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Effect of aging on lazabemide binding, monoamine oxidase activity and monoamine metabolites in human frontal cortex

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Summary. Age-related modifications of monoamine oxidase-A and -B (MAO-A and MAO-B) and amine metabolite concentrations were studied in human frontal cortex taken postmortem from 22 subjects of various ages (21–75 years). Qualitative and quantitative analysis for MAO-B was provided by kinetic studies with a specific radioligand, [^{3}H]lazabemide. The data demonstrated a significant ($P < 0.05$) positive correlation between the density of [^{3}H]lazabemide binding sites (B_{max}) and age of the subject, without showing an apparent modification in the dissociation constant (K_D) of the radioligand. In parallel experiments, MAO-B but not MAO-A activity was shown to correlate with age ($P < 0.05$). The concentrations of the amine metabolites 4-hydroxy-3-methoxyphenylacetic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylglycol (MHPG) and 3,4-dihydroxyphenylglycol (DHPG) were all devoid of a correlation with age. Neither did the concentrations of these metabolites relate to the respective subject's MAO-B enzymatic activity nor to [^{3}H]lazabemide B_{max}. A correlation, though rather weak, was obtained between MAO-A activity and MHPG concentration ($P = 0.045$). The MAO-A and -B enzyme characteristics in subjects who had committed suicide ($n = 9$) did not differ from those of subjects deceased for other causes ($n = 13$). Among the measured monoamine metabolites the concentrations of DOPAC and HVA were higher in the suicide versus control group ($P < 0.05$). The present data confirm in a direct manner that the increase in MAO-B activity in aging brain is due to an enhancement of the number of active sites of the enzyme and not through modifications of its kinetic characteristics. Furthermore, that neither the characteristics nor the activity of the enzyme are changed in the frontal cortex of suicide victims compared to control subjects.

Keywords: Monoamine oxidase, aging, human brain, monoamine metabolites, suicide.

Introduction

Monoamine oxidase (MAO, E.C. 1.4.3.4.) exists in two forms, MAO-A and MAO-B, which are known to be structurally different proteins coded by distinct genes (Cawthon et al., 1981; Bach et al., 1988; Chen et al., 1991). These two isoenzymes that catalyze the oxidative deamination of various endogenous and exogenous amines, show distinct affinities towards various substrates and are unevenly distributed in several extracerebral tissues and in brain (for review see Cesura and Pletscher, 1992). The synthesis of potent, reversible and selective inhibitors of MAO-B, has allowed further biochemical characterization of this isoenzyme (Fowler and Ross, 1984; Da Prada et al., 1990; Haefely et al., 1990; Cesura et al., 1993). In previous studies the MAO-B inhibitors Ro 16-6491 and lazabemide (Ro 19-6327) have been used as high affinity binding ligands for localization and quantitative determination of MAO-B molecules in various tissues (Cesura et al., 1987, 1988, 1989; Saura Marti et al., 1992). These two inhibitors are devoid of significant affinity for MAO-A, and thus bind selectively and reversibly to the active site of MAO-B behaving as false substrates (mechanism-based inhibition; Walsh, 1984). Since the high affinity binding of these inhibitors allows the precise quantitative determination of the MAO-B molecules present in tissue, these inhibitors can also be utilized in evaluating possible changes of MAO-B in disease states.

Previous studies utilizing human post-mortem brain tissue, have demonstrated that MAO-B enzymatic activity increased with age (Fowler et al., 1980; Strolin Benedetti et al., 1980, 1989; Cao et al., 1984; Oreland et al., 1986). These findings were obtained with radiochemical methods using radiolabelled amine substrates for the estimation of the enzyme activity. In a recent study Sastre and Garcìa-Sevilla (1993) have demonstrated in 11 subjects a positive correlation between the number of brain MAO-B binding sites and age. In the present study, we have determined the number of MAO-B binding sites and, in parallel experiments, the MAO-A and MAO-B activities in post-mortem cerebral cortex obtained from 22 subjects of various ages at autopsy. Furthermore, some monoamine metabolites were assayed by reversed phase high-performance liquid chromatography (HPLC) methodology.

Several studies have been devoted to the possible relationship between disturbances in monoaminergic function and suicidal behaviour. In particular, decreased serotoninergic activity, as well as lowered MAO-B activity in blood platelets, have been suggested to be related to suicidal and impulsive behaviour (for review see Fowler et al., 1982). Thus, we felt it of interest to examine whether there was any difference in the MAO characteristics or monoamine metabolite concentrations in the frontal cortex from suicide victims compared to subjects deceased for other causes.

Materials and methods

Lazabemide (Ro 19-6327, N-2-aminoethyl-5-chloro-2-pyridine carboxamide HCl) and [^{3}H]lazabemide (18.9 Ci/mmol) were obtained from Hoffmann-La Roche, Basel, Switzerland. [^{14}C]phenylethylamine (PEA; 50.2 mCi/mmol) was purchased from New England

Nuclear, Boston, MA., U.S.A. [^{14}C]5-hydroxytryptamine (5-HT; 57.4 mCi/mmol) was from Amersham, England. The following drugs were used: L-deprenyl HCl (Selegiline, Chinoin, Budapest, Hungary), clorgyline HCl (May & Baker, London, England), PEA HCl, 5-HT creatinine sulfate, 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 3,4-dihydroxyphenilacetic acid (DOPAC, Fluka, Buchs, Switzerland), 4-hydroxy-3-methoxyphenylglycol (MHPG) hemipiperazine salt, DL-3,4-dihydroxyphenylglycol (DHPG) and 5-hydroxyindol-3-acetic acid (5-HIAA, Sigma Chemical Co., St. Louis, MO., U.S.A.), isoMHPG (Hoffmann-La Roche, Basel, Switzerland). All the other chemicals were of analytical grade and were purchased from different sources.

Subjects

Human cerebral cortex gyrus cinguli was obtained from 22 subjects (19 males and 3 females) at autopsy within 48 h. Previous studies have demonstrated that the enzyme activity is stable during this post-mortem period (Gottfries et al., 1975; Mac Kay et al., 1978). These subjects ranged from 21 to 75 years of age and the cause of death in nine cases (23–72 years) was suicide (eight by hanging and one by jumping), whereas in 13 cases (21–75 years) either an accident or a natural cause of death. In the case of suicide, a detailed psychiatric history was collected from the families in order to make a DMS-III-R diagnosis (American Psychiatric Association, 1987), and for some subjects a clinical history was available. Four subjects had suffered from mood disorder, whereas the remaining five subjects had not shown any psychiatric disorder. Additional information obtained from the autopsy findings showed traces of acutely taken benzodiazepines in three subjects of the suicide group. Subjects that were known to have been exposed to chronic pharmacological treatments with drugs were excluded from the study.

Membrane preparation

The samples of frontal cortex were kept frozen at −70°C until they were utilized. In order to prepare crude membrane homogenates, the brain tissue was thawed and homogenized in 4 volumes ice-cold 0.32 M sucrose including 5 mM HEPES pH 7.4 utilizing a teflon-glass homogenizer. Thereafter the homogenate was centrifuged at 800 × g for 20 min and the supernatant was aliquoted and kept frozen at −70°C until used. Protein content was measured according to Lowry et al. (1951).

Biochemical determinations

Binding assay of [^{3}H]lazabemide was performed essentially as previously described by Cesura et al. (1989). Briefly, frontal cortex membranes suspended in 0.3 ml of 50 mM Tris pH 7.4 containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, were incubated for 2 h at 20°C at a protein concentration of 0.125 mg/ml with concentrations of [^{3}H]lazabemide ranging from 6 to 150 nM. Following the incubation period, 1 ml of ice-cold Tris buffer was added to each sample, and thereafter the samples were centrifuged (12,000 × g for 3 min at 4°C). The resulting membrane pellets were washed once with 1 ml of ice-cold Tris buffer and resuspended in 0.2 ml of sodium dodecylsulfate (20%, w/v). After solubilization, aliquots of the solubilized membranes were counted for radioactivity after the addition of 4 ml of Instagel (Packard, Downers Grove, IL, U.S.A.). The non-specific binding of [^{3}H]lazabemide was measured in the presence of 0.1 mM L-deprenyl.

Measurements of MAO-A and MAO-B activities in the same homogenates of frontal cortex were performed according to the conventional radiochemical method of Wurtman and Axelrod (1963). [^{14}C]5-HT (200 uM) and [^{14}C]PEA (10 uM) were utilized as substrates for MAO-A and MAO-B, respectively.

Free (unconjugated) monoamine metabolites in frontal cortex homogenates were measured by HPLC with electrochemical detection, after extraction into an organic solvent. The brain tissue (100 mg) was homogenized in 550 ul of 0.1 M citrate-0.2 M

phosphate buffer, pH 3. An aliquot of 200 ul of the homogenate, containing 1 ng of isoMHPG as internal standard, was extracted twice with 800 ul of ethylacetate. In parallel, tissue sample with standard amounts of each monoamine metabolite were processed to yield a calibration curve. After extraction, the organic phase was pooled, evaporated to dryness and reconstituted with 150 ul of the chromatographic mobile phase, which was prepared according to Gerlo and Malfait (1985). Thereafter, 50 ul were injected into the HPLC system. This system consisted of a Waters (Milford, MA, U.S.A.) PM-30 pump, a Gilson (Villiers Le Bel, France) model 231 automatic sample injector and a stainless steel column (300 × 3.9 mm) packed with Nucleosil C_{18} reversed phase, 7 um particle size (Macherey-Nagel, Duren, F.R.G.). The analytical mobile phase was delivered at a flow rate of 1.0 ml/min. The electrochemical detection system was a Coulochem 5100A (Environmental Sciences Associates, Bedford, Ma. U.S.A.), equipped with a model 5014 high sensitivity analytical cell. The applied potential to the electrodes was +0.35 V/ −0.35 V, respectively. The compounds were quantified recording the current obtained and determining the peak areas using a Shimadzu (Kyoto, Japan) C-R3A Chromatopack integrator. The concentrations of monoamine metabolites in each sample were determined by calculating the ratio between the area of each analyte peak and the area of the internal standard peak in that particular chromatogram. These ratios were then introduced into the standard calibration curve equation obtained by linear regression analysis. The chromatographic retention time and recovery for each analyte was: DHPG, 4 min, 47%; MHPG 7 min, 79%; DOPAC, 14 min, 100%; HIAA, 26 min, 63%; HVA, 37 min, 98%.

Statistical analysis

The data are expressed as mean ± S.E. The analysis of the data for the binding measurements has been performed using the Ligand computer program (McPherson, 1985) utilizing both linear and non-linear regression methods. The significance of differences between two groups was assessed by Student's t test. Linear regression analysis was performed according to the least square method.

Results

[^{3}H]lazabemide showed high affinity binding to crude membranes prepared from human frontal cortex (Fig. 1). The non-specific binding, estimated in the presence of 0.1 mM L-deprenyl, was negligible and even at a high ligand concentration (150 nM) it was only ca. 5% of the specific binding. Scatchard analysis of the saturation curves resulted in a straight line configuration indicating that [^{3}H]lazabemide labelled a single homogeneous population of sites. All the experiments were performed utilizing a crude membrane preparation of frontal cortex, since a further purification (4 fold) of the homogenate yielding a mitochondrial fraction (B_{max} 0.68 ± 0.13 and 2.65 ± 0.47 pmol/mg protein, n = 4, for homogenates and mitochondrial fractions, respectively) did not cause any relative changes in respect to the results obtained using the crude homogenate. Furthermore, establishing the experimental conditions, incubation at 20°C was shown to be favourable. The equilibrium at 20°C was reached in 2 h, whereas incubation at 4°C for the same period of time let to ca. 80% lower total labelling (data not shown).

When the [^{3}H]lazabemide B_{max} values, obtained from saturation curves for each of the subject used in the study, were correlated with the subjects'age, a positive relationship was obtained (Fig. 2). In contrast, the respective K_D values did not yield any correlation with the subjects' age (r = −0.147;

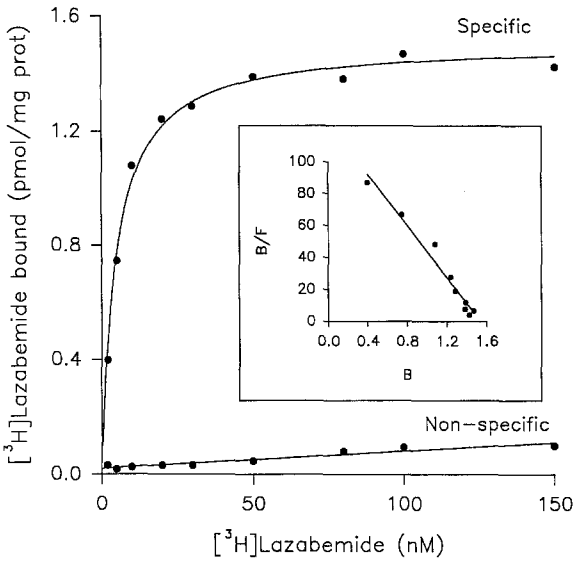

Fig. 1. Saturation curve and Scatchard analysis (inset) of the specific binding of [^{3}H]lazabemide to human frontal cortex homogenate after incubation for 2 h at 20°C with various concentrations of the ligand. Non-specific binding, as determined in the presence of 0.1 mM L-deprenyl, was ca. 5% of the specific binding. The data represents mean of one experiment performed in triplicate. K_D = 4.79 nM; B_{max} = 1.51 pmol/mg protein. Inset: The value of B/F is expressed X 10^3. *B* bound (pmol/mg prot), *F* free ligand

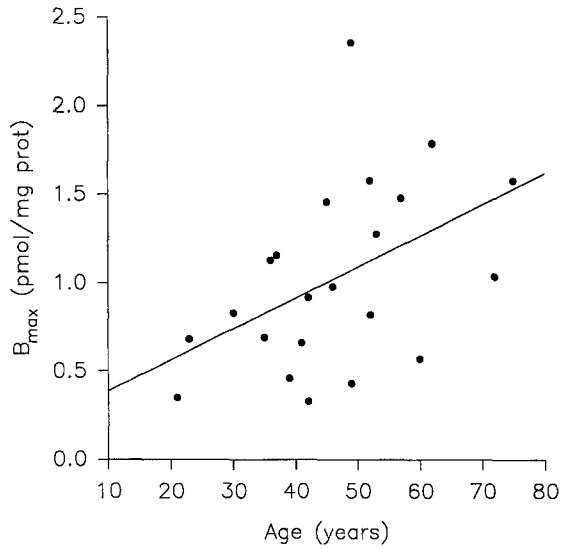

Fig. 2. Correlation between monoamine oxidase (MAO) concentrations and subjects' age. The concentrations of MAO-B were determined by kinetic analysis of the saturation curves from the binding of [^{3}H]lazabemide to homogenates of human frontal cortex (B_{max}). The saturation curves were determined at 9 different ligand concentrations (from 2 to 150 nM) in preparations each deriving from a different subject. Linear regression analysis, r = 0.459, $P < 0.05$, n = 22

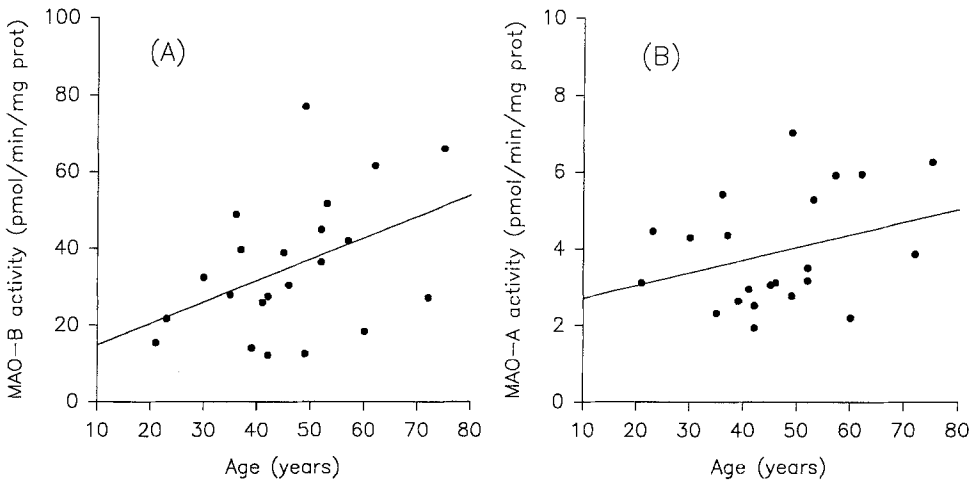

Fig. 3. Correlation between monoamine oxidase (MAO) activity in the homogenates of human frontal cortex and subjects' age. **A** MAO-B activity measured using 10 uM [^{14}C]beta-phenylethylamine as a substrate. Linear regression analysis, r = 0.424, P < 0.05, n = 22. **B** MAO-A activity measured using 200 uM [^{14}C]serotonin as a substrate. Linear regression analysis, r = 0.303, P = 0.170, n = 22

P = 0.514; n = 22). As for the B_{max} values, a positive correlation was found comparing MAO-B activity with the subject's age (Fig. 3a). In addition, the relationship between [^{3}H]lazabemide B_{max} and MAO-B activity was found to be highly significant (r = 0.954; P < 0.0001; n = 22). On the other hand, no correlation between MAO-A enzymatic activity and age was found (Fig. 3b).

The effect of aging on MAO-B in the frontal cortex was also evident when the subjects were divided into two groups: ≤45 years and >45 years, n = 11 in both groups. Both the B_{max} of [^{3}H]lazabemide binding and the MAO-B activity were significantly higher in the group represented by the elder subjects (Table 1). Neither the K_D of [^{3}H]lazabemide binding nor MAO-A activity differed significantly in the two age groups.

The concentrations of free amine metabolites (DHPG, MHPG, DOPAC, HVA and 5-HIAA) evaluated in the study were all devoid of a correlation with age (data not shown). Neither did the concentrations of these metabo-

Table 1. B_{max} (pmol/mg prot) and K_D (nM) of [^{3}H]lazabemide binding and activity of MAO-B and MAO-A (pmol/min/mg prot) in homogenates of human frontal cortex of subjects deceased at the age ≤45 years (n = 11) or >45 years (n = 11)

	≤45 years	>45 years
B_{max}	0.79 ± 0.11	1.26 ± 0.17*
K_D	6.29 ± 0.50	7.20 ± 0.99
MAO-B activity	27.7 ± 3.51	42.6 ± 6.10*
MAO-A activity	3.37 ± 0.33	4.46 ± 0.50

Mean ± S.E. Statistical significance: *P < 0.05 in respect to ≤45 years (Students' t test)

lites relate to the respective subject's MAO-B enzymatic activity nor to lazabemide B_{max}. However, a rather weak correlation was obtained between MAO-A activity and MHPG concentration ($r = 0.431$, $P = 0.045$, $n = 22$). Furthermore, correlations between the concentrations of different metabolites themselves, were observed with HVA and 5-HIAA ($r = 0.678$, $P < 0.01$, $n = 22$) and to a lesser extent, between DHPG and MHPG ($r = 0.489$, $P = 0.02$, $n = 22$).

In the nine subjects who had met their end by suicide, the MAO-A and MAO-B activities as well as [^{3}H]lazabemide B_{max} and K_D values were compared with the respective values from the group where the cause of death did not include possible mental instability. However, no significant difference in the measured parameters was revealed in the suicide group ($n = 9$) compared to the control group ($n = 13$). MAO-B activity: 35.9 ± 6.4 and 34.6 ± 4.9 pmol/mg protein/min; MAO-A activity: 4.1 ± 0.6 and 3.76 ± 0.38 pmol/mg protein/min; [^{3}H]lazabemide B_{max}: 1.08 ± 0.19 and 0.99 ± 0.14 pmol/mg protein; [^{3}H]lazabemide K_D: 5.54 ± 1.23 and 7.59 ± 2.86 nM for suicide and control subjects, respectively. A further analysis regarding the possible differences between the suicide and the control group was performed by comparing the concentrations of amine metabolites. Here, the only significant difference between the suicide and the control group was found in the concentrations of the dopamine metabolites DOPAC and HVA, which were both higher in the former group (DOPAC: 3.38 ± 1.12 and 1.31 ± 0.27 ng/g tissue; HVA: 55.2 ± 11.2 and 31.1 ± 2.93 ng/g tissue, for the suicide and control group, respectively; $P < 0.05$). The data concerning the subjects that had been exposed to benzodiazepines were also analyzed separately, without showing any differences when compared to the rest of the suicide group (data not shown).

Discussion

The binding method used in the present study allows a direct determination of the amount of MAO-B molecules, as well as a sensitive and specific evaluation of possible modifications at the level of the active site of the MAO-B isoenzyme (Cesura et al., 1989). The kinetic analysis of [^{3}H]lazabemide binding to homogenates of human frontal cortex indicates an increase in the amount of MAO-B active sites (B_{max}) as a function of age. On the other hand, no variation in the affinity of the ligand to the active site of MAO-B was found in our study, suggesting the absence of modifications of the enzyme molecule.

The significant correlation between MAO-B and age was confirmed by parallel measurements of the enzyme activity with a conventional radiochemical method utilizing [^{14}C]PEA as a substrate. These results are in line with previous literature reports. In fact, an increase in MAO-B activity has been evidenced in various studies in brain autopsy specimen from elderly subjects (Robinson, 1975; Oreland and Fowler, 1979; Carlsson et al., 1980; Fowler et al., 1980; Gottfries et al., 1983).

To determine whether the elevated MAO-B activity in the aging brain would be due to an increase in the affinity of the enzyme towards its substrate or an increase in the enzymatic efficiency, or to simply increased number of

the active sites, some authors have utilized kinetic studies (Fowler et al., 1980; Carlsson et al., 1980). These studies have demonstrated, though indirectly, that the increase in MAO-B activity is essentially caused by increased MAO-B concentration. In the present study, like in the recent study by Sastre and Garcìa-Sevilla (1993) the above data were confirmed in a direct manner showing that, at least in human frontal cortex, there was an increase in MAO-B active sites, and thus enzyme molecules.

The reasons for this phenomenon are not all clear, but a most probable hypothesis is that in aging brain the number of neurons decreases causing a concomitant proliferation of glial tissue which is known to be rich in MAO-B (Saura Marti et al., 1990). Support for this hypothesis derives from the studies by Oreland and Gottfries (1986) where they showed that the increase in MAO-B activity in preparations of synaptosomes from aged rats' extrapyramidal tissue was limited to extrasynaptosomal mitochondria.

As far as the activity of MAO-A is concerned, our data on the frontal cortex, similar to the data from other studies in the literature, did not show any significant change in the enzyme activity with age (Robinson, 1975; Oreland and Fowler, 1979; Carlsson et al., 1980; Fowler et al., 1980; Gottfries et al., 1983). The fact that the modifications of MAO-A activity as a function of age are less consistent than those concerning MAO-B activity, could have its interpretation in an increase in MAO-A activity of glial origin balanced with decreased MAO-A activity due to specific degeneration of noradrenergic neurons (Westlund et al., 1985, 1988). In the study by Sparks et al. (1991), MAO-A activity has been shown to increase with age, but only in certain brain areas, e.g. temporal lobe and nucleus basalis of Meynert. Instead, MAO-B activity was increased in all tissue studied. It seems worth mentioning in this respect that a generalized age-related increase in the activity of MAO-B, but not MAO-A, throughout the brain, has been proposed to be the result of neuronal degeneration associated with increased formation of hydrogen peroxide, which in studies by Konradi et al. (1986) was shown to selectively increase the MAO-B activity in human brain homogenates.

All the concentrations of deaminated monoamine metabolites assessed in this study, i.e. the noradrenaline (and adrenaline) metabolites DHPG and MHPG, the dopamine metabolites DOPAC and HVA and the 5-HT metabolite 5-HIAA, were devoid of any significant correlation with subjects' age. These results are consistent with previous reports (Adolfsson et al., 1979; Ohmori et al., 1992). However, in the study by Palmer et al. (1987) 5-HIAA concentrations in frontal cortex and MHPG concentrations in the neocortex were shown to be increased with age.

When the values of all the different parameters measured in the present study were compared between suicides and subjects deceased for other causes, no significant differences in either MAO-A and -B activities or [^{3}H]lazabemide B_{max} and K_D were found. These data are consistent with the findings by Gottfries et al. (1975), who reported no differences in brain MAO activity between controls and suicide victims. On the other hand, examining MAO-B in a peripheral tissue, like blood platelets, several authors have demonstrated low enzyme activity in individuals who had attempted suicide

by active methods such as hanging (Gottfries et al., 1980; Oreland et al., 1981; Simonsson et al., 1991), thus confirming that platelet MAO-B activity may be linked to personality traits (Fowler et al., 1982; Strolin Benedetti and Dostert, 1992).

Biochemical evidence for disturbances in central monoaminergic function in suicide attempters, as well as in patients suffering from depressive mood disorders, is mainly based on the findings of altered concentrations of CSF monoamine metabolites. In fact, reports showing lowered CSF 5-HIAA concentrations have suggested a link between decreased serotoninergic activity and suicidal behaviour (Asberg et al., 1976; Brown et al., 1979; Banki et al., 1984). However, human post-mortem studies have provided conflicting results, since low values of brain 5-HIAA were found in some groups of patients who had committed suicide, but not in others (Bourne et al., 1968; Beskow et al., 1976; Lloyd et al., 1974; Ohmori et al., 1992). Concerning the dopamine metabolite HVA, some studies have also shown decreased concentrations in CSF of depressed suicide attempters (Traskman et al., 1981; Roy et al., 1986), whereas no differences in brain HVA concentrations in suicide victims compared to controls have been reported by other authors (Beskow et al., 1976; Cochran et al., 1976). In the present study, the concentrations of the dopamine metabolites DOPAC and HVA were higher in the suicide group compared to controls, whereas 5-HIAA, DHPG and MHPG concentrations were unchanged. This result is consistent with the recent study by Ohmori et al. (1992), who also found increased HVA concentrations in the frontal cortex of suicide victims. As suggested by these authors, among the possible causes of this apparent sign of increased dopamine turnover, the physical or mental state prior to death, or a condition of acute or chronic stress, might play a role. Whatever are the causes, the present study indicates that the increase in cortical dopamine metabolites in our suicide group was not associated with changes in MAO enzyme activity.

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