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INTRA- AND EXTRA-DOPAMINE-SYNAPTOSOMAL LOCALIZATION OF MONOAMINE OXIDASE IN STRIATAL HOMOGENATES FROM FOUR SPECIES

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Abstract—MAO-A and MAO-B activities within and outside dopaminergic synaptosomes in homogenates of striatal tissue from pig, cat, rat and human brains have been studied by using a specific "low substrate concentration technique" with dopamine. It was found that within the synaptosomes, MAO-A activity predominated in all species. Outside the synaptosomes there were more pronounced differences and only in the rat did MAO-A predominate, while in the other species MAO-B predominated. When estimating MAO-A and -B activities with a conventional method the activity of MAO-B predominated in man, cat and pig. Thus, also in species where the MAO-B activity (as estimated in a conventional way) was dominating, the intrasynaptosomal deamination of dopamine was brought about mainly by MAO-A.

The "low substrate concentration technique", more adequately reflects physiological conditions by taking into account the higher concentration of monoamine transmitter substrates within the monoamine neurons. With this technique it was found that in all species (with the possible exception of man) the oxidation rate was higher within than that oustide the DA-synaptosomes. In man the unavoidable longer time between death and estimation of the enzyme activity may be the cause of the deviating result.

Monoamine oxidase (MAO: EC 1.4.3.4) is a well studied enzyme associated with central monoamine transmitter systems [1, 2]. It occurs in two forms termed A and B, which differ in sensitivity to some inhibitors and in substrate specificity. The A-form of MAO is defined as the form highly sensitive to inhibition by clorgyline, while the B-form is sensitive to clorgyline only at very high concentrations [3].

In the rat brain, serotonin (5-HT) is a substrate mainly for the A-form, while dopamine (DA) is an equally well preferred substrate for both forms of the enzyme [4–6]. In the human brain a similar substrate specificity has been found [7, 8].

The occurrence of the two forms of the enzyme varies considerably between various species [9, 10]. In human brain, MAO-B is present in higher concentrations than MAO-A, while in the rat brain MAO-A dominates [5, 7, 9]. Some investigators have reported that certain tissues seem to contain only one form of MAO. Thus, Hall *et al.* [10] studied the MAO activity in liver and brain from several mammals and were not able to demonstrate more than one form, the B-form, of the enzyme in pig brain and liver. Tipton and Spires [11] also found some criteria for homogeneity of pig brain MAO. It could later be demonstrated, however, that probably there is also a small fraction of MAO-A present in these tissues [12]. In the cat, the brain appears to contain both the A-form and the B-form of the enzyme [10].

Brain tissue MAO has been shown to be located in neurons as well as in glial cells [13–15] and information about the partition between neuronal and other tissues in the deaminating process is accumulating. Thus, for example, the DA-synaptosomal fraction of the enzyme was found to be less than 1% of the total amount in rat and human striatal tissues, when using a conventional method for estimation of the activities [5]. However, using a technique for estimation, which takes into account the fact that the active uptake mechanism makes the intraneuronal enzyme operate at a considerably higher substrate (DA) concentration and hence is more likely to represent physiological conditions, the activities towards DA inside and outside the DA-synaptosomes were found to be of the same order of magnitude [5, 16]. In the DA-synaptosomes mainly MAO-A is present. This seems to be true for both human and rat striatal homogenates [5, 17]. Also rat forebrain noradrenaline synaptosomes seem to contain only MAO-A [18]. With respect to central serotonin neurons in the rat, there are, however, indications that MAO-B might be present [19]. In extra-monoamine-neuronal brain tissue in rat and man, MAO-A as well as MAO-B are present [5, 20]. The molecular ratio for MAO-B/-A in the human brain is, however, higher than in the rat brain [5, 7].

We have now conducted a series of experiments aimed at studying the MAO activity inside and outside the DA-synaptosomes in striatal homogenates from pig, cat and man, of which in particular the pig has been claimed to be devoid of MAO-A. Rat striatal tissue was included as a reference.

MATERIALS AND METHODS

Male Sprague–Dawley rats (weight about 330 g, N = 6) were killed by decapitation. Brains from pigs

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(6–7 months old, N = 4) were obtained from the slaughterhouse and chilled on ice within 30 min after the death of the animals. The cats (weight about 2.5 kg, N = 3) were killed by a high dose of pentobarbital (60 mg/kg) injected i.p. and as soon as they had fallen asleep they were decapitated. In all cases the brains were quickly removed, placed on an ice-cold Petri dish and the corpora striata dissected out. The human brains were collected less than 24 hr *post mortem* from individuals who had died suddenly without neurological complications. Corpora striata were dissected fresh and then slowly frozen to −70° in 0.32 M sucrose in a manner known to allow for the isolation of metabolically active synaptosomes [21, 22].

In each experiment the striata were homogenized by six strokes at 600 rpm in 9 vol. of ice-cold isotonic sucrose in a glass–Teflon pestle system.

In order to make sure that pentobarbital in high doses did not affect the active uptake mechanisms and hence also the intrasynaptosomal MAO activity, experiments were done on rats (N = 4) which were injected i.p. with mebumal 60 mg/kg body weight. When unconscious the rats were killed by decapitation and their brains quickly removed. The striata were dissected out and uptake rates of DA and intra- and extrasynaptosomal MAO activities analysed. Control rats were analysed at the same time.

When checking the effect of sodium-pentobarbital on the rates of uptake of DA, aliquots of the homogenates were put in centrifuge tubes containing Krebs–Henseleit's bicarbonate buffer. Ascorbic acid (0.2 mg/ml) as antioxidant and Nialamid (1×10^{-6} M) as MAO inhibitor were also included. The samples were then preincubated at 37° for 5 min. The radioactive amine, ^{14}C-dopamine; {3,4 -(8-^{14}C)-dihydroxyphenylethylamine hydrobromide; 49.7 mCi/mmol; New England Nuclear, Boston, MA} was then added to a final concentration of 10^{-7} M. The tubes were mixed and incubated for 2 min. The incubations were terminated by putting the tubes into an ice water bath. To collect the particulate matter, including the synaptosomes, the tubes were centrifuged (7000 *g* 5 min). After the centrifugation the pellets were rinsed twice with 3 ml of ice-cold saline. Soluene 350® (0.5 ml) was added to each tube to solubilize the pellet and radioactivity measured in a liquid scintillation spectrometer. Similar experiments were carried out in the presence of the monoamine uptake inhibitor cocaine (2.5×10^{-5} M) and active uptake defined as the difference in radioactivity accumulated in the absence and presence of cocaine [23]. In separate experiments we have found the inhibition of 2.5×10^{-5} M cocaine on uptake of dopamine to be about 80% and in itself not significantly to affect the MAO activity. Furthermore, in rat striatal homogenate the selective DA uptake blocker amphoneliic acid (3×10^{-7} M) was found to give the same result with regard to MAO activity as 2.5×10^{-5} M cocaine.

For estimation of intra- and extrasynaptosomal MAO-A and -B activities aliquots of the 10% homogenate were preincubated for 5 min in the Krebs–Henseleit's buffer, in the presence and absence of cocaine (2.5×10^{-5} M), as well as in the presence and absence of (+)-FLA 336 (10^{-5} M) as uptake inhibitor and MAO-A inhibitor, respectively. Incubations were then carried out for another 10 min at 37° under an atmosphere of air with ^{14}C-DA (10^{-7} M). The metabolites formed were extracted into 6 ml of toluene–etylacetate (1/1, v/v), saturated with water and radioactivity counted in 10 ml of Econofluor® (NEN, Boston, MA).

Total MAO-A and -B activities were also assayed radiochemically by a conventional method (high substrate concentration method), with ^{14}C-5-hydroxytryptamine binoxalate (5-HT) and ^{14}C-phenethylamine hydrochloride (2-PEA), respectively, as substrates, at concentrations of 50 μM (5-HT) and 13.8 μM (PEA) [24].

Specific activities were in all cases corrected for the efficiency of extraction of the deaminated metabolites into the organic layers used [25]. In experiments where clorgyline was used to inhibit MAO, the homogenates were preincubated for 30 min with the inhibitor to allow for the irreversible inhibition of MAO-A. All labelled compounds were obtained from New England Nuclear, Boston, MA. Amiflamine ((+)-FLA 336) was a gift from Astra Läkemedel AB, Södertälje, Sweden, and clorgyline hydrochloride a gift from May & Baker Ltd., Dagenham, U.K.

Protein concentrations were assayed by the method of Markwell *et al.* [26].

RESULTS

The possibility that pentobarbital could inhibit the active uptake of ^{14}C-DA and hence the activity of MAO intra-DA-neuronally, was examined. No significant difference was found with respect to the rate of uptake of ^{14}C-DA in striatal homogenates when comparing rats given mebumal (60 mg/kg body weight, i.p.) with control rats (mean 27.9 pmol/mg prot/min compared to 22.5 pmol/mg prot/min). Neither were there any significant differences with regard to total oxidation of ^{14}C-DA, using the low substrate concentration method (Table 1; T), nor when the oxidation rate was separated into the component catalysed by MAO-A and the one catalysed by MAO-B. This was true for intra- as well as extrasynaptosomal MAO. (Table 1; I_A, I_B and E_A, E_B, respectively). Estimation of the MAO-A and MAO-B activities in striatal homogenates by the conventional "high substrate concentration technique" (Table 2) showed that the rat has the highest MAO-A activity (about 1500 pmol/mg prot/min) while the pig, cat and man had considerably lower activities (about 340–440 pmol/mg prot/min). The MAO-B activity, on the other hand, was highest in man (about 1700 pmol/mg prot/min) and lowest in cat (about 430 pmol/mg prot/min).

The distribution of striatal MAO activities in the four species, taking into account separately the intra- and extrasynaptosomal activities, is summarized in Table 3.

The total oxidation (T in Table 3) was most rapid in rat striatal homogenates (16.1 ± 1.9 pmol/mg prot/min) followed by pig (10.1 ± 2.2 pmol/mg prot/min). The activity in the homogenates from cat and man were roughly similar, being about 20% of the activity in the rat.

Table 1. Effect of mebumal on intra- and extraneuronal MAO activity in rat striatum.

	T	E	I	E_A	E_B	I_A	I_B
Controls N = 4	15.54 ± 1.36	2.85 ± 0.36	12.68 ± 1.53	2.18 ± 0.32	0.68 ± 0.05	11.94 ± 1.43	0.75 ± 0.18
Mebumal inj. N = 4	16.85 ± 0.41	2.54 ± 0.59	14.30 ± 0.80	1.94 ± 0.49	0.60 ± 0.11	13.55 ± 0.61	0.76 ± 0.21

For estimation of extra- and intra-synaptosomal MAO activities, 10% homogenates were incubated for 10 min with 0.1 μM dopamine ^{14}C, after 5 min preincubation with or without 25 μM cocaine as uptake inhibitor and with or without 10 μM FLA-336 (+) as MAO-A inhibitor. After incubation, metabolites were extracted into toluene: ethylacetate and the radioactivities counted by LSC. Activities are given as pmoles/mg protein/min. Values represent the means ± SD. Abbreviations; T, Total DA oxidation by extra- and intra-synaptosomal MAO; E, extrasynaptosomal DA oxidation; I, intrasynaptosomal DA oxidation; E_A, DA oxidation by extrasynaptosomal MAO-A; E_B, DA oxidation by extra-synaptosomal MAO-B; I_A, DA oxidation by intrasynaptosomal MAO-A; I_B, DA oxidation by intrasynaptosomal MAO-B. There were no significant differences as determined by two-tailed *t*-test.

An analysis of the distribution of the MAO activities intra- and extrasynaptosomally (I and E in Table 3) revealed that in the cat, pig and rat most of the DA was oxidized within the DA-synaptosomes while in man the reverse was the case.

When separating the oxidation of DA into the portion catalysed by MAO-A and the one catalysed by MAO-B, most of the oxidation in the human striatal homogenate was found to be catalysed by MAO-B (about 3.1 pmol/mg prot/min; $E_B + I_B$ in Table 3), mainly being localized outside the DA-synaptosomes (2.9 ± 0.4 pmol/mg prot/min; E_B in Table 3).

In the striatal homogenate from cat, MAO-A was found to catalyze the oxidation of DA somewhat faster (about 2.4 pmol/mg prot/min; $E_A + I_B$ in Table 3). The activity of the A-form was predominantly within the DA-synaptosomes (2.2 ± 1.0 pmol/mg prot/min; I_A in Table 3).

In pig striatal homogenate the oxidation of DA was carried out by MAO-A at a rate of about 5.4 pmol/mg prot/min ($E_A + I_A$ in Table 3), which is of about the same order of magnitude as that of MAO-B (about 4.7 pmol/mg prot/min; $E_B + I_B$ in Table 3). As was also found in the cat and the rat, but not in man, the MAO-A activity was predominantly intrasynaptosomally localized (I_A; 5.2 ± 1.8 pmol/mg prot/min; Table 3).

Table 2. MAO-A and -B activities in striatal tissue from four species

	MAO-A	MAO-B
Rat	1496.39 ± 101.58	1082.96 ± 79.59
Pig	440.83 ± 56.50	1388.86 ± 272.15
Homo	420.96 ± 78.98	1707.72 ± 196.61
Cat	336.78 ± 53.73	429.74 ± 92.47

For estimation of MAO activities, homogenates were incubated with ^{14}C-5-HT (50 μM) and ^{14}C-B-PEA (12.8 μM). Activities are given as pmoles/mg protein/min (means ± SD).

DISCUSSION

If a preparation of MAO contains both forms of the enzyme, a plateau-shaped inhibition curve will be obtained when the activity towards a substrate, common for both forms, is inhibited by increasing concentrations of, for example, an MAO-A selective inhibitor such as clorgyline. If, on the other hand, the preparation contains only one form of the enzyme, the inhibition curve will assume a sigmoid pattern [3]. Tyramine is an equally well preferred substrate for both forms of the enzyme in most tissues [4, 6]. The inhibition curves, with clorgyline, towards tyramine oxidation, for striatal tissue homogenates from the different species are demonstrated in Fig. 1. For rat brain striatal homogenate, the inhibition curve towards tyramine oxidation was plateau-shaped with one part highly sensitive and one part

Table 3. MAO activities, intra- and extrasynaptosomally from four species

Striatum	T	E	I	E_A	E_B	I_A	I_B
Rat (N = 6)	16.10 ± 1.90	2.50 ± 0.47	13.68 ± 1.90	1.63 ± 0.20	0.87 ± 0.34	13.06 ± 1.73	0.63 ± 0.20
Pig (N = 4)	10.13 ± 2.22	2.67 ± 0.95	7.46 ± 2.53	0.22 ± 0.30	2.46 ± 1.09	5.22 ± 1.80	2.24 ± 0.84
Homo (N = 10)	4.13 ± 0.71	3.48 ± 0.77	0.61 ± 0.45	0.42 ± 0.32	2.92 ± 0.44	0.48 ± 0.55	0.17 ± 0.21
Cat (N = 3)	3.67 ± 1.32	0.50 ± 0.08	3.16 ± 1.33	0.13 ± 0.01	0.37 ± 0.08	2.22 ± 0.97	0.94 ± 0.36

For estimation of extra- and intra-synaptosomal MAO activities, 10% homogenates were incubated for 10 min with 0.1 μM dopamine-^{14}C, after 5 min preincubation with or without 25 μM cocaine as uptake inhibitor and with or without 10 μM FLA-336 (+) as MAO-A inhibitor. After incubation, metabolites were extracted into toluene: ethylacetate and the radioactivities counted by LSC. Activities are given as pmoles/mg protein/min. Values represent the means ± SD. Abbreviations: T, total DA oxidation by extra- and intra-synaptosomal MAO; E, extrasynaptosomal DA oxidation; I, Intrasynaptosomal DA oxidation; E_A, DA oxidation by extrasynaptosomal MAO-A; E_B, DA oxidation by extra-synaptosomal MAO-B; I_A, DA oxidation by intrasynaptosomal MAO-A; I_B, DA oxidation by intrasynaptosomal MAO-B.

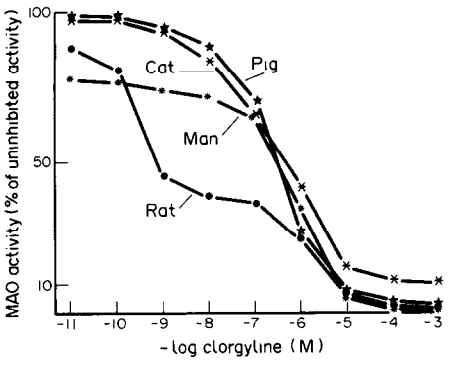

Fig. 1. The effect of *in vitro* inhibition of tyramine deamination by clorgyline in homogenates of striatum from rat, pig, cat and man. Samples of the different striatal homogenates were preincubated in the presence of indicated concentrations of clorgyline in a total volume of 300 μl of 0.01 M potassium phosphate (pH 7.4) for 30 minutes at 37° before adding 25 μl of ^{14}C-tyramine (50 μM e.c.). Data are means from six rats, three cats, four pigs and ten men.

less sensitive towards clorgyline. The plateau was found at about 65% inhibition of the enzyme activity. This result indicates the presence of the two forms of MAO(-A and -B), with the -A form being responsible for about 65% of the oxidation, which is in good agreement with earlier results (e.g. 3,5,7,9,10).

For pig and cat striatal homogenates, however, the curves obtained were single sigmoidally shaped with a rather low sensitivity of the enzyme for clorgyline. This indicates only little contribution of MAO-A in the oxidation of tyramine in homogenates of pig and cat striatal tissue. The inhibition curve for human striatal MAO was, as has been reported earlier [3, 9, 10], similar to those of the pig and cat.

With conventional methods for estimation of MAO activity, using substrate concentrations close to saturation, the pool of enzyme within monoamine synaptosomes is too small to be recognized [5]. Therefore, in order to estimate the MAO activity within the DA-synaptosomes we have used our modification [5] of the technique first applied by Waldmeier *et al.* [27] and later by Urwyler and von Wartburg [28] and Demarest *et al.* [17]. This technique takes into account the fact that, physiologically, the intra-monoamine-neuronal MAO activity is increased because of the higher intraneuronal monoamine concentration created by the uptake mechanisms. In fact, the MAO activity intrasynaptosomally may still be underestimated, since it is likely that part of the intraneuronal mitochondriae will not be contained within the synaptosomes after homogenization. The higher intrasynaptosomal concentration of monoamine substrate may further add to an underestimation of the intrasynaptosomal MAO activity, since the reaction is carried out closer to substrate saturation. Dopamine utilized in the present method has, besides being accumulated by the DA-synaptosomes, the advantage of being an equally good substrate for both forms of the enzyme [5, 8]. However, the use of DA makes the results valid only for the monoamine neurons capable of accumulating this compound, i.e. dopamine and noradrenaline neurons [29]. In the present study, this limitation of the technique was of little relevance, since striatal homogenates were used, which predominantly contain monoaminergic nerve terminals originating from DA-cell bodies [30].

Considerable species differences were observed concerning the total oxidation rate of DA; rat striatal homogenate being most efficient (T in Table 3). The experiments further show that roughly 80% of the MAO activity, estimated in this way, is localized within the striatal DA nerve terminals (I/T in Table 3) in three of the species investigated. In man the oxidation rate within the DA-synaptosomes was proportionally lower, but this might be a result of the unavoidably longer *post-mortem* storage time of human autopsy material. Thus, we have previously shown that the efficiency of the high affinity uptake system of DA starts to decline after about one hour of storage at +4° [22]. MAO activity, on the other hand, seems to be very resistant against storage [31, 32]. However, in order to verify that "the slow freezing and rapid thawing procedure" [21, 22] had no effect on MAO activity, separate experiments on rat brain tissue (striatum and forebrain) were done. As shown in Table 4 no significant effect on the separate MAO activities could be detected, either in striatum or forebrain. Thus, freezing *per se* has no deleterious effect on MAO activity.

In pig, cat and man roughly 75% of the DA-synaptosomal MAO activity could be attributed to MAO-A, while the corresponding figure was about 95% in the rat (I_A/I in Table 3). Since this synaptosomal MAO-A is localized behind an avid uptake mechanism, it is understandable that after exposure of the homogenate to DA in a concentration, which does not saturate the high affinity (low K_m) uptake mechanisms, a significant fraction of the deamination occurs within the DA-synaptosomes, in spite of the fact that this synaptosomal MAO represents less than 1% of the total amount [5]. Outside the dopaminergic synaptosomes, it appears that in pig, cat and man, the deamination of DA is brought about mainly by MAO-B. In rat striatal homogenate, however, this deamination by MAO-B seems to be rather small, as is also the case within the DA-synaptosomes (see also refs 5 and 33).

In all species examined the ratio of the activities of MAO-A/MAO-B was higher inside than outside the DA-synaptosomes. As a matter of fact, in the rat the intrasynaptosomal MAO-B activities were so low that they did not contribute significantly to the deamination. Furthermore, while we were preparing this paper, Fagervall and Ross [34], using selective uptake inhibitors, have shown that only MAO-A was responsible for the deamination of DA intrasynaptosomally in rat striatal tissue. In cat, pig and human, however, there is a contribution of MAO-B of about 30% to the total intrasynaptosomal MAO activity. This would be in agreement with the results of Demarest *et al.* [17], Schoepp and Azzaro [35] and Oreland *et al.* [5] and also in agreement with those of Riederer *et al.* for human striatal homogenate [36].

The main conclusion which can be drawn from the present results is, that even in species where MAO-

Table 4. Effect of "slow freezing and rapid thawing" on MAO activities in rat brain.

	Forebrain				Striatum		
	T	E	I		T	E	I
Fresh (N = 4)	16.9 ± 1.5	8.16 ± 1.2	7.7 ± 1.4	Fresh (N = 5)	20.5 ± 2.3	4.0 ± 0.7	16.5 ± 1.9
Frozen (N = 5)	14.3 ± 3.9	6.9 ± 2.8	7.4 ± 1.7	Frozen (N = 4)	17.4 ± 3.2	3.2 ± 0.8	17.2 ± 2.4

MAO activities in homogenates (10%) from fresh and frozen rat brain tissue (striatum and forebrain) were compared.

For estimation of extra- and intra-synaptosomal MAO activities aliquots of the homogenates were incubated for 10 min with either 0.1 μM DA (striatum) or 0.1 μM 5-HT (forebrain) with or without cocaine (25 μM) as uptake inhibitor. Metabolites formed were extracted in toluene: ethylacetate and radioactivity counted by LSC. Activities are given as pmoles/mg prot/min (means ± SD).

T, total DA or 5-HT oxidation by extra- and intrasynaptosomal MAO: E, extrasynaptosomal DA or 5-HT oxidation; I, intrasynaptosomal DA or 5-HT oxidation.

There were no significant differences as determined by two-tailed *t*-test.

B is predominating (pig, cat and man) the DA-synaptosomal deamination of dopamine is mainly brought about by MAO-A. It might well be, however, that this small pool of MAO is of major importance for the activity of the dopaminergic neurons. This would be in line with the recent findings that intra-neuronal MAO-A in the locus coeruleus in rat, which exclusively contains cell bodies of noradrenaline neurons [37], is of major importance for the spontaneous firing of these cells [18]. However, when studying the biochemical effects of, for example, MAO inhibitors, rat brain is shown not to be a very good model for the human situation.

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