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Sparing by Rasagiline (TVP-1012) of Cholinergic Functions and Behavior in the Postnatal Anoxia Rat

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SPEISER, Z., O. KATZIR, M. REHAVI, T. ZABARSKI AND S. COHEN. *Sparing by rasagiline (TVP-1012) of cholinergic functions and behavior in the postnatal anoxia rat.* PHARMACOL BIOCHEM BEHAV **60**(2)387–393, 1998.—Rasagiline (N-propargyl-1(R)aminoindan) is a selective and potent MAO-B inhibitor currently under development as the mesylate salt (TVP-1012) for the treatment of various neurologic disorders. Preliminary work in adult and senescent rats, either normal or hypoxia-lesioned, showed that chronic rasagiline treatment improved performance in memory and learning tasks, suggesting some beneficial effect on central cholinergic function. We have now used the postnatal anoxia-lesioned rat as a model of cholinergic dysfunction. In the neonatal rat, anoxia strongly affects the cholinergic system, which has not yet reached full maturation at this state of life. Rasagiline mesylate was administered from day 1 to completion of the study (day 60), first through nursing mother milk until weaning (day 21), then in drinking water, at the rate of 0.5 mg/kg/day. Drug access to the CNS was verified by analysis of MAO activity in brain (at 21 days). Treatment improved the juvenile hyperactivity syndrome associated with anoxia (at day 28). It improved performance in the passive avoidance test to normal control level (at day 40). It improved spatial memory performance in the Morris water maze to normal control level (at day 50). The untreated anoxia group failed in these tasks and was significantly inferior to either the normal control and rasagiline-treated anoxia groups. Determination of ChAT activity in the caudate and hippocampus of rats from each of these groups gave the following results (pmol ACh/mg protein/min). Caudate: normal control, *588* ± 56; anoxia, 398 ± 54; rasagiline-treated anoxia, 536 ± 35. Hippocampus: normal control, 380 ± 31; anoxia, 275 ± 47; rasagiline-treated anoxia, 325 ± 35. Results are mean ± SD from each of seven to nine different donors in a group. Thus, improvement in memory and learning tasks of the rasagiline-treated anoxia group finds correspondence in the activity of the cholinergic marker ChAT in two brain regions that have prominent cholinergic innervation. © 1998 Elsevier Science Inc.

Rasagiline TVP-1012 Anoxia ChAT Hyperactivity Passive avoidance Water maze

RASAGILINE [(N-propargyl-1-(R)aminoindan] is a potent and selective monoamine oxidase B inhibitor (MAOI-B), currently under development for the treatment of various neurologic disorders. In preliminary preclinical studies, acute treatment with rasagiline was reported to reverse or correct behavior ascribed to dopaminergic hypofunction induced with haloperidol, or α-methyl-*p*-tyrosine (44,45), or MPTP (39). Chronic treatment with rasagiline evoked behavioral and cognitive effects such as improvement in passive and active avoidance of hypoxia-lesioned adult or senescent rats to the unlesioned control level (45). It is generally agreed that integrity of central dopaminergic function is essential for motor activity and successful active avoidance, but successful passive avoidance seems to depend on undisturbed cholinergic function (4,25,28). The evidence for the latter is the induction of memory and learning deficits by lesioning prominently cholinergic structures as the nucleus basalis of Meynert or the medial septum (9,37,38,52,58); or by pharmacological intervention with either cholinergic agonists or antagonists (12,58); or by use of a synthetic neurotoxin, AF-64A (10,47). The cholinospecific immunotoxin 192-IgG-saporin gave different results in the hands of different authors: in adult rats, passive avoidance was impaired significantly (21,56), or slightly (51), or not at all (55).

The preliminary beneficial effects of rasagiline in tests of learning and memory, and the recent reports on neuronal res-

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cue by selegiline in the face of chemical or surgical-induced stress (2,50) suggested that rasagiline, in analogy with selegiline, might protect neurons exposed to stress. Indeed, preliminary findings seem to corroborate this view (40,46,59). To explore this hypothesis in the specific context of cholinergic deficits and their behavioral and biochemical correlates, we have used the postnatal anoxia-lesioned rat model developed and used here and elsewhere (5,8,43). Specifically, such rats are hyperactive at age 10–42 days (43), and fail in passive and active avoidance at maturity (42). This pattern of behavior was accompanied by an early and transitory increase in QNB-binding sites in the hippocampus (14) and a long-lasting decrease in ChAT activity but also a compensatory increase in choline uptake in the hippocampus and caudate throughout the age of 20–60 days (48). An almost similar pattern of behavior could be elicited by ICV injection of the neurotoxin AF-64A (10), and the evoked behavioral disorders could be corrected by treatment with tacrine or arecoline (42,47).

We now show that chronic rasagiline treatment of postnatal anoxia-lesioned rats blunted or suppressed the juvenile hyperactive syndrome associated with anoxia, and improved passive avoidance and spatial performance in the water maze at adulthood almost to the unlesioned control level. At the same time, hippocampal and caudate activity of the cholinergic marker ChAT was significantly higher in the rasagiline-treated group than in the untreated one.

METHOD

Animals

Wistar rats (Harlan-Olac, Jerusalem) were used throughout this study. All rats were kept at the animal house at 24 ± 2°C and a 12 L:12 D cycle (lights on at 0600 h), with free access to food (Purina) and water. For breeding, a male 4–8-month-old was kept with a pair of females 2–5-month-old for 10 days. The male was withdrawn, the females kept together for an additional 10-day period, then housed in individual cages where they had a litter of about 9–12 pups. Mother and five to seven male offspring were kept together until weaning (21 days) then separated and housed five to seven to a cage. At the conclusion of an experiment, rats were asphyxiated with carbon dioxide.

Anoxia

Only male pups were used. On the second postnatal day, five to six pups were placed in a glass container equipped with an inlet and outlet for the displacement of air with 100% nitrogen at near atmospheric pressure. After a stay of 30 min, the pups were removed to room air and resuscitated by soft intermittent massage to the chest, then returned to their respective mothers.

Drugs

Rasagiline mesylate (TVP-1012) was donated by Teva Pharmaceutical Industries. To avoid handling which could affect behavior, the drug was administered in drinking water to the nursing mothers at a rate estimated as 0.5 mg/kg/24 h, starting on the first day from delivery. Fresh solutions were prepared once in 3 days. Rasagiline in aqueous solution is stable over many days at room temperature (Manufacturer's data). The daily dosage was assessed from prior knowledge of the water volume consumed daily by the mother (about 30 ml) and spillage. Drug concentration was adjusted accordingly. After weaning and to completion of the study, the young rats were given access to drug in drinking water with continuous adjustment of drug concentration to match the volume consumed. To ascertain the transfer of drug through milk from nursing mother to the young, two pups from each of the control, drug-untreated anoxia, and drug-treated anoxia groups, were sacrificed at age 21 days, and MAO activity was assayed in whole brain. Percent activity of MAO was as follows: anoxia, 92% of control, both MAO-A and -B; drug-treated anoxia, MAO-A 70-78%, MAO-B 8-12% of control.

Biochemistry

Choline acetyl transferase (ChAT) was determined using [^{14}C]-acetyl-CoA (Amersham), specific activity 60 mCi/mmol (11). Rats at age 21 days from each of three groups, control, untreated anoxia, and rasagiline-treated anoxia, were decapitated, and the hippocampus and caudate carefully removed and placed on ice. The organ from each individual rat was weighed, then homogenized in 10 vol of 50 mM phosphate buffer, pH 7.4 containing 300 mM NaCl, 20 mM EDTA, and 0.5% Triton B. Ten microliters quadruplicates of each homogenate were treated with 10 μl of a stock solution prepared from phosphate buffer 930 μl, physostigmine salicylate 50 μl of a 5 mM solution, acetyl-CoA 9.5 ml of a 20 mM solution in 0.1 M phosphate citrate buffer, choline chloride 10 μl of 1.6 M solution, and [^{14}C]-acetyl-CoA (3.3 nmol equivalen to about 12,000 dpm). A parallel quadriplicate set was run with the ommission of choline chloride. After incubation at 37°C for 15 min, the reaction was terminated by addition of 50 μl of a solution of tetraphenylboron in 3-heptanone, 15 mg/ml. Vigorous vortex mixing was followed by centrifugation for 2 min at 15,000 rpm. Twenty microliters of the upper layer were pipetted and added to 3.5 ml of a scintillation fluid (Optifluor) then read in a scintillation counter (Packard Tricarb 1600). The difference in dpm between sample pairs in the presence and absence of choline gave the rate of formation of [^{14}C]-acetylcholine. The rate was related to the protein content of the sample (24). MAO activity was determined as given (19).

Behavior

Motor activity was determined simultaneously for eight rats in each of eight fully computerized cages, 28 × 28 cm, which recorded the crossings over time of a grid of infrared beams at 4-cm intervals. Crossings could be differentiated into "big movements" corresponding to the instant crossings of more than two beams; hence, reflect ambulation, and "small movements" corresponding to a more stationary activity as grooming and sniffing. Recordings were taken at ages 14, 21, 28, and 35 days over 15 min at 9–12 h.

Passive Avoidance

Retention of one-trial step-through passive avoidance was determined at the age of 45 days. The apparatus consisted of two compartments separated by a sliding door, one being lit while the other was kept dark. At training, the rat was placed in the lit compartment for 30 s, then the door was opened, whereupon the rat escaped to the dark compartment with a latency that was recorded. The door was shut closed and a 0.3 mA foot shock was delivered for 5 s by a Grass (S-88) stimulator. Retention of the experience after 48 h was determined by repeating the test and recording the latency of step through.

Water Maze

The apparatus consisted of a circular water tank 140 cm across, filled with water to a depth of 38 cm. The water was

made opaque with milk powder. A clear Plexiglas platform 15 cm square supported by a movable stand was submerged to a depth of 2 cm from the water surface. In the working memory paradigm, performed at age 50–60 day, a rat was initially placed on the platform and allowed for 1 min to get familiarized with distal cues. Then it was removed to a given point of entry and allowed to locate the platform at a maximum latency of 120 s (run I). In case of failure, the rat was manually placed on the platform. The test was repeated after 60 s from the same point of entry (Run II). The test was repeated daily for 5 consecutive days, but each day the platform and points of entry were relocated. Shorter latencies in run II are indicative of acquisition from recent experience. During the trial, ambient orientation clues remained unchanged.

Analgesia

In passive avoidance, a change in pain threshold could possibly affect performance. It became necessary to show that rasagiline was devoid of analgetic effects. To this end, three to five rats from each of a control, anoxia-lesioned rasagiline-treated and untreated groups were tested for latency of response in the hot plate (49) and tail-flick (53) tests.

Statistics

The water maze data were analyzed by ANOVA for repeated measures, using a SPSS program run on a PC. All other data were analysed by either ANOVA or the unpaired or paired two-tailed Student's *t*-test.

RESULTS

Motor Activity

At age 28 days, the anoxia group was significantly more active than either control or rasagiline-treated groups, which were not different from each other (Fig. 1). The "big movement" score was the major contributing parameter of hyperactivity. At age 35 days, the difference in motor activity among groups disappeared. This finding is consistent with earlier reports on this model (43).

Passive Avoidance

At age 45 days, both the normal control and rasagiline-treated anoxia groups displayed significantly better retention than the anoxia group as evidenced by their longer step-through latencies with respect to the untreated anoxia group (Fig. 2). This difference disappeared when the test was repeated 72 h following experience of electroshock.

Water Maze

This series of tests was performed at age 50–60 days. The mean latencies in either run I and run II at each of 5 consecutive days are shown in Fig. 3. Differences in latencies among groups were compared separately for run I and run II, using ANOVA. The differences among groups were significant in either run I, $F(2, 48) = 4.16$, $p = 0.022$, and run II, $F(2, 48) = 5.06$; $p = 0.010$. The untreated anoxia group was inferior to control in either run I, $F(1, 32) = 7.42$, $p = 0.01$, and run II, $F(1, 32) = 9.58$, $p = 0.004$. The rasagiline-treated anoxia group had significantly shorter latencies than the untreated anoxia group on run I, $F(1, 31) = 5.22$, $p = 0.029$, but this difference was not significant on run II, $F(1, 31) = 1.58$, $p = 0.219$, unless comparison is restricted to Session 5, $F(2, 48) = 6.97$, $p = 0.002$. There was no group by trial interaction between any of the groups on runs I, $F(8, 192) = 0.82$, $p = 0.58$, and II, $F(8, 192) = 1.83$, $p = 0.073$. Difference in latencies between runs I and II was highest in session 5 for the untreated anoxia group (26.69 ± 4.70) and was significantly different from the corresponding differences for control (7.22 ± 4.43) or the rasagiline-treated anoxia group (4.00 ± 4.55), $F(2, 48) = 6.89$, $p = 0.002$).

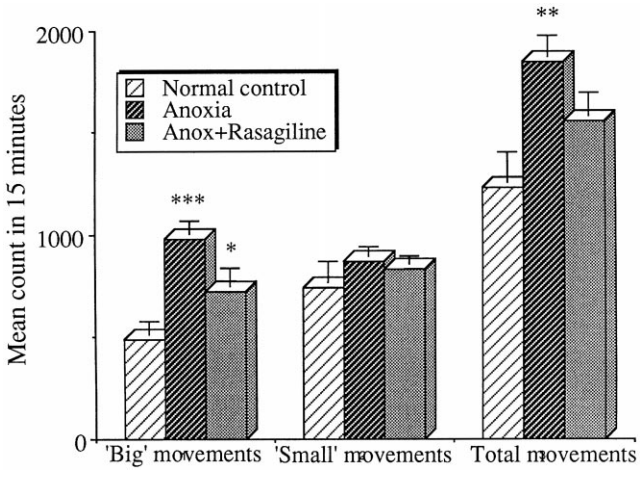

FIG. 1 Locomotor activity of postnatal anoxia-lesioned rats at age 28 days, untreated or treated with rasagiline mesylate (TVP-1012) at 0.5 mg/kg/day administered in drinking water. Statistical significance: the anoxia group is compared to normal controls; the rasagiline-treated group is compared to the anoxia group; vertical bars are SEM; $*p < 0.05$; $*p < 0.01$; $**p < 0.001$ by unpaired Student's *t*-test; $n = 16$–18 per group, in this and following figures.

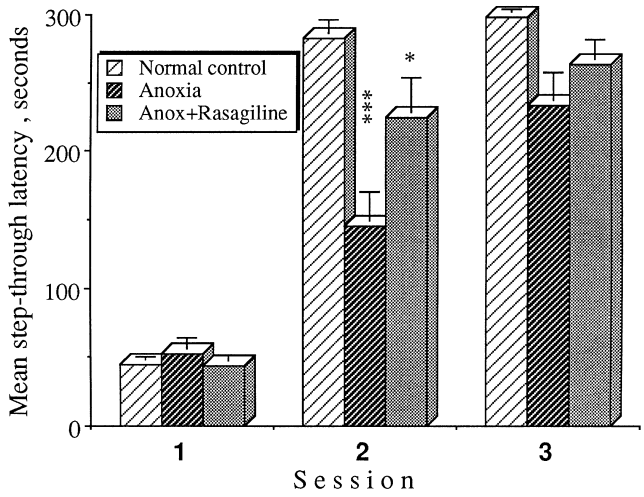

FIG. 2. Passive avoidance retention latencies of postnatal anoxia-lesioned rats at age 40 days, untreated or treated with rasagiline mesylate (TVP-1012) at 0.5 mg/kg/day, with respect to normal controls, to a maximum cutoff of 300 s.

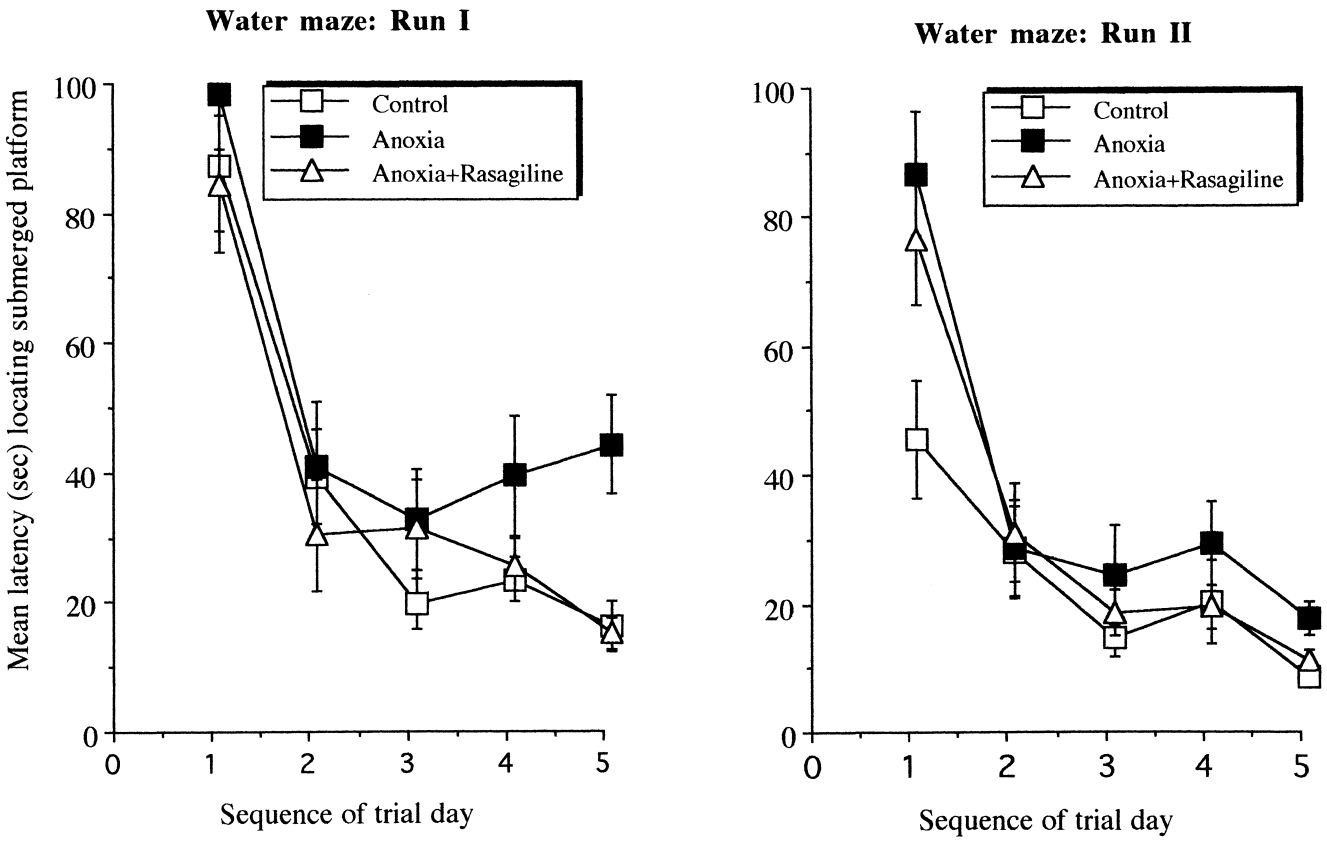

FIG. 3. Water maze place navigational performance of postnatal anoxia-lesioned rats at age 50–60 days, untreated or treated with rasagiline mesylate (TVP-1012) at 0.5 mg/kg/day, with respect to normal controls. Vertical bars are SEM. Mean latency ± SEM is given for runs I and II in a given session. In run I, both the normal control and rasagiline-treated groups had significantly shorter latencies than the untreated anoxia group by ANOVA with repeated measures. Latencies in run II were not significantly different from run I.

ChAT Activity

At age 21 days, anoxia caused a significant decrease in ChAT activity in the caudate and hippocampus (68 and 74% of normal control). At the same time, rasagiline treatment caused a significantly higher level of activity in the caudate and hippocampus than found in matching anoxia group, nearing the level of normal control in the caudate (91%), but less so in the hippocampus (86% of normal control) (Fig. 4)

Analgesia

There was no difference in response to pain among groups. In the tail-flick test, the reaction time in seconds was: control, 2.25 ± 0.6; anoxia, 2.67 ± 0.51; anoxia, rasagiline-treated, 2.26 ± 0.37. In hot plate test: control, 4.37 ± 0.55; anoxia, 3.70 ± 1.35; anoxia, rasagiline-treated, 4.28 ± 1.47, for $n = 3$–5 per group.

Body Weight Gain

The untreated anoxia group showed reduced weight gain with respect to either its rasagiline-treated counterpart or to control, but only during the first month of life. Eventually, the rate of growth was the same in all groups.

DISCUSSION

Relevance of the current model of neonatal anoxia to cholinergic dysfunction in the rat stems from a number of independent reports on the subject. The relatively late ontogenic maturation of the cholinergic system (7,15,31,54) renders it more vulnerable to anoxia at a stage where sprouting and synaptogenesis are still in progress. The hippocampus with its otherwise rich cholinergic innervation is especially sensitive to hypoxia or anoxia in the neonatal period and is among the first sites to suffer damage (5,8,34). A persisting imbalance between the catecholaminergic and cholinergic systems in the juvenile is expressed in hyperactivity due to lack of arousal inhibition (6,13,18,26). Indeed, the postnatal anoxia-lesioned rats or AF-64A–lesioned rats share a common profile of hyperactive syndrome (47). Concurrently, a reduced cholinergic output to the hippocampus has been related to deficits in learning and memory tasks (3,13,37,38). Failure in passive avoidance and spatial orientation in the water maze are the experimental correlates of such deficit (30,32,37,57) as indeed found in the current anoxia model. In either case, chronic rasagiline seemed to correct or prevent the deficit. At this point, it is noteworthy that such impairments in behavior were not observed in adult rats after postnatal injections of an optimal dose of the highly specific cholinotoxin 192-IgG-saporin (20,35). Still, the water maze place navigational performance of the neonatally lesioned animals was impaired after administration of atropine or a second dose of the immunotoxin. In either model, anoxia- and cholinotoxin-elicited, working memory (short-term retention) seemed to be less vulnerable to the lesion induced.

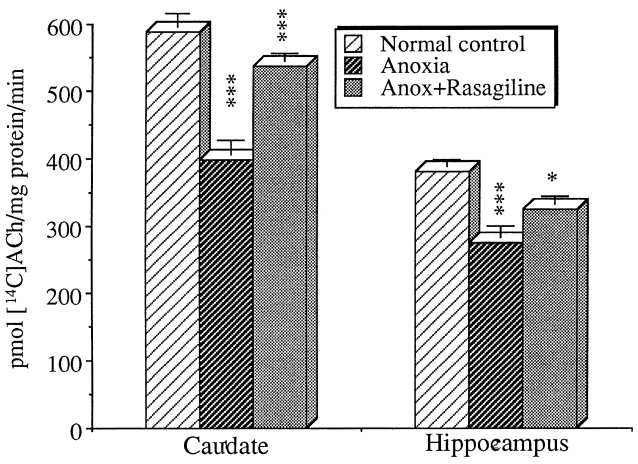

FIG. 4. Mean ex vivo ChAT activity ± SEM (n 7–8 per group) in caudate and hippocampus of postnatal anoxia-lesioned rats at age 21 days (weaning), untreated, or exposed to rasagiline mesylate (TVP-1012) through milk from nursing mothers treated with the drug at 0.5 mg/kg/day. The anoxia group is compared to normal controls. The rasagiline-treated group is compared to the anoxia group. Levels of significance as in Fig. 1.

Obviously, the anoxia and the 192-IgG-saporin models of induced cholinergic hypofunction are not equivalent. Anoxia is expected to elicit a lesion of a more diffuse nature and may affect additional areas such as the basal ganglia (42), while the immunotoxin-elicited lesion targets specific, well-defined areas. This premise is corroborated by the finding that anoxia could cause a significant decrease in ChAT activity in the caudate (68% of control), while 192-IgG-saporin caused no decrease in this organ (35). The behavioral and biochemical effects of rasagiline in the neonatal anoxia-lesioned rats are now considered over this background.

Drug administration to the nursing mothers was initiated 1 day prior to anoxia. The dose of rasagiline reaching the suckling immediately following anoxia is a matter of conjecture, but cannot be considerable. It depends on the one hand on the steady-state concentration of the drug in the nursing mother, and on the other on the feeding ability and sucking force that are usually ineffective after anoxia. The best estimate is about 0.5 mg/kg/24 h. Because administration of this dose is being stretched over 24 h, one expects rasagiline to achieve a steady-state level in about four times its $t_{1/2}$ in the rat, which is about 1 h (private communication). Thus, at 4 h from initiation of feeding, one expects a steady-state level in the suckling resulting from a mean input not exceeding 21 μg/kg/h or less. Evidence for transfer of rasagiline from mother to offspring is seen at weaning on day 21, when brain MAO activity was reduced to about 75% of control for MAO-A and 15% for MAO-B. Brain MAO-B activity in newborn rodents is usually low with respect to that of MAO-A (61), and one may anticipate little or no metabolic contribution from MAO-B in the face of added inhibition by rasagiline. Thus, MAO-B inhibition as such is not likely to account for the protective effect seen in passive avoidance, water maze, and locomotor activity tests. The recovery of ChAT activity in the caudate and hippocampus in the rasagiline-treated group of anoxia-lesioned rats seems to suggest a different mechanism of action. In this context, we recall the work (17) showing that cholinergic cell bodies in 7-day-old rats are more resistant to hypoxic and ischemic injury than their axons. On the other hand, it was shown recently (2) that axotomized facial neurons in the immature rat can be rescued from eventual death by treatment with selegiline (L-deprenyl) at a dose of 0.001 to 10 mg/kg.

Selegiline, like rasagiline, is a selective but less potent MAO-B inhibitor. The authors offered the hypothesis that neural rescue by selegiline is related in some way to MAO-B inhibitory potential but is not a direct consequence of MAO-B inhibition as such. Neuroprotective effects were also reported for some aliphatic MAOI-B (60). In the light of current knowledge, one can assume that rasagiline could have stimulated sprouting of cholinergic fibers from surviving cell bodies. The observed preservation of ChAT activity may possibly provide an indication of the density of spared cholinergic nerve endings in the affected region. An increase in cholinergic activity, in turn, is expected to favor neuronal outgrowth, plasticity, and survival as found in vivo (23) and in vitro (36).

One could still argue that a possible increase in dopamine levels following MAO-B inhibition, low as it maybe, might trigger a compensatory release of ACh (1,22,29,33). Very recently, use of microdialysis (19) showed that chronic treatment with either rasagiline or selegiline increased the release of striatal dopamine, possibly by intervention from increased levels of endogenous phenylethylamine (PEA). The latter is a major substrate of MAO-B. A possible link between dopamine and ACh levels has been reported recently by use of microdialysis (41). An acute dose of selegiline (10 mg/kg) increased twofold ACh release in the frontal cortex. The effect was mimicked by D_1 agonists but blocked by D_1 antagonists. On the other hand, there is also evidence that an increase in dopamine is not a prerequisite for an increase in ACh release. Certain propargylamines as clorgyline and selegiline, but not pargyline or tranylcypromine, can modulate ACh release by binding to a site on the sigma opioid receptor (16,27). Remarkably, it was also shown that this effect is not due to an increase in ChAT activity (27). Thus, not all propargylamines are equal in the context of neuronal protection. In conclusion, evidence suggests that in the neonatal anoxia-lesioned rat, rasagiline is neuronally protective rather than restorative of neurotransmitter function through MAO inhibition.

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