CHARACTERIZATION OF [³H]DESIPRAMINE BINDING ASSOCIATED WITH NEURONAL NOREPINEPHRINE UPTAKE SITES IN RAT BRAIN MEMBRANES¹

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Abstract

A variety of evidence indicates that [³H]desipramine can label neuronal norepinephrine uptake sites in brain membranes. Pretreatment of rat cerebral cortical membranes with 0.3 M KCl increases the ratio of high affinity to low affinity saturable [³H]desipramine binding. With this improved tissue preparation, we have confirmed our earlier observation that the high affinity [³H]desipramine binding component ($K_D = 2$ to 4 nM) is associated with norepinephrine neuronal uptake sites. The potencies of various antidepressant drugs in reducing [³H]desipramine binding correlate with their inhibition of neuronal [³H]norepinephrine accumulation. Like the norepinephrine uptake system, high affinity [³H]desipramine binding is dependent both on sodium and chloride, with half-maximal stimulation by 10 mM chloride. Although bromide can substitute for chloride to stimulate binding, other anions, including iodide, fluoride, acetate, citrate, and phosphate, are inactive. Comparable sodium and anion regulation of [³H]imipramine binding to serotonin uptake recognition sites also is observed.

The association of [³H]desipramine binding sites with neuronal norepinephrine uptake sites is supported further by the selective abolition of high affinity [³H]desipramine binding following the destruction of central norepinephrine neurons by intraperitoneal administration of DSP-4 (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine). *In vitro* incubation of cerebral cortical membranes with DSP-4 also selectively abolishes the high affinity [³H]desipramine binding, an effect which cannot be reversed by repeated washing of the membranes, suggesting that DSP-4 alkylates neuronal norepinephrine uptake sites.

Synaptic inactivation of biogenic amine neurotransmitters, such as norepinephrine (NE), is associated primarily with high affinity uptake into nerve terminals which release the transmitter. Tricyclic antidepressants are thought to exert their therapeutic actions by inhibiting the reuptake inactivation of biogenic amines, such as NE and serotonin. The neuronal uptake processes for these amines generally are studied by monitoring the accumulation of radioactive amines into peripheral tissues or into nerve terminals in brain slices or synap-

somal preparations. A specific amine recognition site on the neuronal membrane presumably initiates the process of translocating the amine into the nerve terminal. Uptake recognition "receptors" for serotonin have been labeled in platelets (Talvenheimo et al., 1979; Langer et al., 1981a; Paul et al., 1981) and brain membranes (Langer et al., 1981a; Paul et al., 1981) through the binding of the antidepressant [³H]imipramine. Recently, we reported and characterized the binding of the antidepressant [³H]desipramine ([³H]DMI), which has a unique high affinity for NE uptake sites, to brain membranes (Lee and Snyder, 1981). In preliminary communications (Hrdina et al., 1981; Langer et al., 1981b; Rehavi et al., 1981), others have obtained similar results. The properties of the [³H]DMI binding sites appear to reflect an association with NE neuronal membrane uptake sites.

In our initial study, it was difficult to characterize detailed properties of the [³H]DMI binding sites, since only half of the total saturable "specific" binding appeared to involve the NE uptake "receptors." In the

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present study, we describe a modified tissue preparation which amplifies the component of [3 H]DMI binding associated with NE uptake sites. This permitted us to characterize in detail saturation properties, kinetic features, regulation of the binding sites by anions as well as cations, and alterations in binding following the destruction of NE neurons.

Materials and Methods

DSP-4 (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine) was a generous gift from Astra Läkemedel A. B. (Södertälje, Sweden). DITA (3',4'-dichloro-2(2-imidazolin-2-yl-thio)acetophenone hydrobromide) was kindly supplied by R. Heikkilä, (Rutgers Medical School, Piscataway, NJ), mazindol was from Sandoz Pharmaceuticals (East Hanover, NJ), and chlordesipramine was from Ciba-Geigy (Basel, Switzerland). Nisoxetine was kindly provided by Lilly Laboratories (Indianapolis, IN). Sources of all other drugs used in the present study have been described previously (Lee and Snyder, 1981). ($\pm$)-[3 H]NE (7.5 Ci/mmol), [3 H]DMI (57.0 Ci/mmol), [3 H]imipramine (74.3 Ci/mmol), [3 H]WB-4101 (25.4 Ci/mmol), and [3 H]*p*-aminoclonidine (56.6 Ci/mmol) were obtained from New England Nuclear (Boston, MA).

[3 H]DMI and [3 H]imipramine binding were assayed as previously described (Lee and Snyder, 1981) with the following modifications. Rat brain tissue was homogenized in 30 vol of ice cold assay buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) with a Brinkmann Polytron PT-10 at a setting of 5 for 10 sec. The homogenate was incubated with 300 mM KCl on ice for 1 hr with intermittent mixing and centrifuged at $50,000 \times g$ for 10 min. The pellet was resuspended in 30 vol of the assay buffer by a Polytron at setting 5 for 5 sec and centrifuged again. This washing procedure was repeated twice and the final pellet was resuspended in 30 vol of assay buffer.

Unless otherwise mentioned, the binding of [3 H]DMI was routinely measured in triplicate at 0°C for 1 hr using 200 μ l of the membrane preparation with 0.2 to 10 nM [3 H]DMI in the presence or absence of various tested drugs in a final volume of 250 μ l. At the end of the incubation period, 5 ml of ice cold assay buffer was added to each tube and its content was filtered immediately under reduced pressure through Millipore or Whatman GF/F glass fiber filters. Each of the filters then was washed three times with 5 ml of ice cold buffer and radioactivity was measured by liquid scintillation spectrometry. Total nonspecific binding was defined as binding in the presence of 100 μ M DMI. High affinity nonspecific binding was defined as binding in the presence of 0.1 μ M DMI or 1 mM (–)-NE, both of which gave similar results. Total and high affinity specific binding of [3 H]DMI were calculated by subtracting the respective nonspecific binding from total binding and were expressed as femtomoles per gm of tissue, wet weight or femtomoles per mg of protein.

The binding of [3 H]imipramine to serotonin uptake recognition sites was assayed by a similar procedure. Specific binding of [3 H]imipramine was calculated as described by Langer et al. (1981a) by subtracting nonspecific binding in the presence of 100 μ M DMI from the total binding.

Binding to α_1 - and α_2 -adrenergic receptors was measured at room temperature according to minor modifications of the previously published method (U'Prichard and Snyder, 1979) using [3 H]WB-4101 (2 nM) and [3 H]*p*-aminoclonidine (2 nM), respectively. Nonspecific binding was defined with 100 μ M (–)-NE.

To study the effect of various anions on [3 H]DMI and [3 H]imipramine binding, the KCl-pretreated rat cerebral cortical membranes were washed and resuspended three times in 300 mM sodium phosphate buffer (pH 7.4). Specific binding was measured in this phosphate buffer at 2 nM of the respective radioligands in the presence of various concentrations of sodium chloride, bromide, iodide, fluoride, acetate, citrate, or sulfate.

The uptake of ($\pm$)-[3 H]NE by crude rat cortical synaptosomes was studied by a modification of the procedure of Coyle and Snyder (1969). Male adult Sprague-Dawley rat cerebral cortex was homogenized in 10 vol of ice cold 0.3 M sucrose in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at $1000 \times g$ for 10 min. The pellet was discarded, and the supernatant was centrifuged at $17,000 \times g$ for 20 min. The supernatant was discarded, the pellet was resuspended with a pestle in 10 vol of 0.3 M sucrose in a glass homogenizer, and the suspension was used for measuring NE uptake. One hundred microliters of the suspension was added to 1.8 ml of oxygenated (95% O₂, 5% CO₂) Krebs bicarbonate buffer (composition in millimolar concentrations: NaCl, 118; CCl₂, 5; CaCl₂, 2.5; MgSO₄, 1.2; NaHCO₃, 25; KH₂PO₄, 1.2; and glucose, 11) containing 0.2 mg/ml of ascorbic acid, 0.05 mg/ml of EDTA, 10 μ M nialamide, and various tested drugs. The mixtures were preincubated in a shaking water bath at 37°C for 5 min and the uptake of NE in triplicate samples was initiated by the addition of 100 μ l of [3 H]NE to give a final concentration of 5×10^{-8} M. After 5 min incubation at 37°C the uptake process was terminated by filtration of the mixture under reduced pressure through Whatman GF/B glass fiber filters. Each of the filters then was washed twice with 5 ml of ice cold buffer. Radioactivity was measured by liquid scintillation spectrometry. To correct for passive diffusion and adsorption to membranes and filters, control samples were incubated at 0°C for 5 min. Active uptake of [3 H]NE was calculated by subtracting the 0°C control and was expressed as femtomoles per mg of tissue per 5 min. In our earlier study (Lee and Snyder, 1981), most tricyclic antidepressants displayed shallow inhibition curves for inhibiting the uptake of [3 H]NE. In these experiments, rat cerebral cortical homogenates were centrifuged at $50,000 \times g$ for 10 min, resuspended, and used for uptake studies. Nonspecific uptake was assayed at 0°C, in the presence of 100 μ M DMI. In the present study, the use of a crude synaptosomal preparation and incubating samples for the determination of nonspecific uptake at 0°C in the absence of DMI resulted in steeper inhibition curves by drugs and greater inhibitory potencies.

To destroy NE neurons in the brain, male adult Sprague-Dawley rats weighing 150 to 200 gm were injected intraperitoneally with 0.5 ml of freshly prepared DSP-4 (50 mg/kg) in 0.9% NaCl as described (Jonsson et al., 1981). Control animals received the same volume of saline. Control and treated animals were decapitated 3

days after injection and each cerebral cortex was homogenized in 10 vol of ice cold 0.3 M sucrose. The homogenate was centrifuged at $50,000 \times g$ for 10 min, the pellet was resuspended in 10 vol of 0.3 M sucrose, and aliquots were assayed for $[^3\text{H}]$ NE uptake. The rest of the homogenate was diluted in 30 vol of the assay buffer, homogenized with a Polytron, and incubated with 300 mM KCl on ice for 1 hr. The homogenate was centrifuged and washed. $[^3\text{H}]$ DMI and $[^3\text{H}]$ imipramine binding were assayed as described in the previous section using 2 nM of the respective radioactive ligand.

Results

Effects of potassium chloride treatment of brain membranes on $[^3\text{H}]$ DMI binding. In our initial study, only about half of the saturable $[^3\text{H}]$ DMI binding appeared associated with the NE uptake sites (Lee and Snyder, 1981). The component of binding that labeled the NE uptake sites displayed higher affinity for DMI than did the other portion of the saturable $[^3\text{H}]$ DMI binding. In attempts to reduce selectively the "low affinity specific" binding, we treated cerebral cortical membranes with reagents which might alter membrane proteins and/or lipids. Treatment of membranes with the enzymes chymotrypsin and phospholipase A_2 as well as the detergents Triton X-100, deoxycholate, and Nonidet decreases high and low affinity specific binding to a similar extent. However, treatment of membranes with 0.3 M KCl does reduce low affinity specific binding selectivity (Table I). While nonspecific binding, measured in the presence of 100 μM DMI, is the same in control and KCl-treated membranes, nonspecific binding measured with 0.1 μM DMI or 1 mM NE is reduced almost 50% by KCl preincubation. Thus, whereas in control membranes, the high affinity specific binding is only about one-third of the total binding, in KCl-treated membranes, it is 50% of the total binding. In control membranes, only half of the total specific binding is high affinity, while in KCl-treated membranes, about 80% of the total specific binding is high affinity. Pretreatment of membranes with 0.3 M NaCl or LiCl is not as effective as pretreatment with KCl in augmenting the high affinity specific binding of $[^3\text{H}]$ DMI. Accordingly, most of our studies have been conducted in KCl-pretreated membranes. Unless otherwise indicated, specific $[^3\text{H}]$ DMI binding henceforth will refer to the high affinity binding of KCl-pretreated cerebral

cortical membranes. Interestingly, $[^3\text{H}]$ imipramine binding displays only a single high affinity component, which is unaffected by KCl treatment (data not shown).

Saturation and kinetics of $[^3\text{H}]$ DMI binding. In our earlier study, the difficulty in differentiating high and low affinity specific binding precluded a thorough analysis of the saturation and kinetic properties of the binding sites, which is possible now with the KCl-treated membranes. Total specific binding of $[^3\text{H}]$ DMI does not fully plateau even at concentrations as high as 20 nM, whereas high affinity specific binding begins to plateau at 10 nM (Fig. 1). Scatchard analysis shows that the total specific binding involves at least two components, while high affinity specific binding reflects a single component with an equilibrium dissociation constant (K_D) of 2 nM and a maximal number of binding sites (B_{max}) of 6.3 pmol/gm of tissue.

High affinity specific $[^3\text{H}]$ DMI binding is time dependent (Fig. 2). At 0°C, binding increases gradually to attain equilibrium levels at about 80 min, with half-maximal binding apparent at about 16 min. At 20°C, binding occurs more rapidly, reaching maximal levels at about 40 min and half-maximal values at about 5 min. In repeated experiments, some decline is observed between 40 and 120 min at 20°C. Maximal binding levels at 20°C are about 30% lower than at 0°C. At 37°C, we have been unable to demonstrate substantial levels of high affinity specific binding.

Plotting the data from the initial phase of association at 0°C and 20°C according to a pseudo-first-order reaction gives straight lines with slope of 5.4×10^{-4} (0°C) and 1.5×10^{-3} (20°C), respectively (Fig. 3A). The association rate constant (k_1) determined from such analysis is 9.5×10^4 and $8.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for 0°C and 20°C, respectively.

Dissociation of $[^3\text{H}]$ DMI from the binding sites was analyzed by incubating cerebral cortical membranes with $[^3\text{H}]$ DMI (2 nM) to equilibrium at 0°C (90 min), whereupon dissociation was initiated by adding 100 or 0.1 μM or 1 mM NE and the residual binding was examined at different time points. The exponential dissociation pattern is identical with all three displacing conditions, with a half-life for dissociation at 0°C of 38 min and at 20°C of 12 min. Both at 0°C and 20°C, dissociation is monophasic when plotted on semilogarithmic paper. The slope which corresponds to the rate constant for dissociation (k_{-1}) is 3.5×10^{-4} and $1.3 \times 10^{-3} \text{ s}^{-1}$ at 0°C and 20°C,

TABLE I
Effect of KCl pretreatment on $[^3\text{H}]$ DMI binding to rat cerebral cortical membranes

The binding of $[^3\text{H}]$ DMI was assayed at 2 nM radioligand concentrations according to the procedure described under "Materials and Methods." Data are typical means of triplicate assays. The experiment was replicated three times with less than 20% variation. Total nonspecific binding was defined as binding in the presence of 100 μM DMI. High affinity nonspecific binding was defined as binding in the presence of 0.1 μM DMI or 1 mM (–)-NE. Total and high affinity specific binding of $[^3\text{H}]$ DMI were calculated by subtracting the respective nonspecific binding values from the total binding.

Membrane Preparation	$[^3\text{H}]$ DMI Bound				
	Total	Total Nonspecific	High Affinity Nonspecific		Total Specific
		100 μM DMI	0.1 μM DMI	1 mM NE	
Control	3200	900	2000	2000	2300
KCl pretreated	2300	900	1200	1200	1400

Figure 1. A, Saturation of specific [^3H]DMI binding to KCl-pretreated rat cerebral cortical membranes with increasing concentrations of [^3H]DMI. Each point represents the mean of five separate experiments (vertical bars indicate SEM). B, Scatchard analysis of the saturation curve of a typical experiment. Total specific binding ($\bullet$) and high affinity specific binding ($\circ$) of [^3H]DMI was calculated by subtracting the nonspecific binding in the presence of $100 \mu\text{M}$ and $0.1 \mu\text{M}$ or 1 mM NE, respectively, from total binding. Since the nonspecific binding defined with $0.1 \mu\text{M}$ DMI or 1 mM NE did not differ significantly, these results were pooled.

Figure 2. Time-dependent high affinity binding of [^3H]DMI (2 nM) to rat cerebral cortical membranes at 0°C ($\circ$) and 20°C ($\bullet$). Total and nonspecific binding (defined with either $0.1 \mu\text{M}$ DMI or 1 mM NE) were determined at the indicated time intervals to yield the specific binding values. Since the nonspecific binding defined with either DMI or NE did not differ significantly from each other, these results were pooled. The results are the mean of two separate experiments performed in triplicate (vertical bars indicate SD).

respectively (Fig. 3B). The K_D value calculated from the ratio of k_{-1}/k_1 is 3.7 nM from the 0°C experiments and 16.3 nM from the 20°C experiments. The reasons for this discrepancy are unclear but could be related to the limited degradation of the binding sites at 20°C , with diminished affinity for DMI.

Regional distribution of [^3H]DMI and [^3H]imipramine binding in rat brain. The regional distribution of specific [^3H]DMI binding tends to parallel that of NE

Figure 3. Kinetic analyses of the association (A) and dissociation (B) time course of high affinity specific binding of [^3H]DMI to cerebral cortical membranes at 0°C ($\circ$) and 20°C ($\bullet$). See the legend to Figure 2 for details of the association experiments. For dissociation experiments, 2 nM [^3H]DMI was incubated with cortical membranes on ice for 90 min. Dissociation then was initiated by adding 100 or $0.1 \mu\text{M}$ DMI or 1 mM NE and the residual binding was examined at indicated time points. Binding at time zero (B) was determined by filtering immediately after the addition of DMI or NE. The results are the means of three separate experiments which varied less than 15%. The lines were fitted by linear regression analysis.

uptake (Snyder and Coyle, 1969). The highest binding occurs in the hypothalamus, with the second highest levels in the cerebral cortex (Table II). The lowest levels of binding occur in the cerebellum and corpus striatum which have very low levels of NE uptake. In the corpus striatum [^3H]NE accumulation into synaptosomes can be demonstrated, but this involves accumulation by the dopamine neuronal uptake process (Coyle and Snyder, 1969). The regional distribution of [^3H]imipramine binding is very different. Thus, the corpus striatum, which had the lowest levels of [^3H]DMI binding, has the second

highest levels of $[^3\text{H}]$ imipramine binding. This finding is consistent with the high levels of endogenous serotonin and serotonin uptake in the corpus striatum. The binding of $[^3\text{H}]$ imipramine is highest in the hypothalamus and

TABLE II

Regional distribution of $[^3\text{H}]$ DMI and $[^3\text{H}]$ imipramine binding in rat brain

Brain membranes were pretreated with 300 mM KCl as described under "Materials and Methods." The specific binding of $[^3\text{H}]$ DMI was measured at 2 nM $[^3\text{H}]$ DMI using 1 mM (–)-NE to define the nonspecific binding. Very similar results were obtained when the nonspecific binding was defined with 0.1 μM DMI. The specific binding of $[^3\text{H}]$ imipramine was measured at 2 nM $[^3\text{H}]$ imipramine using 100 μM DMI to define the nonspecific binding. The values represent the mean $\pm$ SEM of four to six separate experiments, each performed in triplicate. The relative percentage of ^3H -ligand binding in various regions, with hypothalamus being 100%, is given.

Regions	Specific Binding			
	$[^3\text{H}]$ DMI		$[^3\text{H}]$ Imipramine	
	fmol/mg protein	%	fmol/mg protein	%
Hypothalamus	72.1 $\pm$ 16.1	100	54.0 $\pm$ 4.9	100
Cerebral cortex	48.7 $\pm$ 4.9	68	31.1 $\pm$ 5.2	58
Medulla	32.2 $\pm$ 11.5	45	38.6 $\pm$ 2.3	71
Cerebellum	24.5 $\pm$ 3.2	34	14.1 $\pm$ 1.5	26
Striatum	17.0 $\pm$ 9.6	24	43.7 $\pm$ 6.3	81

lowest in the cerebellum, which is in good agreement with reports from other laboratories (Kinnier et al., 1981; Palkovits et al., 1981).

Drug effects on $[^3\text{H}]$ DMI binding. In our earlier studies, most tricyclic antidepressants displayed extremely shallow displacement curves for the inhibition of $[^3\text{H}]$ DMI binding (Lee and Snyder, 1981). In the present study, we have confirmed these shallow curves (Fig. 4A). DMI reduces $[^3\text{H}]$ DMI binding very gradually over about five orders of magnitude. Displacement patterns become less complex in KCl-treated membranes, with less shallow curves for all drugs examined and apparently higher affinity for most of the drugs (Fig. 4B). While it is difficult to define the potency for DMI inhibition of binding in control membranes, in KCl-treated membranes, the displacement curve has a Hill coefficient of approximately unity and an IC_{50} of about 4 nM. Other tricyclic antidepressants are less potent, with imipramine, doxepin, and chlorimipramine displaying similar potencies with IC_{50} values of 90 to 150 nM. Mianserin and iprindole are much less potent, with IC_{50} values of 950 and 16,000 nM respectively.

The affinity of antidepressants for the $[^3\text{H}]$ DMI binding sites correlates closely with their potencies in inhibiting $[^3\text{H}]$ NE uptake into synaptosomal fractions (Fig. 5). By contrast, while some of the antidepressants are quite

Figure 4. Effect of KCl (300 mM) pretreatment (1 hr, on ice) of rat cerebral cortical membranes on the inhibition of the specific binding of $[^3\text{H}]$ DMI (2 nM) by representative antidepressant drugs including: desipramine, imipramine, chlorimipramine, doxepin, mianserin, and iprindole. The results are the mean of three or four separate experiments performed in triplicate which varied less than 15%.

Figure 5. Correlation of the inhibition of [^3H]DMI cortical binding sites by antidepressant drugs with the (A) inhibition of synaptosomal [^3H]NE uptake, (B) inhibition of [^3H]spiroperidol binding to serotonin (5-HT $_2$) receptors, (C) inhibition of [^3H]mepyramine binding to histamine (H $_1$) receptors, and (D) inhibition of [^3H]quinuclidinyl benzilate (QNB) binding to muscarinic cholinergic receptors. The data are from Table III and Peroutka and Snyder (1981). The following drugs were analyzed: desipramine, protriptyline, nortriptyline, chloridesipramine, imipramine, amitriptyline, chlorimipramine, mianserin, iprindole, doxepin, and trazodone. The correlation coefficients were 0.97 (A), 0.03 (B), -0.14 (C), and 0.55 (D).

potent in competing at binding sites associated with histamine (H $_1$), serotonin (5-HT $_2$), and muscarinic cholinergic receptors, their affinities for these sites do not correlate with their influences on [^3H]DMI binding or [^3H]NE uptake. Interestingly, mazindol and nisoxetine, which are potent inhibitors of NE uptake but are structurally unrelated to the tricyclic antidepressants, have almost identical potencies at [^3H]DMI sites and in affecting [^3H]NE accumulation. These results support the conclusion that [^3H]DMI labels recognition sites associated with the NE uptake process.

For some drugs, potencies at [^3H]DMI binding sites do not match their effects on [^3H]NE uptake (Table III). In our earlier study, in the absence of KCl treatment, NE

and amphetamine were extremely weak inhibitors of [^3H]DMI binding with respective IC_{50} values of 1,000 to 10 μM . In KCl-treated membranes, they are substantially more potent, with IC_{50} values of 8 and 1 μM for NE and amphetamine, respectively. However, NE is still 40 times more potent in reducing [^3H]NE uptake into synaptosomal fractions than in inhibiting [^3H]DMI binding, while both (+)- and (-)-amphetamine, DITA, and benztropine are about 10 times more potent inhibitors of NE uptake than of [^3H]DMI binding. A similar discrepancy occurs in the case of dopamine, 6-hydroxydopamine, and serotonin which are about 50 times more potent at decreasing [^3H]NE uptake than in competing for [^3H]DMI binding.

The neurotransmitter specificity of the [^3H]DMI sites

TABLE III

Drug inhibitory potencies on $[^3\text{H}]$ NE uptake and $[^3\text{H}]$ DMI binding in the rat cerebral cortex

Drug effects were examined on high affinity specific $[^3\text{H}]$ DMI binding to KCl-treated rat cerebral cortical membranes. The following compounds had no significant effect on $[^3\text{H}]$ DMI (2 nM) binding to KCl-pretreated cerebral cortical membranes when tested at 10 μM : clonidine, yohimbine, phenylephrine, scopolamine, atropine, reserpine, metanephrine, normetanephrine, harmalol, nifedipine, somatostatin, prolly-leucyl-glycineamide, substance P, and ouabain. GABA and EDTA also had no effect on $[^3\text{H}]$ DMI binding even when tested at 1 mM. The values are the mean of three to six separate experiments, which varied less than 20% in all cases.

Drugs	IC ₅₀	
	[³ H]NE Uptake	[³ H]DMI Binding
	μM	
Tricyclic antidepressants		
Desipramine	0.009	0.004
Chlordesipramine	0.019	0.012
Nortriptyline	0.023	0.028
Protriptyline	0.013	0.037
Amitriptyline	0.130	0.072
Imipramine	0.079	0.090
Doxepin	0.185	0.130
Chlorimipramine	0.255	0.150
Atypical antidepressants		
Mianserin	0.41	0.95
Iprindole	5.0	16.0
Trazodone	34.0	30.0
Others		
Benztrapine	0.29	2.4
Chlorpromazine	0.21	0.19
(+)-Amphetamine	0.09	0.70
(-)-Amphetamine	0.14	1.20
Nisoxetine	0.011	0.014
DITA	0.016	0.07
Mazindol	0.003	0.002
6-Hydroxydopamine	10.0	450
Neurotransmitters		
(-)-NE	0.14	8.0
Dopamine	0.10	8.0
Serotonin	4.3	160.0

is apparent in the very weak activity of serotonin. The similar potency of NE and dopamine at the binding sites fits with their similar affinity for the $[^3\text{H}]$ NE accumulation mechanism.

Regulation of $[^3\text{H}]$ DMI binding by anions. In our initial study, we reported a marked dependence of $[^3\text{H}]$ DMI binding on sodium ions (Lee and Snyder, 1981). While profound enhancement of binding was observed with sodium, lithium and potassium failed to reproduce this effect. Besides having a strict dependency on sodium, neurotransmitter uptake processes also are regulated by anions (Simon and Kuhar, 1976; Sanchez-Armass and Orrego, 1977; Kuhar and Zarkin, 1978). $[^3\text{H}]$ DMI binding appears to have a dependence on chloride similar to that on sodium. Thus, the levels of binding in the presence of Tris-HCl or choline chloride are about the same as with sodium phosphate buffer (data not shown). Deletion of both sodium and chloride, as in potassium phosphate buffers, virtually abolishes specific $[^3\text{H}]$ DMI binding. To

evaluate the influence of anions, we have employed KCl-treated cerebral cortical membranes that are washed thoroughly with 300 mM sodium phosphate buffer and then added to varying concentrations of sodium salts of different anions (Figs. 6 and 7). In preliminary experiments, we have shown that maximal stimulation of $[^3\text{H}]$ DMI binding occurs with 300 mM sodium and that no further change occurs with increases up to 450 mM sodium. We also have observed that phosphate, citrate, sulfate, and acetate ions do not stimulate $[^3\text{H}]$ DMI binding. Thus, the 300 mM sodium phosphate buffer appears to be a suitable control condition for evaluating differential effects of various anions.

Increasing concentrations of chloride markedly enhance high affinity specific $[^3\text{H}]$ DMI binding with negligible effect on low affinity specific binding (Fig. 6). Maximal augmentation of high affinity binding (about a 4-fold increase) occurs with 50 mM chloride and half-maximal binding is apparent with about 10 mM chloride. This effect is selective. While bromide produces a maximal augmentation of $[^3\text{H}]$ DMI binding similar to that of chloride, it is less potent, with half-maximal stimulation apparent only at about 30 mM bromide. By contrast, iodide and fluoride are essentially inactive (Fig. 7A).

We were interested in determining whether the anion selectivity of serotonin uptake recognition sites labeled with $[^3\text{H}]$ imipramine resembles that of $[^3\text{H}]$ DMI binding. Chloride stimulates $[^3\text{H}]$ imipramine binding to a lesser extent that it does $[^3\text{H}]$ DMI binding, producing only a 2.5-fold maximal increase in binding (Fig. 7B). Moreover, chloride is less potent at the $[^3\text{H}]$ imipramine binding

Figure 6. Chloride dependency of $[^3\text{H}]$ DMI binding to rat cerebral cortical membranes. The specific binding of $[^3\text{H}]$ DMI (2 nM) was measured in 300 mM sodium phosphate buffer (pH 7.4) in the presence of various concentrations of sodium chloride. High affinity specific binding was defined with 0.1 μM DMI ($\bullet$) or 1 mM NE ($\circ$). Low affinity specific binding (Δ) was calculated by subtracting the high affinity binding from the total specific binding defined, with 100 μM DMI as a blank. The specific binding of $[^3\text{H}]$ DMI in the absence of sodium chloride was 551 ± 84 fmol/gm of tissue. The results are expressed as a percentage of the binding relative to that of the no chloride control and are the mean of three to six experiments performed in triplicate.

Figure 7. Halide dependency of [3 H]DMI binding (A) and [3 H]imipramine binding (B) to rat cerebral cortical membranes. The binding experiments were performed with 2 nM of the respective radioligand. High affinity specific binding of [3 H]DMI and [3 H]imipramine was determined in 300 mM sodium phosphate buffer (pH 7.4) in the presence of various concentrations of sodium chloride (●), bromide (○), iodide (Δ), and fluoride (▲). The high affinity specific binding of [3 H]DMI and [3 H]imipramine was defined as binding displaceable with 0.1 μ M DMI and 100 μ M DMI, respectively. The specific binding levels of [3 H]DMI and [3 H]imipramine in the absence of halide were 551 ± 84 and 809 ± 124 fmol/gm of tissue, respectively. The results are expressed as a percentage of the binding relative to that of the no halide control and are the mean of three to six experiments performed in triplicate.

sites, with half-maximal stimulation requiring about 20 mM chloride. Bromide produces less of an effect than chloride, while iodide and fluoride are essentially inactive at [3 H]imipramine binding sites.

Influence of NE neuron destruction by DSP-4 on [3 H]DMI binding. Earlier, we showed that the destruction of NE neurons with 6-hydroxydopamine selectively reduces high affinity specific [3 H]DMI binding, indicating that the binding sites occur on NE neurons (Lee and Snyder, 1981). 6-Hydroxydopamine also destroys dopamine neurons and can be employed in adult animals only by direct administration into the brain. Recently, DSP-4 (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine) has been reported to destroy selectively NE and not dopamine or serotonin neurons in the brains of rats following peripheral administration (Ross, 1976; Jaim-Etcheverry and Zieher, 1980; Jonsson et al., 1981). There is evidence that the neurotoxicity of DSP-4 follows from an initial alkylation of NE neuronal uptake sites (Ross, 1976). Accord-

ingly, it was of interest to explore the effects of DSP-4 on [3 H]DMI binding both *in vitro* and *in vivo*.

Preincubation *in vitro* of DSP-4 (100 μ M) with cerebral cortical membranes on ice markedly reduces [3 H]DMI binding (Fig. 8). The effect is time dependent, with maximal reduction of [3 H]DMI binding requiring about 60 min incubation. The inhibitory effect of DSP-4 on [3 H]DMI binding is dose dependent, with an IC_{50} value of 39 μ M. DSP-4 selectively abolishes the high affinity component but not low affinity binding. By contrast, very little reduction in [3 H]imipramine binding is observed upon incubation with DSP-4. As reported previously (Ross et al., 1973; Jonsson et al., 1981), DSP-4 also reduces, to some extent, the binding of [3 H]*p*-aminoclonidine and [3 H]WB-4101 to α_2 - and α_1 -adrenergic receptor binding sites, respectively. The time-dependent reduction of [3 H]DMI binding suggests that this might be an effect resulting from alkylation of the binding sites. Such an irreversible action is supported by the finding that washing membranes up to seven times at 0°C with excess buffer fails to reverse the DSP-4-elicited reduction in [3 H]DMI binding.

Three days after the intraperitoneal injection of DSP-4 in rats, both [3 H]NE uptake into synaptosomal fractions and [3 H]DMI high affinity binding are depleted markedly to a similar extent (Table IV). By contrast, low affinity specific [3 H]DMI binding is unaffected by this treatment. The specificity of this action is apparent in the finding that [3 H]imipramine, [3 H]WB-4101, and [3 H]*p*-aminoclonidine binding are not affected by DSP-4 treatment in the same animals.

Further evidence that only the high affinity binding of [3 H]DMI is associated with NE uptake sites is apparent from our observation that DSP-4 treatment abolishes the high affinity, but not the low affinity, component of DMI competition for [3 H]DMI binding (Fig. 9). Moreover, inhibition of high affinity, but not low affinity, [3 H]

Figure 8. Effect of preincubation with DSP-4 (100 μ M) for various time intervals on ice on specific receptor binding in rat cerebral cortical membranes. At the indicated time points, the membranes were centrifuged and washed three times with 30 vol of assay buffer. The specific binding of [3 H]DMI, [3 H]imipramine, [3 H]*p*-aminoclonidine, and [3 H]WB-4101 was measured at 2 nM concentrations of the respective radioligand. The results are expressed as a percentage of the binding relative to that of the no DSP-4 control and are the mean of two separate experiments performed in triplicate.

TABLE IV

Effect of DSP-4 lesions on rat cerebral cortical $[^3\text{H}]$ NE uptake and $[^3\text{H}]$ DMI and $[^3\text{H}]$ imipramine binding

DSP-4 (50 mg/kg) was injected intraperitoneally and the animals were killed 3 days after the treatment. The results represent the mean $\pm$ SD of three separate determinations, each performed in triplicate. The specific binding of $[^3\text{H}]$ DMI and $[^3\text{H}]$ imipramine was measured at 2 nM radioligand concentrations. Nonspecific binding was assayed with 100 μM DMI for low affinity sites and 0.1 μM DMI or 1 mM NE for high affinity sites. The uptake of $[^3\text{H}]$ NE was determined at 0.05 μM $[^3\text{H}]$ NE.

Animals	³ H]NE Uptake		Specific [³ H]DMI Binding						Specific [³ H]Imipra- mine Binding	
			Low Affinity		High Affinity					
			100 μM DMI		0.1 μM DMI		1 mM NE			
	fmol/mg tis- sue/5 min	%	fmol/gm tissue	%	fmol/gm tissue	%	fmol/gm tissue	%	fmol/gm tissue	%
Saline control	97.7 ± 9.1	100	2516 ± 302	100	2832 ± 777	100	2692 ± 267	100	6826 ± 294	100
DSP-4, 50 mg/kg	19.3 ± 9.5	20 ^a	2104 ± 386	84	799 ± 620	28 ^a	356 ± 252	13 ^a	6137 ± 103	90

^a $p < 0.01$ compared to saline control.

Figure 9. Displacement of $[^3\text{H}]$ DMI (2 nM) binding by unlabeled DMI in rat cerebral cortical membranes following DSP-4 lesion (50 mg/kg, intraperitoneal, 3 days). Specific binding was calculated by subtracting the nonspecific binding in the presence of 100 μM DMI from the total binding and is expressed as femtomoles per gm of tissue (A) or as a percentage of control binding in the absence of unlabeled DMI (B). The results are the mean of three separate determinations performed in triplicate (vertical bars indicate SEM). $\circ$, Control; $\bullet$, DSP-4.

DMI binding by NE is abolished following DSP-4 treatment.

Discussion

The major finding of the present study is that, in KCl-treated membranes, one can identify reliably the high affinity binding of $[^3\text{H}]$ DMI to brain membranes which is associated with NE uptake recognition sites. Evidence

in support of this conclusion includes the close similarity of drug effects on binding sites and on NE uptake, the depletion of the binding sites when NE neurons are destroyed by 6-hydroxydopamine or DSP-4, and the similarity of the regional distribution of the binding sites with that of NE uptake.

In our earlier study (Lee and Snyder, 1981), the high affinity component of $[^3\text{H}]$ DMI associated with NE up-

take sites was detectable, but [^3H]DMI also labeled another lower affinity saturable site. In this situation, one could not readily differentiate a test drug's potency in competing for the high and low affinity sites. Removing the lower affinity sites with KCl now permits a more accurate estimate of drug affinities for the NE uptake-linked sites, indicating a much greater and more physiologic potency for NE.

One interesting observation of the present study is the regulation of [^3H]DMI binding as well as [^3H]imipramine binding by anions. The anion influences resemble the actions of anions on the synaptosomal uptake of NE, GABA, glycine, serotonin, and choline (Kuhar and Zarbin, 1978). Thus, phosphate, citrate, and acetate are unable to stimulate the accumulation of these ^3H substances, while bromide can substitute for chloride, though iodide and fluoride are ineffective. Furthermore, the transport of NE is maximal at 50 mM chloride and half-maximal at about 6 mM chloride (Sanchez-Armass and Orrego, 1977).

The mechanisms accounting for the differences in the anion regulation of [^3H]imipramine and [^3H]DMI binding are unclear. While sodium-dependent binding of radioligands to serotonin (Briley and Langer, 1981), GABA (Enna and Snyder, 1975), and glutamate (Baudry and Lynch, 1979; Vincent and McGeer, 1980) uptake recognition sites have been reported, GABA is the only other transmitter whose uptake recognition site has been shown to be regulated by anions. A preliminary report describes a chloride requirement for the sodium-dependent binding of [^3H]GABA associated with GABA uptake (Krause et al., 1981).

The extent of dependence of [^3H]DMI binding on the cation sodium is similar to that on the anion chloride. It has been suggested that the sodium dependence of NE uptake reflects a linkage of the NE uptake pump to the sodium potassium ATPase system (White, 1976). Our observation (C. M. Lee, J. A. Javitch, and S. H. Snyder, unpublished data) that ouabain (1 to 100 μM) fails to affect [^3H]DMI binding suggests that the sodium potassium ATPase might not be responsible for the sodium dependency of [^3H]DMI binding. The absolute dependence of [^3H]DMI binding on sodium and chloride certainly suggests a linkage of this binding site to ionic sites which could be of importance for the NE uptake process.

DSP-4 appears to be a valuable tool for examining the role of NE neurons in various brain processes. Earlier studies suggested that its neurotoxic effects might derive from alkylation of NE uptake sites (Ross, 1976). Such a conclusion is supported by our preliminary evidence that, after administration *in vivo*, DSP-4 depletes [^3H]DMI binding much earlier than it reduces the enzyme markers of NE neurons (data not shown). These observations suggest that the NE uptake mechanism may be crucial for adrenergic neuronal functioning, with the loss of the uptake recognition sites subsequently resulting in destruction of the neuron.

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