The effects of rivastigmine plus selegiline on brain acetylcholinesterase, (Na^+, K^+)-, Mg^{2+}-ATPase activities, antioxidant status, and learning performance of aged rats

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Abstract: We investigated the effects of rivastigmine (a cholinesterase inhibitor) and selegiline ((-)deprenyl, an irreversible inhibitor of monoamineoxidase-B), alone and in combination, on brain acetylcholinesterase (AChE), (Na^+, K^+)-, Mg^{2+}-ATPase activities, total antioxidant status (TAS), and learning performance, after long-term drug administration in aged male rats. The possible relationship between the biochemical and behavioral parameters was evaluated.

Methods: Aged rats were treated (for 36 days) with rivastigmine (0.3 mg/kg rat/day ip), selegiline (0.25 mg/kg rat/day im), rivastigmine plus selegiline in the same doses and way of administration as separately. Aged and adult control groups received NaCl 0.9% 0.5 ml ip.

Results: TAS was lower in aged than in adult rats, rivastigmine alone does not affect TAS, decreases AChE activity, increases (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activity of aged rat brain and improves cognitive performance. Selegiline alone decreases free radical production and increases AChE activity and (Na^+, K^+)-ATPase activity, improving cognitive performance as well. In the combination: rivastigmine seems to cancel selegiline action on TAS and AChE activity, while it has additive effect on (Na^+, K^+)-ATPase activity. In the case of Mg^{2+}-ATPase selegiline appears to attenuate rivastigmine activity. No statistically significant difference was observed in the cognitive performance.

Conclusion: Reduced TAS, AChE activity and learning performance was observed in old rats. Both rivastigmine and selesiline alone improved performance, although they influenced the biochemical parameters in a different way. The combination of the two drugs did not affect learning performance.

Keywords: aged rat, brain enzymes, TAS, learning, rivastigmine, selegiline

Introduction

Brain aging in most cases is characterized by cognitive deficits and a central cholinergic hypofunction (Bartus et al 1982). Alzheimer's disease (AD), a neurodegenerative disorder, is characterized by loss of memory and other cognitive abilities. It is also characterized by a prominent loss of cholinergic neurons in the basal forebrain (Davis and Maloney 1976), leading to decreased amounts of acetylcholine and decreased activities of cholinacetyltransferase (ChAT) and acetylcholinesterase (AChE) in almost the entire neocortex (Coyle et al 1983). The observed association between the loss of cholinergic neurons, receptors, reduction of cholinergic markers, cognitive and executive function impairments in AD was the base for the development of cholinergic hypothesis (Bartus 2000) and the introduction of AChE inhibitors as the main therapeutic approach for this disease. Rivastigmine is a second-generation carbamate-based pseudo-irreversible AChE and butyrylcholinesterase (BuChE) inhibitor, indicated for treatment of mild to moderate AD (Anand et al 1996; Corey-Bloom et al

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1998; Eskander et al 2005; Caltagirone et al 2005; Takeda et al 2006; Gonzalez-Gutierrez and Gobbart 2007) or for patients with rapid disease progression (Farlow et al 2005). As the disease and age progress, significant loss of other neurons (noradrenergic, dopaminergic) is observed as well (Davies and Wolozin 1987; Strong 1998). The pathology of the disorder may involve oxidative stress and accumulation of free radicals, leading to excessive lipid peroxidation and neuronal degeneration in the brain (Smith et al 1991; Strong 1998; Pratico and Delanty 2000). Selegiline,(-)deprenyl, an irreversible monoamineoxidase-B (MAO-B) inhibitor, has been used in depression and in Parkinson's disease in combination with L-dopa (Birkmayer et al 1985; Lieberman and Fazzini 1991; Knoll 2000; Negrotti et al 2001; Kitani et al 2002). Selegiline enhances the release of dopamine, blocks the reuptake of dopamine and produces an amphetamine-like effect (Ebadi et al 2002). Pretreatment with selegiline can protect neurons against a variety of neurotoxins as MPTP, DSP-4, 5,6-dihydroserotonin and AF64A, which damage dopaminergic, adrenergic, serotonergic, and cholinergic neurons respectively (Walsh et al 1984; Ricci et al 1992; Mayar and Haberle 1999; Matsubara et al 2001). In patients with moderate impairment from AD, treatment with selegiline slowed the progression of disease (Sano et al 1997; Filip and Colibas 1999; Knoll 2003). However, according to recent data from a meta-analysis, selegiline significantly improved cognition and activities of daily living at an earlier time point, but not at a later assessment time (Wilcock et al 2002; Birks and Flicher 2003). Selegiline prevents the effects of oxidative stress in a variety of models both in vitro and in vivo (Youdim et al 2001).

The underlying mechanism of the beneficial effect of selegiline on neuronal function is believed to be associated with enhanced activity of free radical scavenging enzymes (Carrillo et al 1994; Kitani et al 2002; Kiray et al 2006), diminished production of hydrogen peroxide through MAO-B inhibition (Cohen and Spina 1989; Takahata et al 2006), some trophic-like effects that increase the survival of degenerating motoneurons (Ju et al 1994), restoration of ChaT reduced activity (Koutsilieri 2001), the number of neurons in the hippocampus (Kiray et al 2006), or enhancement of neuroplastic status (Murphy et al 2006). Recently Ono and colleagues (2006) also reported an in vitro antiamyloidogenic activity of selegiline.

We have previously shown that the administration of selegiline in a dose of 0.25 mg kg^{-1} rat/other day for 50 days in old rats increased whole brain TAS, stimulated (Na^+, K^+)-ATPase activity and although it increased AChE activity, it improved the learning performance of aged rats (Carageorgiou et al 2003).

The (Na^+, K^+)-ATPase, or Na^+ pump, is an energy transducting ion pump first described by Skou in 1952 (Skou 1998). In recent years, research on (Na^+, K^+)-ATPase revealed that interactions of (Na^+, K^+)-ATPase with other proteins not only are important for regulation of pumping function, but also make it possible for the enzyme to function as a single transducter (Xie and Cai 2003). Long-term pharmacological interruption of cholinergic transmission can decrease the postsynaptic membrane potential by altering (Na^+, K^+)-ATPase activity. This can be seen as a decline in [^{3}H] ouabaine binding (Henning et al 1994). Age-associated impairments in a test of attention and evidence of involvement of cholinergic systems was referred by Jones and colleagues (1995). It is known that inhibition of (Na^+, K^+)-ATPase induces neurotransmitter release in several experimental models (Rodríguez de Lores Arnaiz and Pellegrino de Iraldi 1991). Furthermore, studies suggest that (Na^+, K^+)-ATPase might play a role on memory formation (dos Reis-Lunardelli et al 2007). According to Gorini and colleagues (2002) (Na^+, K^+)-ATPase is a particular age-related enzyme. The (Na^+, K^+)-ATPase activity is lower in all plasma membrane subfractions (rat frontal cerebral cortex) at 22 months of age, than in 5 months (where reduction is already evident at 10 months of age). Similar reductions in (Na^+, K^+)-ATPase activity were observed by Kaur and colleagues (1998) in different brain regions of 24-month-old rats. However, old age and selective loss of cholinergic basal frontal cells did not significantly alter the presynaptic second messenger system that influences (Na^+, K^+)-ATPase activity. On the other hand the lesions had a much greater effect on performance than old age alone did (Stoehr et al 1997).

Little is known about the activity of Mg^{2+}-ATPase in old age, an enzyme that is of primary importance in phosphorylation reactions and the maintenance of high brain intracellular Mg^{2+}. Its change can control rates of protein synthesis and growth of the cell (Sanui and Rubin 1982). Decreased Mg^{2+}-ATPase activity in the frontal cortex of old age rats was reported by Gorini and colleagues (2002).

Oxidative stress has been implicated in aging and age-related neurodegenerative diseases (Atamna and Frey 2007; Tahirovic et al 2007; Weinreb et al 2007) and the proposal of the "free radical theory of aging" (Harman 1956). Impaired total antioxidant capacity in different structures from aged rat brain was observed by Siqueira and colleagues (2005). A decrease of the total antioxidant status in the brain of old male rats has been observed by Carageorgiou and colleagues (2003).

Based on the aforementioned selegiline study in aged rats, the foreseen trend of combining drugs with a different mechanism of action in AD therapy (Youdim and Weinstock 2002; Ucar et al 2005; Dantoine et al 2006; Groner et al 2007; Tahirovic et al 2007) and since multiple factors contribute to AD pathology (van Dyck 2004; Liu and Ames 2005) we decided to investigate the effect of the combination of the two agents, rivastigmine and selegiline, on brain TAS, AChE, (Na^+, K^+)-ATPase, Mg^{2+}-ATPase activities and on cognitive capacity of aged rats. We also considered evaluating the possibility of correlations between biochemical and behavioral data. It should be mentioned that there were no previous in vivo data about the effect of a) rivastigmine alone on the activities of brain (Na^+, K^+)-ATPase, Mg^{2+}-ATPase or TAS. and b) rivastigmine plus selegiline combined administration on all the biochemical and behavioral parameters studied.

Materials and methods

Animals

Fifty two (52) aged male Wistar rats (24 months old) and 485 ± 23 g BW were used. A group of 11 adult rats (8 months old) and 391 ± 12 g BW was also used as an adult control. The rats were housed five or six in a cage at a constant room temperature (22 ± 1 °C) under a 12-h light: 12-h dark (light 08:00–20:00 h) cycle. Food and water were provided *ad libitum*. Animals were cared for in accordance with the principles of the *Guide for the Care and Use of Experimental Animals* (Committee on Care and Use of Laboratory Animals 1985).

Drugs in vivo administration

Rats were divided into five groups, according to the procedure followed in the object recognition test: 1) Group (R) was treated with rivastigmine (0.3 mg kg^{-1} rat day^{-1} ip) for 36 consecutive days, 2) Group (S) was treated with selegiline (0.25 mg kg^{-1} rat day^{-1} im) for the same period, 3) Group (R + S) was treated with the combination of the two drugs at the doses and way of administration mentioned before for each drug separately and for the same period of time, 4) a group was treated with equal volumes (0.5 ml) of NaCl 0.9% ip (aged control group) and 5) a group was also treated with equal volumes (0.5 ml) of NaCl 0.9% ip (adult control group) for every of the 36 consecutive days.

Tissue preparation

Animals were sacrificed by decapitation (right after the last performance test and 90 minutes after the last drug administration) and the whole brain was rapidly removed. The tissue was homogenized and centrifuged as described earlier (Tsakiris et al 2000; Antoniades et al 2002). In the resulting supernatant, the protein content was determined according to the method of Lowry and colleagues (1951) and the enzyme activities and TAS were evaluated.

Determination of enzyme activities

AChE activity was determined according to Ellman and colleagues (1961) and (Na^+, K^+)-ATPase, Mg^{2+}-ATPase activities according to Bowler and Tirri (1974). The enzyme reaction mixture and assay conditions of these enzyme activities were previously described in detail (Tsakiris et al 2000; Antoniades et al 2002).

Determination of brain total antioxidant status

TAS was evaluated in each fresh homogenized rat brain. The total antioxidant capacity was measured spectrophotometrically by a commercial kit (Randox Laboratories Ltd., Cat. No. NX2332) as previously reported (Tsakiris et al 2000). 2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) was incubated with a peroxidase (metmyoglobin) and H_2O_2 in order to produce the radical cation $ABTS^+$. The latter had a relatively stable blue-green color, which was measured at 600 nm. Inhibited values of TAS reflect the increase of brain free radical production whereas stimulated TAS values show the decrease of free radical production and the protective antioxidant effect of the drug in the brain.

Cognitive capacities tests

Cognitive capacities were evaluated using two different tasks: object recognition test (ORT) and passive avoidance conditioned response (PA). The ORT was carried out according to the procedure described by Vannucchi and colleagues (Ennaceur and Delacour 1988; Scali et al 1994; Vannucchi et al 1997). The apparatus was an open white polyvinylchloride arena (70 × 60 × 30 cm^3) illuminated by a 75 W lamp suspended 50 cm above the arena. The objects to be distinguished were made of polyvinylchloride, grey-colored and were in two different shapes: cubes (8 × 8 cm^2 side) or pyramids (8 cm height). Apparently they had no significance for the rats. For the procedure, the rat was submitted to a session of two trials, each of which had a 5-min duration. The intertribal interval (ITI) was 60 min. In the first trial (T1) two identical objects were presented in two opposite corners of the box and the amount of time spent by each animal for the object exploration was recorded. Exploration was considered

to be directing the nose at a distance <2 cm to the object and/or touching it with the nose. During the second trial (T2), one of the objects presented in T1 was replaced by a new (differently-shaped) one. To reduce place preference effects, the positions of the two different objects were randomly changed during T2 for each rat. The times spent on exploration of the familiar (F) and new (N) object during T2 were recorded separately and a discrimination index (D) was calculated (N - F/N + F). An animal was defined as impaired if the D was <0.20. This procedure took place twice for each animal. The first session was one day before the beginning of drugs administration. Among the 52 aged rats studied, 42 (81%) showed impaired performances with a D <0.10, four rats were unimpaired (D <0.50), while six rats were discarded because they did not explore. The 42 impaired aged rats were subdivided into the four aforementioned groups: Aged control group (10 rats), Group (R) (10 rats), Group (S) (11 rats), and Group (R + S) (11 rats). Among the 11 adult rats studied none was discarded, as all of them sufficiently explored. The second session was on the 34th day of the drugs administration, in order to evaluate the cognitive capacity of the animals practically at the end of the experiment. The task took place one hour after the drugs' administration.

The passive avoidance training was started 24 h after the last object recognition session. It was carried out according to the procedure described by Riekkinen and colleagues (1997) with some modifications (the testing trial took place 24 h after the training trial and not 72 h after it) and consisted of two trials. The passive avoidance box had a light and a dark compartment of equal size, which were separated by a sliding guillotine door. During the first trial (training trial), which took place at the 35th day of drugs administration, the rats were placed in the light compartment. Thirty seconds later the door was opened. After the rat entered the dark compartment, the door was closed and a foot shock of 1.0 mA (3 s) was given. The latency to enter the dark compartment was measured (360 s maximum latency). During the second trial (testing trial), which took place on the 36th day of drugs administration (last day of the experiment), the rat was placed in the light compartment again and the latency to enter the dark compartment was measured. Passive avoidance most likely involves both working memory and reference memory (Myhrer 2003).

Statistical analysis

The biochemical data were analyzed by a two-tailed Student's t-test. The object recognition data were analyzed by a non-parametric Mann-Whitney test and the passive avoidance data were analyzed by an one-way ANOVA test and a post-hoc test (Bonferroni test).

Drugs

Rivastigmine; Novartis Ltd., Basle, Switzerland. Selegiline; Sigma-Aldrich. St. Louis, MO, USA.

Results

Total antioxidant status (TAS)

Group (S) (received selegiline) showed a significant increase in TAS compared with the control group of aged rats (+30%, $P < 0.001$, $t_{value} = 9.66$). Groups (R) (received rivastigmine) and (R + S) (received rivastigmine + selegiline) did not show any difference in comparison to the aged control group ($t_{values} = 0.48$ and 1.13 respectively). TAS was significantly decreased in rivastigmine + selegiline-treated rats compared with selegiline-treated rats (-21%, $P < 0.001$, $t_{value} = 16.66$) (Figure 1).

Brain AChE activity

Selegiline-treated rats revealed a significant increase in brain AChE activity compared with the aged control rats (+25%, $P < 0.001$, $t_{value} = 10.73$). Contrary to this, rivastigmine alone and rivastigmine+selegiline co-administration induced a significant decrease in brain AChE activity in comparison to the aged saline-treated rats (–20%, $P < 0.001$, $t_{value} = 10.80$ and –22%, $P < 0.001$, $t_{value} = 11.08$, respectively). Brain AChE activity was also significantly decreased in rats treated with rivastigmine+selegiline in comparison to the rats treated with selegiline alone (-38%, $P < 0.001$, $t_{value} = 36.99$) (Figure 2).

(Na^+, K^+)-ATPase activity

(Na^+, K^+)-ATPase activity was significantly increased in rats which received selegiline (+50%, $P < 0.001$, $t_{value} = 11.08$) or rivastigmine (+36%, $P < 0.001$, $t_{value} = 8.48$) in comparison with aged saline-treated rats. Furthermore, in the case of rivastigmine+selegiline co-administration, an additive action of the two drugs concerning the increase of (Na^+, K^+)-ATPase activity was revealed in comparison to the aged control group (+88%, $P < 0.001$, $t_{value} = 15.62$). A significant increase of (Na^+, K^+)-ATPase activity in (R+S) group in comparison to the (S) group was also observed (+25%, $P < 0.001$, $t_{value} = 14.84$) (Figure 3).

Mg^{2+}-ATPase activity

Mg^{2+}-ATPase activity was significantly increased in (R) group and (R + S) group in comparison to the aged

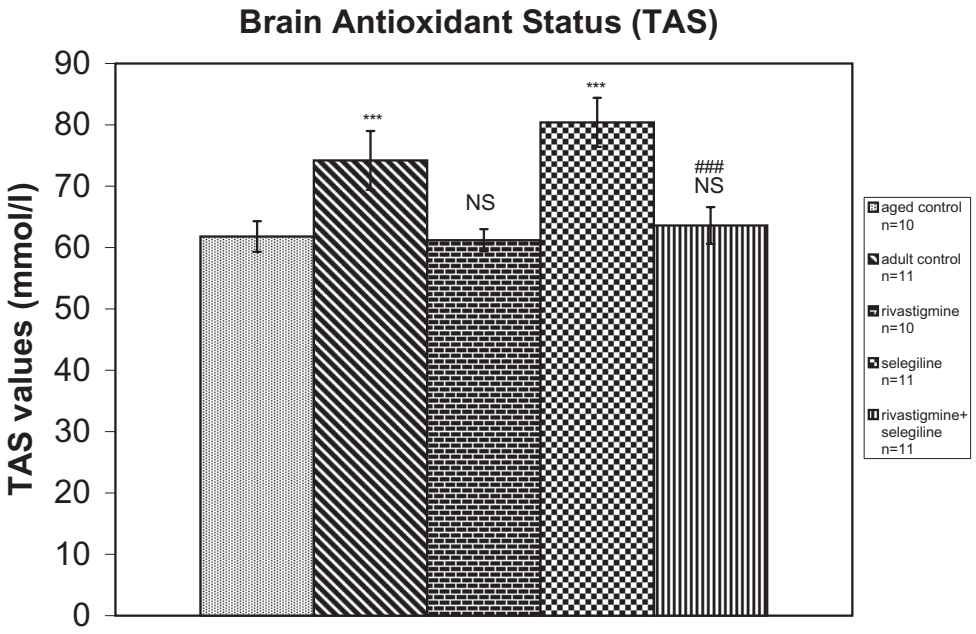

Figure 1 Effects of rivastigmine, selegiline, and rivastigmine + selegiline on brain antioxidant status (TAS). TAS values were determined in each homogenized rat whole brain. Values of the groups of aged control rats and of rivastigmine indicate the mean ± standard error (SE) of ten independent experiments (ten rats). Values of the groups of adult control rats, of selegiline, and of rivastigmine + selegiline indicate the mean ± SE. of eleven independent experiments (eleven rats). The average value of each experiment arises from three determinations.

Notes: NS, nonstatistical significance; $^{***}P < 0.001$ compared with aged control group; $^{###}P < 0.001$ compared with selegiline-treated group.

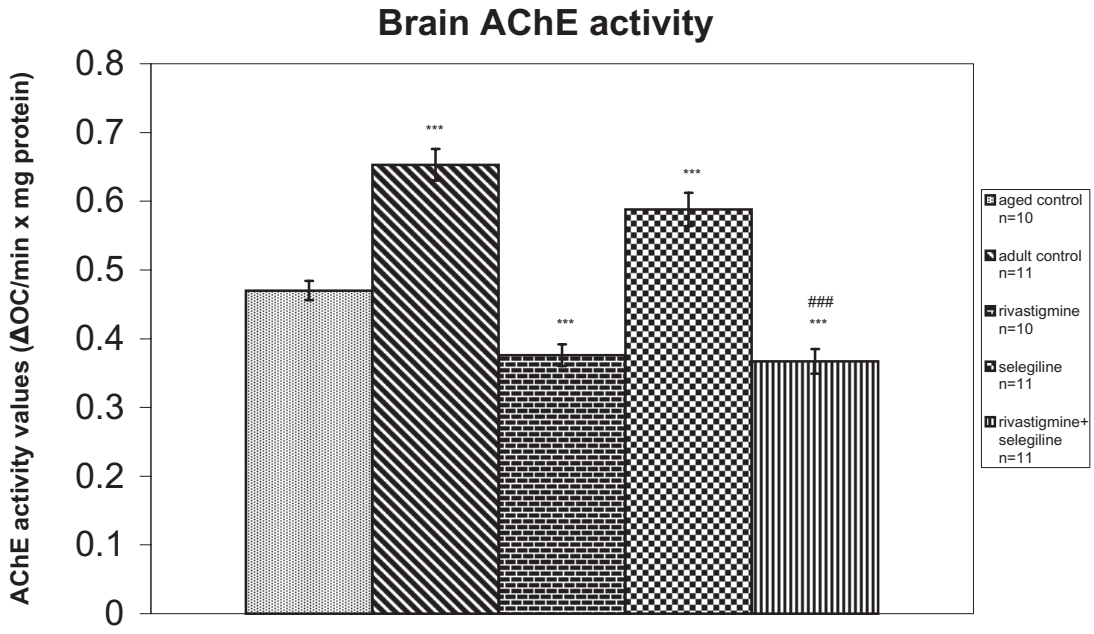

Figure 2 Effects of rivastigmine, selegiline, and rivastigmine + selegiline on brain AChE activity. AChE activities were determined in each homogenized rat whole brain. Values of the groups of aged control rats and of rivastigmine indicate the mean ± standard error (SE) of ten independent experiments (ten rats). Values of the groups of adult control rats, of selegiline and of rivastigmine + selegiline indicate the mean ± SE of eleven independent experiments (eleven rats). The average value of each experiment arises from three determinations.

Notes: $^{***}P < 0.001$ compared with aged control group; $^{###}P < 0.001$ compared with selegiline-treated group.

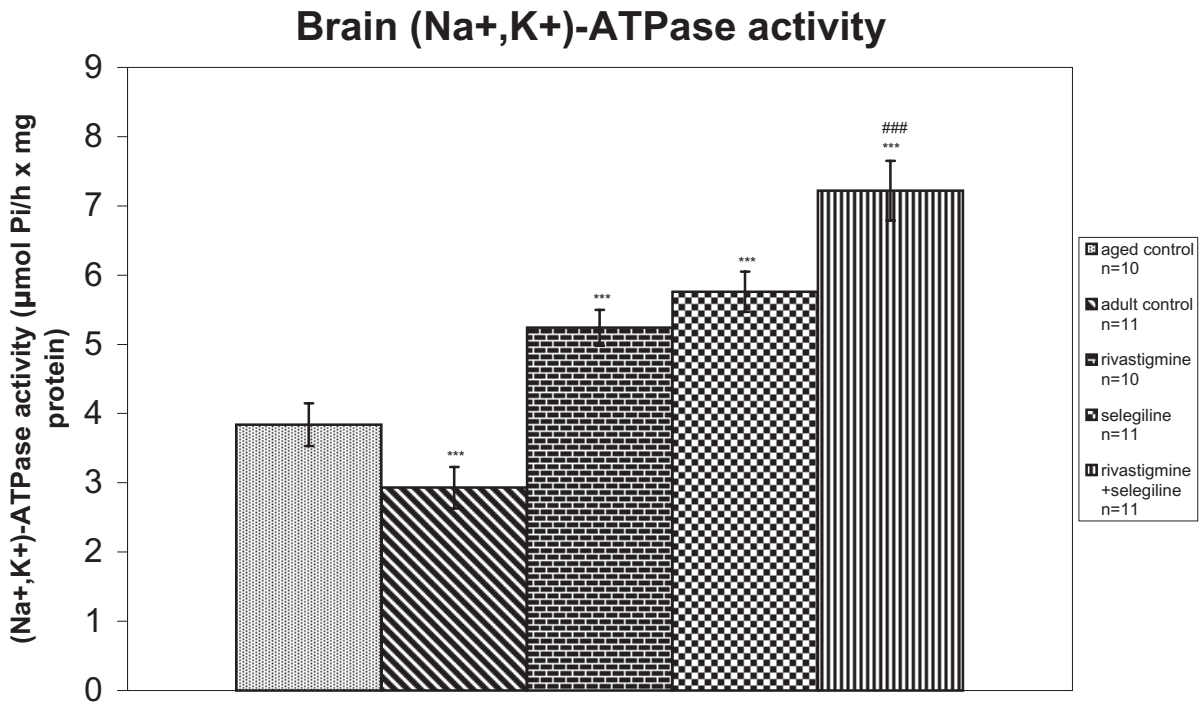

Figure 3 Effects of rivastigmine, selegiline, and (rivastigmine + selegiline) on brain (Na^+, K^+)-ATPase activity. (Na^+, K^+)-ATPase activities were determined in each homogenized rat whole brain. Values of the groups of aged control rats and of rivastigmine indicate the mean ± standard error (SE) of ten independent experiments (ten rats). Values of the groups of adult control rats, of selegiline and of rivastigmine + selegiline indicate the mean ± SE of eleven independent experiments (eleven rats). The average value of each experiment arises from three determinations.

Notes: $^{***}P < 0.001$ compared with aged control group; $^{\#\#\#}P < 0.001$ compared with selegiline-treated group.

control group (+40%, $P < 0.001$, $t_{value} = 8.78$ and +23%, $P < 0.001$, $t_{value} = 8.15$, respectively), while no difference in Mg^{2+}-ATPase activity was observed in the selegiline-treated group($t_{value} = 0.55$). The rivastigmine+selegiline co-administration induced a significant increase in Mg^{2+}-ATPase activity in comparison to selegiline alone administration (+26%, $P < 0.001$, $t_{value} = 13.45$) (Figure 4).

Object recognition test

In the object recognition test the discrimination between familiar and novel objects was significantly better in selegiline-treated ($P < 0.001$, Mann-Whitney U = 4.50) or rivastigmine-treated rats ($P < 0.001$, Mann-Whitney U = 0.50) than aged saline-treated rats. There was no statistically significant difference in the discrimination index between (R + S) group (Mann-Whitney U = 12.00) and aged control group (Figure 5).

Passive avoidance procedure

In passive avoidance procedure, during the training trial the mean latency (42 s) was not significantly different among the groups. During the testing trial, a significantly better performance was observed in groups (R)($F = 59.18$) and (S)($F = 59.18$), in comparison with the aged control group. The combination of rivastigmine + selegiline did not show any statistically significant difference from the aged control group ($F = 59.18$) (Figure 6). These results are similar to those of the object recognition test.

Adult versus aged rats

An increase of total antioxidant status (TAS) was observed in adult control group compared with the aged control group (+20%, $P < 0.001$, $t_{value} = 5.62$) (Figure 1). Adult controls revealed a significant increase in brain AChE activity compared with the aged control rats (+39%, $P < 0.001$, $t_{value} = 25.07$) (Figure 2). (Na^+, K^+)-ATPase activity was significantly decreased in adult controls compared to the aged control group (–24%, $P < 0.001$, $t_{value} = 5.17$) (Figure 3). Mg^{2+}-ATPase activity was significantly increased in adult control group in comparison with the aged control group (+34%, $P < 0.001$, $t_{value} = 10.69$) (Figure 4). In the object recognition test the adult control group performed significantly better than the aged one ($P < 0.05$, Mann-Whitney U = 9.00) (Figure 5). In passive avoidance procedure, during the testing trial, a better performance was observed in adult control group in comparison with the aged control group ($F = 59.18$) (Figure 6).

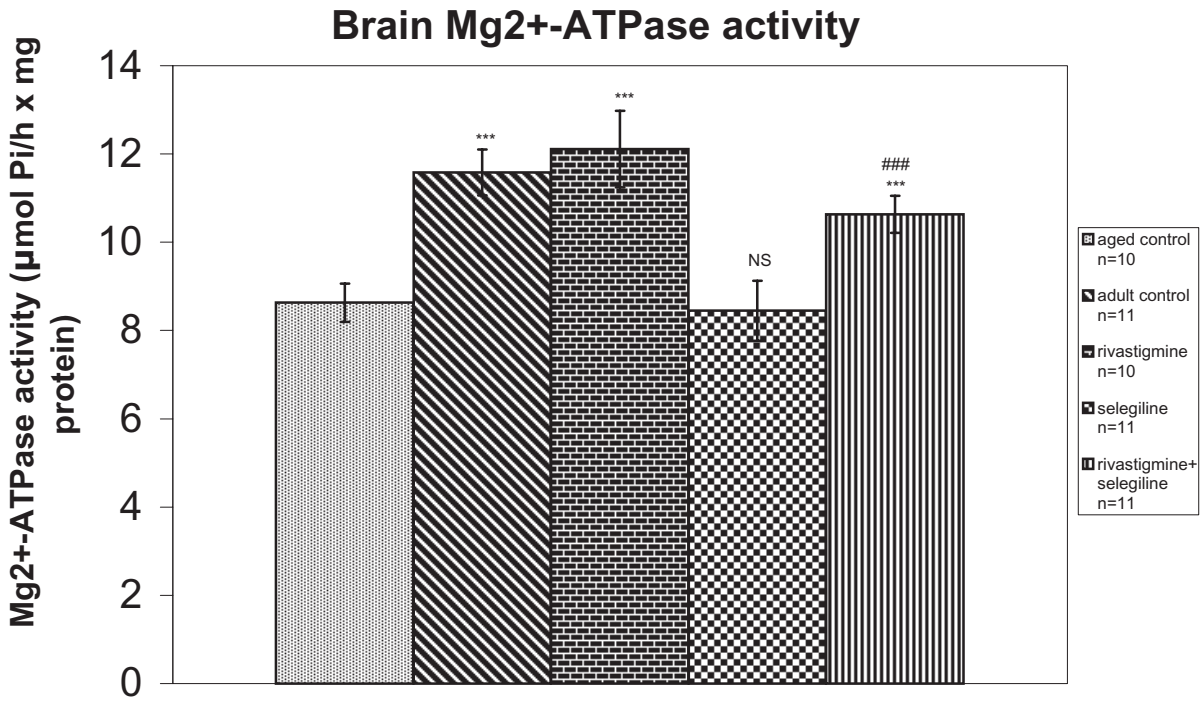

Figure 4 Effects of rivastigmine, selegiline, and (rivastigmine + selegiline) on brain Mg^{++}-ATPase activity. Mg^{++}-ATPase activities were determined in each homogenized rat whole brain. Values of the groups of aged control rats and of rivastigmine indicate the mean ± standard error (SE) of ten independent experiments (ten rats). Values of the groups of adult control rats, of selegiline and of rivastigmine + selegiline indicate the mean ± SE of eleven independent experiments (eleven rats). The average value of each experiment arises from three determinations.

Notes: NS, nonstatistical significance; $^{***}P < 0.001$ compared with aged control group; $^{\#\#\#}P < 0.001$ compared with selegiline-treated group.

Discussion

We have examined: (a) the long term effects of rivastigmine in combination with selegiline on the activity of AChE, (Na^+, K^+)-ATPase, Mg^{2+}-ATPase and TAS in whole brain homogenate of aged male rats, as well as their cognitive capacity; and (b) the possible relationship between biochemical and behavioral findings.

Rivastigmine decreased AChE activity in the aged rat whole brain as expected (Anand et al 1996; Corey-Bloom et al 1998), in comparison with the adult and aged control group (Figure 2). It is worth noticing that the adult control has the highest value of AChE activity. On the contrary, selegiline alone significantly increased AChE activity in the aged rats. The latter data are in accordance with other studies (Ricci et al 1992; Lakshmana et al 1998; Zhu et al 2000; Carageorgiou et al 2003). In particular Zhu and colleagues (2000) found increased AChE activity in the rat brain following 1 week of selegiline administration and Lakshmana and colleagues (1998) observed increased AChE activity in certain brain areas of adult monkeys following selegiline treatment with most pronounced effect in the region of hippocampus. Enhanced AChE activity in rat hippocampus was also reported by Ricci and colleagues (1992) following intracerebroventricular administration of selegiline. In the combination of rivastigmine + selegiline, the rivastigmine effect appears to prevail leading to a significant decrease in brain AChE activity compared with the (S) group and with the two control groups (Figure 2).

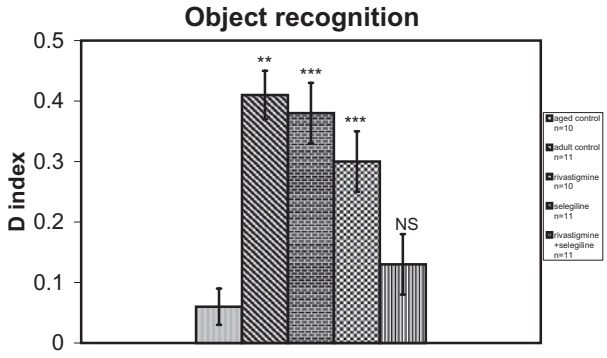

Figure 5 Effects of rivastigmine, selegiline, and rivastigmine + selegiline on object recognition task on day 34 of the administration of the drugs. Values of the groups of aged control rats and of rivastigmine indicate the mean ± standard error (SE) of ten independent experiments (ten rats). Values of the groups of adult control rats, of selegiline and of rivastigmine + selegiline indicate the mean ± (SE) of eleven independent experiments (eleven rats).

Notes: NS, nonstatistical significance; $^{**}P < 0.05$; $^{***}P < 0.001$ compared with aged control group.

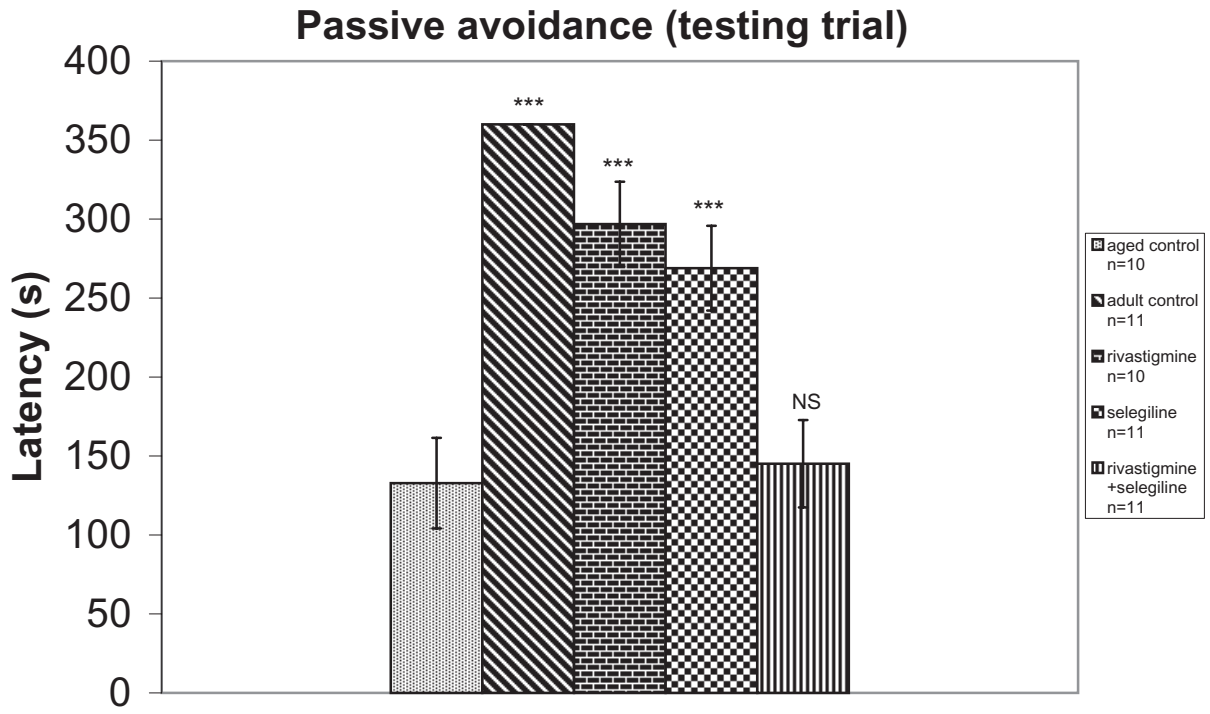

Figure 6 Effects of rivastigmine, selegiline, and rivastigmine + selegiline on passive avoidance test in testing trial (day 36 of the administration of the drugs). Values of the groups of aged control rats and of rivastigmine indicate the mean ± standard error (SE) of ten independent experiments (ten rats). Values of the groups of adult control rats, of selegiline, and of rivastigmine + selegiline indicate the mean ± SE of eleven independent experiments (eleven rats).
Notes: NS, nonstatistical significance; ***$P < 0.001$ compared with aged control group.

Reduced activity of AChE and ChAT has been reported in the cortex and the hippocampus of AD patients (Fishman et al 1986) and is also associated with the decline in cognitive function (DeKosky et al 1992). In our study, brain AChE activity was decreased in aged rats, in parallel with the object recognition and passive avoidance performance (Figure 2, 5–6). On the contrary, while rivastigmine (as expected) decreases AChE activity, it improves cognitive performance. Here a question could arise as to whether the 20% inhibition of AChE by rivastigmine is enough to produce an increase in cortical and hippocampal ACh release. Many studies in the literature using different doses of rivastigmine revealed a strong correlation between AChE inhibition and ACh increase in the aforementioned rat brain areas (Tanaka et al 1994; Chen et al 1998; Kozaka 1999; Trabace et al 2000; Scali et al 2002; Amenta et al 2006; Liang and Tang 2006). Considering the improved performance in learning and memory tests by rivastigmine group of rats, we suggest that this percentage reduction of AChE was enough for the needed cortical and hippocampal ACh release. Also, while selegiline increases AChE activity, it improves cognitive performance. In the combination of rivastigmine + selegiline treatment no improved performance was observed despite decreased AChE activity. Concerning AChE activity, it is obvious that the mechanism by which a better performance is attained in the behavioral tests following administration of rivastigmine or selegiline (given separately) is different. Rivastigmine is used in AD according to the cholinergic hypothesis, which improves or delays to some extent the deterioration of AD patients (Anand et al 1996; Corey-Bloom et al 1998; Bartus 2000). At the same time selegiline was found to improve cognitive function after long-term administration in rats (Knoll 2000) and to slow the progression of AD in man (Sano et al 1997). In our past experiments, although selegiline increased AChE activity, it improved avoidance performance (Carageorgiou et al 2003). The present study also shows improvement in both learning parameters. One can only speculate that the increased expression of ChAT and AChE in the hippocampus by selegiline (Ricci et al 1992) may have also caused an increase of ACh (which is mainly involved in learning and memory) provided that ACh release is not affected by selegiline in the hippocampus (Knoll 1989; Nowakowska et al 2001; de Lima et al 2005). Furthermore, it has been reported that increases in dopamine levels enhance a compensatory release of acetylcholine in the frontal cortex (Nilsson et al 1992; Shimazu et al 1996) and that the forebrain dopaminergic system is related to cognitive function (Marie and Defer 2003; Remy and Samson

2003). According to Koutsilieri and colleagues (2001) selegiline (given in a dose of 2 mg/kg) completely restores ChAT activity deficits in simian immunodeficiency infection in brain regions containing cholinergic neurons. It is unclear whether selegiline acted on the expression of ChAT (directly or through increased dopamine availability) thereby increasing protein synthesis or as a neuroprotective agent on cholinergic and other neurons through its antioxidant effects (Kitani et al 2002). In addition, Appleyard (1995) reported that AChE induced long-term potentiation in hippocampal pyramidal neurons, suggesting that AChE per se might enhance cognitive performance. We could refer to Shen´s (1994, 2004) hypotheses on the design of agents that could enhance the neuronal AChE activity in order to delay the degeneration of brain AChE system in the development of dementia and AD. Furthermore, Frolich (2002) has come to a similar conclusion concerning the cholinergic hypothesis in its present form and the use of AChE-inhibitors. According to Kaduszkiewicz and colleagues (2005), the scientific basis for recommendations of cholinesterase inhibitors for the treatment of Alzheimer´s disease is questionable because of flawed methods and small clinical benefits; even though the AChE inhibitors and memantine is the only available drug treatment until now (Lane 2006; Birks 2006).

In any case, one cannot exclude the neuronal adaptations to diminished synaptic ACh metabolism in acetylcholinesterase knockout mice (Volpicelli-Daley et al 2003) and the possibility of AChE involvement of the senile plaque (Rees and Brimijoin 2003; Castro and Martinez 2006).

The synaptic plasma membrane enzyme (Na^+, K^+)-ATPase is very important for neurotransmission-neuronal excitability (Sastry and Philips 1977), metabolic energy production (Mata et al 1980), the uptake and release of catecholamines (Bogdanski et al 1968; Swann 1984), serotonin (Hernandez 1987), glutamate (Lees et al 1990), and at least partial ACh release (Meyer and Cooper 1981). Studies suggest that (Na^+, K^+)-ATPase might play a role on memory formation (dos Reis-Lunardelli et al 2007) and that it is a particular age-related enzyme (Gorini et al 2002).

Increased activity of whole brain (Na^+, K^+)-ATPase was observed after the administration of rivastigmine, selegiline, or their combination (Figure 3). In the latter, the effect seems to be additive. Similar results concerning increased enzyme activity in aged and selegiline-treated rats were observed in our previous studies (Tsakiris et al 1996; Carageorgiou et al 2003).

Dickey and colleagues (2005) reported a decreased overall (Na^+, K^+)-ATPase enzyme activity in the amyloid containing hippocampi of the APP + PSI mice. They also reported absence of (Na^+, K^+)-ATPase staining in the zone surrounding congophilic plaques, which was occupied by dystrophic neurites and that cerebral (Na^+, K^+)-ATPase can be directly inhibited by high concentrations of soluble Ab. It has been also reported that (Na^+, K^+)-ATPase protein levels are decreased in AD tissue but not in normal aged tissue (Harik et al 1989; Liguri et al 1990). Our study deals with normally aged rats in which (Na^+, K^+)-ATPase activity was actually and significantly increased in comparison with the adult control, but their performance was decreased. Considering that ouabain, a (Na^+, K^+)-ATPase inhibitor, has been shown to impair memory consolidation, it was suggested that increasing (Na^+, K^+)-ATPase activity and maintaining ionic balance of the neurons may benefit AD patients by delaying the onset of neuritic dystrophia and memory dysfunction (Watts and Mark 1971; Mark and Watts 1971).

In our study with aged rats, in spite of enhanced (Na^+, K^+)-ATPase activity in comparison with the adult control, the aged animals had an impaired learning performance. In addition, in the (S) and (R) groups (with enhanced (Na^+, K^+)-ATPase activity) a better learning performance was observed, while in the combination group (in which an even higher increase of (Na^+, K^+)-ATPase activity was observed) no statistically significant difference in its learning performance was noticed (Figures 3, 5–6). According to all the above, we come to the conclusion that increased (Na^+, K^+)-ATPase activity is not relevant to the cognition enhancement by rivastigmine, selegiline, or their combination, at least not under our experimental conditions.

The role of Mg^{2+}-ATPase is to maintain high brain intracellular Mg^{2+}, the changes of which can control rates of protein synthesis and growth of the cell (Sanui and Rubin 1982). Decreased Mg^{2+}-ATPase activity in the frontal cortex of old age rats was reported by Gorini and colleagues (2002). In our experiments, decreased Mg^{2+}-ATPase activity was observed in old rats in parallel with decreased learning performance. Mg^{2+}-ATPase activity was found to be higher in the groups (R), (R+S) and young controls (Figure 4). Consequently, a positive association between increased Mg^{2+}-ATPase activity and learning performance could be supported for rivastigmine, while selegiline improves learning performance without affecting brain Mg^{2+}-ATPase activity. In the combination, although there was an increased Mg^{2+}-ATPase activity, no significant improvement in the behavioral parameters was observed. It is likely that the addition of selegiline in some way reduced the effect of rivastigmine alone on Mg^{2+}-ATPase activity and this could influence the better performance of rivastigmine alone. This association is questionable as well.

The observed decrease of brain TAS during aging is in accordance with our previous studies (Tsakiris et al 1996; Carageorgiou et al 2003) and is associated with the decreased learning performance (object recognition and passive avoidance test) (Figures 1, 5–6). Although rivastigmine administration in aged rats did not affect TAS, it resulted in a better learning performance. On the contrary, selegiline administration (as previously shown by Carageorgiou and colleagues [2003]) increased TAS and this effect can be linked with better learning performance (Figures 1, 5–6). Similar results were also reported by Kiray and colleagues (2006): increase of spatial memory performance in aged male rats after selegiline administration for 21 days, suppression of lipid peroxidation, and alleviation of the age-related decrease of the number of neurons in the hippocampus. Improved performance after long term selegiline administration has also been observed by Knoll (1989) and other investigators (Nowakowska et al 2001; de Lima et al 2005). In the combination of rivastigmine + selegiline no statistically significant differences were observed either in TAS or in learning performance although there is a tendency in the object recognition test (Figures 1, 5). It is rather obvious that selegiline effect on TAS is blunted by rivastigmine (Figures 1, 5–6). Selegiline is a MAO-B inhibitor enhancing dopamine levels and after its chronic administration long-term postsynaptic changes have most likely occurred. The dopaminergic activity for example could be inhibitory to cholinergic striatal inter neurons. This probably explains the negation of the behavioral effects of rivastigmine by selegiline. In the study of Sagi and colleagues (2005) the chronic treatment of rats with ladostigil, a novel drug derived from the combination of rivastigmine and a MAO-B inhibitor rasagiline, resulted in activation of both striatal cholinergic and dopaminergic activity, attenuation of stereotyped motor behavior and maintenance of normal spontaneous motor performance. However, Takahata and colleagues (2005) (donepezil and selegiline in acute and high dose in scopolamine + chlorophenylalanine – induced memory deficits) and Dringenberg and colleagues (2000) (tacrine and selegiline with electroencephalographic and behavioral evidence) who used totally different experimental protocols observed that the combination of selegiline and another AChE inhibitor (donepezil or tacrine) acted synergistically and improved reversal of memory impairment in rats.

Conclusions

The overall analysis of our data revealed that rivastigmine when given alone decreases AChE, does not influence TAS, increases (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activities, and improves learning performance of the aged rats. In the combination the effect of rivastigmine on AChE activity (reduced) appears to prevail that of selegiline (increased) and the result is reduced activity of AChE. In the case of TAS, although rivastigmine when given alone, did not affect TAS, in the combination decreases the enhanced by selegiline old rat brain TAS and increases Mg^{2+}-ATPase activity. There is also an improved learning performance by each drug alone, but not in the combination. It is obvious that the better performance of rivastigmine and selegiline given separately in the object recognition and in the passive avoidance test is attributed to a different mechanism of action: Selegiline possibly acts through its antioxidant effect and increased levels of catecholamines and rivastigmine by its anticholinesterase activity and increased levels of acetylcholine. Finally, the combination of the two drugs does not appear to be beneficial for the declining memory of aged rats at least not under our experimental conditions. Reduced Mg^{2+}-ATPase activity is correlated with old age and reduced learning performance. Rivastigmine is correlated with increased Mg^{2+}-ATPase activity and increased learning performance. Decreased TAS is correlated with old age and in parallel with decreased performance. Selegiline is correlated with increased TAS and increased performance. In the combination, rivastigmine+selegiline did not affect either TAS or learning performance. Several transmitter systems can probably have a primary function in some cognitive processes and among which are the cholinergic and dopaminergic ones, but the extent of interactions is difficult to be elucidated. The subject therefore requires further investigation.

To our knowledge this is the first report about a) the effect of rivastigmine on (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activities, TAS, on rat whole brain and b) the combined administration of rivastigmine and selegiline and its effect on all the studied parameters.

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