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EFFECT OF NISOXETINE ON UPTAKE OF CATECHOLAMINES IN SYNAPTOSOMES ISOLATED FROM DISCRETE REGIONS OF RAT BRAIN

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Abstract—Nisoxetine inhibits the high affinity uptake of norepinephrine by synaptosomes hypothalamus, brain stem and cerebral cortex of rat brain with inhibitor constants, K_i values, of 1, 2 and 6 nM respectively. The drug also inhibits the high affinity uptake of dopamine by hypothalamic synaptosomes with a K_i value of 9 nM. In corpus striatum, however, the K_i values for nisoxetine to inhibit the uptake of dopamine and norepinephrine are 360 and 220 nM respectively. Thus, nisoxetine has about 200-fold greater affinity for the uptake sites of norepinephrine than for those of dopamine. Nisoxetine administered at 10 mg/kg i.p. to rats reduces the high affinity uptake of catecholamines by synaptosomes of brain regions. A low affinity process for uptake of catecholamines also occurs in synaptosomes of hypothalamus, brain stem and cerebral cortex but is not blocked by 10^{-8} M nisoxetine. The significance of the low affinity uptake processes is discussed.

Nisoxetine hydrochloride (Lilly 94939) has been selected from a series of phenoxyphenylpropylamines for clinical studies as a potential antidepressant [1]. The compound shows pharmacological properties characteristic of the tricyclic antidepressant, desipramine, such as the reversal of reserpine- or apomorphine-induced hypothermia* and the enhancement of the vasopressor effect of norepinephrine (NE). Furthermore, nisoxetine is as potent as desipramine in blocking the high-affinity uptake of [^{3}H]NE by synaptosomes isolated from rat brain and by heart [2, 3]. In the present study, we report the effects of nisoxetine on the high affinity uptake of [^{3}H]NE by synaptosomes obtained from cerebral cortex, brain stem, hypothalamus and corpus striatum and on uptake of [^{3}H]dopamine by synaptosomes from corpus striatum and hypothalamus. We also have observed a low affinity uptake of [^{3}H]NE in these crude synaptosomal fractions.

METHODS AND MATERIALS

Male Sprague–Dawley rats (110–150 g) from Harlan Industries, Cumberland, Ind., were fed Purina Chow *ad lib* for at least 3 days in our laboratories before being killed by decapitation. Whole brains were removed and dissected following the method of Schubert and Sedvall [4]. Crude synaptosomal preparations were prepared from 10 per cent homogenates of brain tissues in 0.32 M sucrose and 10 mM glucose by differential centrifugation [5].

The active uptake of [^{3}H]monoamines by synaptosomes was determined by the modified method of Wong *et al.* [6]. Synaptosomes equivalent to 1 mg protein [7] were added to 1 ml of Krebs bicarbonate buffer containing 10 mM glucose, 0.1 mM iproniazid, 1 mM ascorbic acid, 0.17 mM ethylenediamine tetraacetic acid (EDTA) and [^{3}H]monoamine (0.05 to 2 μM) and incubated at 37° for 3 min. The incubation mixtures were immediately diluted with 2 ml of ice-chilled Krebs bicarbonate buffer containing 1 mM nonradioactive monoamine. Synaptosomes were harvested by centrifugation and rinsed with 5 ml of cold buffer and transferred to counting vials containing 10 ml of scintillation fluid [Permafluor (Packard)–Triton X-100–toluene, 1:8:16]. Radioactivity was measured using a Packard liquid scintillation spectrometer. The amount of [^{3}H]monoamine accumulated by synaptosomes and effects of nisoxetine on uptake of [^{3}H]monoamines were analyzed using the method of Lineweaver and Burk [8] or Dixon [9]. All experiments were repeated two to three times in order to validate the kinetics and the inhibitor constant (K_i) of nisoxetine.

Nisoxetine [*dl*-*N*-methyl-3-(*o*-methoxyphenoxy)-3-phenyl-propylamine, hydrochloride] was prepared by Dr. B. B. Molloy at the Lilly Research Laboratories. [7-^{3}H]*l*-norepinephrine ([^{3}H]*l*-NE), 15 Ci/m-mole; [7-^{3}H]*dl*-NE, 15 Ci/m-mole; and [^{3}H (G)]dopamine ([^{3}H]DA), 10 Ci/m-mole, were purchased from New England Nuclear Corp. [^{14}C]nisoxetine labeled at the *N*-methyl group was kindly supplied by Dr. F. J. Marshall of our laboratories.

RESULTS

Effect of nisoxetine on uptake of [^{3}*H*]*NE in synaptosomes of hypothalamus, cerebral cortex and brain stem.* The uptake of [^{3}H]NE in synaptosomes of hypothalamus shows a biphasic phenomenon which indicates the participation of two components, a high-affinity component and a low-affinity component having K_m values of 0.15 ± 0.01 μM (K_{m_1}) and 0.49 ± 0.06 μM (K_{m_2}) respectively (Fig. 1A).

Nisoxetine at 2 nM increased the K_{m_1} value from 0.15 ± 0.01 to 0.24 ± 0.03 μM (Fig. 1A) giving a K_i

* R. Rathbun and B. Molloy, personal communication.

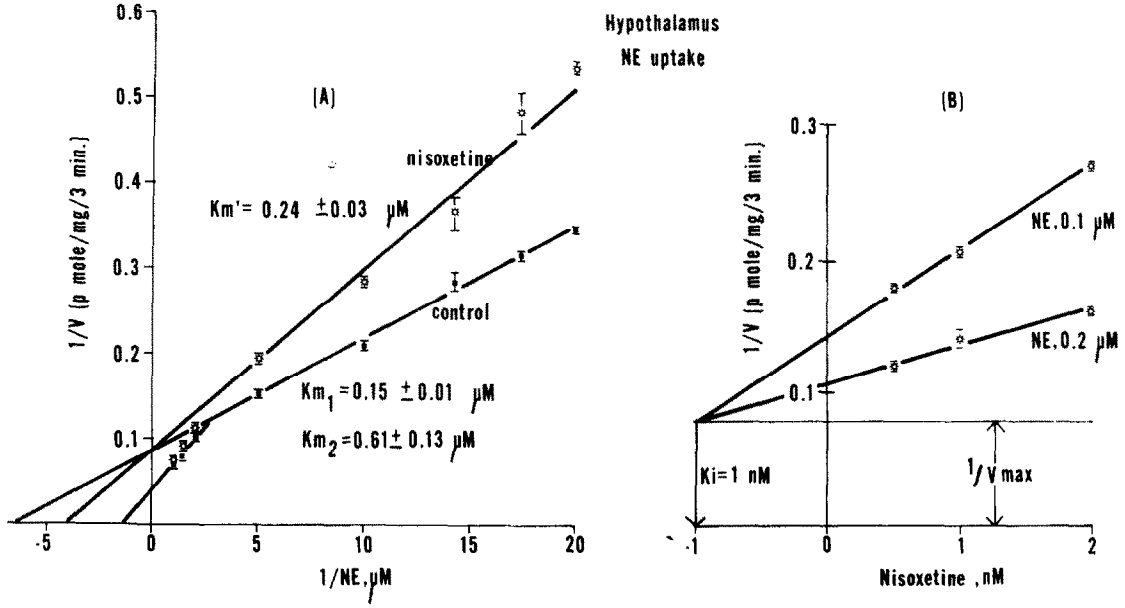

Fig. 1. Biphasic uptake of [^{3}H]NE in synaptosomes of hypothalamus competitively inhibited by nisoxetine as illustrated by the Lineweaver–Burk plot [8]. (A) Synaptosomes in triplicate samples of 1 mg protein were incubated in 1 ml of Krebs bicarbonate buffer containing [^{3}H]*l*-NE (0.05 to 2 × 10^{-6} M *l*-NE), 10 mM glucose, 0.1 mM iproniazid, 1 mM ascorbic acid and 0.17 mM EDTA at 37° for 3 min. Synaptosomes were sedimented from the reaction mixture by centrifugation, rinsed and measured for radioactivity by liquid scintillation. The experiment was repeated three times. $K_{m_1} \pm$ S.E.M. and $K_{m_2} \pm$ S.E.M. are the dissociation constants for the high- and low-affinity NE uptake processes respectively. (B) [^{3}H]*l*-NE at 0.1 and 0.2 μM was used to study the effect of three concentrations of nisoxetine according to the method of Dixon [9]. The bars represent S.E.M.

constant of 3.4 nM for inhibition of [^{3}H]NE uptake by synaptosomes from the hypothalamus. The K_i constant directly obtained from an experiment designed according to the method of Dixon [9] yielded a value of 1 nM (Fig. 1B).

The biphasic phenomenon for uptake of [^{3}H]NE also occurred in synaptosomes of cerebral cortex (Fig. 2) and brain stem (Fig. 3). The K_{m_1} values were about the same as in hypothalamus, whereas the K_{m2} values were greater in cerebral cortex and brain stem, 1.47 ± 0.11 and 1.34 ± 0.24 μM respectively. Nisoxetine at 10 nM competitively inhibited the high affinity uptake component in both cerebral cortex and brain stem with calculated K_i values of 6 and 2 nM.

Effects of nisoxetine on the uptake of [^{3}H]NE and [^{3}H]DA by synaptosomes from the corpus striatum. The kinetics for uptake of [^{3}H]NE into synaptosomes of corpus striatum were resolved by a double reciprocal plot into a single straight line which indicated

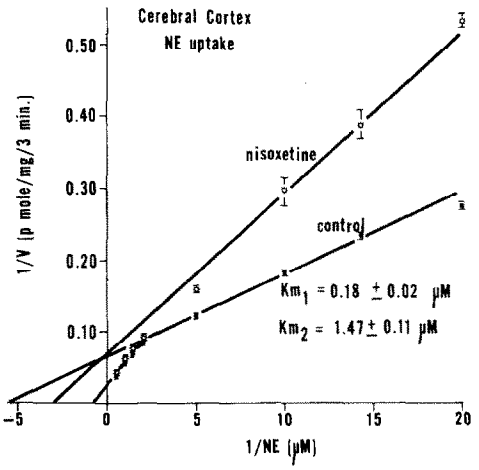

Fig. 2. Biphasic uptake of [^{3}H]NE in synaptosomes of cerebral cortex and inhibitory effect of nisoxetine as illustrated by the Lineweaver–Burk plot [8]. Experimental conditions were identical to those in Fig. 1 except that synaptosomes from cerebral cortex and 10 nM nisoxetine were used.

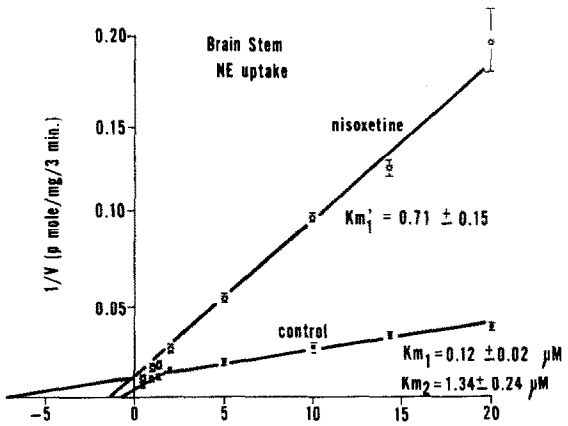

Fig. 3. Biphasic uptake of [^{3}H]NE in synaptosomes of a brain stem and inhibitory effect of nisoxetine as illustrated by the Lineweaver–Burk plot [8]. Experimental conditions were the same as in Figs. 1 and 2 except that synaptosomes from brain stem and nisoxetine at 10 nM were used.

the participation of a single uptake component (Fig. 4A). The K_m value of 0.47 ± 0.08 μM was significantly increased to 3.15 ± 0.69 μM by 0.5 μM nisoxetine. The K_i value was calculated to be 220 nM nisoxetine in the blockade of [^{3}H]NE uptake in corpus striatum.

The uptake of [^{3}H]DA in striatal synaptosomes was also resolved into a single straight line in the double reciprocal plot yielding a K_m value of 0.09 ± 0.01 μM (Fig. 4B). Nisoxetine (0.5 μM) competitively inhibited the uptake of [^{3}H]DA with a higher K_m value of 0.27 ± 0.02 μM of [^{3}H]DA. The calculated K_i value for nisoxetine was 360 nM in blocking the uptake of [^{3}H]DA in striatal synaptosomes.

Uptake of [^{3}H]DA by synaptosomes of hypothalamus and effect of nisoxetine. The uptake of [^{3}H]DA by synaptosomes of hypothalamus shows biphasic kinetics. Two straight lines were resolved in a double reciprocal plot (Fig. 5) which provided the evidence of two uptake components for the uptake of [^{3}H]DA as seen earlier for [^{3}H]NE uptake. The K_m values for [^{3}H]DA were 0.24 ± 0.1 (K_{m_1}) and 1.82 ± 0.10 μM (K_{m_2}). Only the high affinity uptake of [^{3}H]DA was inhibited by 10 nM nisoxetine with an inhibitor constant of 9 nM.

Effect of nisoxetine in vivo *on [^{3}H]NE uptake into synaptosomes of brain regions.* The effect of nisoxetine *in vivo* on uptake of catecholamines was examined in synaptosomes isolated from four anatomical regions of rat brain. The uptake of [^{3}H]NE was reduced most in synaptosomes of hypothalamus, followed by brain stem and cerebral cortex (Table 1). The uptake of [^{3}H]DA in corpus striatum was least influenced by the administration of nisoxetine.

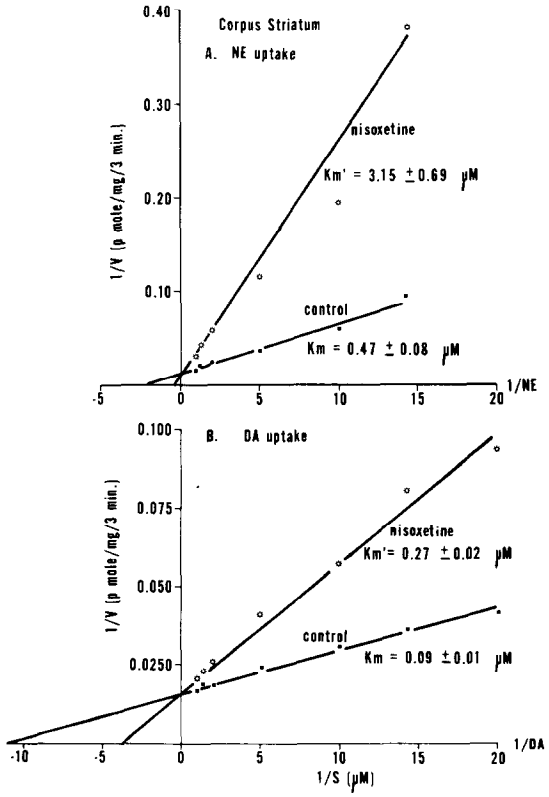

Fig. 4. Uptake of [^{3}H]NE (A) and [^{3}H]DA (B) in synaptosomes of corpus striatum and inhibitory effect of nisoxetine as illustrated by the Lineweaver–Burk plots [8]. Synaptosomes from corpus striatum were incubated in an identical manner as in Figs. 1 and 2 except that nisoxetine at 0.5 μM and [^{3}H]DA instead of [^{3}H]*l*-NE in (B) were used.

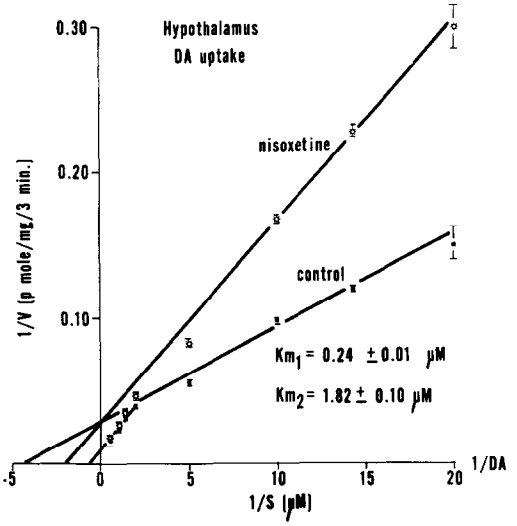

Fig. 5. Biphasic uptake of [^{3}H]DA in synaptosomes of hypothalamus and inhibitory effect of nisoxetine as illustrated by the Lineweaver–Burk plot [8]. Experimental conditions were the same as in Figs. 1 and 2 except nisoxetine at 10 nM was used.

Distribution of [^{14}C]nisoxetine in brain regions. In view of the selectivity of nisoxetine *in vivo* in the blockade of [^{3}H]NE uptake in brain regions, we wanted to know if the inhibition simply reflects the tissue distribution of the compound. Table 2 showed that [^{14}C]nisoxetine, within 10 min of intravenous injection, was almost evenly distributed in all regions, with a slightly higher concentration in cerebellum. At least 80 per cent of the radioactivity in the extract of cerebral cortex was identified as authentic nisoxetine by thin-layer chromatography. Similar regional distribution of radioactivity was observed at the end of 1 hr after the administration of [^{14}C]nisoxetine.

DISCUSSION

The uptake of NE by synaptosomes from hypothalamus, brain stem and cerebral cortex is effectively blocked by nisoxetine *in vitro*. The K_i values of nisoxetine (Table 3) for hypothalamus and brain stem were about the same (1–2 nM), suggesting similar affinities of the drug for NE uptake sites in both regions. The affinity of nisoxetine for the NE uptake sites of cerebral cortex was about 5-fold weaker with a K_i value of 9 nM. One hr after its administration, nisoxetine also produced the largest reduction in NE uptake by synaptosomes from hypothalamus and less reduction in NE uptake by synaptosomes of brain stem and cerebral cortex. The various degrees of drug effect *in vivo* did not correlate with levels of the drug, which was evenly distributed among brain regions. The affinity of the drug for the NE uptake sites as revealed by the K_i value was more associated with its potency *in vivo*.

Table 1. Effects of nisoxetine administration on uptake of catecholamines in synaptosomes of four brain regions*

Brain regions	Catecholamine tested	Catecholamine uptake (pmoles/mg protein) Control	Nisoxetine treated	Inhibition (%)
Hypothalamus	NE	5.93 ± 0.35	1.79 ± 0.12†	69.8
Cerebral cortex	NE	2.11 ± 0.07	0.94 ± 0.13†	55.5
Brain stem	NE	3.16 ± 0.30	1.34 ± 0.14†	57.6
Corpus striatum	DA	38.96 ± 2.48	29.32 ± 1.48‡	24.8

* Four rats were treated with either saline or nisoxetine (10 mg/kg, i.p.) for 1 hr before being killed by decapitation. Each brain was dissected in four regions [4] for the preparation of synaptosomes. Synaptosomes in duplicate samples were incubated at 37° for 3 min in Krebs bicarbonate buffer containing 0.1 μM [^{3}H]*l*-NE or [^{3}H]DA. Mean values from four rats ± S. E. M. of each brain region were compared between the saline- and nisoxetine-treated animals by Student's *t*-test.

† $P < 0.001$.

‡ $P < 0.025$.

The *o*-methoxy group in the phenoxy ring of nisoxetine is essential in directing the affinity of the molecule for the NE uptake sites. This affinity is reduced upon substituting a more electron-withdrawing group, such as a trifluoromethyl-group, to the same ring as in fluoxetine hydrochloride (Lilly 110140) and its related compounds. [10]. Fluoxetine is a poor inhibitor for the uptake of NE with a K_i value of 10 μM [10].

Nisoxetine also inhibited the uptake of 5-hydroxytryptamine (5-HT) with a K_i value of 0.5 μM in synaptosomes of rat brain [3] and 1 μM in synaptosomes of hypothalamus (unpublished results). Thus, in hypothalamus, nisoxetine was 1000 times better as an inhibitor of NE uptake than of 5-HT uptake. The replacement of the *o*-methoxy group by a *p*-trifluoromethyl group in the phenoxy ring of the molecule as in fluoxetine increases the affinity for the 5-HT uptake site with a K_i value of 0.1 μM for blocking the uptake of 5-HT in synaptosomes of rat brain [10]. Thus, nisoxetine and fluoxetine constitute the most interesting series of phenoxyphenylpropylamines as selective inhibitors of monoamine uptake in nerve endings of the central nervous system.

Corpus striatum contains mainly dopaminergic nerve terminals [11]. This conclusion is supported by the present finding that there is a greater affinity for DA uptake in this region than for NE with a 5-fold difference in K_m values, 0.09 and 0.47 μM respectively (Table 4). The corresponding values of 0.4 and 2 μM have been reported by Snyder and Coyle [11] for a particulate fraction of striatal homogenate. The similar K_i values of nisoxetine to inhibit uptake of DA and NE also support such explanation. Furthermore, nisoxetine was about 200-fold less effective in blocking the uptake of catecholamines by striatal synaptosomes than by synaptosomes of hypothalamus and brain stem.

Snyder and Coyle [11] also reported that the uptake of DA in homogenates of brain regions other than corpus striatum was biphasic, while the uptake of NE was monophasic in all brain regions. In partial agreement with these authors, we have observed the biphasic uptake of DA in synaptosomes of hypothalamus. However, we have also found that the kinetics for uptake of NE in hypothalamus, brain stem and cerebral cortex are all biphasic. Thus, the uptake of NE in these three brain regions involves the participation of a high-affinity (K_{m_1}) and a low-affinity (K_{m_2}) component (Table 4). The K_{m_1} and the corresponding V_{max_1} values for all three regions are approximately equal in magnitude, indicating some similarity in the chemical nature of these high-affinity uptake sites.

It has been suggested that the low-affinity uptake of DA in synaptosomes of hypothalamus may represent the uptake of DA by DA neurons of the region [12]. In the present study, the K_{m_2} value for DA uptake in hypothalamic synaptosomes is two times

Table 2. Distribution of [^{14}C]nisoxetine in brain regions*

Brain regions	[^{14}C]nisoxetine equivalent (μg/g tissue)	Estimated concn (10^{-6} M)
Hypothalamus	0.326 ± 0.008	1.06 ± 0.03
Cerebral cortex	0.396 ± 0.222	1.29 ± 0.07
Brain stem	0.435 ± 0.013	1.42 ± 0.04
Midbrain	0.361 ± 0.013	1.17 ± 0.04
Corpus striatum	0.394 ± 0.030	1.28 ± 0.10
Cerebellum	0.478 ± 0.015	1.55 ± 0.05

* Eight rats (100 g) were injected with [^{14}C]nisoxetine (*N*-methyl-^{14}C-labeled, 3.48 μCi/mg) at 3.65 mg/kg i.v. for 10 min before being killed by decapitation. Tissues of brain regions except cerebral cortex from two animals were pooled and digested in 1.5 ml of 27% H_2O_2 and 7% perchloric acid. Radioactivity was measured by the liquid scintillation technique.

Table 3. Inhibitor constants (K_i values) of nisoxetine in the blockade of catecholamine uptake by synaptosomes of brain regions

Brain regions	Catecholamine	K_i values* (nM)
Hypothalamus	NE	1
	DA	9
Brain stem	NE	2
Cerebral cortex	NE	6
Corpus striatum	NE	220
	DA	360

* K_i values are estimated from experiments illustrated in Figs. 2–6.

Table 4. Kinetic parameters for uptake of norepinephrine (NE) and dopamine (DA) by synaptosomes from various rat brain regions*

Brain regions	Substrates	High affinity		Low affinity	
		K_{m_1}	V_{max_1}	K_{m_2}	V_{max_2}
Hypothalamus	*l*-NE	0.15 ± 0.02	13.8 ± 1.0	0.49 ± 0.06	25.6 ± 1.24
	l-NE†	0.12 ± 0.02	10.54 ± 0.88	0.70 ± 0.12	30.93 ± 2.77
	DA	0.24 ± 0.01	37.20 ± 1.00	1.82 ± 0.10	117.3 ± 3.80
Cerebral cortex	*l*-NE	0.18 ± 0.02	15.4 ± 0.80	1.47 ± 0.11	44.74 ± 1.80
Brain stem	*l*-NE	0.12 ± 0.02	8.43 ± 0.72	1.34 ± 0.24	23.4 ± 2.30
Corpus striatum	*l*-NE	0.47 ± 0.08	82.68 ± 5.86		
	DA	0.09 ± 0.01	64.54 ± 1.38		

* K_m (μM) and V_{max} (pmoles/mg of protein/3 min) ± S. E. M. were obtained from experiments shown in Figs. 1–5.
† [^{3}H]*dl*-NE was diluted with *l*-NE and the specific activity had been divided by 2.

larger than the K_{m_1} value and half the K_{m_2} value for NE uptake in the same tissue. However, the V_{max_1} value for DA uptake is in the same order of magnitude as the V_{max_2} value for NE uptake. Thus, in agreement with the earlier suggestion, the low affinity uptake of NE in hypothalamus may be dopaminergic in origin. Furthermore, only the high affinity but not the low affinity uptake of NE in hypothalamus and cerebral cortex was reduced after an intraventricular administration in a limited dose of 6-hydroxydopamine (unpublished results), which presumably caused degeneration specifically at NE neurons [13].

The low-affinity NE uptake process in cerebral cortex and brain stem may also represent a separate uptake process specific for other amines. The K_{m_2} values for NE uptake in synaptosomes of cerebral cortex and brain stem are two times larger than the K_{m_2} value for NE uptake in hypothalamic synaptosomes. Thus, a third process for the uptake of catecholamines may exist in cerebral cortex and brain stem. The presence of epinephrine (E) in the central nervous system has been detected [14–16]. An uptake process specific for E is not yet known. Preliminary data in our laboratory show that a sodium-dependent uptake of E follows monophasic and saturable kinetics with K_m values of about 1 μM E in synaptosomes of brain stem, cerebral cortex and hypothalamus (unpublished results). The affinity of E resembles the low affinity (K_{m_2}) for NE in brain stem and cerebral cortex.

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