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[^{3}H]Nisoxetine: A Radioligand for Quantitation of Norepinephrine Uptake Sites by Autoradiography or by Homogenate Binding[1]

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ABSTRACT

The uptake sites for norepinephrine (NE) in brain have not been studied in much detail, probably due to the absence of an adequate radioligand for labeling these sites. This study describes the binding properties of [^{3}H]nisoxetine to uptake sites for NE in rat brain homogenates and in tissue slices analyzed by quantitative autoradiography. The binding of [^{3}H]nisoxetine was found to be saturable and sodium-dependent to a single class of binding sites (K_d = 0.8 nM). The potencies of drugs to inhibit the uptake of NE correlated highly with their potencies to inhibit the binding of [^{3}H]nisoxetine. Studies using [^{3}H]nisoxetine for mapping of sites associated with uptake of NE by quantitative autoradiography indicated that the pattern of binding of [^{3}H]nisoxetine is consistent with the pattern of noradrenergic innervation. Destruction of central noradrenergic neurons by 6-OH-dopamine or DSP-4 resulted in large decreases in the binding of [^{3}H]nisoxetine in almost all areas of the brain regions examined. [^{3}H]Nisoxetine should prove to be a useful tool to study the regulation of uptake sites for NE as well as a useful marker for noradrenergic innervation in the study of various neurological diseases.

Attempts to label uptake sites for NE have not met with the same success associated with the labeling of the uptake site for serotonin (5-HT). In the early 1980s, [^{3}H]DMI was reported by several investigators to bind to uptake sites for NE in rat brain (Lee and Snyder, 1981; Rehavi *et al.*, 1981). However, [^{3}H]DMI exhibits a high amount of nonspecific binding (Lee and Snyder, 1981; Lee *et al.*, 1982; Slater *et al.*, 1982; Rapp *et al.*, 1987; Backstrom *et al.*, 1989) and binds to a low affinity site in addition to a high affinity one (Lee and Snyder, 1981; Lee *et al.*, 1982; Backstrom *et al.*, 1989). [^{3}H]MAZ has also been used to label uptake sites for NE in the rat brain (Javitch *et al.*, 1984, 1985). However, [^{3}H]MAZ also binds with high affinity to DA uptake sites (Javitch *et al.*, 1984, 1985), necessitating the use of selective inhibitors of the uptake of DA when measuring uptake sites for NE.

NIS [N-methyl-3-(*o*-methoxyphenoxy)-3-phenylpropylamine] (fig. 1) is a potent and selective inhibitor of the uptake of NE, being approximately 1000-fold more potent in blocking the uptake of NE than that of serotonin and 400-fold more potent in blocking the uptake of NE than that of dopamine (Wong and Bymaster, 1976; Wong *et al.*, 1982; Richelson and Pfenning, 1984). Furthermore, NIS has little or no affinity for many neurotransmitter receptors (Richelson and Nelson, 1984; Wander *et al.*, 1986). The relatively simple chemical structure of NIS coupled with the above pharmacological characteristics would make radiolabeled NIS a suitable candidate for use in studies associated with the measurement of uptake sites for NE. [^{11}C]-labeled NIS was synthesized by Haka and Kilbourn (1989) and the regional distribution *in vivo* of [^{11}C]NIS in mice was reported. Their data indicated that because nonspecific binding of [^{11}C]NIS was high, it would limit the use of [^{11}C]NIS as a ligand for positron emission tomography. By contrast, in a preliminary report (Tejani-Butt *et al.*, 1990) we found that [^{3}H]NIS is a suitable radioligand for labeling uptake sites for NE in rat brain homogenates. This study further characterizes the binding of [^{3}H]NIS in rat brain and demonstrates that it is not only useful for measuring uptake sites for NE in homogenates, but also in tissue slices by quantitative autoradiography.

Methods

Subjects. Male Sprague-Dawley rats (Ace Animals, Boyertown, PA) weighing 225 to 275 g were housed in group cages in a room maintained at constant temperature and humidity and illuminated 12 hr/day. Rats had free access to food and water.

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ABBREVIATIONS: DSP-4, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine; DA, dopamine; 6-OHDA, 6-hydroxydopamine; [^{3}H]NIS, [^{3}H]nisoxetine; B_{max}, maximum binding site density; K_d, concentration of ligand at half-maximal saturation; NE, norepinephrine; [^{3}H]DMI, [^{3}H]desipramine; [^{3}H]MAZ, mazindol; 5,7-DHT, 5,7-dihydroxytryptamine.

Homogenate studies. In studies using homogenates, rats were decapitated and brains removed rapidly and dissected on ice. Cerebral cortex was homogenized for 15 sec in 30 volumes of ice-cold buffer (50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM KCl) and centrifuged at 40,000 × *g* for 10 min at 4°C. The pellet was resuspended in 30 volumes of buffer and centrifuged. This procedure was repeated twice with the final pellet being suspended in 30 volumes of ice-cold buffer (50 mM Tris, pH 7.4, 300 mM NaCl, 5 mM KCl; incubation buffer).

The reaction mixture consisted of 400 µl of homogenate (300–550 µg of protein); 50 µl of [^{3}H]NIS and 50 µl of buffer or MAZ (1 µM to define nonspecific binding) or another competing drug. After an incubation time of 4 hr at 0°C, the reaction was terminated by the addition of 5 ml of ice-cold incubation buffer to each tube and the membranes were collected under vacuum onto glass fiber filters (Schleicher and Schuell, #25). Radioactivity collected on the filters was measured by liquid scintillation spectrometry at 50% efficiency.

The kinetics of dissociation of [^{3}H]NIS from its binding site(s) was studied by the addition of 1 µM MAZ after binding had reached steady state. The reaction was then stopped at different times by the method described above. The observed association rate constant, K_{obs} was obtained from a plot of ln ($B_{max}/B_{max} - B$) *vs.* time *t*, where B_{max} is the amount of [^{3}H]NIS specifically bound at equilibrium and *B* is the amount of [^{3}H]NIS specifically bound at time *t* (Limbird, 1985). The first-order rate constant for dissociation, K_2, was obtained from a plot of ln (B/B_o) *vs.* time *t*. The initial rate constant for association, K_1, was obtained from the equation $K_1 = (K_{obs} - K_2)/\mathrm{D}$, in which D represents the free concentration of [^{3}H]NIS.

Autoradiographic Studies

Preparation of tissue sections. Rats were decapitated and brains removed rapidly and dipped into ice-cold saline. Attached meninges and external blood vessels were removed carefully. Brains were dipped into cold 2-methylbutane (−25°C) for 5 sec (to maintain gross morphology) and then placed onto powdered dry ice for 10 min. Brains were stored at −80°C until sectioning. Coronal sections of brain (20 µm) were cut using a cryostat microtome (Reichert Histostat, Reichert Scientific Instruments, Buffalo, NY). Sections were taken at different levels: plates 18, 25, 30, 49 and 57 according to the Stereotaxic Atlas of the Rat Brain (Paxinos and Watson, 1986). Sections were thaw-mounted onto gelatin-coated microscopic slides and dried under vacuum for 12 hr.

Incubation procedure. Duplicate sections were incubated with [^{3}H]NIS in the presence or absence of competing compounds in Tris buffer (Tris 50 mM; NaCl 300 mM; KCl 5 mM, pH 7.4). Nonspecific binding was defined using 1 µM mazindol. After incubation, the sections were washed three times in fresh ice-cold buffer for 5 min each, and dipped into ice-cold water to remove buffer salts. Sections and calibrated standards were exposed against LKB ^{3}H-Ultrofilm (LKB Instruments, Gaithersburg, MD) at room temperature for 4 to 8 weeks to generate the autoradiograms or the sections were wiped off the glass slides individually with Whatman No. 1 filter disks and their radioactivity counted using a liquid scintillation counter (Searle Isocap, Des Plaines, IL). The autoradiograms were analyzed on a DUMAS (Drexel Unix-based Microcomputer Image Analysis System) densitometer using the BRAIN software package (Feingold *et al.*, 1986). Plastic-embedded tritium standards (American Radiolabeled Chemicals, St. Louis, MO) calibrated using brain-mash sections according to the method of Geary and Wooten (1985) were used for quantitation. The sections were stained with thionin and the brain areas were identified using the atlas by Paxinos and Watson (1986).

Lesion Studies

Norepinephrine system. 6-OHDA. Male Sprague-Dawley rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and placed on a stereotaxic instrument (David Kopf, Tujunga, CA). The intraventricular injections were performed by lowering the tip of a 25-µl Hamilton syringe stereotaxically to a point within the lateral ventricle, 4 mm below the skull and 1.5 mm lateral to the midline at bregma (Uretsky and Iversen, 1970). 6-OHDA (200 µg/15 µl) dissolved in 0.9% saline containing 0.1% ascorbic acid was delivered into the right lateral ventricle over a period of 5 min. The wounds were closed with autoclips. Control rats received vehicle under the same conditions. The rats were sacrificed by decapitation 14 days later and the brains removed for autoradiographic studies. Initial experiments were done in which rats were given bupropion (100 mg/kg, i.p.) 30 min before surgery, in order to protect DA neurons from the neurotoxic effects of 6-OHDA (Cooper *et al.*, 1980). However, this pretreatment resulted in a <20% survival rate and was therefore discontinued.

DSP-4. Male Sprague-Dawley rats were injected i.p. with 0.5 ml of DSP-4 (50 mg/kg) freshly prepared in saline (Ross, 1976). Control rats received the same volume of saline. The rats were sacrificed 3 days later and the brains assayed for binding of [^{3}H]NIS by quantitative autoradiography.

Serotonin system. Male Sprague-Dawley rats were given 25 mg/kg DMI i.p. 45 min before surgery in order to protect noradrenergic neurons from the neurotoxic effects of 5,7-DHT (Bjorklund *et al.*, 1975). The rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and placed on the stereotaxic instrument (David Kopf, Tujunga, CA). The intraventricular injections were performed by lowering the tip of a 25-µl Hamilton syringe stereotaxically to a point within the lateral ventricle, 4 mm below the skull and 1.5 mm lateral to the midline at bregma. Ten µl of 5,7-DHT (100 µg) were infused in 0.1% ascorbic acid in saline over 5 min. The injections were done bilaterally so that the total dose was 200 µg/brain. The wounds were closed with autoclips. Control animals were injected with the vehicle alone under the same experimental conditions. The rats were sacrificed by decapitation 6 days later and the brains removed for autoradiographic studies.

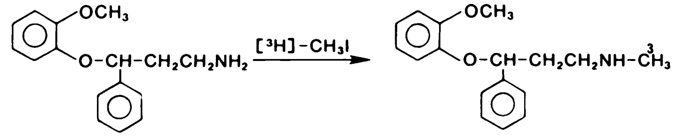
Protein measurement. Protein was measured by the method of Lowry *et al.* (1951).

Statistics. Saturation and competition experiments were analyzed by nonlinear regression analysis using RS1 (BBN Research Systems, Cambridge, MA). K_i values were obtained for competing drugs by the method of Cheng and Prusoff (1973). Data obtained from quantitative autoradiography of [^{3}H]NIS binding following treatment with 6-OHDA, DSP-4 or 5,7-DHT were analyzed by Student's *t* test. The level of significance was set a priori at $P < .01$. Results are expressed as the mean ± S.E.M.

Materials used. [^{3}H]NIS was synthesized by Amersham Corporation (Arlington Heights, Il) with a specific activity of 82.6 Ci/mmol. Drugs were obtained from the following sources: DSP-4 and GBR-12909 (Research Biochemicals Incorporated, MA), desmethylimipramine hydrochloride (Merrell Dow Pharmaceuticals, Cincinnati, OH), sertraline (Pfizer Pharmaceuticals Inc., Groton, CT) fluoxetine and NIS (Eli Lilly and Co., Indianapolis, IN), protriptyline (Merck, Sharp and Dohme Research Labs, West Point, PA), diazepam (Roche, Inc., Nutley, NY), haloperidol (McNeil Laboratories, Inc., Fort Washington, PA), maprotiline, oxaprotiline and phentolamine (Ciba-Geigy Corp., Summit, NJ), scopolamine (A. H. Robins, Richmond, VA), 5,7-DHT creatine sulfate, 6-hydroxydopamine, (−)norepinephrine, mazindol, L-propranolol hydrochloride and cocaine were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Kinetic properties of the binding of [^{3}H]NIS in cortical homogenates. The initial experiment measured the time for



[^{3}H]-NISOXETINE

Fig. 1. Chemical structure of [^{3}H]NIS [^{3}H-(±)N-methyl-3-(0-methoxyphenoxy)-3-phenylpropylamine].

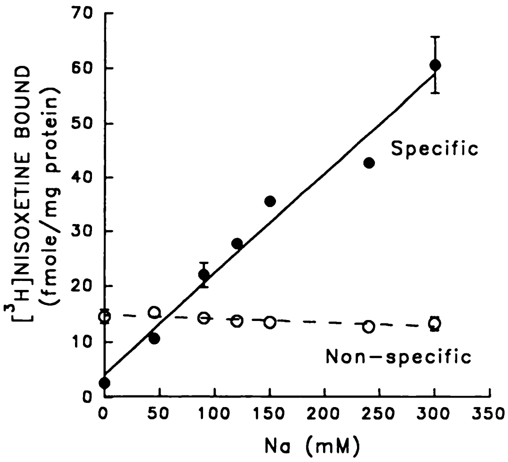


Fig. 2. Effect of sodium cations on the binding of [^{3}H]NIS (2.0 nM) to rat cortical membranes. Nonspecific binding was defined using 1 μM MAZ. Each point represents the mean ± S.E.M. of three separate experiments performed in triplicate. For some points, error bars are not seen because they were too small to be visualized.

the binding of [^{3}H]NIS (1 nM) to reach equilibrium at 0°C. Equilibrium was reached in about 2 hr and remained unchanged up to 6 hr. An incubation time of 4 hr at 0°C was chosen for all subsequent experiments. At this time and concentration of radioligand, specific binding (defined by 1 μM mazindol) represented about 85% of total binding (Tejani-Butt *et al.*, 1990).

The specific binding of [^{3}H]NIS was dependent on the concentration of sodium cations (Tejani-Butt *et al.*, 1990). Both total and specific binding of [^{3}H]NIS increased as the concentration of Na^+ was raised; by contrast, nonspecific binding remained unchanged and was independent of the concentration of Na^+ (fig. 2). In the absence of Na^+, specific binding was almost undetectable.

Under these conditions, we have shown previously that [^{3}H]NIS binds with high affinity to a single class of saturable sites (Tejani-Butt *et al.*, 1990). Analysis of four saturation isotherms on cerebral cortical homogenates using [^{3}H]NIS concentrations ranging from 0.1 to 12 nM, yielded a K_d of 0.80 ± 0.11 nM and a B_{max} of 97 ± 12 fmol/mg protein.

The graph of the time course of association of [^{3}H]NIS (1.0 nM) (plotted as ln $B_{max}/B_{max} - B$ *vs.* time) is shown in figure 3, A and B. The slope of the straight line obtained is the observed rate constant (K_{obs}) of the association of [^{3}H]NIS, assuming simple reversible bimolecular binding kinetics (see Limbird, 1985). Results of three separate experiments yielded a K_{obs} value of 0.014 ± 0.001 min^{-1}. Dissociation of [^{3}H]NIS from its binding sites was initiated by the addition of mazindol (1 μM) to the incubation medium after equilibrium had been reached. The time course of dissociation is shown in figure 3, C and D. The negative slope of the straight line obtained is the

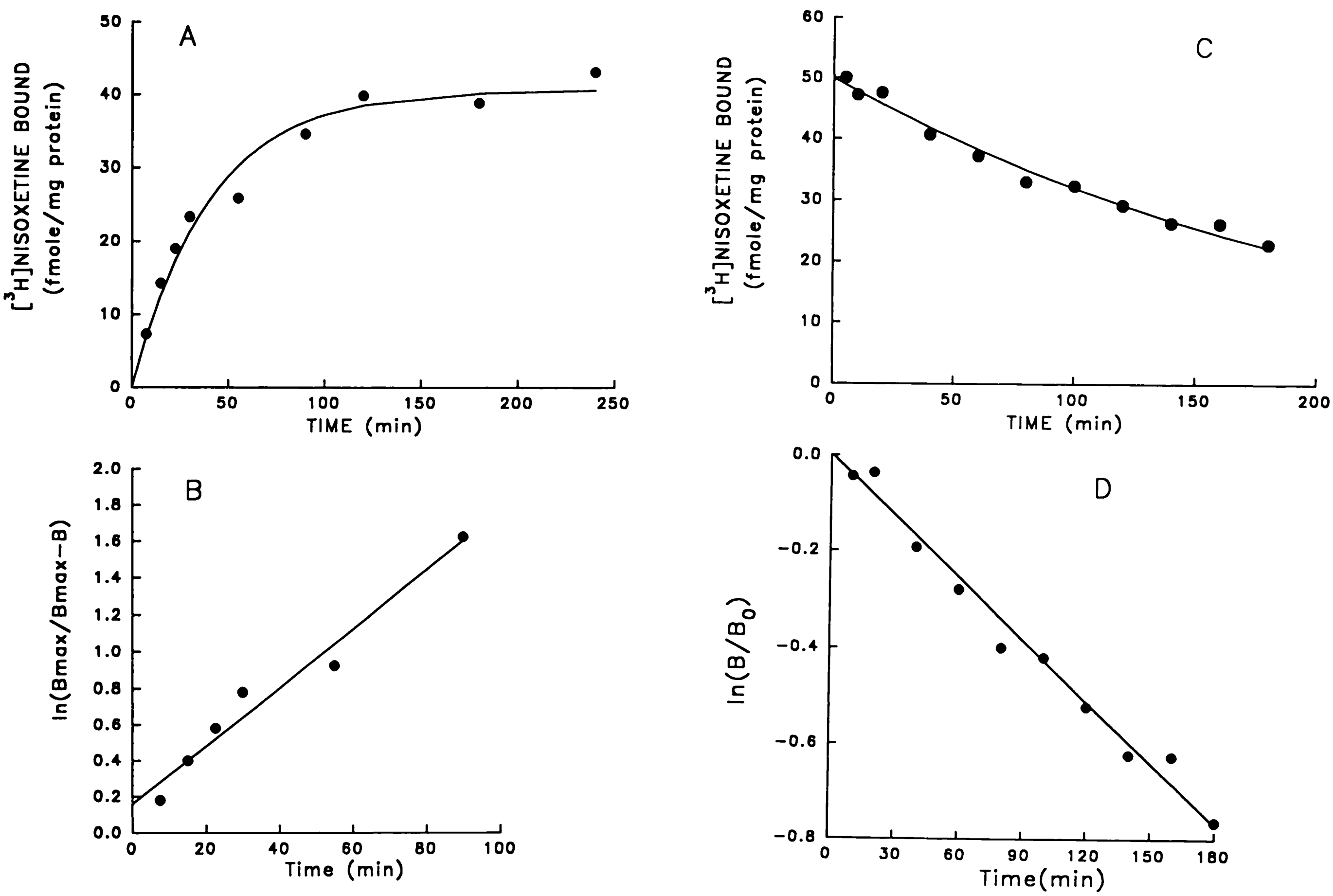


Fig. 3. Kinetic analyses of association (A and B) and dissociation (C and D) time course of specific binding of [^{3}H]NIS (1.0 nM) to rat cortical membranes. Total and nonspecific binding (defined by 1 μM MAZ) were determined at the indicated time intervals to yield specific binding. The data were plotted according to pseudo-first order reaction kinetics and the lines fitted to the data points by linear regression analysis. The points are shown from a typical experiment that was performed three times.

dissociation rate constant K_2. Results of three separate experiments yielded a K_2 value of 0.005 ± 0.0009 min^{-1}. The rate constant of association (K_1) was 0.009 $M^{-1} \cdot min^{-1}$. The calculated equilibrium dissociation constant (K_d) determined kinetically from the ratio of the rate constant for dissociation to the rate constant for association was 0.60 nM. This value is in agreement with the K_d value obtained in saturation experiments, 0.80 nM.

Pharmacological specificity. The ability of various drugs to compete for the binding of [^{3}H]NIS (2.0 nM) to rat cortical membranes was studied. Displacement curves for desipramine, mazindol, nisoxetine, maprotiline and protriptyline are shown in figure 4. The affinities and the Hill coefficients of the compounds for inhibiting the binding of [^{3}H]NIS are shown in table 1. Drugs known to have high affinity for NE uptake sites, such as desipramine, mazindol, nisoxetine, maprotiline, protriptyline and (+)oxaprotiline, were potent inhibitors of the binding of [^{3}H]NIS. Cocaine, which is a relatively weak inhibitor of NE uptake (Koe, 1976), was inactive at inhibiting the binding of [^{3}H]NIS at concentrations up to 1 μM. (−)Oxaprotiline was also inactive at inhibiting the binding of [^{3}H]NIS at concentrations up to 1 μM. Compounds that are reported to bind selectively to 5-hydroxytryptamine or DA uptake sites (fluoxetine, sertraline and GBR 12909) had no effect at inhibiting the binding of [^{3}H]NIS at concentrations up to 1 μM. Compounds that bind to various post-synaptic receptors (1-propranolol for *beta* adrenoceptors; prazosin for *alpha*-1 adrenoceptors; phentolamine for *alpha*-1 and -2 adrenoceptors; scopolamine for muscarinic receptors; haloperidol for DA-2 receptors; diazepam for benzodiazepine receptors and promethazine for histamine-1 receptors) were all found to be inactive at inhibiting the binding of [^{3}H]NIS at concentrations up to 1 μM.

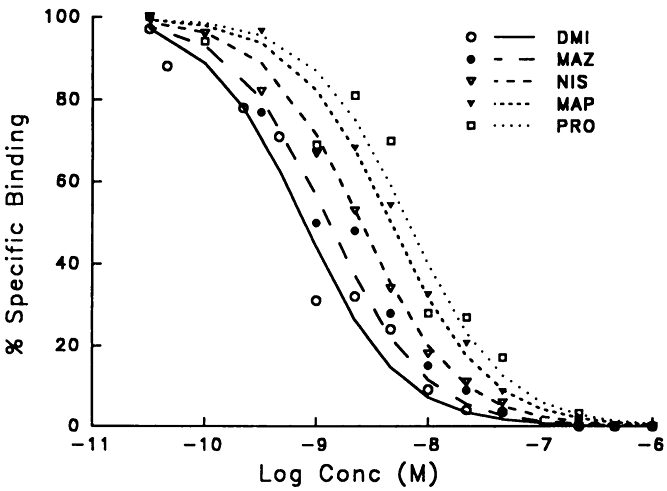


Fig. 4. Displacement of [^{3}H]NIS (2.0 nM) binding by desipramine, MAZ, NIS, maprotiline and protriptyline in rat cortical membranes. Specific binding was obtained by subtracting nonspecific binding in the presence of 1 μM MAZ from total binding. The data are expressed as a percentage of specific binding. The figure represents one of four experiments performed.

TABLE 1

Drug inhibitory potencies for [^{3}H]NIS binding in rat cerebral cortex

Drug effects were examined on specific [^{3}H]NIS binding as described under "Methods." K_i values were determined by Cheng and Prusoff equation. The values are the mean ± S.E.M. of four separate experiments performed in triplicate.

Drugs	[^{3}H]NIS K_i (nM ± S.E.M.)	Hill Coefficient (Mean ± S.E.M.)
Desipramine	0.2 ± 0.08	0.88 ± 0.03
Mazindol	0.4 ± 0.003	0.80 ± 0.05
Nisoxetine	0.8 ± 0.1	0.85 ± 0.03
Maprotiline	1.8 ± 0.25	0.90 ± 0.05
(+)Oxaprotiline	3.4 ± 0.4	0.96 ± 0.04
(−)Oxaprotiline	2000 ± 225	
Protriptyline	4.0 ± 0.6	1.1 ± 0.05
Cocaine	a	
(−)Norepinephrine	a	
Fluoxetine	a	
Sertraline	a	
GBR 12909	a	
Scopolamine	a	
L-Propranolol	a	
Phentolamine	a	
Haloperidol	a	
Diazepam	a	
Promethazine	a	
Prazosin	a	

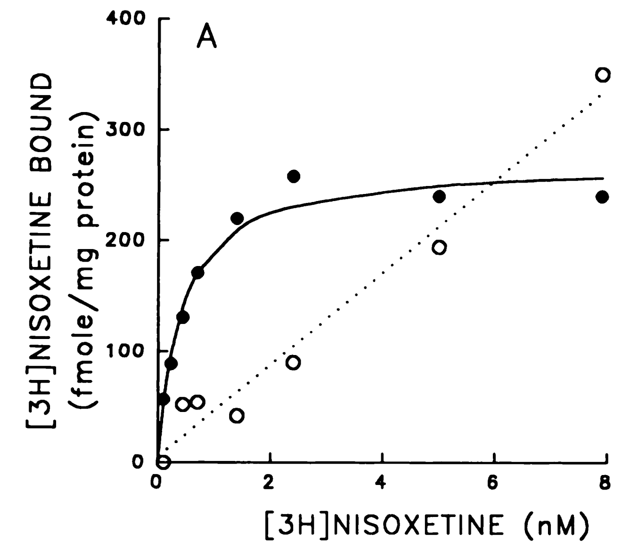
[a] The following compounds were inactive at inhibiting the binding of [^{3}H]NIS at concentrations up to 1 μM.

Autoradiography studies. Preliminary experiments were performed to determine the optimal conditions for measuring the binding of [^{3}H]NIS to slide-mounted tissue sections by liquid scintillation counting. The optimal wash time was determined by incubating the sections with [^{3}H]NIS (3.0 nM) for 4 hr and washing for various times to remove unbound ligand. Sections were washed two, three or four times for either 2, 5 or 10 min. Some tissue sections were incubated with [^{3}H]NIS in the presence of mazindol (1 μM) to define nonspecific binding. The decrease in tissue radioactivity seen with three washes for 5 min each was due to a reduction in nonspecific binding with little or no change in specific binding (data not shown); consequently, a wash time of 5 min (three washes) was chosen for further experiments. The optimal time to reach equilibrium was next examined at ligand concentrations of 0.5 and 3.0 nM. Maximum binding was reached by 1 hr at a radioligand concentration of 3.0 nM, but reached equilibrium by 3 hr at ligand concentration of 0.5 nM (data not shown). At both radioligand concentrations, the binding remained stable up to 6 hr. An incubation time of 4 hr was chosen for subsequent experiments.

Saturation analysis of [^{3}H]NIS binding. Using the technique of quantitative autoradiography, saturation curves were obtained for the binding of [^{3}H]NIS (0.1–8.0 nM) in the frontoparietal cortical region in rat brain slices taken approximately at the level of plate 30 (Paxinos and Watson, 1986). Computer analysis of the data using nonlinear regression analysis indicated that the binding was to a single class of binding sites (K_d = 0.73 ± 0.28 nM; B_{max} = 280 ± 9.4 fmol/mg protein, fig. 5A). Scatchard transformation of the data gave no indication of a second site being present up to a ligand concentration that was 11 times greater than the K_d value (fig. 5B) The K_d value obtained by quantitative autoradiography was similar to that obtained using homogenates.

Stereoselectivity of [^{3}H]NIS binding. The ability of the stereoisomers of oxaprotiline to displace [^{3}H]NIS binding to slide-mounted sections was examined (fig. 6). Ki values obtained were 3.0 ± 0.13 nM for the pharmacologically more active (+)isomer and 2,400 ± 170 nM for the weaker (−)isomer of oxaprotiline. The affinities obtained for the stereoisomers by the slide wipe technique were similar to those obtained in cerebral cortical membrane preparations (see table 1).

Autoradiographic localization. To assess the anatomic relationship of the binding of [^{3}H]NIS to noradrenergic neu-



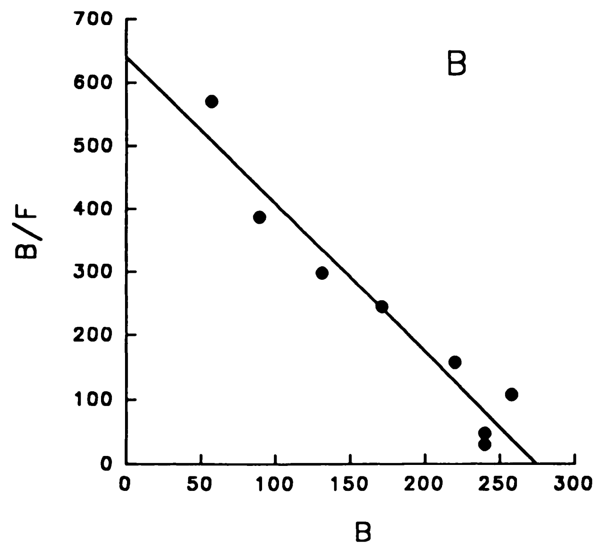


Fig. 5. A) Saturation analysis of [^{3}H]NIS binding in the frontoparietal region of the cortex in rat brain slices by quantitative autoradiography. Specific binding is shown by filled circles and nonspecific binding by (○). Specific binding is defined as the difference between binding in the presence and absence of 1 μM MAZ. Points shown are from a typical experiment which was performed four times in duplicate. B) Scatchard plot of the specific binding.

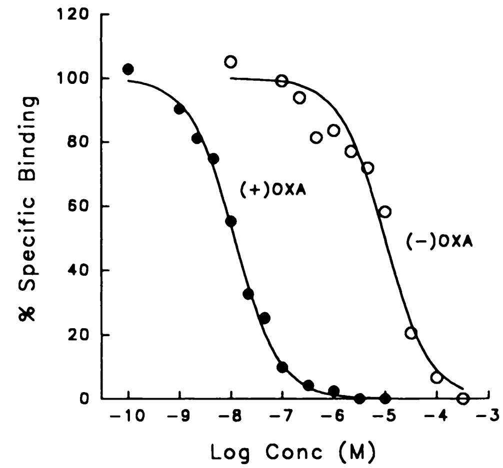


Fig. 6. Displacement of [^{3}H]NIS (3.0 nM) binding by the stereoisomers of oxaprotiline on slide-mounted sections. Results are based on total radioactivity per section as determined by liquid scintillation spectroscopy. Specific binding was calculated by subtracting nonspecific binding in the presence of 1 μM MAZ from total binding. The data are expressed as a percentage of specific binding. K_i values were 3.0 ± 0.13 nM for the (+)isomer and 2.4 ± 1.7 μM for the (−)isomer. The figure represents one of four experiments performed.

TABLE 2

Regional distribution of specific binding of [^{3}H]NIS to NE uptake sites in rat brain

Autoradiograms were quantitated using the Drexel University Image Analysis System (DUMAS). Binding of [^{3}H]NIS (3.0 nm) and nonspecific binding (defined by 1 μM mazindol) was determined as described under "Methods." Each value is the mean ± S.E.M. of measurements from four rats with determinations made in duplicate sections from each brain.

Brain Region	[^{3}H]NIS Binding
	(fmol/mg protein)
Locus coeruleus	1526 ± 39
Anteroventral thalamic n., dorsomedial	1444 ± 147
Bed n. of the stria term., lat. div., ventral	1348 ± 57
Dorsomedial hypothalamic n.	1062 ± 37
Paraventricular hypothalamic n.	847 ± 81
Paraventricular thalamic n.	845 ± 46
Dentate gyrus	716 ± 40
Medial zona incerta	672 ± 50
Dorsal raphe	639 ± 27
Mammillothalamic tract	544 ± 86
Bed n. of the stria term., lat. divl., dorsal	529 ± 10
Median raphe	500 ± 77
Basolateral amygdaloid n.	497 ± 30
$CA_{2,3}$ layers of hippocampus	375 ± 32
Frontoparietal cortex	358 ± 25
Ventral posterior thalamic n.	321 ± 21
Central amygdaloid n.	309 ± 42
Centromedial thalamic n.	307 ± 10
Ventromedial hypothalamic n.	301 ± 12
Lateral dorsal thalamic n.	297 ± 26
Lateral amygdaloid n.	267 ± 18
Centrolateral thalamic n.	205 ± 7
CA_1 layer of hippocampus	204 ± 15
Medial amygdaloid n.	180 ± 10
Caudate putamen	54 ± 11

ronal systems, the autoradiographic localization of [^{3}H]NIS binding to rat brain sections was evaluated. Table 2 shows the level of specific binding of [^{3}H]NIS (3.0 nM) in different brain regions. The specific binding of [^{3}H]NIS is highly heterogenous in the rat brain (table 2). Nonspecific binding (as defined by 1 μM MAZ) was constant but low (not more than 50–75 fmol/mg protein throughout the brain). The highest density of binding of [^{3}H]NIS of all areas examined was found in the locus coeruleus. The anteroventral nucleus of the thalamus, ventral portion of the bed nucleus of the stria terminalis and dorsomedial nucleus of the hypothalamus were also densely labeled. The paraventricular nucleus of the hypothalamus, the paraventricular nucleus of the thalamus, dentate gyrus and medial zona incerta also showed high levels of binding. Interestingly, relatively high binding was observed in the area of dorsal raphe nucleus. The mammillothalamic tract, dorsal aspect of the bed nucleus of stria terminalis, median raphe nucleus and basolateral nucleus of the amygdala showed moderate levels of binding. The $CA_{2,3}$ layer of the hippocampus, frontal cortex, ventral posterior nucleus of the thalamus, central nucleus of the amygdala, centromedial nucleus of the thalamus, ventromedial nucleus of the hypothalamus, lateral dorsal nucleus of the thalamus and lateral nucleus of the amygdala showed relatively low levels of binding. The lowest levels of binding were seen in the centrolateral nucleus of the thalamus, CA_1 layer of the hippocampus, medial nucleus of the amygdala and the caudate putamen. Representative autoradiograms showing the high binding in the locus coeruleus (plate 57) and the heterogenous distribution of binding at plate 30 are shown in figure 7, A and B.

Effect of noradrenergic neuronal lesions on the binding of [^{3}H]NIS. Treatment with 6-OHDA resulted in a very marked reduction of the binding of [^{3}H]NIS (3.0 nM) in all brain regions examined (fig. 8). 6-OHDA produced a nearly complete loss of binding in the cortex, hippocampus, thalamus, amygdala and hypothalamus (plate 30) and also caused a marked reduction in binding of [^{3}H]NIS in the locus coeruleus (plate 57). Representative autoradiograms showing loss of binding after treatment with 6-OHDA are shown in figure 9, A and B.

Treatment with DSP-4 has been previously reported to selectively destroy noradrenergic terminals without any effect on serotonergic or dopaminergic neurons in the rat brain after peripheral administration (Ross, 1976). Treatment with this selective noradrenergic neurotoxin resulted in significant decreases in the binding of [^{3}H]NIS (3.0 nM) in many areas examined (fig. 10). Similar to the effects of 6-OHDA, treatment with DSP-4 produced a nearly complete loss of binding in the cortex, hippocampus, thalamus and the amygdala (except for the central nucleus). The decreases seen in the hypothalamus were much less than those produced in the cortex or hippocampus and much less than those caused by 6-OHDA. DSP-4 did not cause a reduction in binding of [^{3}H]NIS in the locus coeruleus. Representative autoradiograms showing the effect of DSP-4 are shown in figure 11, A and B.

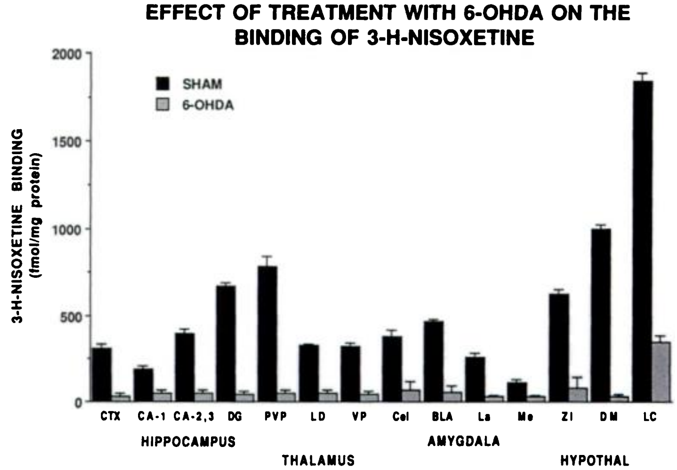


Fig. 8. Effect of 6-OHDA lesions on specific binding of [^{3}H]NIS (3.0 nM) in rat brain slices taken at the level of plates 30 and 57 by quantitative autoradiographic technique. Each bar and bracket represents the mean ± S.E.M. of four to six rats in each group. Nonspecific binding was defined by 1 μM MAZ. A significant effect of the lesion was seen in all brain regions (Student's t test, $P < .01$). Regions of the brain analyzed and their abbreviations are: frontoparietal cortex, (CTX); CA-1, CA-2,3 layers of the hippocampus; dentate gyrus (DG); paraventricular (PVN), lateral dorsal (LD), ventral posterior (VP) nuclei of the thalamus; central (Cel), basolateral (BLA), lateral (La), medial (Me) nuclei of the amygdala; zona incerta (ZI), dorsomedial (DM) nuclei of the hypothalamus and locus coeruleus (LC).

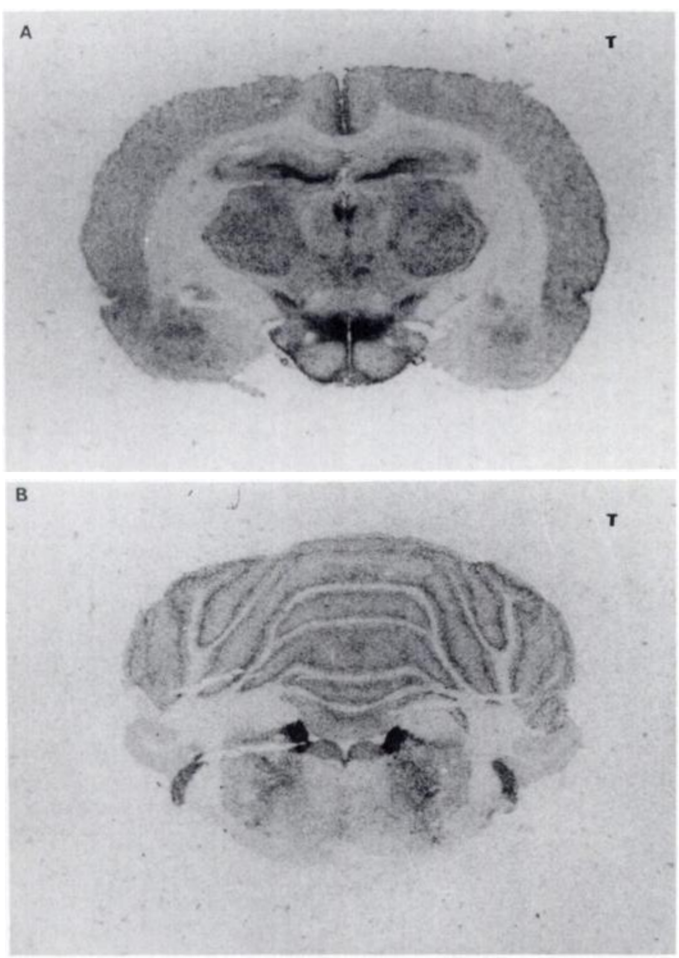


Fig. 7. Autoradiographic images of [^{3}H]NIS binding to rat coronal brain slices taken at the levels of plate 30 (A) and 57 (B) (Paxinos and Watson, 1986). Nonspecific binding was defined in the presence of 1 μM MAZ and was less than 15% throughout the brain regions analyzed.

Effect of serotonergic neuronal lesions on the binding of [^{3}H]NIS. Injections of 5,7-DHT into the lateral ventricle had no effect on the binding of [^{3}H]NIS (3.0 nM) in any of the 15 brain regions examined at the level of plate 30 (data not shown). By contrast, in these same rats the binding of [^{3}H] cyanoimipramine to the 5-hydroxytryptamine uptake site (Kovachich *et al.,* 1988) was reduced very substantially (80%) throughout the brain (unpublished observations).

Discussion

The results described in this study demonstrate that [^{3}H] NIS is a suitable ligand for measurement *in vitro* of uptake sites for NE. [^{3}H]NIS binds with high affinity to a single class of sites having the pharmacological characteristics of the uptake site for NE; the binding of [^{3}H]NIS is dependent on the presence of Na^+ ions (Iversen and Kravitz, 1966); [^{3}H]NIS binding is lost upon destruction of NE neurons; desipramine, mazindol, nisoxetine, maprotiline, (+)oxaprotiline and protriptyline inhibited the binding of [^{3}H]NIS with high affinities. The respective K_i values agreed within an order of magnitude with the literature values for the ability of these compounds to inhibit the uptake of [^{3}H]NE (Hyttel, 1982; Lee *et al.,* 1982). Similarly, compounds that failed to inhibit the binding of [^{3}H] NIS also had poor ability to inhibit the uptake of [^{3}H]NE (Hyttel, 1982; Lee *et al.,* 1982).

The regional distribution of binding sites for [^{3}H]NIS in rat brain (see table 2) is in good agreement with the distribution of NE terminals (Dahlstrom and Fuxe, 1964; Brownstein *et al.,* 1974; Palkovits *et al.,* 1974; Versteeg *et al.,* 1976). Interestingly,

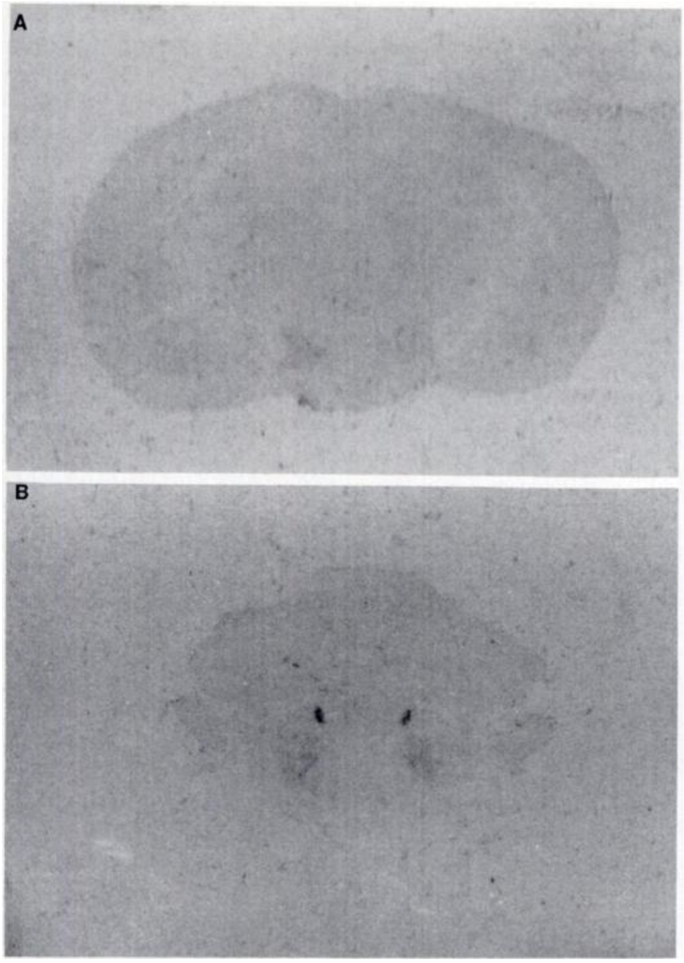


Fig. 9. Autoradiographic images of [^{3}H]NIS binding to rat coronal brain slices taken at the levels of plates 30 (A) and 57 (B) after treatment with 6-OHDA.

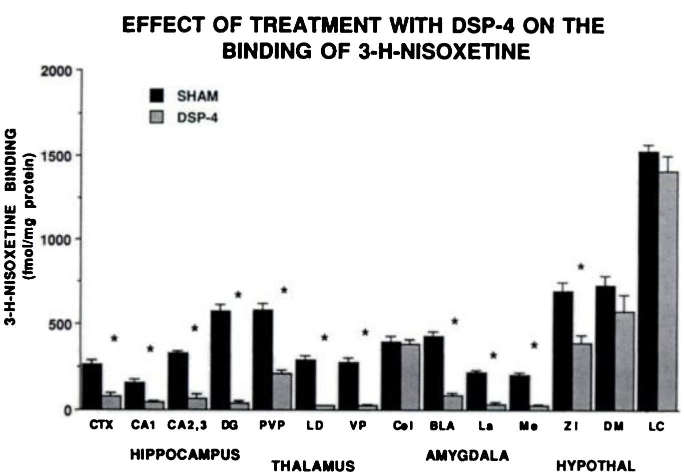


Fig. 10. Effect of DSP-4 lesions on specific binding of [^{3}H]NIS (3.0 nM) in rat brain slices taken at the level of plates 30 and 57 by quantitative autoradiography. Each bar and bracket represents the mean ± S.E.M. of four to six rats in each group. Nonspecific binding was defined by 1 μM MAZ. A significant effect of the lesion was seen in most of the brain regions analyzed (Student's *t* test; *$P < .01$).

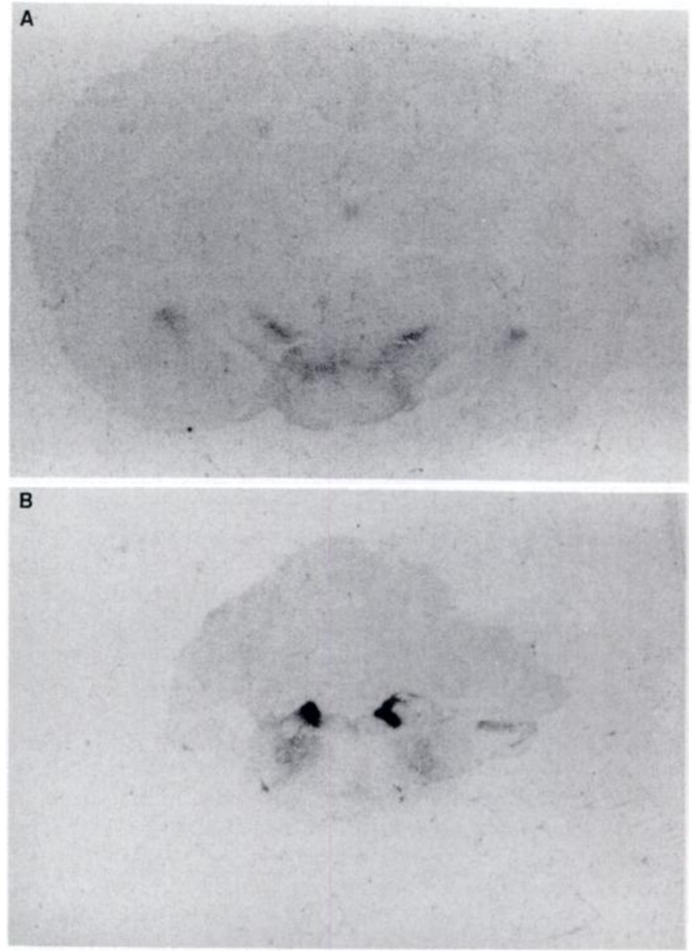


Fig. 11. Autoradiographic images of [^{3}H]NIS binding to rat coronal brain slices taken at the levels of plates 30 (A) and 57 (B) after treatment with DSP-4.

the highest concentration of binding sites for [^{3}H]NIS were found in the locus coeruleus, the largest group of NE containing soma in brain (Dahlstrom and Fuxe, 1964; Palkovits and Jacobowitz, 1974). Several nuclei in the limbic system also showed high binding: the ventral portion of the bed nucleus of the stria terminalis, the anteroventral nucleus of the thalamus, the paraventricular nucleus of the hypothalamus and the dorsomedial nucleus of the hypothalamus. Intermediate to high levels of [^{3}H]NIS binding were seen in brain regions such as the dorsal aspect of the bed nucleus of the stria terminalis, the mamillary tract, the dentate gyrus of the hippocampus, the peraventricular nucleus of the thalamus and the medial zona incerta.

The presence of NE within the dorsal raphe nucleus has been demonstrated previously using histofluorescent (Fuxe, 1965), biochemical (Saavedra *et al.*, 1976) and immunocytochemical techniques (Grzanna and Molliver, 1980). Electron microscopic autoradiographic methods have also provided evidence that noradrenergic terminals innervate the 5-hydroxytryptamine neurons within the dorsal raphe nucleus (Baraban and Aghajanian, 1981). Consistent with this, a relatively high concentration of [^{3}H]NIS binding sites in the dorsal raphe nucleus, as well as the median raphe nucleus, was found in this study.

Intermediate to low levels of [^{3}H]NIS binding were observed in brain regions with a lower content of NE (Kobayashi *et al.*, 1974). These regions were the cortex, CA_1, $CA_{2,3}$ layers of the hippocampus, lateral dorsal nucleus, ventral posterior nucleus, centrolateral, and centromedial nucleus of the thalamus, the ventromedial nucleus of the hypothalamus and the amygdaloid nuclei. Using radiochemical assays, Ben-Ari *et al.* (1975) have reported that the central and basolateral nuclei of the amygdala have higher concentrations of NE than the lateral and medial nuclei; the same pattern of [^{3}H]NIS binding in the amygdaloid nuclei was found.

The lowest level of [^{3}H]NIS binding was found in the caudate putamen, a region which receives only a sparse noradrenergic innervation (Brownstein *et al.*, 1974; Swanson and Hartman, 1975).

The projections of noradrenergic pathways to the forebrain are divided into a dorsal and ventral bundle. The dorsal bundle arises from axons of the locus coeruleus (A6), whereas the ventral bundle is formed from axons of the A1, A2, A5 and A7 nuclei (non-locus coeruleus) (Dahlstrom and Fuxe, 1964; see Jacobowitz, 1978). In the cortex, hippocampus and cerebellum, noradrenergic axon terminals arise almost exclusively from the locus coeruleus (see Foote *et al.*, 1983). By contrast, noradrenergic terminals in the ventral forebrain and hypothalamus originate primarily in non-locus coeruleus cells. Sawchenko and Swanson (1982) have found that locus coeruleus neurons constitute only a minority of retrogradely labeled NE neurons from the hypothalamus.

Lesion studies further confirm the association of [^{3}H]NIS binding with presynaptic noradrenergic terminals. Lesioning with 6-OHDA caused a complete loss of [^{3}H]NIS binding in all the brain regions analyzed. Iversen and Uretsky (1970) examined the effects of 6-OHDA on endogenous levels of NE and tyrosine hydroxylase activity in various brain regions of the rat central nervous system. In their study, there was a 95% reduction in NE content and enzyme activity in the cerebral cortex, but only a 50% reduction in the medulla-pons region. It was suggested that the large amounts of NE and tyrosine hydroxylase remaining in the medulla-pons may reflect resistance of cell bodies to the destructive effect of 6-OHDA. Their interpretation agrees with the morphological evidence provided by Bloom *et al.* (1969), suggesting that the perikarya of NE neurons in rat brain survive 6-OHDA treatment. In an autoradiographic study utilizing [^{3}H]proline, Jones and Moore (1977) reported that the compact portion of the locus coeruleus showed a significant loss of neuronal perikarya, whereas the surviving cell bodies were found to be shrunken and pyknotic after 6-OHDA treatment. The dorsal pathway arising from the locus coeruleus was not labeled by [^{3}H]proline; however, a large projection from the ventral tegmental pathway existed, suggesting that 6-OHDA was more neurotoxic to the dorsal pathway. Other studies (see Kostrzewa and Jacobowitz, 1974; McBride *et al.*, 1985; Commins *et al.*, 1989) suggest that depending on the route of administration of 6-OHDA, age of animal (neonatal *vs.* adult) and method of analysis, 6-OHDA may be neurotoxic to both the dorsal pathway as well as the ventral tegmental pathway. The present study, using [^{3}H]NIS to quantitate neuronal degeneration by autoradiography, indicates that 6-OHDA (200 μg) given i.c.v. into adult rats leads to a loss of noradrenergic terminals from both the locus coeruleus as well as the non-locus coeruleus pathways, and also causes a loss of [^{3}H]NIS binding in the locus coeruleus, a cell body area.

Several investigators have reported that the destructive effects of DSP-4 are more pronounced in brain regions that are innervated by projections arising from the locus coeruleus (Loy *et al.*, 1980; Jonsson *et al.*, 1981; Logue *et al.*, 1985; Fritschy and Grzanna, 1989). Previous methods that have been used to assess the neurotoxic effects of DSP-4 do not provide a quantitative measure of the lesion in different regions of the brain. Using the technique of quantitative autoradiography which allowed for quantitation as well as anatomical resolution, the present study looked at the effects of DSP-4 on the binding of [^{3}H]NIS in different regions of the brain.

Lesions with DSP-4 caused a marked loss of [^{3}H]NIS binding in most of the brain regions analyzed, with prominent exceptions being the central nucleus of the amygdala, the dorsomedial nucleus of the hypothalamus and the locus coeruleus. Areas that are severely depleted by DSP-4 include the cortex, hippocampus, thalamus, cerebellum and spinal cord; areas such as the hypothalamus and brain stem appear to be less affected (Jaim-Etcheverry and Zieher, 1980; Jonsson *et al.*, 1981; Hallman and Jonsson, 1984; Logue *et al.*, 1985). Using immunohistochemical analysis of dopamine β-hydroxylase, after DSP-4 treatment, Fritschy and Grzanna (1989) have identified two populations of noradrenergic terminals. Regions depleted of NE are those innervated by locus coeruleus cells. Noradrenergic terminals not affected by DSP-4 treatment as those in the central nucleus of the amygdala and the dorsomedial nucleus of the hypothalamus receive their innervation from the non-locus coeruleus cells. DSP-4 treatment had no effect on the locus coeruleus cell bodies. The pattern of [^{3}H]NIS binding after DSP-4 treatment matches closely the pattern of loss seen with DA-β-hydroxylase immunohistochemistry (Fritschy and Grzanna, 1989).

The distribution of noradrenergic projections from the locus coeruleus has previously been studied by examining changes in the content or uptake of NE using histofluorescence (Anden *et al.*, 1966; Ungerstedt, 1971; Olson and Fuxe, 1972), biochemical methods (Brownstein *et al.*, 1974; Kobayashi *et al.*, 1974; Korf *et al.*, 1973) autoradiography (Segal *et al.*, 1973; Jones and Moore, 1977) and more recently by immunohistochemical analysis of NE or dopamine-β-hydroxylase (Swanson and Hartman, 1975; Grzanna *et al.*, 1989; Fritschy and Grzanna, 1989). Although histofluorescence and immunohistochemical techniques provide anatomical localization of cell bodies and distribution of NE-containing terminals, they do not permit precise quantification of NE levels in different regions of the brain. Biochemical measurements of NE or tyrosine hydroxylase activity do not provide detailed anatomical resolution. Previous autoradiographic techniques involving the injection of tritiated amino acid into the locus coeruleus region (Jones and Moore, 1977) have had problems associated with them; in particular, the exact location of the injection and the precise definition of labeled neurons.

The present study has used quantitative autoradiography to measure [^{3}H]NIS binding to uptake sites associated with NE. This technique has several advantages in that it provides a quantitative measure of the density of uptake sites as well as a detailed anatomical map of the location of these sites.

In previous studies, [^{3}H]DMI has been the ligand of choice for labeling uptake sites for NE in brain homogenate preparations and in autoradiographic studies (Lee and Snyder, 1981; Rehavi *et al.*, 1981; Lee *et al.*, 1982; Slater *et al.*, 1982; Biegon and Rainbow, 1983; Rapp *et al.*, 1987; Backstrom *et al.*, 1989). However, there are problems associated with the use of [^{3}H]DMI. This radioligand binds to two sites; a high-affinity binding site which is the uptake site for NE and a low-affinity site which is not (Lee *et al.*, 1982). Lee *et al.* (1982) have reported that only a small fraction of [^{3}H]DMI binding, as defined by 1 μM DMI or 1 mM NE could be considered as specific binding to NE uptake sites. Backstrom *et al.* (1989) have also demonstrated heterogeneity of [^{3}H]DMI binding sites in the rat brain. If nonspecific binding was properly defined so that only the high-affinity site was measured, then the percentage of specific binding was found to be low (40% at twice the K_d concentration). This observation caused Backstrom *et al.* (1989) to conclude that only a small fraction of [^{3}H]DMI binding sites in rat brain should be regarded as "specific" binding. By contrast, [^{3}H]NIS binds to a homogeneous population of sites and the percentage of specific binding is about 80% at twice its K_d concentration.

When the effect of lesioning on the binding of [^{3}H]DMI was examined, it was found that DSP-4 or 6-OHDA abolished only the high-affinity component of [^{3}H]DMI binding (Lee and Snyder, 1981; Lee *et al.*, 1982; Backstrom *et al.*, 1989). Rehavi *et al.* (1982) and Hrdina *et al.* (1981) noted a discrepancy between the extent of lesion caused by 6-OHDA (as measured by either the uptake or content of NE) and the decrease in [^{3}H] DMI binding. Both these groups observed only a 36 to 50% decrease in cortical binding of [^{3}H]DMI in contrast to a 90% depletion of cortical uptake or content of NE. Using [^{3}H]NIS, we find very marked reductions in its binding caused by 6-OHDA, a result more consistent with the data for uptake and content of NE (Rehavi *et al.*, 1982; Hrdina *et al.*, 1981). Using [^{3}H]DMI for autoradiographic studies, Biegon and Rainbow (1983) observed that the reduction in [^{3}H]DMI binding after 6-OHDA treatment was not uniform through all the brain regions and this was probably due to the fact that low-affinity binding of [^{3}H]DMI accounted for a certain proportion of total binding.

More recently, [^{3}H]MAZ has been reported to label both NE and DA uptake sites in rat brain (Javitch *et al.*, 1984). We have found that [^{3}H]MAZ is a suitable radioligand for labeling uptake sites for DA in the striatum (unpublished observations); however, it does not appear to be a suitable ligand for labeling NE uptake sites in the rat brain as measured by quantitative autoradiography. Depending on the brain region analyzed, specific binding ranged from 5 to 40% of total binding (unpublished observations). Furthermore, Donnan *et al.* (1989) have observed that except for high binding in the locus coeruleus, [^{3}H]MAZ binding to NE uptake sites were more poorly visualized in the rest of the mouse brain than DA uptake sites.

In summary, we have shown that [^{3}H]NIS is a useful radioligand for the measurement of uptake sites for NE in rat brain homogenates as well as in tissue slices by quantitative autoradiography. It possesses several properties that make it superior to other radioligands for the analysis of these sites. First, [^{3}H] NIS binds to a single class of binding sites. Second, it possesses high affinity for NE uptake sites. Third, the amount of nonspecific binding seen with [^{3}H]NIS is very low. Fourth, [^{3}H] NIS binds with high selectivity to NE uptake sites, as evidenced by competition experiments and lesion studies. Fifth, the regional distribution of [^{3}H]NIS corresponds highly to the regional distribution of noradrenergic innervation. These properties should, therefore, make [^{3}H]NIS a useful marker for the NE transporter in the study of various neurological diseases in which the noradrenergic system is implicated.

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