^3$H]MDMA to rat brain membranes. However, a subsequent study (Wang et al. 1987) suggested that these apparent binding sites represent [$^3$H]MDMA association with glass fiber filter paper. The present study reexamines the possibility of [$^3$H]MDMA as well as [$^3$H]MDA association with rat brain membranes. Also characterized is the nature of [$^3$H]MDA association with brain membranes to evaluate the possible importance of the binding site in mediating MDA's neurochemical and behavioral effects and to examine similarities between apparent [$^3$H]MDA binding sites and those labeled by [$^3$H]amphetamine and [$^3$H]fenfluramine.

Centrifugation assays were employed in all our studies to circumvent the problem of [$^3$H]MDA and [$^3$H]MDMA absorbing onto glass filters.

## ASSOCIATION TO RAT BRAIN MEMBRANES

### Assay for [$^3$H]MDA and [$^3$H]MDMA Association

Assays were performed using a crude synaptosomal preparation of rat brain. Male Sprague-Dawley rats were sacrificed by decapitation, and brains were immediately dissected on ice for membrane preparation. Telencephalon containing cerebral cortex, striatum, and hippocampus was used in all experiments except in studies examining the regional distribution of the binding sites. Brain areas were homogenized in 20 volumes of ice-cold 0.32 M sucrose using a smooth glass homogenizer equipped with a motor-driven teflon pestle. The homogenate was centrifuged at 800 x g for 10 minutes at 4 °C to remove large particulate material, and the supematant was removed and centrifuged at 20,080 x g. The resultant pellet was resuspended in 20 volumes of the original wet weight in either ice-cold 50 mM Tris-HCl buffer (pH 7.1) alone or in the same buffer containing 0.27 M sucrose. Binding assays were performed in Beckman minivials containing either 0.9 mL of 50 mM Tris-HCl (pH 7.1) or the same buffer containing 0.27 M sucrose. The vials also contained 2 nM [$^3$H]MDA (54 Ci/mmol) or [$^3$H]MDMA (74 Ci/mmol), competing drugs at various concentrations, and 100 µL of each of the homogenates as indicated above. Nonspecific binding was assessed by measuring the [$^3$H]MDA or [$^3$H]MDMA incorporated into boiled tissue. All incubations were performed for 90 minutes at 4 °C: unless indicated otherwise. Assays were terminated by centrifugation at 32,008 x g at 4 °C. The vials were removed and the supernatant fluid discarded. The pellets were superficially washed with ice-cold water (7 mL three times), after which the excess water was wiped from the inside of the vial and 5.0 mL of scintillation fluid was added to each vial. The pellets were allowed to solubilize overnight, and the vials were assessed for radioactivity by scintillation counting. Data from saturation isotherms were analyzed by the nonlinear curve-fitting program LIGAND (Munson and Rodbard 1980). Protein was measured by the method of Lowry et al. (1951).

### [$^3$H]MDA and [$^3$H]MDMA Association to Rat Brain Membranes

Preliminary experiments to delineate the optimum conditions for [$^3$H]MDA and [$^3$H]MDMA binding to rat brain membranes indicated that the best signal-to-noise ratio was found using a crude synaptosomal preparation incubated at 4 °C. While most experiments employed 50 mM Tris-HCl (pH 7.1) as the incubation medium, we found that the addition of 0.27 M sucrose to the incubation medium increased the specific binding approxi-mately fivefold. Subsequent experiments were carried out under both conditions. Other preliminary experiments suggested that [$^3$H]MDA and

[$^3$H]MDMA interacted with multiple sites in rat brain. A low affinity [$^3$H]MDA binding site (apparent $K_d$>1.0 mM) was found to be resistant to boiling of the synaptosomal preparation for 15 minutes. This site was saturable, as indicated by a 30 percent inhibition of [$^3$H]MDA binding to boiled synaptosomes by 1.0 mM MDA and a 56 percent inhibition of the binding by 0.1 mM of the serotonin uptake blocker paroxetine. The indication of a saturable, nonspecific binding site for [$^3$H]MDA in boiled membranes necessitated that we use boiled tissue to assess nonspecific binding in all subsequent experiments.

The saturation profiles of [$^3$H]MDA and [$^3$H]MDMA recognition sites in rat brain synaptosomes in the presence of 0.27 M sucrose are shown in figure 1. Both ligands exhibited shallow saturation curves, which extended over 4 log units from approximately 100 nM to 1.0 mM MDA, suggesting the presence of multiple binding sites. Data from [$^3$H]MDA saturation experiments fit significantly ($p<0.015$) better to a two-site model, indicating both high ($K_d$=887 nM, $B_{max}$=23 pmol/mg protein) and low ($K_d$=45 μM, $B_{max}$=3.2 nmol/mg protein) affinity sites upon iterative nonlinear curve-fitting analysis. Analysis of saturation data of apparent [$^3$H]MDMA binding in the presence of sucrose also fit significantly better to a two-site model ($p<0.02$; high affinity $K_d$=2.9 μM, $B_{max}$=79 pmol/mg protein; low affinity $K_d$=128 μM, $B_{max}$=7.4 nmol/mg protein).

The effect of eliminating sucrose from the incubation medium of [$^3$H]MDA binding assays is shown in figure 2. While the Eadee-Scatchard plot of the data from [$^3$H]MDA saturation experiments performed in the absence of sucrose was linear, suggesting [$^3$H]MDA binding to one population of sites, the plot representing the data from experiments performed in the presence of 0.27 M sucrose was curvilinear, consistent with [$^3$H]MDA binding to multiple populations of sites (see above). Nonlinear curve-fitting analysis of the data suggested a single apparent binding site for [$^3$H]MDA, when incubated with synaptosomes in the absence of sucrose ($K_d$=2.8 μM, $B_{max}$pmol/mg protein). Thus, removal of sucrose from the incubation medium led to the elimination of low-affinity specific [$^3$H]MDA binding. Similar to observations of [$^3$H]MDA binding, removal of sucrose from the incubation medium led to a 61 percent decrease in the apparent specific binding at 100 nM [$^3$H]MDMA.

The affinity ($K_d$ values) observed for [$^3$H]MDA and [$^3$H]MDMA binding were similar to the effective doses (i.e., $ED_{50}$ or $K_1$ values) of MDA and MDMA reported for various pre- and postsynaptic monoamine markers, such as serotonin and dopamine release (Johnson et al. 1986), monoamine transport (Steele et al. 1987), and multiple brain, ligand binding sites (Battaglia et al. 1988).

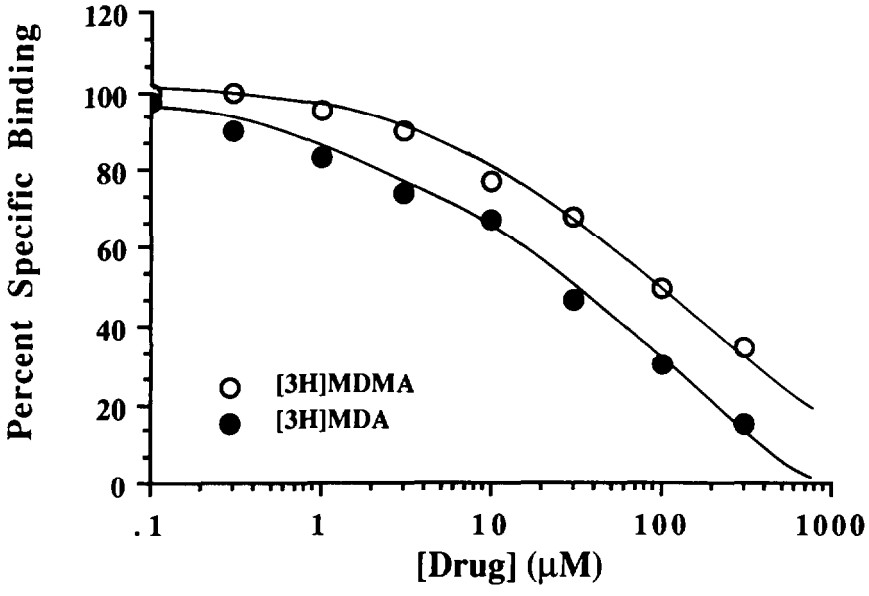


**FIGURE 1.** *Saturation of [$^3$H]MDA and [$^3$H]MDMA binding in rat brain synaptosomes*

NOTE: lncreasing amounts of unlabeled MDA or MDMA were added to 1.0 mL of incubation buffer containing 2 nM [$^3$H]MDA or 2 nM [$^3$H]MDMA, respectively. Experiments were performed on tissue in the presence of 0.27 M sucrose. Data are expressed as percent of [$^3$H]MDA or [$^3$H]MDMA bound to tissue in the absence of added nonradioactive drug. Nonspecific binding was assessed by measuring the amount of [$^3$H]ligand bound to boiled synaptosomes incubated in the presence of 0.27 M sucrose.

The high capacities (i.e., $B_{max}$ value) of [$^3$H]MDA and [$^3$H]MDMA binding sites, as well as those that have been reported for [$^3$H]amphetamine binding sites (60 pmol/mg protein) (Hauger et al. 1984) and [$^3$H]fenfluramine binding sites (63 pmol/g tissue) (Garattini et al. 1987), argue against bimolecular interactions of these drugs with monovalent protein-binding sites. Although the mechanism by which sucrose acts to preserve low affinity [$^3$H]MDA binding is yet to be determined, a similar phenomenon has been observed for [$^3$H]amphetamine binding (Hauger et al. 1984). In the latter study. a wash of tissue in isotonic sucrose prior to incubation was reported to increase nearly threefold the specific binding over a wash with 50 mM Tris-HCl alone (Hauger et al. 1984).

### Pharmacology of Specific [$^3$H]MDA Binding in Rat Brain

The pharmacology of [$^3$H]MDA binding was determined by examining the effects of other monoamine reuptake blockers and related amphetamine

analogs on the inhibition of [$^3$H]MDA binding. The pattern of paroxetine, desipramine (DMI), dimethoxymethamphetamine (DOM), and MDA inhibition of specific [$^3$H]MDA binding is shown in figure 3. Experiments were performed in the presence of 0.27 M sucrose using 2 nM [$^3$H]MDA, conditions under which both high- and low-affinity [$^3$H]MDA binding sites are labeled. Paroxetine was the most potent inhibitor ($IC_{50}$= 1.6 μM) followed by DMI ($IC_{50}$= 5.9 μM), DOM ($IC_{50}$ = 17 μM), and MDA ($IC_{50}$ = 43 μM). Analysis of paroxetine and DMI inhibition curves revealed Hill coefficient values ($n_H$) close to 1.00 (paroxetine, $n_H$ = 0.85; DMI, $n_H$ = 0.91). MDA and DOM gave rise to much shallower inhibition curves (MDA, $n_H$ = 0.56; DOM, $n_H$ = 0.53), providing additional evidence for the existence of multiple apparent [$^3$H]MDA binding sites.

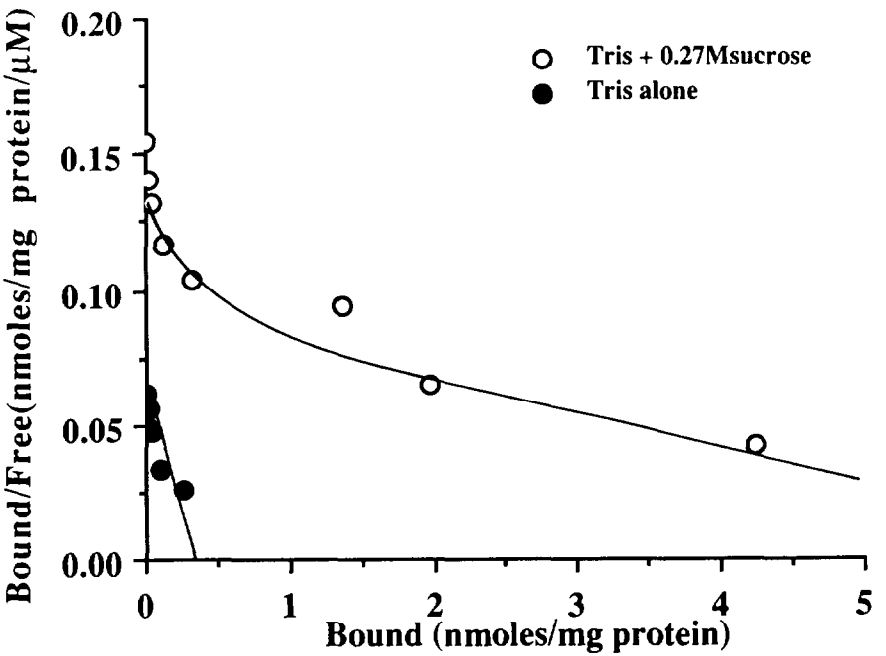


**FIGURE 2.** *Eadee-Scatchard transformation of saturation data of [$^3$H]MDA binding in rat brain synaptosomes*

NOTE: Increasing amounts of unlabeled MDA were added to 1.0 mL of incubation buffer containing 2 nM [$^3$H]MDA. Experiments were performed on tissue in the presence (open circles) and absence (closed circles) of 0.27 M sucrose. Nonspecific binding was assesed by measuring the amouut of [$^3$H]MDA bound to boiled synaptosomes incubated in the presence of 0.27 M sucrose.

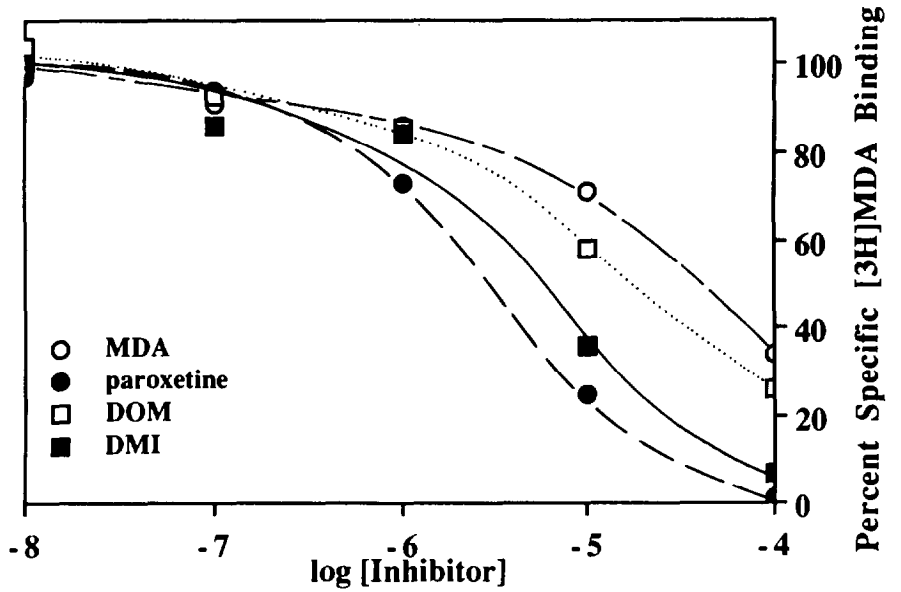


**FIGURE 3.** *Inhibition of [$^3$H]MDA incorporation into synaptosomes*

NOTE: [$^3$H]MDA binding assays were performed in 50 mM Tris-HCl (ph 7.1) containiag 0.27 M sucrose and 2.0 nM [$^3$H]MDA as described in the text. Results are expressed as percent inhibition of specific [$^3$H]MDA incorporation in the absence of inhibitors. Boiled tissue blanks were performed at each concentration of drug.

The inhibition of [$^3$H]MDA binding by several other related compounds is seen in table 1. All compounds were tested at a concentration of 10 μM under conditions that favored the labeling of the high-affinity [$^3$H]MDA binding site (zero sucrose, 2 nM [$^3$H]MDA) and at 100 μM concentration under conditions designed to favor the study of the low-affmity [$^3$H]MDA binding site (0.27 M sucrose, 3 μM [$^3$H]MDA). A significant positive correlation ($r^2$=0.80, $p<0.01$) between the relative inhibition potencies of the test drugs at the high- and low-affinity [$^3$H]MDA binding sites was observed upon linear regression analysis (figure 4A).

## EFFECTS OF OSMOLARITY AND DETERGENTS

A possible explanation for the large capacity (i.e., high $B_{max}$ values) of [$^3$H]MDA binding sites and stimulation of [$^3$H]MDA binding by isotonic sucrose is intrasynaptosomal internalization and sequestration of [$^3$H]MDA. Studies of apparent chloride-dependent [$^3$H]glutamic acid binding (Pin et al. 1984; Zaczek et al. 1987) have demonstrated this type of phenomenon for labeled glutamate. This possibility was examined by measuring [$^3$H]MDA binding in the presence of varying concentrations of sucrose and

estimating the relative synaptosomal volume by measuring [$^3$H]$H_2O$ incorporation into synaptosomes in parallel experiments. As shown in figure 5, decreasing the concentration of sucrose in the incubation medium led to a decrease in the level of [$^3$H]MDA binding. This contrasted with an increase in intrasynaptic volume, as indicated by an increase in the capacity of the synaptosomes to retain [$^3$H]$H_2O$. A significant negative correlation ($r^2$=0.84; $p<0.02$) was obtained when [$^3$H]$H_2O$ incorporation was correlated with [$^3$H]MDA incorporation by linear regression analysis. These data argue against a sequestration phenomenon, since the amount of [$^3$H]MDA binding should increase with increasing vesicular volume, if intrasynaptosomal internalization and sequestration was occurring.

**TABLE 1.** *Pharmacology of inhibition of [$^3$H]MDA binding*

| Drug | High-Affinity Binding | Low-Affinity Binding |
|---|---|---|
| Amphetamine | 47 ± 21 | 31 ± 13 |
| Mescaline | 36 ± 4 | 12 ± 6 |
| MDMA | 48 ± 12 | 32 ± 3 |
| N,N-DMT | 59 ± 11 | 56 ± 20 |
| PCA | 84 ± 7 | 56 ± 8 |
| DOM | 70 ± 3 | 35 ± 8 |
| Fenfluramine | 63 ± 6 | 34 ± 9 |
| Paroxetine | 100 ± 1 | 91 ± 8 |
| Desipramine | 96 ± 5 | 83 ± 9 |
| Imipramine | 96 ± 3 | 61 ± 15 |

NOTE: Inhibition of high-affinity [$^3$H]MDA binding was performed in 50 mM Tris-HCl (pH 7.1) in the presence of 2 nM [$^3$H]MDA to preferentially label the high-affinity site. Drugs were tested at 10 μM concentrations for inhibition of high-affinity binding. Low-affinity [$^3$H]MDA binding inhibition was performed in 50 mM Tris-HCl (pH 7.1) containing 0.27 M sucrose in the presence of 3.0 μM [$^3$H]MDA to preferentially label the low-affinity site. Inhibition was peformed using 100 μM concentrations of the drugs tested. Values represent percent inhibition of specific [$^3$H]MDA binding (mean ± SEM) performed in the absence of inhibiting drugs. Boiled tissue was no in simultaneous assays to assess nonspecific binding.

Another approach used to examine the possible existence of [$^3$H]MDA sequestration into synaptosomes was to investigate the effects of the detergents Triton X-100 and digitonin on the level of [$^3$H]MDA incorporation into rat brain synaptosomes (table 2). Concentrations of detergents lower than 0.01 percent did not affect specific [$^3$H]MDA binding. Digitonin. at a concentration of 0.01 percent, caused a 30 percent decrease ($p<0.05$) in the level of apparent [$^3$H]MDA binding as compared to control, and 0.01 percent Triton caused a 71 percent decrease ($p<0.01$). These data provide additional evidence against intrasynaptosomal internalization and sequestration of [$^3$H]MDA since relatively high concentrations (0.01 percent)

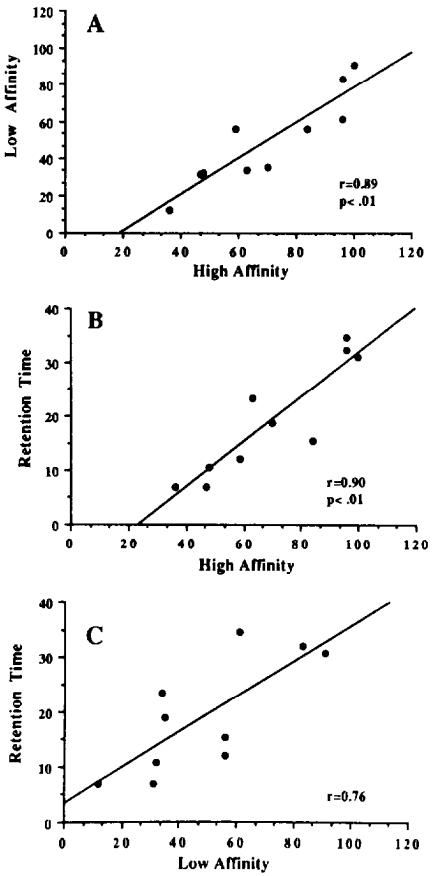


**FIGURE 4.** *Correlation between the relative inhibitory potencies of various drugs at high- and low-affinity [$^3$H]MDA binding and between drug lipophilicities and inhibition potencies of [$^3$H]MDA binding*

NOTE: Panel A represents the relationship between the relative inhibitory potencies of various drugs at high- and low-affinity [$^3$H]MDA binding. Percent inhibition by test drugs of low-affinity [$^3$H]MDA binding is plotted vs. the inhibition of high-affinity binding. Panels B and C represent the relation between the lipophilicity of test drugs and their ability to inhibit high and low [$^3$H]MDA binding, respectively. In both cases, the retention times of test drugs on reverse-phase HPLC are plotted vs. percent inhibition of [$^3$H]MDA binding. Pearson's r values and levels of significance are derived from linear regression analysis of the data.

of the detergents were required to cause significant decreases in [$^3$H]MDA incorporation into the tissue. Furthermore, these decreases were only partial, which is in contrast to what is generally observed when labeled

substances am released from a membrane-intemalized pool by pore-forming detergents, which abruptly release the total contents of membrane-sequestered compounds.

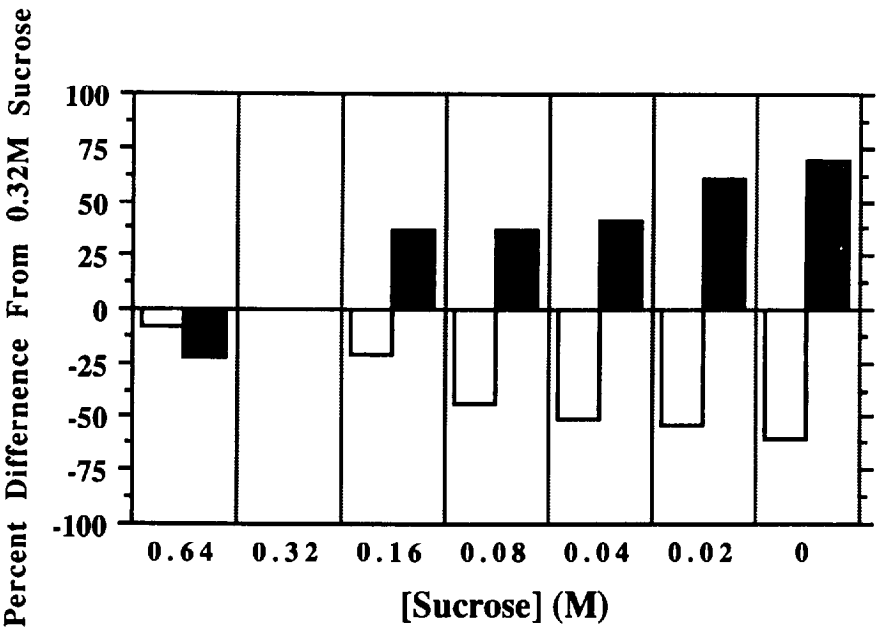


**FIGURE 5.** *Effects of varying sucrose concentration on [$^3$H]MDA and [$^3$H]water incorporation into rat brain synaptosomes*

NOTE: Synaptosomes were prepared and incubated under standard procedures for [$^3$H]MDA incorporation. The osmolarity of the incubation buffer was changed by the addition of various concentrations of sucrose to 50 mM Ttis-HCl (pH 7.1). The incubation medium contained either 100 nM [$^3$H]MDA or 2 million cpm of [$^3$H]water. After a 90-min incubation at 4 °C, the assays were terminated and assessed for radioactivity. Bars represent the percent change in the level of radioactive $H_2O$ (shaded bars) end [$^3$H]MDA (open bars) incorporated from the respective incorporation at 0.32 M surcrose.

The results of the osmolarity and detergent experiments indicate that MDA is not internalized into synaptosomes to any appreciable degree. In addition, the [$^3$H]MDA binding assays were performed at 4 °C, demonstrating a lack of dependence on physiological temperatures; this lack is characteristic of membrane internalization phenomena. Since other investigators have shown that [$^3$H]MDMA is not taken up into synaptosomes by a sodium-dependent mechanism (Wang et al. 1987), the internalization of MDA, MDMA, and related compounds does not appear to be necessary for their presynaptic actions to enhance the release and to inhibit the reuptake of monoamines. Furthermore, the intraneuronal internalization of MDA or

MDMA is not likely to be involved in the ability of these compounds to cause serotonin terminal degeneration. The preponderance of the evidence supports the hypothesis that the association of [$^3$H]MDA with synaptosomes is primarily with membrane elements.

**TABLE 2.** *Effects of detergents on apparent [$^3$H]MDA binding*

| Detergent | Weight/Vol (Percent) | Percent Control |
|---|---|---|
| Triton X-100 | .0001 | 95 ± 9 |
| | .001 | 98 ± 8 |
| | .01 | 37 ± 9** |
| Digitonin | .0001 | 89 ± 9 |
| | .001 | 85 ± 11 |
| | .01 | 70 ± 4* |

Difference significant at $p<0.05$ by ANOVA and Duncan's multiple range test.

**Difference significant at $p<0.01$ by ANOVA and Duncan's multiple range test.

NOTE: Synaptosomes were incubated for 90 min at 25 °C in 50 mM Tris-HCl (pH 7.1) containing 0.27 M sucrose and 100 nM [$^3$H]MDA. Results are expressed as percent of specific [$^3$H]MDA binding in the absence of detergent. Results are the means ± SEM from three seperate experiments done in duplicate.

### Role of Lipophilicity in the Incorporation of [$^3$H]MDA Into Rat Brain Synaptosomes

Compounds that were included in the pharmacologic profile of [$^3$H]MDA binding were subjected to reverse-phase HPLC analysis to assess their relative lipophilicity. Briefly, each compound (10 µg) was injected onto a Waters Nova-Pak C18 column and eluted with a linear gradient from 95 percent buffer A:5 percent buffer B to 20 percent buffer A:80 percent buffer B (buffer A=95 percent water, 5 percent acetonitrile, 0.1 percent ammonium acetate; buffer B=20 percent water, 80 percent acetonitrile, 0.1 percent ammonium acetate). Detection was performed using a Waters Model 441 UV detector at 214 nm. Figure 6 shows the reverse-phase chromatographic elution pattern of MDA and related compounds, which are listed in table 1. Since separation of compounds by reverse-phase chromatography is based upon the aqueous/organic partition coefficients of the substances separated, this method gives an index of the relative lipophilicity of various compounds. The more lipophilic a compound is, the greater its retention time on C18 material. The retention times, in minutes, of the compounds tested in the present study were: amphetamine, 6.93; mescaline, 6.93; MDMA, 10.6; N,N-dimethyltryptamine (N,N-DMT), 12.1;

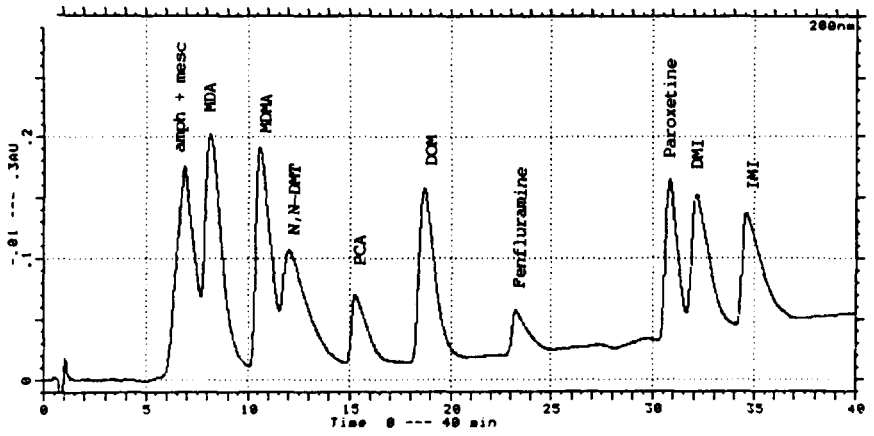


**FIGURE 6.** *Elution pattern of monoamine uptake blockers and amphetamine analogs from reverse-phase HPLC*

KEY: amph=amphetamine; mesc=mescaline; N,N-DMT=N,N-dimethyltryptamine
PCA=parachloroamphetamine; DOM=dimethoxymethamphetamine; DMI = desipramine; IMI=imipramine.

NOTE: HPLC detection of test drugs at 200 nm UV. Peak are named for the drugs they represent. Peak identity was discerned by analyzing each drug individually and observing its retention time and UV spectrum by photodiode array detection between 190 and 360 nm using a Waters Model 990 PDA detector. Retention times are listed in the text.

parachloroamphetamine (PCA), 15.4; DOM, 18.8; fenfluramine, 23.4; paroxetine. 30.9; DMI, 32.2; and imipramine, 34.7. Figure 4B and 4C show the correlations that were found between the retention times of the tested drugs and their levels of inhibition of high- and low-affinity [$^3$H]MDA binding, respectively (table 1). A significant positive correlation ($r^2$=0.81, $p<0.01$) was observed between the HPLC retention times of the tested drugs and their respective levels of inhibition of high-affinity [$^3$H]MDA binding. Although a positive correlation was found between the lipophilicity and the ability of test compounds to inhibit low-affinity [$^3$H]MDA binding ($r^2$=0.56), this correlation did not reach the level of statistical significance.

As stated earlier, the primary site of association of [$^3$H]MDA with brain synaptosomes is with membrane components, not with the intrasynaptic space. While the phenolic ends of these compounds may enable them to interact with hydrophobic environments of brain membrane components, their polar side chains may inhibit the ability of these compounds to move freely across the membranes, thus inhibiting internalization. The pKa of

amphetamine is 9.9, which indicates that over 99 percent of this drug and, most likely, its structural analogs will be ionized at pH 7.4. The ability of these compounds to enter the brain readily, which has been ascribed to their supposed lipophilicity, may be due to the existence of a saturable transport process for these drugs across the blood-brain barrier (Pardridge and Connor 1973). The high capacity for MDA and MDMA incorporation into brain membranes could be explained by their absorption into a hydrophobic membrane environment. We have shown positive correlations between the lipophilicity of several monoamine uptake blockers and amphetamine analogs and their relative inhibition potencies of both high- and low-affinity [$^3$H]MDA binding (figure 4); however, this correlation is not perfect. For instance, MDMA, which has a lower affinity (higher IQ for these sites of interaction, is more lipophilic than MDA; fenfluramine, which is less potent at inhibiting [$^3$H]MDA binding than PCA, is more lipophilic than the latter compound. These data suggest that there may exist some level of structural specificity beyond lipophilicity.

## REGIONAL DISTRIBUTION OF APPARENT [$^3$H]MDA BINDING

The relative distribution of [$^3$H]MDA incorporation into $p_2$ preparations from various regions of rat brain, liver, and kidney is shown in table 3. Apparent [$^3$H]MDA binding had a heterogeneous regional distribution in brain, being highest in neocortex and midbrain followed by medulla-pons, striatum, and diencephalon. Brain levels of [$^3$H]MDA incorporation were lowest in cerebellum, which had 50 percent fewer binding sites than neocortex. Although [$^3$H]MDA incorporation was detected outside the brain, levels were much lower than those found in most of the brain structures studied. The level of [$^3$H]MDA binding in liver was 40 percent and that in kidney 20 percent of that found in neocortex. The regional distribution of [$^3$H]MDA incorporation in the absence of sucrose had a profile similar to that performed in the presence of 0.27 M sucrose (data not shown). There was a significant positive correlation between the regional levels of apparent binding studied under the two conditions ($r^2$=0.84, $p<0.61$). These data, together with those indicating a significant correlation between the relative inhibition potencies of MDA analogs at high- and low-affinity [$^3$H]MDA binding, suggest an intimate relationship between the high- and low-affinity [$^3$H]MDA binding sites. Although there exist some differences in the pattern of regional distribution between [$^3$H]MDA binding found in the present study and that of [$^3$H]amphetamine binding found by Hauger et al. (1984). similarities also exist. For example, the lowest level of binding in brain for both ligands is found in the cerebellum and low levels of binding are found for both ligands in the periphery. The differences among the regional distributions of [$^3$H]amphetamine, [$^3$H]MDA, and [$^3$H]fenfluramine (Garattini et al. 1987) binding may be attributed to the various membrane preparation and assay procedures used in each study.

**TABLE 3.** *Regional distribution of apparent [$^3$H]MDA binding*

| Region | [$^3$H]MDA Bound (pmol/mg protein) |
|---|---|
| Neocortex | 31 ± 4 |
| Striatum | 2 4 ± 3 |
| Diencephalon | 2 4 ± 5 |
| Midbrain | 29 ± 5 |
| Medulla-Pons | 2 6 ± 5 |
| Cerebellum | 15 ± 3 |
| Liver | 10 ± 1 |
| Kidney | 6 ± 2 |

NOTE: A $p_2$ preparation was prepared from each dissected region and assayed for [$^3$H]MDA incorporation as described in the text. Incubation was performed in 50 mM Tris-HCI (pH 7.1) containing 0.27 M sucrose and 2.0 nM [$^3$H]MDA. [$^3$H]MDA incorporation into boiled tissue served as the measure of nonspecific binding. Values are expressed as specific [$^3$H]MDA bound (mean ± SEM; pmol/mg protein) and are the results of three experiments performed in triplicate.

**Concentration of MDA in Brain After Systemic Administratian**

To elucidate further the relevance of the high-nanomolar to low-micromolar affinities of tbe [$^3$H]MDA binding sites, we determined the brain concentrations of MDA following systemic administration of behaviorally active doses (20 mg/kg) of the drug to rats. Rats were injected subcutaneously with 20 mg/kg MDA containing 0.5 μCi of [$^3$H]MDA. Rats were sacrificed at various times after injection, and the hippocampus was removed, weighed, and solubilized overnight in Soluene 100 (Packard). Econoflour (5 mL) (NEN) was then added to the solution, and radioactivity was assessed by liquid scintillation counting. To evaluate the identity of the radioactivity, rats were sacrificed 45 minutes after injection, and brains were removed and homogenized in 0.1 M sodium acetate using a polytron (10 seconds, position 6). After centrifugation at 30,000 x g, the supernatant fluid was collected and 4.0 mL was applied to a C18 Sep Pak cartridge (Waters). The cartridge was washed with 3.0 mL of water, and radioactivity was eluted in 1.0 mL of acetonitrile containing 0.1 percent trifluoroacetic acid. After drying the organic eluate to approximately 200 μL under nitrogen, 50 μL of the extract was injected onto a Waters Nova Pak C18 column and eluted at 1.0 mL per minute with a linear gradient from 3 percent acetonitrile:97 percent 50 mM potassium phosphate buffer (pH 6.4) to 80 percent acetonitrile: 20 percent water over 30 minutes. Eluted compounds were detected by UV absorbance at 214 nm. Fractions (1.0 mL) of the column eluate were collected and added to vials containing 5.0 mL of formula 963, which were then assessed for radioactivity by scintillation counting.

**Accumulation and Clearance Findings**

Figure 7A shows the timecourse of [$^3$H]MDA accumulation and clearance from rat brain after a subcutaneous injection. Peak concentration, which was reached at 45 minutes, was equivalent to 165 μM (36 μg/g). To verify the identity of the radioactivity as [$^3$H]MDA, reverse phase chromatographic analysis was performed on brain extract from a rat 45 minutes after MDA (20 mg/kg) injection (figure 7B). Radioactivity eluted as a single peak at 14 minutes, which coincided with a peak having a retention time of 13.8 minutes observed by UV detection. This peak, which was not observed in naive animals, was isochromatographic with standard MDA. Thus, the injected material reaching the brain at 45 minutes was MDA, not a metabolite. The level of MDA found in brain indicates that the $K_d$ values of MDA's interaction with rat brain synaptosomes are within the range of the brain concentrations of MDA reached following administration of behaviorally active doses of the drug.

**SUMMARY**

Brain recognition sites have been identified for [$^3$H]MDA and [$^3$H]MDMA. The dissociation constants of MDA and MDMA for these sites are similar to the concentrations needed to affect several brain neurochemical parameters and are in keeping with concentrations of MDA in brain (165 μM) following administration of behaviorally active doses (20 mg/kg) of the drug. While the characteristics of these binding sites suggest a possible hydrophobic interaction with brain membranes, this interaction is not without specificity, since it has a unique pharmacology and a heterogeneous distribution in brain.

Similarities have been found between [$^3$H]MDA binding studied in the present report and that of apparent [$^3$H](+)amphetamine binding studied by Hauger et al. (1984). Both have extremely high $B_{max}$ values, are optimal in $p_2$ preparations, are stabilized by sucrose, and share similar patterns of regional distribution. Measuring the specific binding of [$^3$H]amphetamine, [$^3$H]fenfluramine, [$^3$H]MDA, and related compounds under identical conditions will be required to determine the possible relationships among the interactions of these compounds with brain membranes. Further study is also needed to determine the possible importance such interactions of amphetamine and its substituted analogs may have with brain membranes in relation to the pharmacology of these substances.

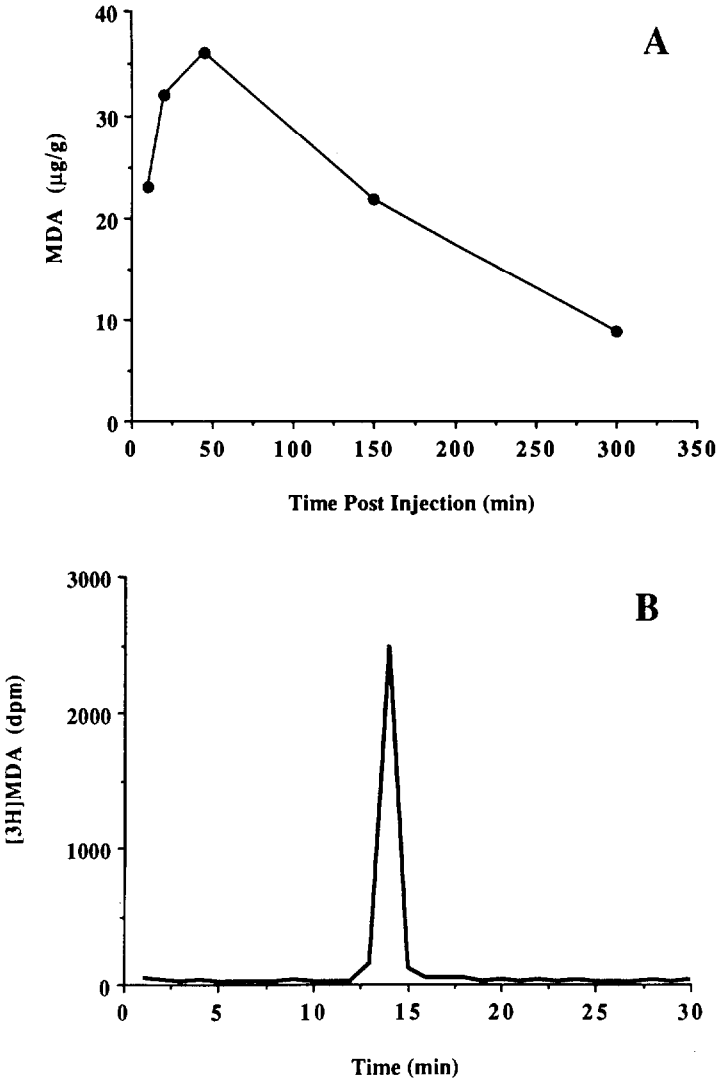


**FIGURE 7.** *Determination of MDA concentration in rat brain following administration of behaviorally active doses of the drug*

NOTE Panel A shows concentration of hippocampal MDA at various timer after adminitration of 20 mg/kg MDA containing 0.5 uCi of [$^3$H]MDA. Each point represents the average MDA concentration of four hippocampi from two animals. No regional variation was observed in the analysis of MDA concentrations in other brain areas. Panel B shows the reverse-phase chromatographic elution profile of radioactivity extracted from MDA-treated rats.

## DISCUSSION

QUESTION: Do you know if there is cell activity at the site?

ANSWER: It has not been tested. We have not done much pharmacology at all.

QUESTION: Do you know if your regional variations in this binding site correlate at all with myelin?

ANSWER: No, I do not know offhand the concentration of myelin in those areas. In fact, I did the profile to prove that it was not lipophilicity, so I was not expecting that. I did not have the foresight to look at the concentration of myelin.

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