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Structural significance of azaheterocyclic amines related to Parkinson's disease for dopamine transporter

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Abstract

We have evaluated the neuronal uptake of 12 neutral and quaternary azaheterocyclic amines that are possible candidates for idiopathic Parkinson's disease via dopamine transporter of striatal synaptosomes. The double-reciprocal plots for dopamine transporter obtained from Wistar rat and C57BL/6 mouse synaptosomes with *N*-methyl-4-phenylpyridinium cation (MPP^+) as a substrate were identical to each other. Neutral β-carbolines and tetrahydroisoquinolines were unfavorable substrates for dopamine transporter. The quarternization of these compounds strikingly increased the affinity for dopamine transporter with 2–10 times greater K_m and 10 times smaller V_{max} values than MPP^+. Although catechol tetrahydroisoquinolines were weak substrates, their quarternization reduced their original properties as substrates for dopamine transporter. These results provide both topographic and electrogenic information of azaheterocyclic amines for the dopamine transporter-mediated influx. The intramolecular distance between the *N*-atom and the centroid of the benzene ring could be an important factor for the recognition of binding site of dopamine transporter, and an adequate net charge similar to dopamine would be further required for translocation into the cells. © 1998 Elsevier Science B.V.

Keywords: Dopamine transporter; Neurotoxin; β-Carboline; Isoquinoline; MPP^+ (*N*-methyl-4-phenylpyridinium); Uptake

1. Introduction

The neurodegenerative process underlying destruction of the dopamine neurons of the substantia nigra pars compacta in Parkinson's disease remains unknown. A synthetic meperidine byproduct, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), induces parkinsonian symptoms in humans as a result of the degeneration of nigrostriatal neurons (Langston et al., 1983). After transport through the blood–brain barrier, MPTP is oxidized intracerebrally in glia or non-dopaminergic neurons by monoamine oxidase type B to an intermediate, *N*-methyl-4-phenyl-dihydropyridinium, before undergoing disproportionate or spontaneous oxidation to form a neurotoxic metabolite, *N*-methyl-4-phenylpyridinium cation (MPP^+) (Chiba et al., 1985; Markey et al., 1984). MPP^+ accumulates in nigrostriatal neurons via dopamine transporter, the Na^+-coupled plasma membrane transporter, before being concentrated in the mitochondria by cationic gradient and thereby shutting down ATP synthesis through inhibition of NADH dehydrogenase at Complex I of the respiratory chain (Javitch et al., 1985; Nicklas et al., 1985).

This MPTP story may lead to possible neurotoxins underlying Parkinson's disease. Several classes of azaheterocyclic molecules structurally related to MPTP have been advanced as possible neurotoxin precursors underlying the nigrostriatal degeneration in parkinsonism; indoleamine-related β-carbolines, catechol isoquinolines and phenethylamine-derived isoquinoline derivatives are the most reasonable candidates (Collins et al., 1992; Kohno et al.,

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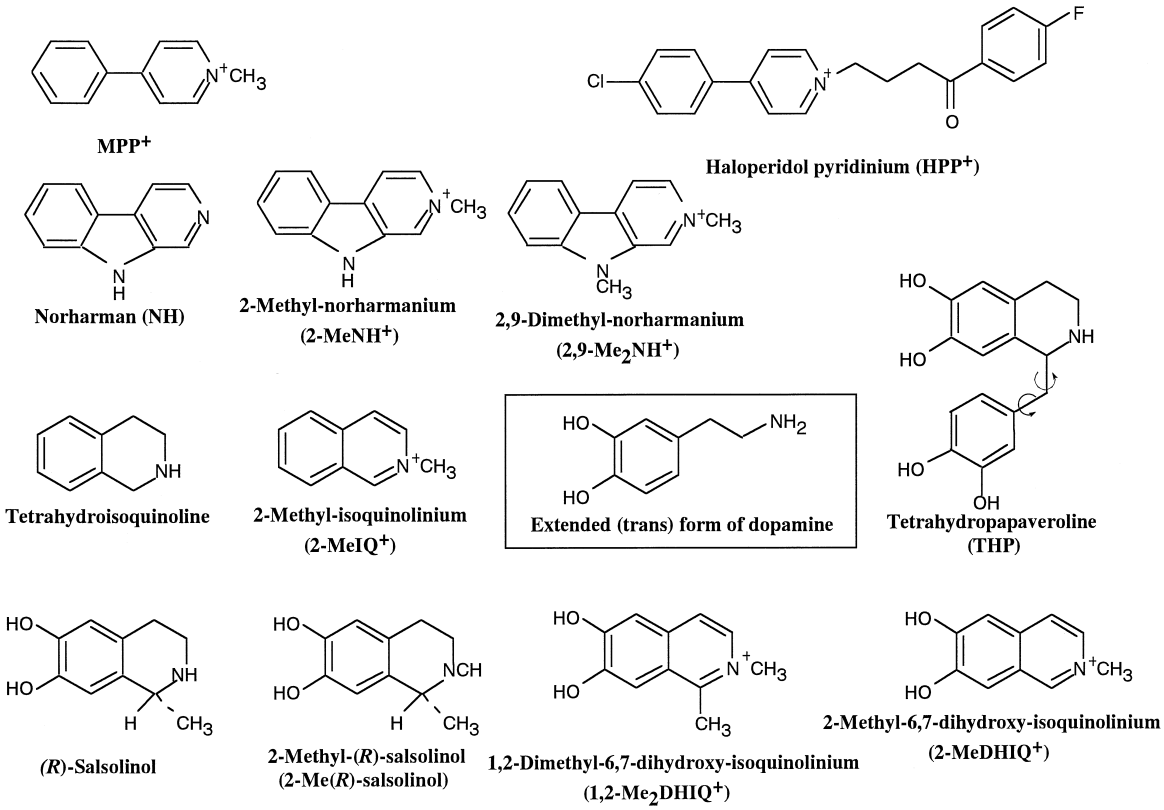

Fig. 1. Structures of the azaheterocyclic amines, MPP^+ and extended (*trans*) dopamine.

1986; Maruyama et al., 1992; Matsubara et al., 1998; Suzuki et al., 1990). These substances are *N*-methylated into quaternary amines and possess neurotoxic potential (Maruyama et al., 1992; Matsubara et al., 1992a,b; Naoi et al., 1989). These cations have been found at higher concentrations in the lumbar cerebrospinal fluid of parkinsonian patients compared with age-matched control subjects (Kotake et al., 1995; Matsubara et al., 1995; Maruyama et al., 1996). In addition, a variety of pyridinium and related charged species (Booth et al., 1989; Michel et al., 1989; Rollema et al., 1990) have been reported to be neurotoxic. The antipsychotic agent haloperidol is a piperidinol derivative which enzymatically produces 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium ion (HPP^+), a structural analog of MPP^+. This cation has been proposed to mediate in part the side effects, such as the tardive dyskinesias, associated with chronic haloperidol use (Subramanyam et al., 1991).

Dopamine transporter uptakes dopamine more rapidly than norepinephrine (Meiergerd and Schenk, 1994), whereas norepinephrine transporter transports both norepinephrine and dopamine very efficiently (Pacholczyk et al., 1991). Dopamine transporter also transports MPP^+ at a velocity twice that of dopamine; in contrast, norepinephrine transporter transports the pyridinium cation at a rate half that of norepinephrine (Buck and Amara, 1994). To induce selective dopaminergic toxicity, candidate toxins for Parkinson's disease must be accumulated into dopaminergic neurons via dopamine transporter. β-Carbolines are potent inhibitors of dopamine uptake system (Drucker et al., 1990; Matsubara et al., 1996) while isoquinolines are weak (McNaught et al., 1996). However, the substrate affinity for the dopamine transporter-mediated translocation into dopaminergic neurons has not been established. Thus, we have studied the dopamine transporter-dependent neuronal uptake of 12 neutral and quaternary azaheterocyclics listed in Fig. 1 using the striatal synaptosomes prepared from Wistar rat and C57BL/6 mouse. We also discuss the structural characteristics of these compounds in relation to those of dopamine transporter.

2. Materials and methods

2.1. Chemicals

MPP^+ and 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]-piperazine dihydrochloride (GBR 12909) were obtained from RBI (Natick, MA, USA). Norharman (NH) and tetrahydroisoquinoline were purchased from Sigma (St. Louis, MO, USA). 1-Octanesulfonic acid was purchased from Nacalai Tesque (Kyoto, Japan). Cation 2-methylated norharmanium (2-MeNH^+), 2,9-dimethylated norharmanium ($2,9\text{-Me}_2\text{NH}^+$), 2-methylated isoquinolinium (2-MeIQ^+) and HPP^+ were prepared in our

Table 1
The HPLC conditions employed in this study

Compounds	Column	Mobile phase	Detector	Wave length (nm) or voltage (eV)
MPP^+	Puresil C18	A	Fluorescence	Ex, 325; Em 375
NH, 2-$MeNH^+$ and 2,9-Me_2NH^+	Puresil C18	A	Fluorescence	Ex, 310; Em 455
2-$MeDHIQ^+$	Puresil C18	B	Fluorescence	Ex, 365; Em 430
1,2-Me_2DHIQ^+	Puresil C18	B	Fluorescence	Ex, 370; Em 500
2-$MeIQ^+$	Puresil C18	B	Fluorescence	Ex, 350; Em 380
HPP^+	μBondapack C18	C	Fluorescence	Ex, 330; Em 375
(*R*)-Salsolinol	MA-5ODS	D	ECD[a]	400
2-Me(*R*)-salsolinol	MA-5ODS	D	ECD	400
THP	μBondapack C18	E	ECD	750

Column: Puresil C18 (4.6 mm × 250 mm, Waters); μBondapack (3.9 mm × 300 mm, Waters) and MA-5ODS (4.6 mm × 150 mm, EICOM).
Mobile phase: A, 0.1 M acetic acid containing 2 mM sodium octanesulfonate hydrochloride (pH 3.0 with triethylamine)–acetonitrile (80:20) B, 90 mM sodium acetate–35 mM citric acid buffer containing 0.13 mM disodium EDTA and 0.23 mM sodium octanesulfonate (pH 4.35)–methanol (80:20); C, 30 mM ammonium acetate containing 15 mM triethylamine (pH 4.25 with acetic acid)–acetonitrile (60:40); D, 90 mM sodium acetate–35 mM citric acid buffer containing 0.13 mM disodium EDTA and 0.23 mM sodium octanesulfonate (pH 4.35)–methanol (89.5:10.5) and E, 75 mM ammonium dihydrogen phosphate containing 0.5 mM sodium octanesulfonate (pH 3.0 with phosphoric acid)–1,4-dioxane (90:10). Flow rate was 0.8–0.9 ml/min in each system.
[a]ECD, electrochemically with a glassy carbon working electrode vs. Ag/AgCl.

laboratory as previously reported (Kikuchi et al., 1991; Matsubara et al., 1992b; Igarashi et al., 1996). (*R*)-Enantiomer of 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline ((*R*)-salsolinol), 2-methylated (*R*)-salsolinol (2-Me(*R*)-salsolinol), 2-methylated 6,7-dihydroxyisoquinoline (2-$MeDHIQ^+$) and 1,2-dimethylated 6,7-dihydroxyisoquinoline (1,2-Me_2DHIQ^+) were kindly provided by Dr. Dostert. Other chemicals were of analytical grade and were from Wako (Osaka, Japan).

2.2. Preparation of synaptosomes

Male Wistar rat (250–300 g, SLC, Japan) or C57BL/6 mouse (20–25 g, SLC, Japan) was decapitated, and both striata were immediately dissected in ice-cold 0.32 M sucrose/5 mM HEPES at pH 7.5. Striata were homogenized in 20 volumes of sucrose/HEPES solution (12 up and down strokes, at 850 rpm, in a 0.25 mm clearance glass Teflon homogenizer). The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C. Supernatant was centrifuged at $12\,000 \times g$ for 20 min at 4°C. The final pellet, consisting of the crude synaptosomal (P2) fraction, was gently resuspended in an equal volume of incubation buffer and then diluted to 0.25 mg protein/ml solution with an appropriate volume of the buffer. The incubation buffer was composed of 128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, 10 mM D-glucose, 1 mM L-ascorbic acid and 0.1 mM pargyline at pH 7.5. Protein concentration was determined by the method of Bradford (1976) with bovine γ-globulin as standard.

2.3. Uptake of neurotoxins

Crude synaptosomes (~0.2 mg protein) were preincubated with or without GBR 12909 (final concentration: 10 μM) for 10 min at 37°C in 900 μl of incubation medium. Then, an aliquot of 100 μl of the tested compound solution was added to the synaptosomes suspension and incubated for 30 min at 37°C (total reaction mixture, 1 ml). The reaction was stopped by the immediate cool-down with an ice-bath, and immediately centrifuged at $15\,000 \times g$ for 10 min at 4°C. The pellet was washed 5 times with 1 ml of the incubation medium. The washed pellet was mixed with 0.5 ml of 0.2 M perchloric acid followed by sonication for 5 min, and the mixture was centrifuged for 10 min. The supernatant was filtered through a cellulose–acetate filter (pore size, 0.45 μm; DISMIC-3, Advantec, Tokyo, Japan), and a portion (200 μl) of the filtrate was injected into an high-performance liquid-chromatograph (HPLC) apparatus (Shimadzu LC-10). In the case of the tetrahydroisoquinoline experiment, the supernatant was mixed with 500 pg anthracene (internal standard), 1 ml of

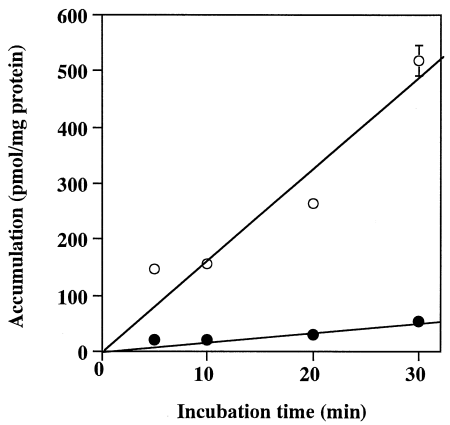

Fig. 2. Time-dependent dopamine transporter mediated accumulation of MPP^+ (open circle) and 2,9-Me_2NH^+ (solid circle) into the rat striatal synaptosomes. Each plot represents the mean ± S.E.M. of five to six experiments.

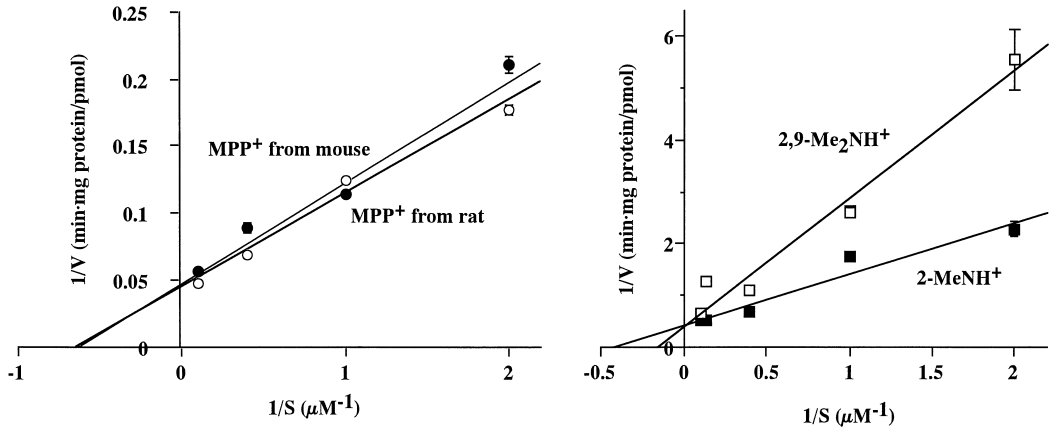

Fig. 3. Double-reciprocal plots of the uptake velocity against the concentrations of MPP^+, 2-$MeNH^+$ and 2,9-Me_2NH^+. The reciprocal of the accumulation velocity $(1/V)$ was plotted against that of the concentration of the substance. Open circle, MPP^+ obtained from rat synaptosome preparations; solid circle, MPP^+ from mouse; open square, 2,9-Me_2NH^+ from rat; solid square, 2-$MeNH^+$ from rat. Each plot represents the mean ± S.E.M. of 8–12 experiments.

0.5 M NaOH and 20 μl of isopropylchloroformate, and vortexed for 1 min. Derivatized tetrahydroisoquinoline was extracted with 3 ml of toluene. The organic solvent was transferred into the glass tube and evaporated under N_2. The residue was dissolved in 50 μl of toluene, and an aliquot of 4 μl was injected to a gas chromatograph/mass spectrometer (GC/MS, HP 5890SRII/HP 5972).

2.4. Calculation of uptake velocity

The dopamine transporter-mediated transport was calculated by the subtraction between concentrations measured in the presence and absence of GBR 12909 ($n = 5–12$ at each point), when statistical significance ($P < 0.01$) by Student's *t*-test was obtained between the two values. To study the kinetics of uptake, the synaptosomes were incubated with four to five different concentrations of the compounds. The apparent Michaelis constant, K_m, and maximal velocity, V_{max}, were obtained by plotting the reciprocal of the uptake velocity against that of the substrate concentration.

2.5. Analytical conditions

The HPLC conditions for the analyses of the compounds except tetrahydroisoquinoline are summarized in Table 1. The GC/MS conditions for tetrahydroisoquinoline analysis were as follows: column, DB-225 (J & W, 30 m × 0.32 mm i.d.); column temperature, 100°C (1 min) programmed to 220°C at 10°C/min; carrier gas, He; pressure, 67 kPa for 0.75 min then reduced quickly to 6 kPa, ion source temperature, 250°C and electron energy, 70 eV. The quantification was done by plotting ion current ratios to the internal standard, *m/z* 132/178 and 176/178 in the

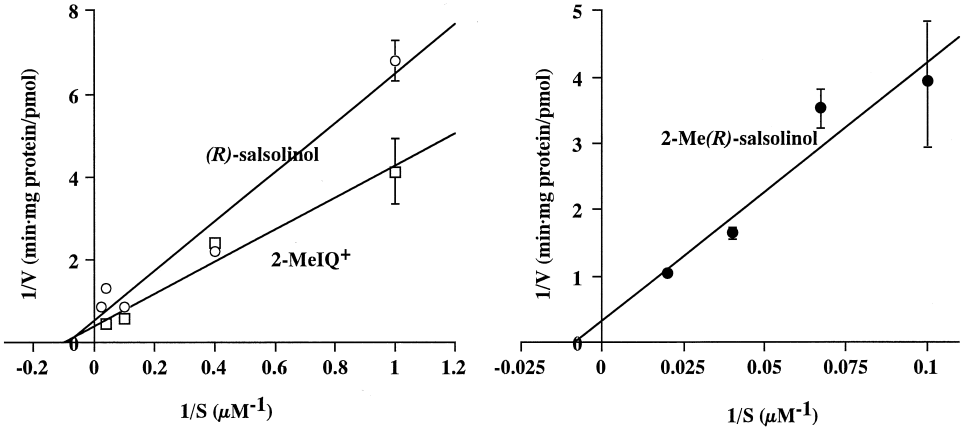

Fig. 4. Double-reciprocal plots of the uptake velocity against the concentrations of (*R*)-salsolinol, 2-$MeIQ^+$ and 2-Me(*R*)-salsolinol obtained form rat synaptosome preparations. The reciprocal of the accumulation velocity $(1/V)$ was plotted against that of the concentration of the substance. Open circle, (*R*)-salsolinol; open square, 2-$MeIQ^+$ and solid circle, 2-Me(*R*)-salsolinol. Each plot represents the mean ± S.E.M. of 7–10 experiments.

selected-ion monitoring mode, against the known concentrations of the compounds.

3. Results

3.1. Time-course of accumulation

The time-course of the accumulation of MPP^+ and 2,9-Me_2NH^+ was studied (Fig. 2). The uptake of these compounds into rat striatal synaptosomes was linear over the time period examined (5–30 min). However, MPP^+ was accumulated much faster than 2,9-Me_2NH^+.

3.2. Kinetic properties for dopamine transporter

The compounds (0.5–10, 1–25 or 10–50 μM) were incubated with rat striatal synaptosomes. Figs. 3 and 4 show the double-reciprocal plots of the uptake velocity against the concentrations of the compounds. MPP^+ was potently taken up into the synaptosomes by dopamine transporter with a K_m of 1.53 μM and a V_{max} of 22.22 pmol/mg protein per min. Significant dopamine transporter-mediated influx of NH and tetrahydroisoquinoline was not observed when they were incubated with the synaptosome preparations at any concentration. 2-$MeNH^+$, 2,9-Me_2NH^+, (*R*)-salsolinol and 2-$MeIQ^+$ were accumulated dose-dependently (0.5–10 or 1–25 μM) by synaptosomes. 2-Me(*R*)-salsolinol was significantly taken up at higher concentrations (>10 μM, $P<0.01$) with a 100 times higher K_m value than that of MPP^+, but significant accumulation at lower doses (0.5–5 μM) was not detected. Cationic 2-$MeDHIQ^+$ and 1,2-Me_2DHIQ^+ were not translocated into the synaptosomes at all. Similarly, the dopamine transporter mediated uptake of THP or HPP^+ was not observed. The K_m and V_{max} values of the tested compounds for dopamine transporter are summarized in Table 2.

Table 2
Apparent K_m and V_{max} for dopamine transporter mediated influx into the rat striatal synaptosome

Compound	K_m (μM)	V_{max} (pmol/mg protein per min)
MPP^+	1.53	22.22
NH	N.A.	N.A.
2-$MeNH^+$	2.59	2.46
2,9-Me_2NH^+	5.34	2.23
Tetrahydroisoquinoline	N.A.	N.A.
2-$MeIQ^+$	10.30	2.65
(*R*)-Salsolinol	23.64	3.57
2-Me(*R*)-salsolinol	160.56	3.88
1,2-Me_2DHIQ^+	N.A.	N.A.
2-$MeDHIQ^+$	N.A.	N.A.
THP	N.A.	N.A.
HPP^+	N.A.	N.A.

N.A., not accumulated through dopamine transporter.

3.3. Difference in dopamine transporter between rat and mouse

To examine species difference in dopamine transporter affinity for neurotoxins, the kinetic properties of MPP^+ were obtained using the synaptosomes prepared from C57BL/6 mice striata. The double-reciprocal plots obtained from rat and mice synaptosomes using MPP^+ as a substrate were very similar to each other (Fig. 3); the K_m and V_{max} values obtained from the mice synaptosomes were 1.66 μM and 21.28 pmol/mg protein per min, respectively. These values are also almost equal to those observed with rat synaptosomes (Table 2).

4. Discussion

The metabolic bioactivation hypothesis, involving neutral azaheterocyclic amines to induce neurotoxic quaternary cationic amines, has been proposed in the etiology of idiopathic Parkinson's disease. In this hypothesis, the dopamine transporter-mediated influx is an essential step in inducing their dopaminergic neurotoxicity. In this study, we evaluated the dopamine transporter-dependent neuronal uptake of 12 neutral and quaternary azaheterocyclic amines that are proposed candidates for inducing Parkinson's syndrome (Collins et al., 1992; Kohno et al., 1986; Maruyama et al., 1992; Suzuki et al., 1990).

GBR 12909 potently inhibits dopamine uptake and demonstrates a nearly 400-fold greater selectivity for the inhibition of dopamine over norepinephrine uptake in the synaptosomal preparation (Andersen, 1989). GBR 12909 prevents the toxicity of MPP^+ in the striatal synaptosomes. This preventive effect appears at 5 μM GBR 12909 in the presence of 2 μM MPP^+ (Bougria et al., 1995). Thus, in this study, we employed 10 μM GBR 12909 to obtain the nonspecific uptake. The kinetic property of MPP^+ for dopamine transporter obtained here was concordant with that reported previously (Chiba et al., 1985). However, the result of (*R*)-salsolinol here was different from that in the previous report (Takahashi et al., 1994), in which 2-Me(*R*)-salsolinol but not (*R*)-salsolinol was accumulated in the human dopaminergic neuroblastoma SH-SY5Y cells by dopamine transporter. The reason for this discrepancy was unclear. Although non-primates are relatively resistant to MPTP toxicity, C57BL/6 strain mice are extremely sensitive to MPTP, in terms of depletion of dopamine content and tyrosine hydroxylase activity (Heikkila and Sonsalla, 1992; Vaglini et al., 1994). This species difference is probably not a reflection of the dopamine transporter-mediated process, because the equal kinetic parameters of MPP^+ for dopamine transporter were obtained from the striatal synaptosome preparations of Wistar rat and C57BL/6 mouse.

The low affinity of isoquinoline derivatives for dopamine transporter compared to MPP^+ was similar to

that in a previous study indicating that isoquinolines are far less potent inhibitors of dopamine uptake (IC_{50}, more than 8 mM) than MPP^+ (IC_{50}, less than 1 μM) (McNaught et al., 1996). β-Carbolines are fairly potent inhibitors of dopamine uptake system, although their IC_{50} values are 20 times less than that of MPP^+ (Drucker et al., 1990). The dopamine conformation at the uptake site of dopamine transporter is the extended (*trans*) form (Fig. 1) (Horn, 1974; Meiergerd and Schenk, 1994). The intermolecular distance between the *N*-atom and the centrinoid of the benzene or catechol ring has been suggested as being an important factor in dopamine uptake inhibition (McNaught et al., 1996; Meiergerd and Schenk, 1994). This distance in MPP^+ or β-carboline is close to that of the extended dopamine conformation (Fig. 1), but in isoquinolines it is considerably shorter than that in dopamine or MPP^+. These topographical differences among azaheterocyclics could be responsible for the different affinities for dopamine transporter. Previous works demonstrated that β-carbolines and isoquinolines inhibit dopamine uptake, but their quarternization does not greatly enhance the degree of inhibition (Drucker et al., 1990; McNaught et al., 1996). However, β-carbolines and isoquinolines were not accumulated into the synaptosome through the dopamine transporter. Therefore, this intramolecular distance should be considered as primarily important for the recognition of the binding site of dopamine transporter.

On the other hand, it is also established that monoamine transport involves the cotransport of additional ions along with the neurotransmitter, a process which is, depending on the net charge of substrates and ions, potentially electrogenic. Dopamine appears to be transported with two sodium ions and one chloride ion, and hence transport processes are predicted to be electrogenic (Povolock and Amara, 1997). Thus adequate net charge of the heterocyclic amine molecules similar to dopamine would be required for translocation into the nerve terminals. Indeed, the present results revealed that quaternary β-carbolines and tetrahydroisoquinoline are potential substrates for dopamine transporter, although the neutral analogs of these cations were not substrates for dopamine transporter. The kinetic study revealed that β-carbolinium cations were transported with apparent 2- to 4-fold greater K_m values and 10-fold smaller V_{max} values than MPP^+. This slower uptake velocity with a relative low K_m value of 2,9-Me_2NH^+ would explain our previous results which indicate that this cation acts as a potent dopamine uptake inhibitor, in vivo, to elicited massive dopamine overflow in the striatum (Matsubara et al., 1996). Both the K_m and V_{max} values of 2-$MeIQ^+$ were 10 times less than those of MPP^+. However, the quarternization of catechol isoquinolines caused the loss of their affinities for the dopamine transporter mediated influx, indicating that catechol tetrahydroisoquinolines would be already charged, similar to dopamine. This would also explain the result that 2-Me(*R*)-salsolinol was less potent than (*R*)-salsolinol, because the former is more lipophilic that the latter. In the dopamine related structure, the amine functionality has steric requirements; there is a decline in transport velocity when it is methylated or substituted with a longer alkyl group (Meiergerd and Schenk, 1994). Similarly, THP, which can take an extended dopamine form in its structure (Fig. 1) and is a much more potent inhibitor of dopamine uptake than salsolinol (Alpers et al., 1975), would not be adequately charged for dopamine transporter because of having two catechol structures. Indeed, 1-(3′,4′-dihydroxy-benzyl)-tetrahydroisoquinoline, which has an extended dopamine and a catechol in its structure, was a substrate for dopamine transporter (Kawai et al., 1998). HPP^+, which has been proposed as mediating in part the side effects associated with chronic haloperidol use (Subramanyam et al., 1991), could also be an inadequate substrate for dopamine transporter for both electrogenic and topographic reasons. This is supported by results on HPP^+ from earlier publications which document that HPP^+ induces nonspecific neurotoxic effects in intracerebral microdialysis (Igarashi et al., 1996; Rollema et al., 1994) and neuronal cell culture (Bloomquist et al., 1994) studies. This compound may cross the neuronal membrane due to its lipophilic property.

5. Conclusion

The quarternization of β-carboline and tetrahydroisoquinoline increased their affinity for the dopamine transporter-mediated transport. Catechol tetrahydroisoquinolines themselves were weak substrates for dopamine transporter, and quarternization further reduced their properties for acting dopamine transporter. However, the present results do not necessarily diminish the possibility that isoquinoline derivatives may be endogenous neurotoxins underlying idiopathic Parkinson's disease, because the levels found in cerebrospinal fluid of parkinsonian patients (Maruyama et al., 1996) are much higher than those of β-carbolines (Matsubara et al., 1995). Present results strongly indicate that there are both topographic and electrogenic limitations for the dopamine transporter-mediated influx. The intramolecular distance between the *N*-atom and the centroid of the benzene ring of azaheterocyclic amines would be an important factor for the recognition of binding site of dopamine transporter, and an adequate net charge similar to dopamine would also be required for translocation into the cells.

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