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Monoamine oxidase-inhibition and MPTP-induced neurotoxicity in the non-human primate: comparison of rasagiline (TVP 1012) with selegiline

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Summary. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to induce parkinsonism in man and non-human primates. Monoamine-oxidase B (MAO-B) has been reported to be implicated in both MPTP-induced parkinsonism and Parkinson's disease, since selegiline (L-deprenyl), an irreversible MAO-B inhibitor, prevents MPTP-induced neurotoxicity in numerous species including mice, goldfish and drosophyla. However, one disadvantage of this substance relates to its metabolism to (−)-methamphetamine and (−)-amphetamine. Rasagiline (R-(+)-N-propyl-1-aminoindane) is a novel irrevesible MAO-B-inhibitor, which is not metabolized to metamphetamine and/or amphetamine. The present study compared the effects of high doses of selegiline and rasagiline (10 mg/kg body weight s.c.) on MPTP-induced dopaminergic neurotoxicity in a non-human primate (Callithrix jacchus) model of PD.

Groups of four monkeys were assigned to the following six experimental groups: Group I: Saline, Group II: Selegiline/Saline, Group III: Rasagiline/Saline, Group IV: MPTP/Saline, Group V: Rasagiline/MPTP, Group VI: Selegiline/MPTP. Daily treatment with MAO-B-inhibitors (either rasagiline or selegiline, 10 mg/kg body weight s.c.) was initiated four days prior to MPTP-exposure (MPTP-HCl, 2 mg/kg body weight subcutaneously, sepa-

rated by an interval of 24 hours for a total of four days) and was continued until the end of the experiment, i.e. 7 days after the cessation of the MPTP-injections, when animals were sacrificed. MPTP-treatment caused distinct behavioural, histological, and biochemical alterations: 1. significant reduction of motor activity assessed by clinical rating and by computerized locomotor activity measurements; 2. substantial loss (approx. 40%) of dopaminergic (tyrosine-hydroxylase-positive) cells in the substantia nigra, pars compacta; and 3. putaminal dopamine depletion of 98% and its metabolites DOPAC (88%) and HVA (96%). Treatment with either rasagiline or selegiline markedly attenuated the neurotoxic effects of MPTP at the behavioural, histological, and at the biochemical levels. There were no significant differences between rasagiline/MPTP and selegiline/MPTP-treated animals in respect to signs of motor impairment, the number of dopaminergic cells in the substantia nigra, and striatal dopamine levels. As expected, both inhibitors decreased the metabolism of dopamine, leading to reduced levels of HVA and DOPAC (by $>95\%$ and 45% respectively).

In conclusion, rasagiline and selegiline at the dosages employed equally protect against MPTP-toxicity in the common marmoset, suggesting that selegiline-derived metabolites are not important for the neuroprotective effects of high dose selegiline in the non-human MPTP-primate model in the experimental design employed.

However, unexpectedly, high dose treatment with both MAO-inhibitors caused a decrease of the cell sizes of nigral tyrosine hydroxylase positive neurons. It remains to be determined, if this histological observation represents potential adverse effects of high dose treatment with monoamine oxidase inhibitors.

Keywords: Parkinson's disease, MPTP, MPP^+, common marmoset, monoamine-oxidase, A and B, selegiline, TVP-1012, rasagiline.

Abbreviations

CNS central nervous system; *DA* dopamine; *DOPAC* 3,4-dihydroxyphenylacetic acid; *5-HIAA* 5-hydroxyindol acetic acid; *HPLC* high pressure liquid chromatography; *5-HT* 5-hydroxytryptamin; *HVA* homovanillic acid; *MAO* monoamine oxidase *MPP^+* 1-methyl-4-phenylpyridinium ion; *MPTP* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; *NaCl* sodium chloride; *NMDA* N-methyl-D-aspartate; *PB* phosphate buffer; *PD* Parkinson's disease; *SNC* substantia nigra pars compacta; *TH-IR* tyrosine hydroxylase immunoreactivity.

Introduction

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that has been extensively used in various animal species to model the symptoms and/or pathology of Parkinson's disease (PD) (for reviews Gerlach and Riederer, 1996; Gerlach et al., 1991, 1996a), a common and disabling neurodegenerative disorder (Marsden, 1994). MPTP causes Parkinsonian

symptoms in humans (Davis et al., 1979; Langston et al., 1983), non-human primates (Burns et al., 1983; Langston et al., 1984a,b; Herkenham et al., 1991), including marmosets (Jenner et al., 1984), and certain rodents (for review cf. Gerlach and Riederer, 1996; Gerlach et al., 1991).

The similarities between the MPTP-model in monkeys and the human disease are reflected by a number of biochemical, molecular, and neuropathological features. These features embrace, for instance, a relatively selective degeneration of mesencephalic nigro-striatal dopamine (DA) projection neurons, including the finding of eosinophilic inclusions resembling Lewy bodies (for review cf. Forno et al., 1993; for distinct differences between the human disease and MPTP-intoxication cf. Gerlach and Riederer, 1996).

It is well established that the neurotoxic effects of MPTP critically depend on its conversion to 1-methyl-4-phenylpyridinium (MPP^+) and/or other neurotoxic metabolites generated by monoamine oxidase B (MAO-B; Chiba et al., 1984; Heikkila et al., 1984; Langston et al., 1984b). MAO-A and MAO-B are the major intracellular enzymes that catalyze the oxidative deamination of DA in the central nervous system. The formation of hydrogen peroxide (H_2O_2) and hydroxyl radicals as by-products of this enzymatic reaction are considered risk factors for cellular defense mechanism against oxidative stress (for review cf. Tatton et al., 1996). It has been repeatedly postulated that a disturbed redox equilibrium contributes to nigral cell loss in PD, which could be prevented by MAO-B inhibitors (for recent reviews cf. Gerlach et al., 1996a; Knoll, 1995).

Selegiline is an irreversible inhibitor of MAO-B and is used as monotherapy or as an adjunctive in the symptomatic treatment of idiopathic PD (for reviews cf. Gerlach et al., 1992; Wu et al., 1994; Knoll, 2000) and more recently in depression (Mann et al., 1989; Sunderland et al., 1994; Quetnik et al., 1984) and Alzheimer's disease (Sano et al., 1997, for review cf. Thomas, 2000). It is generally agreed that selegiline delays the onset of disability requiring levodopa in early PD (Parkinson Study Group, 1993; for review Shoulson, 1998; Przuntek et al., 1999), possibly via its gastrointestinal metabolite desmethylselegiline (Mytilineou et al., 1997a). Furthermore, selegiline has been postulated to slow down the progression of PD (Birkmayer et al., 1985; Olanow et al., 1995), although potential neuroprotective actions of selegiline remain controversial (c.f. Lees et al., 1995; Gerlach et al., 1996b; Olanow et al., 1998). Interestingly, it has been recently shown that cigarette smokers display a 40% decrease in the level of striatal MAO-B in comparison to non-smokers, which may contribute to the lower incidence of PD among cigarette smokers (Fowler et al., 1996). In in vitro and in vivo-models of PD it has been repeatedly postulated that the neuroprotective effects of selegiline against MPTP/MPP^+ in dopaminergic neurons are partially independent of selegiline's inhibition of MAO-B (Mytilineou and Cohen, 1985; Rothblat et al., 1998; Schmidt et al., 1997; Magyar et al., 1999; Shimazu et al., 1999).

Selegiline is partially metabolized to (−)-amphetamine and (−)-metamphetamine (Reynolds et al., 1978; Karoum et al., 1982; Melega et al., 1999), which in turn could even counteract potential neuroprotective actions of selegiline (Stephans and Yamamoto, 1996; Hom et al., 1997; Tatton et al.,

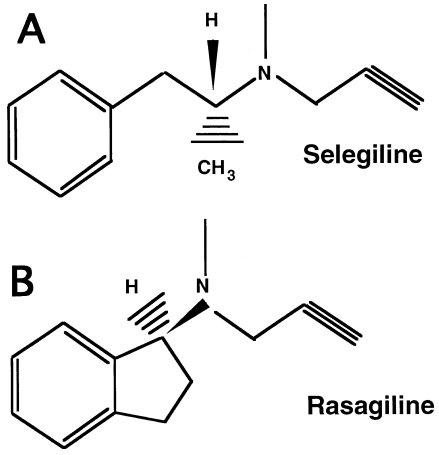

Fig. 1. Structures of selegiline (**A**) and rasagiline (**B**)

1996; Thiffault et al., 1995, 1997; cf. however Sziraki et al., 1994; Yasar et al., 1996) or lead to unwanted side (possibly cardiovascular, Lees et al., 1995) effects. Thus, it appears to be important to develop agents with MAO-blocking activities, which are not metabolized to products with amphetamine-like actions (for review cf. Yu et al., 1995).

Rasagiline, (R(+)-N-propargyl-1-aminoindane, TVP 1012), a potentially anti-Parkinsonian drug, is a restricted analogue of selegiline, which has been developed from the racemic indane derivative AGN 1135 (Kalir et al., 1981; Finberg et al., 1996, 1981; for review c.f. Sterling et al., 1998). Rasagiline is an irreversible MAO-B inhibitor and possesses the same propargyl moiety and high selectivity for human and rat brain MAO-B in vitro and in vivo and is 5–10 times more potent than selegiline in regard to MAO inhibition (for molecular structures cf. Fig. 1). However, it is devoid of either the "cheese effect" and the sympathomimetic or neurotoxic amphetamine-like modes of actions (Finberg et al., 1996). Interestingly, in contrast to selegiline, rasagiline has been reported to exert striking in-vitro protective actions on dopaminergic neurons under serum-free conditions (Finberg et al., 1998) and does not influence – as selegiline – the striatal DA transporter (Lamensdorf et al., 1999). Furthermore in rodents rasagiline has been shown to improve memory and learning tasks, to diminish the sequelae of brain injury (possibly via a cholinergic mechanism; c.f. Speiser et al., 1998a,b; Huang et al., 1999) and to reduce ischemic damage (Speiser et al., 1999).

The present study aimed at comparing the efficacy of high doses of rasagiline versus selegiline against MPTP-induced neurotoxicity in a non-human MPTP-primate model of PD, since high dose treatment with selegiline has been implicated to involve the generation of potential hazardous metabolites, like metamphetamine (in contrast to rasagiline).

Methods

The protocols used in this study were approved by the Government of Upper Bavaria and the Institutional Animal Care and Use Committee.

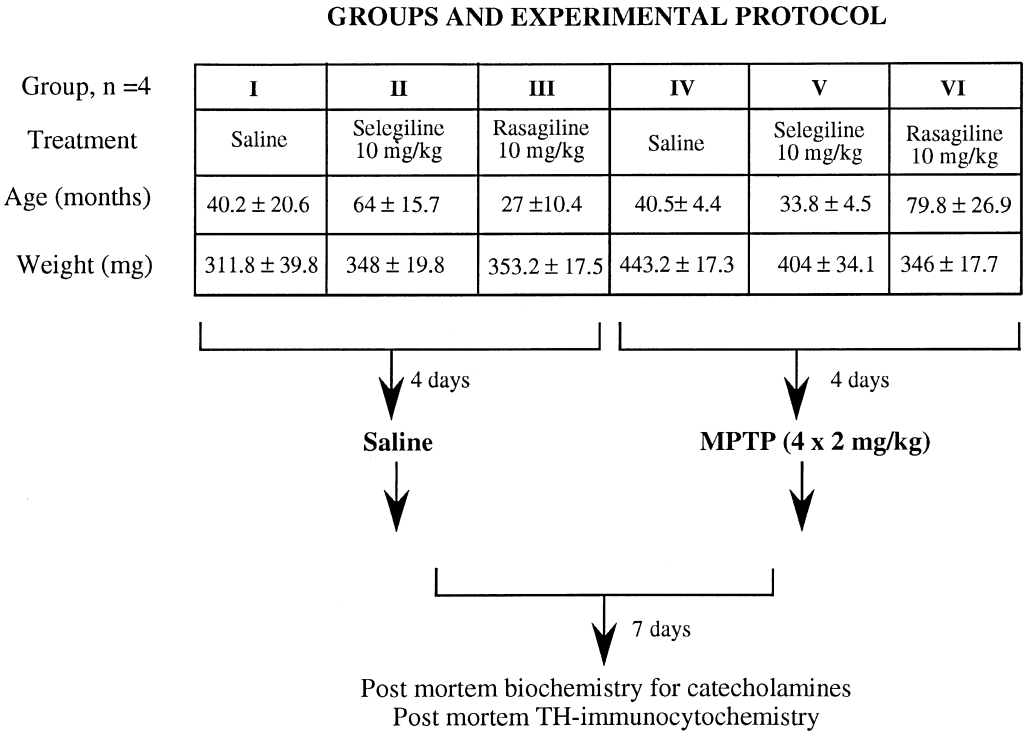

Fig. 2. Monkeys were assigned into 6 groups and treated according to the scheme described in the text

Animals and experimental groups

Twenty-four, both female and male, adult common marmosets (Callithrix jacchus) from a self-sustaining breeding colony, weighing 220–470 g were selected for the study. They were balanced for age differences (Fig. 2), since the sensitivity of common marmosets to the neurotoxic effects of MPTP may be age-related and controversial reports exist on age-related changes in MAO-A and MAO-B in primates (Irwin et al., 1997; for mice cf. e.g. Gupta and Wiener, 1995). There were no significant differences between the mean ages of the different groups (ANOVA, $p = 0.22$).

Animals were kept under standardized conditions (12 hour light-dark-cycle, light on from 6.00–18.00 h, temperature 27°C ± 1°C, relative humidity 50–60%) with free access to primate pellets, water and supplementary food (fruits, vegetables). The experimental design involved six groups of common marmosets (Fig. 2).

Monkeys assigned to group I (control) received s.c. saline injections. Monkeys of groups II (L-selegiline) and III (rasagiline) were treated with selegiline and rasagiline (10 mg/kg body weight per day), respectively, and s.c. saline injections. Marmosets in group IV (MPTP-only) received 4 × MPTP-HCl (2 mg/kg body weight, 24 hours apart on four consecutive days). Monkeys in group V (MPTP/selegiline) and VI (MPTP/rasagiline) were treated with selegiline and rasagiline (10 mg/kg per day), respectively, and received 4 × MPTP-HCl (2 mg/kg, 24 hours apart on 4 consecutive days). Values for age and weight refer to the start of the experiment (first injection) and are given as mean and ± S.E.M. The relatively high mean age of group VI resulted from the inclusion of one very old animal in this group (153 months), however, the differences in the mean age of the different groups did not reach significance (one factor ANOVA, $p > 0.05$). The high dosages of the MAO-inhibitors were chosen in view to a maximal effect of the MAO-inhibitors against MPTP-toxicity in order to facilitate comparison between selegiline and rasagiline.

Four days after the initiation of the treatment with MAO-inhibitors the animals in groups IV, V and VI were treated subcutaneously with four doses of MPTP-HCl, separated by an interval of 24 hours for a total of four days (2 mg/kg body weight per injection on days −4, −3, −2, −1, Kupsch et al., 1996). At the same time animals in groups I, II and III received saline injections.

All drugs were administered in a volume of 1 ml/kg (sterile saline solutions, 0.9% NaCl) prepared on the day of the administration.

Behavioural analyses

For assessment of behavioural activity a Parkinsonian rating scale for marmosets was employed as described previously (Kupsch et al., 1996). Monkeys were rated once daily between 8–9 a.m. on this rating scale in their home cages without drug administration for a period of one hour by an observer, who had been previously trained to evaluate the behaviour of both normal and MPTP-treated marmosets. Additionally, spontaneous locomotor activity (without drug administration) was assessed, using cages identical to the home cages of the marmosets ($60 \times 60 \times 90$ cm in size), except for being equipped with 19 horizontal infrared sensor beams on three different levels (built by EE-A GmbH, E. Ciesinksi, H. Ramthun, Berlin-FRG). The beam interruptions were recorded for 120 minutes. Animals were tested 7 days prior to MPTP-exposure and 4 days after completion of the MPTP-treatment. Animals were allowed to become accustomed to the test environment for one hour prior to each test period.

Fasting animals were weighed daily each morning at 6 a.m. For analysis of weight differences, weights of the animals at the day of sacrifice were substracted from the weights at the beginning of the experiment (prior to first injection).

Biochemical analyses

Seven days after MPTP-administration the monkeys were sacrificed under deep Saffan®-anesthesia (3 ml/kg; alphaxalone/alphadolone acetate, Pitman-Moore, Middlesex-England) and their brains were rapidly removed and immediately placed on ice-cooled Petri-dishes, as previously described (Kupsch et al., 1996). Briefly, brains were divided by a sagittal section across the corpus callosum and by a coronal section at the level of the dorsal tier of the hypophysis (A7 according to a standard stereotaxic marmoset atlas by Stephan et al., 1980). The right putamen (and other brain regions, which were partially used for other studies; Gerlach et al., 1996c; Goetz et al., 1998) were dissected on ice. The isolated tissue samples were wrapped into aluminum foil, transferred to liquid nitrogen, and kept at −196°C until analysis of DA, noradrenaline (NA), serotonin (5-HT) and respective metabolites.

Parts of the right caudate nucleus (approximately 10 mg) were transferred to Eppendorf vials and stored at −196°C for analysis of MAO-A and B activities in the brain.

The right sides of the diencephalon, the midbrain (including mesencephalon and medulla oblangata) and the cerebellum were immersionfixed in 4% paraformaldehyde.

Neurochemical analysis

Monoamine and monoamine metabolite concentrations (DA, DOPAC, HVA, 5-HT, 5-HIAA, MHPG, NA; for abbreviations cf. "Materials") were determined by high pressure liquid chromatography system (HPLC) and electrochemical (amperometric) detection as described previously (Kupsch et al., 1992). Results are expressed as pmol/mg wet tissue weight.

MAO-activity in caudate nucleus

Protein content and MAO-A and -B activity were assayed in parts of the right caudate nucleus as previously described (Wurtman and Axelrod, 1963; Tipton and Youdim, 1983; Tipton and Singer, 1993). Briefly, after homogenizing the tissue an aliquot of the homogenate (300 µg of protein) was preincubated in 200 µl 0.1 M phosphate buffer for 7 minutes at 37°C by shaking in a 6 ml borosilicate open glass tube.

MAO-A activity was assessed by the addition of 50 µl ml 0.1 M phosphate buffer (PB), pH 7.4, containing (2-^{14}C)-5-hydroxytryptamine (5-HT) creatinine sulphate (specific activty 2.04 GBq, 1 mM, Amersham-International, England) followed by shaking for 30 min at 37°C. Following the addition of 250 µl citric acid (2 M) and 5 ml of an extraction mixture (toluene/ethylacetate 1/1 v/v containing 0.6% 2,5,-diphenyloxazole, Merck, Darmstadt-FRG) the reaction mixture was shaken vigorously for 30 min and centrifuged at 1000 g for 5 min. The hydrophilic phase was frozen at −80°C. The lipophilic upper phase was counted for ^{14}C-activity by liquid scintillation. For 5-HT metabolites recoveries of labeled products in human cortex amounted to 33% of total radioactivity used.

For the measurement of MAO-B activity 25 µl 0.1 M PB, pH 7.4, containing (1-^{14}C-ethyl) phenylethylamine (PEA) HCl (specific activity 1.16 GBq, 1 mM, New England Nuclear, Boston-USA) was added to 225 µl of brain homogenate (300 µg protein) in 0.1 M PB (pH 7.4). After 10 min of incubation (continously shaken) 50 µl of 1 M HCl was added. The acidified solution was extracted with 2 ml of ethyl acetate by vigorous shaking for 10 min. The two phases were separated by centrifugation at 1000 g for 5 min and 1 ml of the organic phase containing the deaminated metabolites were combined with 4 ml biosolve cocktail (Roth, Karlsruhe-FRG) and counted by liquid scintillation. Recovery of labeled products amounted to 98% of total radioactivity used.

All assays were performed in triplicate. Values were corrected for blank activity (radioactivity extracted from aciî pre-deactivated reaction mixtures) and counting was quench corrected. MAO activity is given as nmol of products formed per mg protein per minute. 5-HT was used at a final concentration of 200 µM (actual activity 7.2 KBq/assay), whereas PEA was administered at a concentration of 10 µM with an activity of 4.2 kBq/assay.

Choosing these conditions MAO-A or -B activities were checked for linearity with respect to time with 300 µg cerebral protein, up to 45 or 20 min, respectively.

The assays were checked for recovery of 5-HT and PEA metabolites including extraction efficiency and quench correction in liquid scintillation counting. PEA metabolites could be almost quantitatively recovered (98%). In contrast, 5-HT recovery was only 33%. High amounts of oxidation products formed are retained within the aqueous phase (Huether et al., 1990). We could not increase the yield of 5-HT metabolites by additional enzyme or by increaisng time of incubation longer than 90 min above 33% of total substrate added. Thus, this correction factor (67%) has been included for activity calculations of MAO-A.

Immunocytochemistry and microscopic analysis of the substantia nigra

One half of the midbrains were immersion-fixed in 4% paraformaldehyde in 0.1 M PB for four days at 4°C. Following dehydration in PB supplemented with 20% sucrose, coronal sections were cut through the substantia nigra (SNC) at 35 µm on a cryostat (Model 2800 Frigocut Reichert-Jung, Leica Instruments, Nußloch, FRG). Every fourth section per brain was processed for free-floating tyrosine hydroxylase immunoreactivity (TH-IR), as described previously (Kupsch et al., 1996).

For morphological analysis brain sections were analysed at all rostro-caudal levels extending from planes A6 to A3 according to a standard stereotaxic atlas of marmoset brain (Stephan et al., 1980). In order to compare similar rostrocaudal levels of the SNC between animals, only sections on which the ventral tegmental area (A10) and the pars compacta (A9, SNC) were clearly separated by the roots of the third cranial nerve were

selected for the final quantification of nigral TH-IR counts (according to Stephan et al., 1980; AP 5.5 to AP 5 at the level of the interpeduncular nucleus at the widest dimension of the SNC; results did not differ statiscally and qualitatively, if counts from the whole SNC (A6 to A3) were included, ANOVA, data not shown). Nigral nucleated, process-bearing TH-IR cells of the right sides were microscopically counted under bright field illumination (Leitz DM RB, magnification ×100) by three different observers (A.K., J.S., J. Sch.) under "blind" conditions (i.e. unawareness of groups the sections belonged to) in 3–5 sections per animal. Results are expressed as mean TH-IR cell counts per section (± S.E.M.). Cell areas were measured with the help of a Macintosh-based image analysis system (Screen Machine-Camera and Image 1.14., Schwarz et al., 1997).

Materials

MPTP-HCl was purchased from Research Biochemicals (Cologne, F.R.G.). Selegiline (L-deprenyl) and rasagiline (TVP 1012, 2,3-dihydro-N-2-propynyl-1H-inden-1-amine-(1R)-mesylate) were kindly provided by TEVA Pharmaceutical Company. Dopamine (5-hydroxytryptamine hydrochloride, DA), 3,4-dihydroxyphenylacetic acid (DOPAC), (HVA), 5-hydroxytryptophane (5-HT, serotonin), 5-hydroxyindolacetic acid (5-HIAA), norepinephrine (NA), MHPG (3-methoxy-4-hydroxy-phenyl-glycol) as well as all other drugs and chemicals used were obtained from Sigma, F.R.G., at the highest purity grade, unless otherwise indicated in the text.

Data analysis

For statistical evaluation one-way analysis of variance (ANOVA) followed by post-hoc Scheffé's test, Students's paired t-test or Kruskal-Wallis test (Parkinsonian rating scales for marmosets, non-parametric data) were employed. Results were expressed as mean ± standard error of the mean (S.E.M).

Results

Parkinsonian behaviour and locomotor activity

No lethality occured with the treatment regimens chosen. Acutely, MPTP-exposure ensued increased locomotion, salivation, and occasionally a generalized myoclonic syndrome ("shaking dog syndrome"). These acute symptoms subsided within 2 to 3 hours. Subsequently, MPTP-treated marmosets displayed severe Parkinsonian signs, including bradykinesia, rigidity, and stooped posture, requiring intermittent hand-feeding of the monkeys after completion of the MPTP-regimen. Pretreatment with either selegiline or rasagiline attenuated the subacute (during the observation period of 11 days) MPTP-induced behavioural deficits, as assessed by the daily rating numbers and the motor counts (Fig. 3A,B). Figure 3A depicts the effects on the motor disability as assessed by the Parkinsonian rating scale, which started to differ significantly between groups IV versus V and VI on the first day after completion of the MPTP-treatment and continued to improve until termination of the experiment (7 days after the last MPTP-treatment). Animals treated with MAO-inhibitors only (groups II and III) did not display any obvious abnormal behaivoural changes in comparison to preinjection behaviour (data not shown).

Figure 3B shows the results on cumulative light beam interruptions (reflecting locomotor acitivity) one day prior to MPTP-exposure and at the day

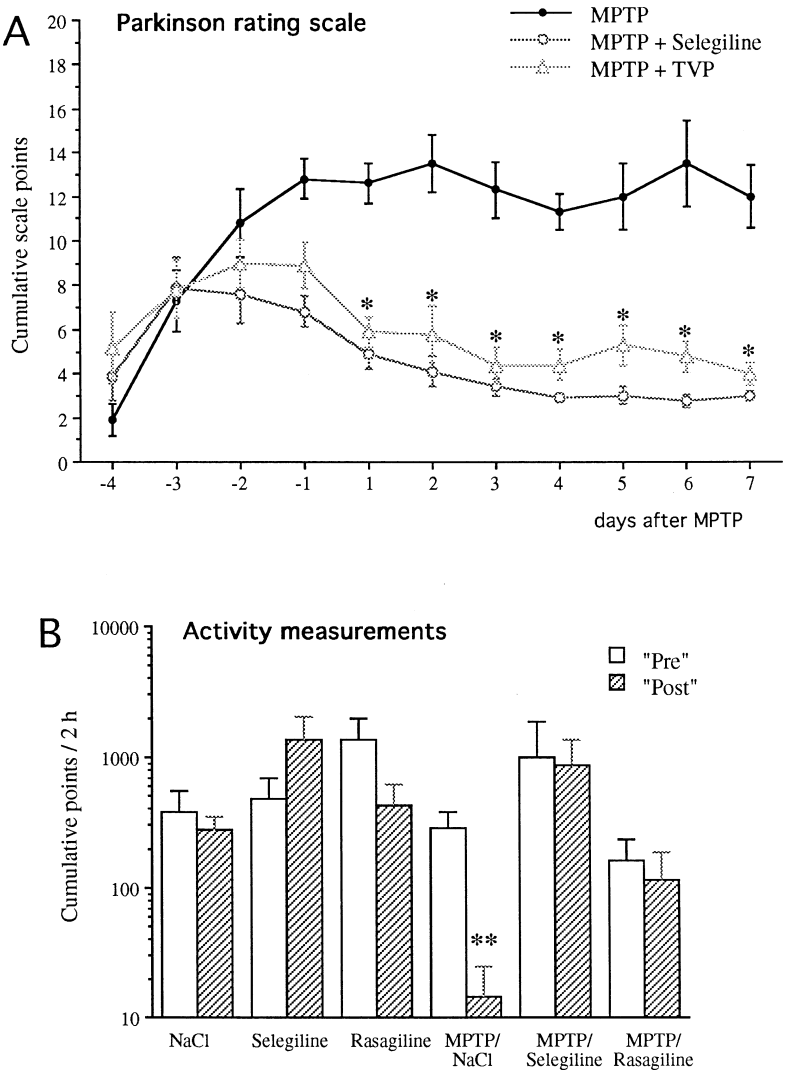

Fig. 3. Effects of selegiline, rasagiline, MPTP, and combined treatments at the behavioural level assessed by a non-human primate PD rating scale (**A**) and by spontaneous locomotor activity (**B**). The figure shows that pretreatment with both MAO-B inhibitors attenuated the behavioural deficits induced by the MPTP-treatment. **A** Cumulative results (±S.E.M.) of Parkinsonian signs in MPTP-treated marmosets assessed by daily evaluation on a PD rating scale for marmosets. The results demonstrate that during the observation period of 11 days, starting after MPTP-exposure, MPTP-selegiline/MPTP-rasagiline-treated monkeys were less severely impaired than MPTP-only treated animals. The asterisk (*) indicates significant differences between mean values of group IV vs. groups V and VI (Kruskal-Wallis, $p \leq 0.05$). There were no differences between MPTP/selegiline- versus MPTP/rasagiline-treated animals. **B** Spontaneous locomotor activity assessed by infrared was significantly decreased in MPTP-only treated animals four days after completion of the MPTP-treatment (paired Student's t-test, $p < 0.05$, denoted by **), but not in MPTP-selegiline/MPTP-rasagiline-treated or control-injected monkeys (paired Student's t-test, $p > 0.05$). Note the logarithmic scale of the ordinate. Mean activity counts of the six groups did not differ prior to initiation of the experimental injections (ANOVA, $p > 0.05$)

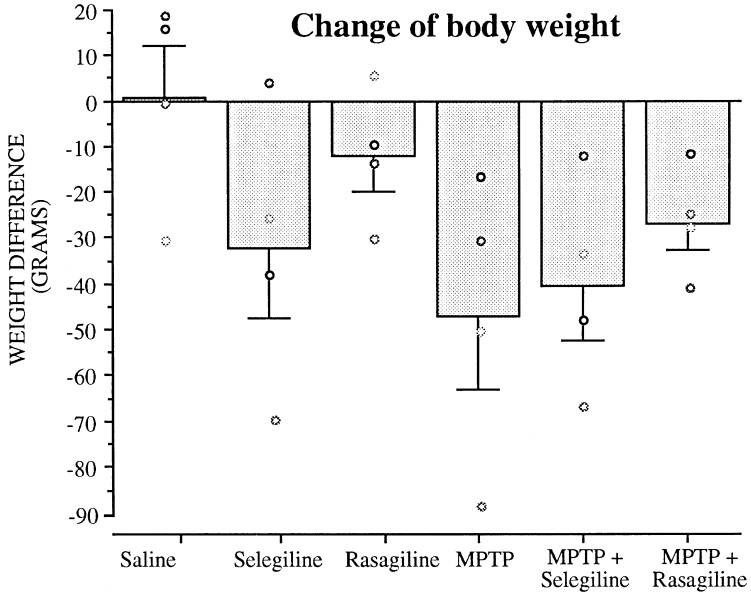

Fig. 4. Mean weight differences (day of sacrifice versus start of the experiment) did not differ significantly in group I vs. groups II, III, IV, V, and VI, although a tendency to a more pronounced weight loss was observed in groups IV and V

of sacrifice. Light beam interruptions prior to and after initiation of the MPTP-injections significantly differed only in group IV-treated animals (MPTP-only).

Body weight

There was a clear tendency for a reduction in body weight in all verum-treated animals (groups II–VI; cf. Fig. 4). This trend was particurlarly evident in (the selegiline and MPTP-only) groups II, IV and V with mean reductions of 32, 47, and 40 grams, respectively, and less pronounced in (the rasagiline-treated) groups III and VI with mean reductions of 12 and 26 g. However, these differences did not reach the level of significance, possibly due to the relatively low numbers of non-human primates available in each group (one factor ANOVA, $p > 0.05$; $F_{(5,23)} = 2.3$, with post-hoc Scheffé-test, $p = 0.092$).

MAO acitivity

The activities of MAO-A and -B in the right caudate nucleus were almost completely inhibited (≥97% for MAO-A and ≥99% for MAO-B; one-factor ANOVA, F-MAO-$A_{(5,23)} = 360.3$, with post-hoc Scheffé-test, $p \leq 0.0001$; F-MAO-$B_{(5,23)} = 83.6$, with post-hoc Scheffé's test, $p \leq 0.0001$; cf. Fig. 5) by either selegiline and rasagiline (each 10 mg/kg body weight for 14 days). Concomittant administration of MPTP did not significantly influence these results, although there was a further decrease in MAO-activity in the MPTP-

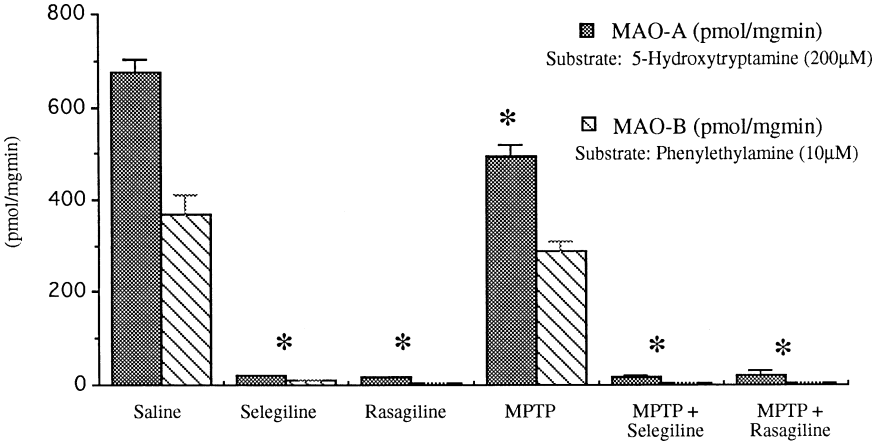

Fig. 5. MAO-A and -B activity in the caudate nucleus were assessed 14 days after the beginning of the first injections in all experimental animals. MAO-A and B activities were reduced by approximately 97% and 99%, respectively, in groups II, III, V, and VI in comparison to saline-treated animals (one factor ANOVA, *$p \leq 0.0001$)

only group which reached the level of significance for MAO-A-values. There were no significant differences between selegiline- and rasagiline-treated groups. Interestingly, MAO-A activities were significantly decreased in the MPTP-only group by approximately 25% (control: 675 vs. MPTP: 496), while a similar trend did not reach the level of significance for MAO-B in the respective groups (control: 370 vs. MPTP: 290).

Concentrations of neurotransmitters and metabolites

The effects of selegiline and rasagiline and respective coapplication of MPTP on DA-, HVA-, DOPAC-, 5-HT, 5-HIAA, NA, and MHPG-levels in the right putamen (one week after exposure to MPTP) are summarized in Fig. 6 and 7.

MPTP-treatment induced pronounced depletions of DA, DOPAC, HVA of approximately 98%, 88%, and 96%, respectively (Fig. 6).

Both selegiline and rasagiline alone (groups II and III) reduced DOPAC concentrations, one of the main metabolites of DA, by approximately 65%; concomittant MPTP-application (groups V and VI) non-significantly attenuated the MPTP-induced DOPAC depletions from 88% to approximately 75% of control values (Fig. 6B; one factor ANOVA, $F_{(5,23)} = 7.3$, with post-hoc Scheffé's test, $p \leq 0.005$, (**)).

Both selegiline and rasagiline alone (groups II and III) reduced HVA concentrations, the other main metabolite of DA, by approximately 96%; which was not affected by concomittant MPTP-application (groups V and VI; cf. Fig. 6C; one factor ANOVA, $F_{(5,23)} = 98.5$, with post-hoc Scheffé's test, $p \leq 0.0001$, (***)).

(DOPAC + HVA)/DA ratios for groups I to VI (0.35 ± 0.005, 0.004 ± 0.0005, 0.0043 ± 0.0004, 1.24 ± 0.29, 0.0017 ± 0.0005, 0.0016 ± 0.0007, respectively, mean ± SEM) differed significantly between the MPTP-only group

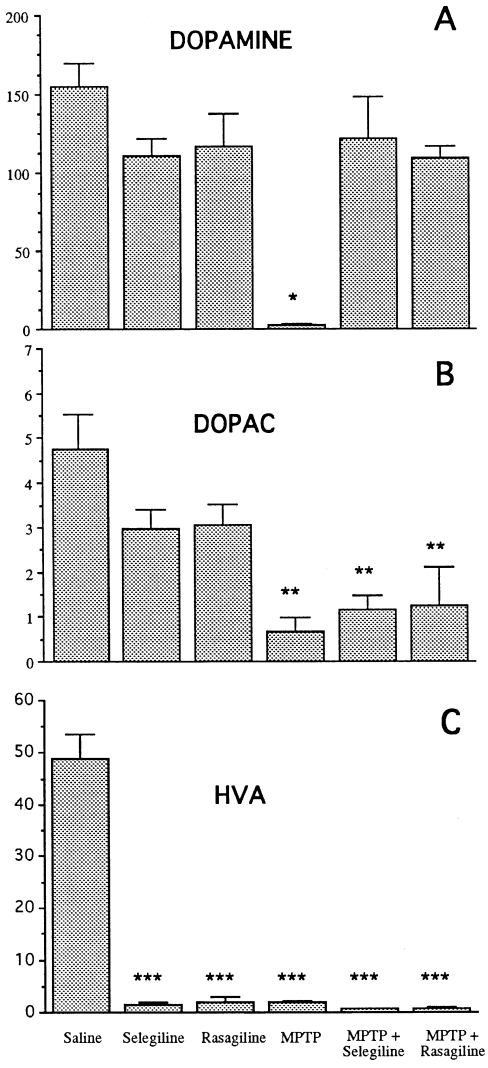

Fig. 6. Effects of the different treatment regimes on DA-, DOPAC-, and HVA-levels in the putamen 7 days after cessation of MPTP-treatment (means ± S.E.M., 4 animals per group, pmol per ng wet tissue weight). **A** DA concentrations were reduced by 98% in the MPTP-treated animals compared to saline-treated animals (one factor ANOVA, $F_{(5,23)} = 10.4$, with post-hoc Scheffé's test, $p \leq 0.0001$ (*)). The means of the other groups did not differ significantly. **B** DOPAC-concentrations were reduced by 88% in the MPTP-treated animals and by approximately 75% in the selegiline/MPTP and rasagiline/MPTP-treated animals compared to saline-treated animals (one factor ANOVA, $F_{(5,23)} = 7.3$, with post-hoc Scheffe's test, $p \leq 0.001$ (**)). There were no significant differences between the other groups. **C** HVA-concentrations were significantly reduced by 95% in the MPTP-treated animals the selegiline- and rasagiline-treated animals compared to saline-treated animals (one factor ANOVA, $F_{(5,23)} = 98.5$, with post-hoc Scheffé's test, $p \leq 0.0001$ (***)). In animals treated with MAO-inhibitors and MPTP (groups V, VI) a further non-significant reduction of HVA-values to 98% of saline-values were observed. There were no significant differences between the other groups apart from the saline group

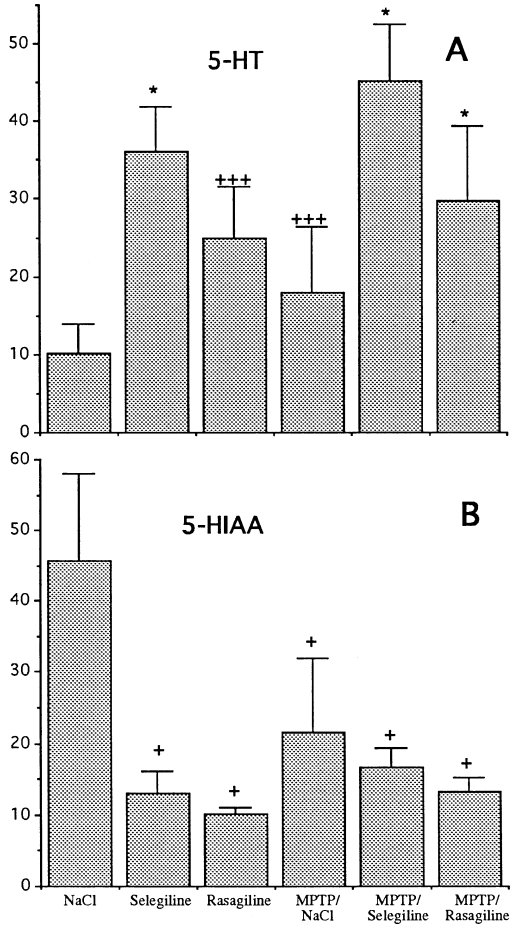

Fig. 7. Effects of the different treatment regimes on 5-HT- and 5-HIAA-levels in the putamen 7 days after cessation of MPTP-treatment (means ± S.E.M., 4 animals per group, pmol per ng wet tissue weight). **A** 5-HT-concentrations were increased in the selegiline-, selegiline/MPTP-, and rasagiline/MPTP-treated animals (groups II, V, VI), compared to saline-treated animals (one factor ANOVA, $F_{(5,23)} = 3.7$, with post-hoc Scheffé's test, $p \leq 0.05$, (*)). Significant differences were also found between groups III, IV vs. V, as depicted by the three crosses (+++). There were no significant differences between the other groups. **B** 5-HIAA-concentrations were reduced in all treated animals (groups II–VI) compared to saline-treated animals (one factor ANOVA, $F_{(5,23)} = 3.7$, with post-hoc Scheffé's test, $p \leq 0.05$ (+)). There were no significant differences between the other groups

(IV) and all other groups (one factor ANOVA, $F_{(5,23)} = 16.6$, with post-hoc Scheffé's test, $p \leq 0.0001$). This is compatible with an increased metabolism of DA in MPTP-treated animals, which was fully reversible by concomittant treatment with MAO-inhibitors.

The effects of the different treatment regimens on 5-HT and 5-HIAA-concentrations are shown in Fig. 7:

Treatment with MAO-inhibitors enhanced 5-HT levels in the putamen in groups II, V, and VI, while MPTP alone did not significantly affect 5-HT

levels (Fig. 7A; one factor ANOVA, $F_{(5,23)} = 3.7$, with post-hoc Fisher PLSD, $p \leq 0.05$, (*)). In contrast, concentrations of 5-HIAA, the main metabolite of 5-HT, were lowered by approximately 70% in animals receiving treatment with MAO-inhibitors, which was not significantly influenced by concomittant MPTP-treatment (Fig. 7B; one factor ANOVA $F_{(5,23)} = 7.3$, with post-hoc Fisher PLSD, $p \leq 0.005$, (+)). MPTP alone reduced 5-HIAA-levels by approximately 51%.

Tyrosine hydroxylase immunocytochemistry in the substantia nigra

Since results of the three independent investigators significantly correlated (simple regression analysis: $r = 0.9$, $p < 0.0001$) and did not differ qualitatively, data presentation is based on the quantification of one of the investigators (Fig. 8). Mean cell numbers of animals of groups I, II, and III did not differ significantly (one-way ANOVA, $p > 0.05$) and have been summarized as "Control" counts in Fig. 8A. Mean numbers of TH-IR neurons in the lateral part of the SNC were distinctly decreased by approximately 40% following MPTP-treatment in comparison to "Control" injections (Fig. 8A). Pretreatment with either selegiline and/or rasagiline attenuated the MPTP-induced decrease of nigral TH-IR (Fig. 8A). However, the differences between mean nigral cell counts between the different experimental groups did not reach the level of significance (ANOVA, $p > 0.05$) probably due to the relatively low number of non-primates available.

The mean size of nigral TH-IR cells was significantly reduced by 47% in MPTP-treated animals (Fig. 8B) which was partially reversed by treatment with MAO-inhibitors. Interestingly, MAO-inhibitors alone also decreased the cell size by about 22%.

Discussion

The aim of the present study was to assess and to compare the behavioural, biochemical, and morphological effects of high, non-selective dosages of selegiline versus rasagiline on the nigrostriatal DA system in the MPTP-non human primate model of PD. Neither of the MAO inhibitors has been investigated before in monkeys (pargyline has been examined in monkeys, Langston et al., 1984; selegiline and rasagiline were studied in mice, Heikkila et al., 1984, 1985a).

The assessment of MAO-B inhibitors' actions embraced three different levels: i. behavioural level, ii. biochemical (DA) level, i.e. at the nigrostriatal synapse, iii. cellular level via TH-immunocytochemistry of the SNC.

MPTP-induced behavioural impairments

Pretreatment with either selegiline and/or rasagiline at 10 mg/kg body weight attenuated MPTP-induced behavioural deficits, as assessed by a Parkinsonian rating scale and spontaneous locomotor activity (Albanese et al., 1993; Hantraye et al., 1993). Interestingly, neither selegiline nor rasagiline affected the acute Parkinsonian MPTP-effects at days −3 till −1 (Fig. 3A). This

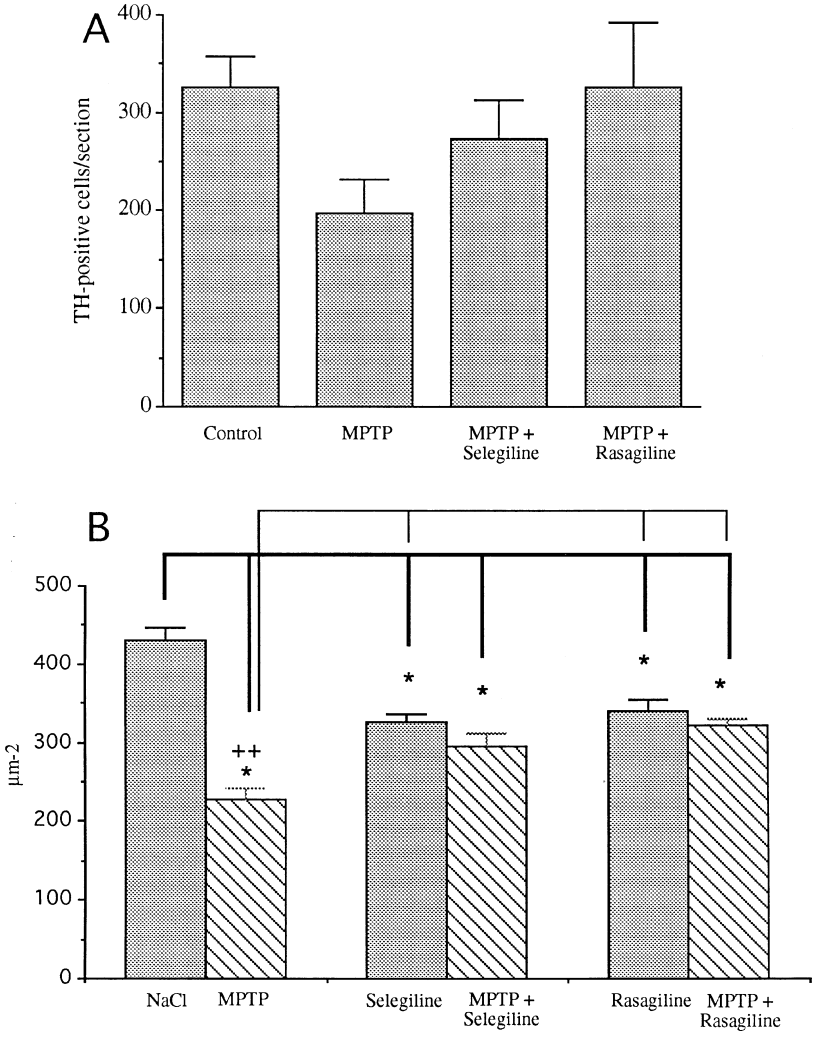

Fig. 8. Effects of treatment with selegiline or rasagiline (10 mg/kg body weight) on MPTP-induced decreases of nigral TH-IR cell counts in common marmosets seven days after exposure to MPTP-HC1 (4 × 2 mg/kg s.c. body weight). **A** TH-IR cell counts from immersion-fixed tissue per section. TH-IR cell numbers were distinctly diminished in MPTP-treated monkeys (groups IV) in comparison to group I, II, III, V, and VI monkeys. However, these differences did not reach the level of significance, possibly due to the relatively low number of non-human primates available (one factor ANOVA, $F_{(4,22)} = 1.7$, with post-hoc Scheffé-test, $p = 0.2$). **B** Effects of the different treatment regimen on sizes of nigral TH-IR cells. Significant decreases in cell size were found in groups II–VI when compared to saline-treated animals ((*); one factor ANOVA, $F_{(5,23)} = 21.7$, with post-hoc Scheffé's test, $p < 0.0001$). Furthermore values differed between group IV (MPTP-only) and group II, III, and VI animals (as indicated by two crosses (++, $p < 0.0001$)

underlines the importance of differentiating between acute, subacute, and chronic effects in the non-human MPTP-primate model of PD. For instance, it is well known that MPTP-induced motor deficits may recover over periods of 2–5 weeks post termination of the MPTP-treatment (Rose et al., 1989a,b; Uiki et al., 1989). This complies with the observation of the present study,

which used 4. × 2 mg/kg body weight of MPTP and where no behavioural recovery was noted during the observation period of 11 days. The behavioural data of the present study suggests that the acute effects of MPTP, i.e. behavioural alterations within the first few days during the MPTP-exposure, may be mediated via a MAO-independent mechanism not necessarily related to the changes in nigrostriatal system (Galvan et al., 1987).

It should also be emphasized that spontaneous motor activity of the monkeys was not altered by treatment with selegiline or rasagiline alone and that there were no obvious behavioural differences between selegiline and rasagiline, 11 days after the start of the treatment with MAO-inhibitors (Fig. 3B). This implies that amphetamine-like actions of selegiline-derived metabolites were not prominent at the behavioural level (except for a non-significant reduction in body weight, which was particularly prominent in selegiline- and MPTP-treated animals).

MAO-activity

Both MAO-A and -B activity in the caudate nucleus were almost completely inhibited by either rasagiline or selegiline (10 mg/kg body weight) treatment (Fig. 4, cf. for distribution of MAO in the marmoset Willoughby et al., 1990). A high dosage of the MAO-inhibitors was chosen for the following reasons: Firstly, amphetamine-like effects of selegiline seem to occur predominantly at higher dosages (for dogs for instance >2 mg/kg body weight, Head and Milgram, 1992). Thus, the present study aimed to compare selegiline vs. rasagiline at dosages where amphetamine-like effects have been previously reported for selegiline (Head and Milgram, 1992). Secondly, it has been postulated that selegiline-derived metabolites contribute to neuroprotective effects of selegiline (Sziraki et al., 1994) Therefore we aimed to compare the neuroprotective actions of selegiline on MPTP-neurotoxicity with agents which are not metabolized to metamphetamine-like substances to verify that the neuroprotective potential of selegiline is indeed completely mediated via MAO-inhibition. Thirdly, in the clinical situation high dosages of selegiline (up to 60 mg daily) seem to be required for antidepressive effects (Sunderland et al., 1994; Quetnik et al., 1984). Yet, the safety of high dose regimen with MAO inhibitors has been questioned, since MAO-inhibition may for instance even enhance metamphetamine neurotoxicity (Wagner and Walsh, 1991).

Non-human primate studies on this matter are not available. In mice selegiline inhibits at 2 mg/kg body weight MAO-A by 23% and MAO-B by 95% (Heikkila et al., 1990), while in rats selegiline and rasagiline inhibits at 0.25 and 0.05 mg/kg body weight, respectively, MAO-B by 90%, with 40% (selegiline) and 15% (rasagiline) inhibition of MAO-A (Lamensdorf et al., 1996). Clinically, it is generally recommended to prescribe selegiline at a dosage of approximately 0.15 mg/kg (equals to approximately 2 × 5 mg per day), which may result in a more selective inhibition of MAO-B.

Importantly, MPTP alone inactivated MAO-A and MAO-B by 27% and 22%, respectively. This was significant only for MAO-A, which complies with previously reported binding of MPP^+ to MAO-A (May, 1993). However,

there was no enhancement of MAO-inhibition by MPTP in selegiline- and rasagiline-treated animals (no differences between group II vs. V and group III vs. VI values). This presumably reflects the distinctly higher affinity of selegiline (Salach et al., 1984) and rasagiline towards the enzymes in comparison to MPTP in the non-human primate brain as compared to rodents (for rats Fuller and Hemrick-Luecke, 1985; for mice Heikkila et al., 1985b; for cats Lin et al., 1995), although studies with lower dosages of selegiline and rasagiline would be necessary to definitely exclude an enhancement of MAO-inhibition by concomittant MPTP-treatment.

Striatal neurotransmitter concentrations

In the saline-treated group DA-concentrations in the putamen were in the range of 120–170 pmol/mg wet tissue, which agrees with previous reports (Perez-Otano et al., 1993; Kupsch et al., 1996). Selegiline- or rasagiline-treatment did not alter post mortem striatal DA concentrations (Fig. 6A; for increased striatal DA levels after selegiline-treatment with concomittant levodopa/carbidopa-treatment as assessed via microdialysis c.f. Kaseda et al., 1999).

The systemic MPTP-injections led to a pronounced depletion (approximately 99%) of striatal DA-content 7 days after MPTP exposure which could be almost completely antagonized by pretreatment with selegiline or rasagiline (Fig. 6). The neuroprotective action of MAO-inhibitors in the MPTP-model is probably mediated by the impaired MAO-dependent generation of the presumably toxic MPTP-metabolite MPP^+ (Herkenham et al., 1991). The almost complete inhibition of the MAO-subenzymes is also reflected by the reduced concentrations of DOPAC and HVA, since both metabolites depend on the intact degradation of DA via MAO (Eisenhofer et al., 1994; c.f. also Lakshmana et al., 1998).

Treatment with selegiline (and less pronounced with rasagiline) enhanced 5-HT levels in the putamen in groups II, V. In parallel, reduced 5-HIAA-concentrations were found in animals, treated with the two MAO-inhibitors, suggesting a decreased metabolism of 5-HT to 5-HIAA by MAO-A (Cumming et al., 1992) (Fig. 7B).

MPTP alone did not affect 5-HT levels (Fig. 7A), but reduced 5-HIAA-levels by approximately 51% (Fig. 7B) which emphasizes that the subacute actions of MPTP are not restricted to the dopaminergic system.

Tyrosine hydoxylase immunocytochemistry in the substantia nigra

In the vehicle-treated group nigral TH-IR cell counts were in the range of 300 cells per section which supports previous findings (Lange et al., 1993; Kupsch et al., 1996). The presently employed MPTP-regimen reduced the number of TH-IR neurons by approximately 30–40% in the SNC (Fig. 8). Coadministration of MAO-B inhibitors almost completely antagonized this reduction of nigral TH-IR. An important caveat, when interpreting data based on TH-immunocytochemistry, relates to the potential discrepancy of TH-protein expression, on-going cell degeneration, and actual cell death,

although TH-IR is necessary to identify DA neurons (Jackson-Lewis et al., 1995). For instance, it has been reported that MPTP ensue a reversible reduction of TH-IR without actually causing neuronal cell loss (TH-IR amacrine cells in the retina, c.f. Tatton et al., 1990). To our knowledge detailed studies concerning this issue have not been conclusively carried out in the non-human MPTP-primate model of PD (Kitt et al., 1986; Lange et al., 1993; Zuddas et al., 1993; Kupsch et al., 1996). However, data from the MPTP-mouse model show that neuronal cell death and loss of TH-IR do coincide 4 days following MPTP-exposure (Jackson-Lewis et al., 1995; Seniuk et al., 1990). Furthermore and more importantly, we have observed a similar reduction (>50%) of nigral TH-IR in common marmosets which were allowed to survive for up to 6–12 months after completion of the presently employed MPTP-regimen (n = 3, mean nigral TH-IR neurons per unilateral section ± S.E.: 119.3 ± 11.6, range 99–139). Thus, it seems likely that the presently observed reduction of nigral TH-IR 7 days after completion of the MPTP-treatment actually correlates with cell death.

Furthermore, we were able to demonstrate a significant reduction in TH-IR cell sizes in MPTP-treated animals by about 60% which was attenuated by concomittant treatment with MAO-inhibitors to approximately 80%. Surprisingly, high-dose treatment with MAO-inhibitors alone also diminished cell sizes in comparison to saline-treated animals by about 20%. Obviously, this finding (decreased TH-IR cell size after MAO-inhibition) is difficult to explain (Rodríguez-Gómez et al., 1997), although it is interesting to note that in-vitro application of selegiline in higher concentrations (>100 μM) has been reported to cause apoptosis (Le et al., 1997). More importantly, our observation complies with a recent in vivo-study by Thiffault et al. (1998), who observed increased striatal lipid peroxidation and decreased nigral TH-IR in mice treated the MAO-B inhibitor MDL 72974. Furthermore trophic or other mechanisms (e.g. glutamatergic, Mytilineou et al., 1997b) may play a role, since both selegiline and rasagiline are irreversible, enzyme-activated inhibitors of MAO (so called "suicide" inhibitors, c.f. Riederer and Youdim, 1986). Thus, selegiline and/or rasagiline have been proposed to influence gene expression (Tatton et al., 1996), anti-apoptotic (Maruyama et al., 2000) and antioxidant activities (Carrillo et al., 2000), cytokines and NGF-synthesis (Semkova et al., 1996; Wilfried et al., 1996).

In conclusion, to our knowledge, this is the first study in non-human MPTP-primate model on the effects of selegiline and rasagiline. We conclude that selegiline and rasagiline are equally potent in preventing MPTP-neurotoxicity and that the metabolites of selegiline are of minor, if any, importance in the experimental design chosen here. Thus, even at high dosages no clear amphetamine responses of selegiline administration were observed in comparison to rasagiline.

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