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EFFECTS OF HETEROCYCLIC AMINES IN FOOD ON DOPAMINE METABOLISM IN NIGRO-STRIATAL DOPAMINERGIC NEURONS

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Abstract—We investigated the effects of 14 heterocyclic amines in food on nigro-striatal dopaminergic neurons. Among 14 compounds tested, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-b]indole (Trp-P-2) caused substantial decreases in 3,4-dihydroxyphenylalanine (DOPA) formation in striatal tissue slice system. When Trp-P-1 or Trp-P-2 was unilaterally infused in the rat striatum by an *in vivo* micro-dialysis technique, both compounds produced a transient increase of dopamine (DA) and continuous decreases in the metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in the perfusate. This suggests that the two compounds inhibit monoamine oxidase (MAO) *in vivo*. Indeed they were found to be very potent inhibitors of MAO *in vitro*. Systemic administration of Trp-P-1 to C57 Black mice caused a marked decrease of DOPAC content and a significant increase of DA in the striatum, indicating inhibition of MAO *in vivo*. These results suggest that Trp-P-1 and Trp-P-2 contained in food could alter the metabolism of DA in the brain.

Some chemical substances are known to induce symptoms mimicking neuronal diseases, for example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in Parkinson's disease [1, 2], and β-*N*-methylamino-L-alanine (BMAA) in amyotrophic lateral sclerosis [3]. MPTP is an artificial substance to be converted to a parkinsonian neurotoxin, 1-methyl-4-phenylpyridinium ion (MPP^+), in the brain, while BMAA is a natural compound found in the seeds of *Cycas circinalis* (Guamanian cycad) [3]. This indicates the importance of effects of environmental compounds on etiology of neuronal diseases.

In this paper, we tested the effects of 14 heterocyclic amines in food to the dopaminergic neurons. These compounds were found as carcinogens in cooked food [4–7] and are related to β-carbolines with structures similar to MPTP. First we measured their effects on tyrosine hydroxylation in striatal tissue slices, and then examined their effects on dopamine (DA) metabolism *in vivo* by *in vivo* brain microdialysis technique or after systemic administration. Their effects on DA metabolism were also analyzed by examining their inhibitory activity of monoamine oxidase (MAO; EC 1.4.3.4, amine:O_2 oxidoreductase).

MATERIALS AND METHODS

The structures, chemical names and abbreviations of the tested compounds are listed in Fig. 1. 2-Amino - 6 - methyldipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-2) were obtained from Katsura Chemical Co. (Tokyo, Japan). All other heterocyclic amines were supplied by Nard Institute (Osaka, Japan). NSD-1055 (4-bromo-3-hydroxy-benzyloxyamine dihydrogen phosphate) was from Nakarai Chemicals (Kyoto, Japan), and aluminium oxide was from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade.

3,4-Dihydroxyphenylalanine (DOPA) formation in rat striatal slices was assayed as described previously [8] with slight modifications. Male Wistar rats (200–300 g) were killed by decapitation and the striata were immediately dissected and cut into slices (0.22 mm in thickness) on a McIlwain tissue chopper. The slices were washed extensively before use in the Krebs–Ringer bicarbonate medium composed of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.1 mM $MgSO_4$, 1.4 mM KH_2PO_4, and 8 mM glucose. The medium was saturated with atmosphere of 95% O_2/5% CO_2. The final pH was 7.2. Incubation was carried out in Thunberg tubes at 37° for 60 min in 1 ml of the medium containing NSD-1055 (an aromatic L-amino acid decarboxylase inhibitor) and the compounds examined. Amounts of DOPA formed from endogenous tyrosine were determined by high-performance liquid chromatography with electrochemical detection (HPLC–ECD).

Protein concentrations were estimated by the method of Bradford [9] with bovine serum albumin as a standard.

The brain micro-dialysis method used was described in detail elsewhere [10]. In brief, male Wistar rats weighing 250–350 g were anesthetized

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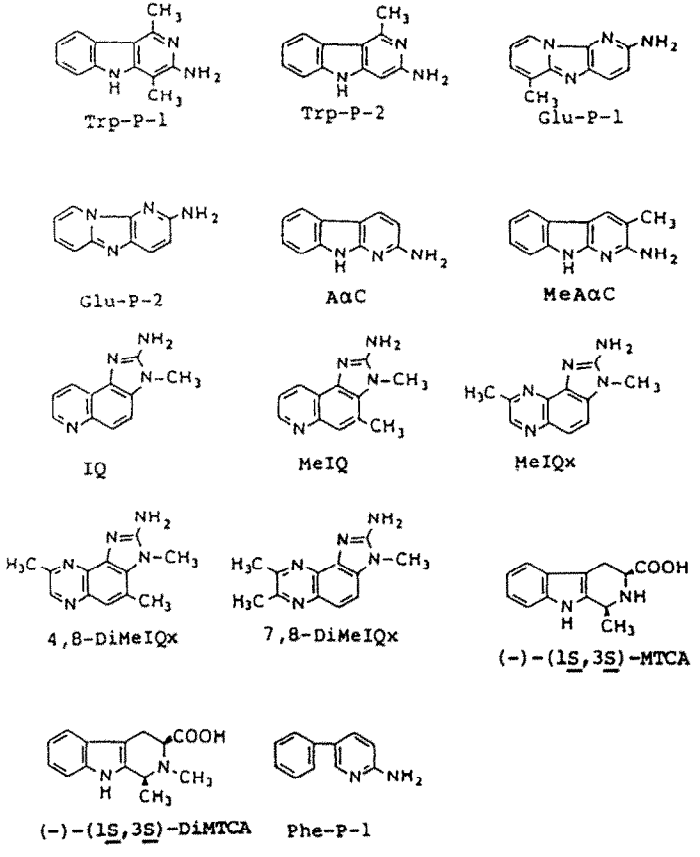

Abbreviation	Chemical Name
Trp-P-1	3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Trp-P-2	3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole
Glu-P-1	2-Amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole
Glu-P-2	2-Aminodipyrido[1,2-*a*:3',2'-*d*]imidazole
Aα C	2-Amino-9*H*-pyrido[2,3-*b*]indole
MeAα C	2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
IQ	2-Amino-3-methylimidazo[4,5-*f*]quinoline
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline
MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
(-)-(1*S*,3*S*)-MTCA	(-)-(1*S*,3*S*)-1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid
(-)-(1*S*,3*S*)-DiMTCA	(-)-(1*S*,3*S*)-1,2-Dimethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid
Phe-P-1	2-Amino-5-phenylpyridine

Fig. 1. Chemical structures, chemical names and abbreviations of tested heterocyclic amines in food.

Table 1. Effects of heterocyclic amines on tyrosine hydroxylation in tissue slices of the rat striatum

		N	% of control (mean ± SEM) [nmol/hr/mg protein]
Control		11	0.68 ± 0.09
Trp-P-1	(2 μg/ml)	4	40 ± 5*
Trp-P-2	(2 μg/ml)	4	40 ± 9*
Glu-P-1	(2 μg/ml)	4	78 ± 9
Glu-P-2	(2 μg/ml)	4	67 ± 9
AαC	(2 μg/ml)	4	89 ± 9
MeAαC	(2 μg/ml)	4	96 ± 7
IQ	(2 μg/ml)	4	70 ± 5
MeIQ	(2 μg/ml)	3	88 ± 15
MeIQx	(2 μg/ml)	3	124 ± 28
4,8-DiMeIQx	(2 μg/ml)	4	105 ± 28
7,8-DiMeIQx	(2 μg/ml)	4	148 ± 16
(−)-(1*S*,3*S*)-MTCA	(2 μg/ml)	6	142 ± 9*
(−)-(1*S*,3*S*)-DiMTCA	(2 μg/ml)	5	106 ± 21
Phe-P-1	(2 μg/ml)	7	105 ± 9

* $P < 0.01$.

with sodium pentobarbital and a Teflon 22-gauge guide cannula was stereotaxically implanted into the left striatum for the subsequent insertion of a dialysis probe. Two days after the surgery, the dialysis experiment was carried out with a freely-moving rat placed in a plastic home cage. The dialysis probe connected to a microinfusion system was perfused at 2 μl/min with a Ringer solution. Perfusates were collected every 20 min into a small test tube and assayed for DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) using HPLC–ECD.

MAO activity was assayed based on the formation of 4-hydroxyquinoline from kynuramine according to the method by Kraml [11]. Mouse brain mitochondria and the homogenate of rat pheochromocytoma PC12h clonal cells [12] were used as enzyme sources. The reaction was carried out at 37° for 20 min in 1.5 ml of the reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.4). 100 μM kynuramine, 1 μM deprenyl for type A MAO or 1 μM clorgyline for type B MAO, and the enzyme preparation.

Mouse striata were homogenized with 9 volumes of 20 mM Tris–HCl (pH 7.2) containing 1 mM dithiothreitol, 0.1 mM EDTA and 8% sucrose. An aliquot of the homogenate was used both for the analysis of DA and DOPAC by HPLC–ECD, and for enzymatic analyses of tyrosine hydrocyclase (TH; EC 1.14.16.2, tyrosine 3-monooxygenase) and MAO activities.

TH activity *in vitro* was measured by HPLC–ECD [13]. Incubation mixture contained (total volume 200 μl, in final concentrations) 0.2 M potassium phosphate buffer (pH 6.3), 1 mM (6*RS*)-methyl-5,6,7,8-tetrahydropterin, 0.1 M mercaptoethanol, 0.02 mg catalase, 0.1 mM L-tyrosine and the enzyme preparation. Incubation was carried out for 10 min at 37°.

Statistical analysis of mean differences were done by the Student's *t*-test or Welch procedure [14] whenever variations were unequal.

RESULTS

Effects of heterocyclic amines on tyrosine hydroxylation in striatal tissue slices

First we used the striatal tissue slice system to investigate the effects of heterocyclic amines on tyrosine hydroxylation in dopaminergic neurons. This system makes it possible for us to screen many compounds rapidly and reproducibly. Table 1 summarizes the effect of each of 14 heterocyclic amines on the hydroxylation of endogenous tyrosine to DOPA in striatal tissue slices with NSD-1055 to inhibit aromatic L-amino acid decarboxylase. The concentration of 2 μg/ml was equivalent to about 1×10^{-5} M. 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-b]indole (Trp-P-2) caused a substantial inhibition. All other compounds tested did not show any significant effect on tyrosine hydroxylation, except (−)-(1S,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid ([(−)-(1S,3S)-MTCA] which significantly activated tyrosine hydroxylation.

Effects of Trp-P-1 and Trp-P-2 on dopamine metabolism in the striatum as determined by in vivo *brain micro-dialysis*

Next we tested whether Trp-P-1 and Trp-P-2 could change the levels of DA and its metabolites in the striatum *in vivo* by a brain micro-dialysis method. Figure 2 shows that the intrastriatal perfusion of 10 mM Trp-P-1 or Trp-P-2 immediately caused a transient increase of DA. Maximum values were observed at 60 min for Trp-P-1 (263 ± 51% of the basal value, mean ± SEM, N = 3), and at 20 min for Trp-P-2 (470 ± 166% of the basal value, N = 3). The concentration of drugs (10 mM) was chosen to compare with our previous report on MPP^+ (8 mM) [10]. HVA and DOPAC concentrations in the striatal perfusates decreased gradually (Fig. 2), and their decrement continued until 180 min after the onset of the drug-perfusion. DOPAC concentration was

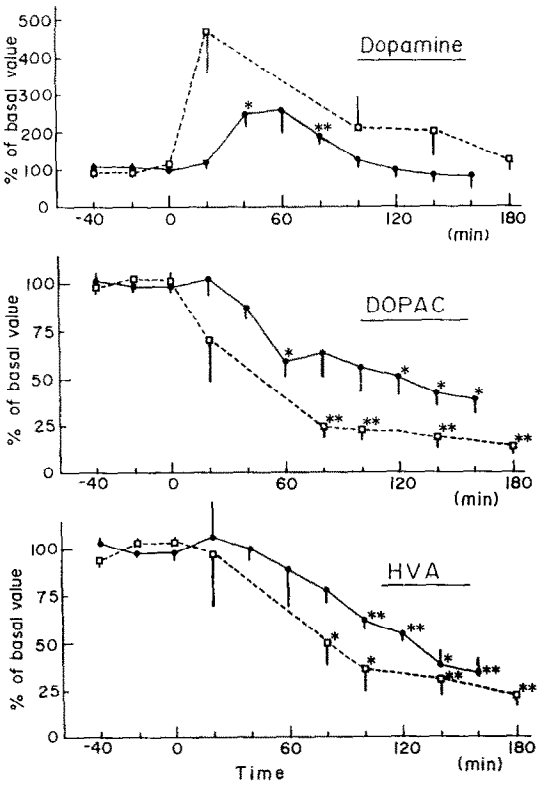

Fig. 2. Changes in the levels of DA, DOPAC, and HVA in the perfusate of rat striatum after 10 mM Trp-P-1 (—●—) or 10 mM Trp-P-2 (---□---) treatment. Administration of drugs was started at 0 min. Each point represents mean values and the SEM from three rats. *P < 0.05, **P < 0.01.

39 ± 7% of the control value, and HVA concentration was 35 ± 7% in Trp-P-1-treated rats at 160 min (mean ± SEM, N = 3). In Trp-P-2-treated rats, DOPAC was 14 ± 3% and HVA was 23 ± 3% at 180 min (N = 3). Basal values for the Trp-P-1-treated rats were: 14.6 fmol/min for DA; 602 fmol/min for DOPAC; and 428 fmol/min for HVA. Basal values for the Trp-P-2-treated rats were: 9.6 fmol/min for DA; 546 fmol/min for DOPAC; and 380 fmol/min for HVA.

Effect of Trp-P-1 on DA metabolism in vivo *in mouse striatum after systemic administration*

Trp-P-1 was administered systemically to mice to examine its *in vivo* effect on DA metabolism in the striatum. As a reference compound, MPTP, a potent dopaminergic neurotoxin, was also administered to mice. Male C57-Black/6N mice (approximately 25 g) were used. Drugs were administered by intraperitoneal injection at a total dose of 80 mg/kg of MPTP (4 injections of 20 mg/kg per injection at 2 hr intervals) [15], and 200 mg/kg of Trp-P-1 (4 injections of 50 mg/kg per injection at 2 hr intervals), respectively. Control animals were treated with the same volume of saline (0.25 ml per injection). The animals were killed 20 hr after the last injection by cervical dislocation, and *in vitro* TH activity and the contents of DA and DOPAC in the striatum were measured. As shown in Table 2, a significant increase of dopamine and marked decrease of DOPAC were observed. TH activity *in vitro* was not altered by Trp-P-1, while MPTP caused a significant reduction of TH activity, DA, and DOPAC, which agrees with our previous report on MPTP by Nagatsu and Hirata [16]. *In vitro* MAO activity was also measured with homogenates, but no significant changes were observed (data not shown).

Inhibition of MAO in vitro *by Trp-P-1 and Trp-P-2*

Effects of Trp-P-1 and Trp-P-2 on MAO activity *in vitro* were examined. As enzyme sources we used homogenates of PC12h cells for type-A MAO and the mitochondrial fraction of mouse brain treated with 1 μM clorgyline (a specific inhibitor of type-A MAO) for type-B MAO. Trp-P-1 and Trp-P-2 inhibited both MAO activity. Kinetic analysis showed that Trp-P-1 and -2 were competitive inhibitors against the substrate (Fig. 3). Inhibition constants (K_i) of Trp-P-1 were 2.1×10^{-7} M for type-A MAO and 5.2×10^{-6} M for type-B MAO, whereas those of Trp-P-2 were 4.3×10^{-8} M for type-A MAO and 3.1×10^{-5} M for type-B MAO (Table 3). Trp-P-2 more specifically inhibited type-A MAO than Trp-P-1 did. As the fluorescence spectrum of Trp-P-1 or Trp-P-2 was not altered at all before and after incubation at 37° for 30 min with mitochondrial fraction of mouse brain, Trp-P-1 or Trp-P-2 could not be a substrate for MAO (data not shown).

Table 2. Effects of systemic administration of Trp-P-1 to mice on *in vitro* TH activity and the levels of DA and DOPAC in the striatum

	In vitro TH activity (nmol/min/g tissue)	DA (nmol/g tissue)	DOPAC (nmol/g tissue)
Control	17.6 ± 0.6	61.1 ± 2.1	72.6 ± 3.7
Trp-P-1	18.5 ± 1.2	79.7 ± 4.4*	16.3 ± 2.9†
MPTP	8.3 ± 0.8†	25.2 ± 3.6†	18.9 ± 0.4†

Male C57-black mice were injected intraperitoneally with each drug four times at 2-hr intervals. The dose for each injection was 50 mg/kg in Trp-P-1 (total dose of 200 mg/kg) and 20 mg/kg in MPTP (total dose of 80 mg/kg). The mice were killed at 20 hr after the last injection. The activity of TH and the contents of DA and DOPAC in the striatum were determined. Data are the mean ± SEM (N = 4). *P < 0.05, †P < 0.01 compared to control.

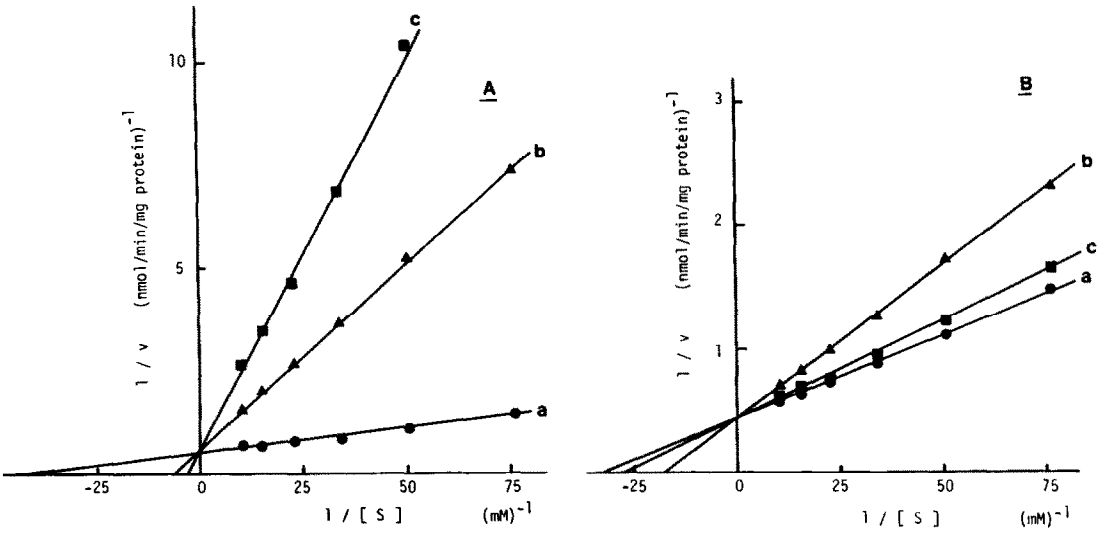

Fig. 3. (A) Lineweaver–Burk plots of type-A MAO activity in the homogenate of PC12h cells with the absence and presence of Trp-P-1 or Trp-P-2. The MAO activity was measured using kynuramine as substrate. (a) Control; (b) in the presence of 1 μM Trp-P-1; (c) in the presence of 0.5 μM Trp-P-2. (B) Lineweaver–Burk plots of type-B MAO activity in mouse brain mitochondria with the absence and presence of Trp-P-1 or Trp-P-2. The MAO activity in mitochondria was measured in the presence of 1 μM clorgyline using kynuramine as substrate. (a) Control; (b) and (c): in the presence of 5 μM Trp-P-1 and Trp-P-2, respectively.

DISCUSSION

Among heterocyclic amines tested Trp-P-1 and Trp-P-2 were found to inhibit tyrosine hydroxylation in striatal tissue slices of rats. This inhibitory effect is similar to an acute effect of MPTP [17] or MPP^+ [18], as reported previously, and it can be observed shortly after a single administration [19]. Repeated systemic administration of MPTP to mice caused inhibition of TH activity due to the decrease of TH protein, as Mogi *et al.* [20] previously reported. In contrast, Trp-P-1 did not inhibit TH activity after repeated systemic administration to mice. This result suggests that Trp-P-1 does not reduce TH protein to reduce TH activity, as MPTP does.

The *in vivo* brain micro-dialysis method provides us with an effective means to assess the acute changes of functional states of a neurotransmitter system induced by pharmacological treatment in freely-moving unanesthetized animals. The increased extracellular DA levels recovered in the dialysis tubing after superfusion of Trp-P-1 or Trp-P-2 are likely to reflect the lack of degradation by MAO of uptaken DA to increase the cytoplasmic level of DA rather than acute release of DA. This assumption is supported by the results on inhibition of MAO by Trp-P-1 and Trp-P-2 *in vitro*, and by the different findings between Trp-P-1 or Trp-P-2 and MPP^+ in micro-dialysis experiments. MPP^+ brings about a massive release of DA after uptaken into DA vesicles. As we reported previously [10], the increase of DA by MPP^+ is more than 100 times basal values, while the increase of DA by Trp-P-1 or Trp-P-2 in this study was only 5 times.

Trp-P-1 was found to be a potent competitive inhibitor of type-A MAO and type-B MAO. However, there were no reductions on type-A MAO

Table 3. Kinetic data on the inhibition of MAO by Trp-P-1 or Trp-P-2

Enzyme sources	Inhibition constants [K_i] (M)		Michaelis constants* [K_m] for kynuramine (M)
	Trp-P-1	Trp-P-2	
Type-A MAO (homogenate of PC12h cells)	2.1×10^{-7}	4.3×10^{-8}	$(2.27 \pm 0.12) \times 10^{-5}$
Type-B MAO (clorgyline-treated mouse brain mitochondria)	5.2×10^{-6}	3.1×10^{-5}	$(3.13 \pm 0.15) \times 10^{-5}$

* These values were calculated from Lineweaver–Burk plot using a least-squares method and expressed with standard deviations. Maximum velocities of used enzymes are 1.94 ± 0.07 (nmol/min/mg protein) for type-A MAO and 2.25 ± 0.08 (nmol/min/mg protein) for type-B.

and type-B MAO activities assayed *in vitro* after repeated systemic administration of Trp-P-1 to mice. This discrepancy may be due to the reversible nature of the MAO inhibition.

The alterations of DA and its metabolites in *in vivo* micro-dialysis and *in vivo* experiments strongly suggest that Trp-P-1 and Trp-P-2 could inhibit the activity of MAO *in vivo*, increasing cytoplasmic DA concentrations. Decrease of tyrosine hydroxylation in tissue slices of the striatum may be ascribed to inhibition of TH activity by increased cytoplasmic DA.

MAO inhibitors are clinically used for neural diseases such as depression and Parkinson's disease [21, 22]. Endogenous inhibitors of MAO were also reported in urine [23, 24], plasma [25], and cerebrospinal fluid [26]. Such endogenous inhibitors could modulate MAO activity *in vivo*. Some environmental compounds may also alter the MAO activity. Cigarette smoke contains MAO inhibitors [27]. Quinoline and quinaldine in coal tar have MAO inhibitory action [28]. Trp-P-1 and Trp-P-2 reported in this paper are the most potent MAO inhibitors in food.

Trp-P-1 and Trp-P-2 were first isolated as mutagens from tryptophan pyrolysis products and found to be present in cooked fish and meat [4–7]. Mutagenicity was shown by means of microbial assays [7]. Trp-P-1 or Trp-P-2 made about 2000 revertants of *Salmonella typhimurium* (TA100) per microgram of them. Beef cooked on naked flame, for example, contains 53 ng/g of Trp-P-1 [5]. This concentration is relatively low. But our results of systemic injection revealed that Trp-P-1 could penetrate the blood–brain barrier and alter the DA metabolism in brain. Brandt *et al.* [29] has also reported that Trp-P-1 could penetrate the blood–brain barrier and was accumulated in the pigmented tissues. These findings and high affinity to MAO suggest that Trp-P-1 and Trp-P-2 could be accumulated in the brain.

In this paper, we found novel and potent inhibitors in food for MAO in the striatum. Further study is expected to clarify the involvement of environmental compounds in neural diseases.

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