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Deprenyl and the Relationship Between Its Effects on Spatial Memory, Oxidant Stress and Hippocampal Neurons in Aged Male Rats

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Summary

Oxidative stress may play a major role in the aging process and associated cognitive decline. Therefore, antioxidant treatment may alleviate age-related impairment in spatial memory. Cognitive impairment could also involve the age-related morphological alterations of the hippocampal formation. The aim of this study was to examine the relationship between the effects of deprenyl, an irreversible monoamine-oxidase B inhibitor, on spatial memory by oxidant stress and on the total number of neurons in the hippocampus CA1 region of aged male rats. In this study, 24-month-old male rats were used. Rats were divided into control and experimental groups which received an injection of deprenyl for 21 days. Learning experiments were performed for six days in the Morris water maze. Spatial learning was significantly better in deprenyl-treated rats compared to saline-treated rats. Deprenyl treatment elicited a significant decrease of lipid peroxidation in the prefrontal cortex, striatum and hippocampus regions and a significant increase of glutathione peroxidase activity in the prefrontal cortex and hippocampus. It was observed that deprenyl had no effect on superoxide dismutase activity. The total number of neurons in the hippocampus CA1 region was significantly higher in the deprenyl group than in the control group. In conclusion, we demonstrated that deprenyl increases spatial memory performance in aged male rats and this increase may be related to suppression of lipid peroxidation and alleviation of the age-related decrease of the number of neurons in the hippocampus. The results of such studies may be useful in pharmacological alleviation of the aging process.

Key words

Deprenyl • Aging • Spatial memory • Lipid peroxidation • Antioxidant enzymes • Hippocampus

Introduction

Reactive oxygen species (ROS) are produced within the body during oxygen metabolism and living organisms have developed several defense mechanisms to protect themselves from oxidative stress (Wickens 2001). Under normal conditions, ROS and antioxidant systems are in balance. If any imbalance occurs between pro-

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oxidant and antioxidant factors, it is called oxidative stress (Yu 1999, Semsei 2000, Floyd and Hensley 2002). The defense mechanisms include antioxidant enzymes like superoxide dismutase (SOD) or glutathione peroxidase (GPx) and several nonenzymatic free radical scavengers (Ashok and Ali 1999, Wickens 2001). It has been proposed that the progressive increase in ROS and consequent oxidative damage play the major role in aging and age-related degenerative disorders (Harman 1993, Barja 2004).

Learning and memory show an age-related decline and this age-associated impairment extends to spatial memory tasks. It has been indicated that the age-related decline in spatial memory is due to functional and morphological changes of the hippocampal formation (Wyss *et al.* 2000, Geinisman *et al.* 2004). Furthermore, the neural circuits between the prefrontal cortex and striatum are also involved in spatial memory (Floresco *et al.* 1997). Since the oxidative damage may play a role in the aging process, including an associated cognitive decline, age-related impairment in spatial learning and memory may be alleviated by antioxidant treatment (D'Hooge and De Deyn 2001).

Cognitive impairment in aging could involve the age-related functional and morphological alterations of hippocampal formation (Driscoll *et al.* 2003, Miller and O'Callaghan 2003). In the previous studies it was reported that the neuron count and number of synaptic connections were decreased in the hippocampus with aging (Uylings and De Brabander 2002). According to these findings, neuronal loss seems to be an inevitable consequence of aging and may cause age-related cognitive impairment.

Deprenyl (selegiline) is an irreversible monoamine-oxidase B (MAO-B) inhibitor which has antioxidant and neuroprotective effects (Kitani *et al.* 1999, 2002a, Maruyama and Naoi 1999, Thomas 2000). Deprenyl is used for treatment of Parkinson's disease because of these effects and can attenuate the progressive degeneration of nigro-striatal dopaminergic neurons during aging and neurodegenerative disorders (Foley *et al.* 2000, Kitani *et al.* 2002b, Maruyama *et al.* 2000). Deprenyl may also improve age-related cognitive deficits in aged rats (Bickford *et al.* 1997). In our previous study, we have reported that deprenyl has a positive effect on spatial memory in aged female rats and this effect may be not only due to its antioxidant properties (Kiray *et al.* 2004). In this study, we aimed to examine the relationship between the effects of deprenyl on spatial memory by oxidant stress and the total neuron number of hippocampus CA1 region in normal aged male rats. We have determined parameters associated with oxidative stress, SOD and GPx enzyme activities in the prefrontal cortex, striatum and hippocampus regions of aged male rats that form interconnected neural circuits for spatial memory, and cellular damage as indicated by lipid peroxidation.

Methods

Animals

This study was performed with the approval of the local ethical committee. In this study, 24 months old Wistar male rats were used. The animals were maintained under standard colony conditions with a 12 h light/dark cycle and *ad libitum* food and water throughout the experiments. Rats were divided into two groups; 1) control group (n=14) was injected sc with physiological saline and 2) deprenyl group (n=14) received an injection sc of deprenyl (Sigma-Aldrich), 1 mg/kg/day for 21 days. During the last six days, learning experiments were performed in a Morris water maze. Twenty-four hours after the last injection, half of the rats in each group were sacrificed by cervical dislocation and used for estimation of oxidant stress parameters, and the other half was perfused with saline followed by 10 % formalin under ether anesthesia and used for histological evaluation.

Morris water maze testing

All rats were tested for six days in the Morris water maze. The maze was 2 m in diameter and 75 cm high. The water level in the maze was 50 cm, which was 1.5 cm above the height of the escape platform. The pool was filled with opaque water to prevent visibility of the platform in the pool. The escape platform was placed in the middle of one of the random quadrants of the pool and was kept in a constant position throughout the experiments (north-east for this study). On each test day, rats were placed in the water (22±1˚C) and trained until they found the hidden platform within 120 s, using extra maze cues. On successive days the start position was randomly altered (south, east, north, west and south, respectively). The swimming was monitored by a video camera, which was positioned directly above the center of the pool. If the animal failed to locate the platform within 120 s, the experimenter placed the rat on the platform and left it there for 30 s. Each rat was tested for four swimming trials daily for 5 consecutive days, with an

inter-trial interval of 60 s (a total of 20 trials). On the sixth day, a probe trial was run in the pool from which the platform was removed and rats were placed into the pool and swam for 60 s. Time in seconds spent in the correct quadrant was recorded. The data were analyzed for latency to find the platform and time spent in the correct quadrant.

Biochemical estimations

Twenty-four hours after the last injection, the rats in half of each group (n=14) were sacrificed by cervical dislocation under ether anesthesia. Hippocampus, prefrontal cortex and striatum tissues were dissected on an ice-cold surface. Tissue homogenates were prepared as described by Carrillo *et al.* (1991). An aliquot of the homogenate and supernatant was stored at –70 ˚C until thiobarbituric acid reactive substances (TBARS) levels, an indicator of lipid peroxidation, and SOD and GPx enzyme activities were determined. Determination of TBARS levels and antioxidant enzyme activities were performed spectrophotometrically.

Determination of TBARS was performed in homogenate according to the method of Rehncrona *et al.* (1980) and expressed as nmol/mg protein. SOD and GPx activities were measured in the supernatant by using RANSOD and RANSEL kits (Randox Labs., Crumlin, UK). The results were expressed as units/mg protein. The protein contents of supernatant and homogenates were determined using a total protein kit (Randox Labs., Crumlin, UK).

Histological evaluation

Twenty-four hours after the last injection half of the rats in each group were anesthetized *via* inhalation of ether. All rats were perfused intracardially by using isotonic sodium chloride followed by 10 % formaldehyde solution. Following the perfusion process, brains were removed and stored in the same solution for 6 days. Brain tissues were processed by routine histological methods and embedded in paraffin blocks. Paraffin blocks were placed in Leica/Reichert-Jung rotary microtome (Köln, Germany) and serial coronal sections of 6 μm thickness were obtained. The boundary of hippocampus was defined in accordance with the atlas of Paxinos and Watson (1986). Pyramidal cells of the CA1 region were counted by the physical disector method and the volumes of hippocampi were estimated by Cavalieri's principle (Gundersen 1986, Schmitz and Hof 2000). The reference and look-up sections required for an unbiased estimation of total neuron numbers from hippocampi were obtained by choosing every eleventh section systematically with a random start within hippocampal sections (systematic random sampling). All sections taken from the right and left hippocampus were stored for 24 h at 60 °C and stained with Cresyl violet staining (Fluka Chemika). After the staining process had been completed, the preparations were examined under a light microscope (Olympus BH-2 Tokyo, Japan) and images transferred to computer using a high-resolution camera (JVC TK-890E, Japan) and Aver TV Studio (Version 4.21.0.0 [Software] Aver Media Technologies, Inc.) and Video Capture [Software]. All sections were digitally photographed and the number of CA1 neurons was counted using a 400 μm^2 counting frame viewed through x40 Nikon lens at the monitor. The counting frame was placed on the image analyzer system monitor and the number of CA1 neurons was counted. All counting and measurement procedures were performed in a blind manner.

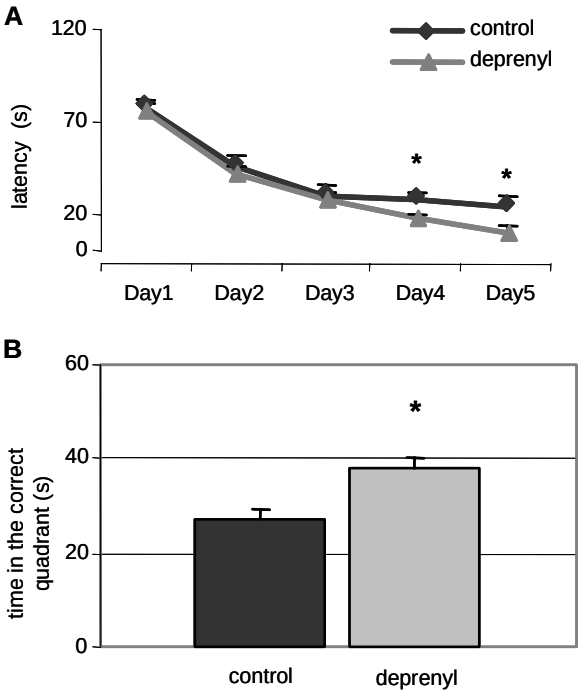

Fig. 1. Effects of deprenyl treatment on spatial learning in the Morris water maze (A) and time spent in the correct quadrant in the probe trial (B). Data are presented as the mean ± SEM. * $p<0.05$ compared with the control group.

The mean number of neurons (N_v) per μm^3 in CA1 area (1) and total number (N) of CA1 neurons (2) were estimated using the following formulas (West and Gundersen 1990, Schmitz and Hof 2000):

$$N_v = \frac{\sum Q}{t. \sum a} \quad (1)$$

$$N = V_{ref} \times N_v \quad (2)$$

where "$\sum$ Q" is the total number of counted particles appeared only in the reference sections, "t" is the section thickness, "a" is the area of counting frame and "V_{ref}" is the volume of the hippocampus region.

Statistical analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed by Mann-Whitney U test. Correlation between spatial memory, oxidant stress markers and the number of CA1 neurons was calculated using Spearman correlation analysis. $P<0.05$ value was considered to be statistically significant.

Results

The effects of deprenyl on latency times in the Morris water maze are presented in Fig. 1A. The latency to reach the platform declined progressively throughout five days in all animals of control and deprenyl groups. The deprenyl-treated rats had significantly shorter latencies on the fourth and fifth day of training trials as compared with saline-treated rats. In the probe trial, the deprenyl group spent a significantly longer time in the correct quadrant than the control group (Fig. 1B). These results indicated that spatial learning was significantly better in deprenyl-treated rats as compared to saline-treated rats (38.28±1.96 s and 26.71±2.46 s, respectively).

Table 1. Total numbers of CA1 neurons in aged male rats.

Group	n	Number of cells ($x10^6$)	CV	Mean CE
Control	5	0.40033 ± 0.03970	0.22	0.048
Deprenyl	5	0.60232 ± 0.04957***** p=0.032	0.18	0.041

*Significantly different from control rats ($p<0.05$). Data are presented as the mean ± S.E.M. n: the number of subjects, CV: the coefficient of variance, Mean CE: mean coefficient of error.

Table 1 shows the total number of hippocampal neurons in the CA1 regions of deprenyl and saline-treated rats. After comparing the estimated number of neurons in the control and deprenyl groups, a significant difference was observed. The total neuron numbers of hippocampus in the deprenyl group were significantly higher than in the control group. Volume estimations were done on the same sections in each group. There was no significant group difference between volumes of the hippocampi. Figures 4A and 4B represent photomicrographs of 6-µm thick coronal sections of the hippocampus of control and deprenyl groups, respectively. We assessed the correlation between the mean latency of probe trials and the total number of neurons in the hippocampus and found a significant correlation ($p=0.037$, $r=0.900$). The scattergram of correlated values is presented in Figure 5A.

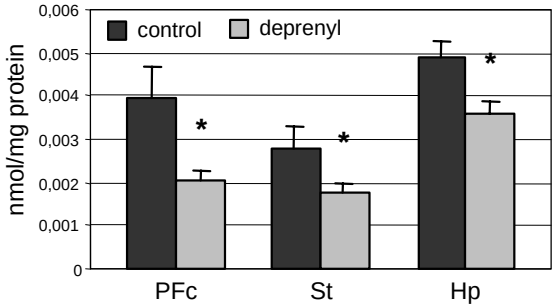

Fig. 2. Effects of deprenyl treatment on TBARS levels in the rat brains. PFc, prefrontal cortex; St, striatum and Hp, hippocampus. Data are presented as the mean ± S.E.M. *p<0.05 compared with the control group.

Figure 2 represents the TBARS levels in control and deprenyl groups. Deprenyl treatment resulted in a significant decrease of lipid peroxidation in all three brain regions. We assessed the correlation between lipid peroxidation and spatial memory performance and found a significant correlation ($p=0.033$, $r=0.793$). The scattergram of the correlated values is presented in Figure 5B.

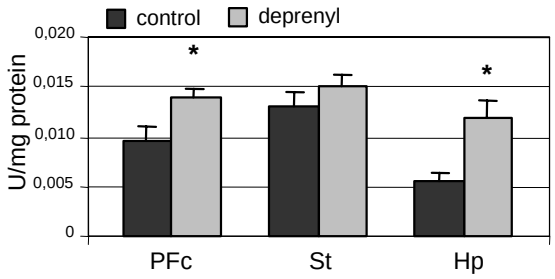

Fig. 3. Effects of deprenyl treatment on GPx activity in rat brains. PFc, prefrontal cortex; St, striatum; Hp, hippocampus. Data are presented as mean ± S.E.M. *p<0.05 compared with the control group.

Deprenyl treatment caused a significant increase in the prefrontal cortex and hippocampus GPx activity (Fig. 3). We found no correlation between spatial memory performance and GPx activity. No effect of deprenyl and no significant changes in the SOD activity were observed (data not shown).

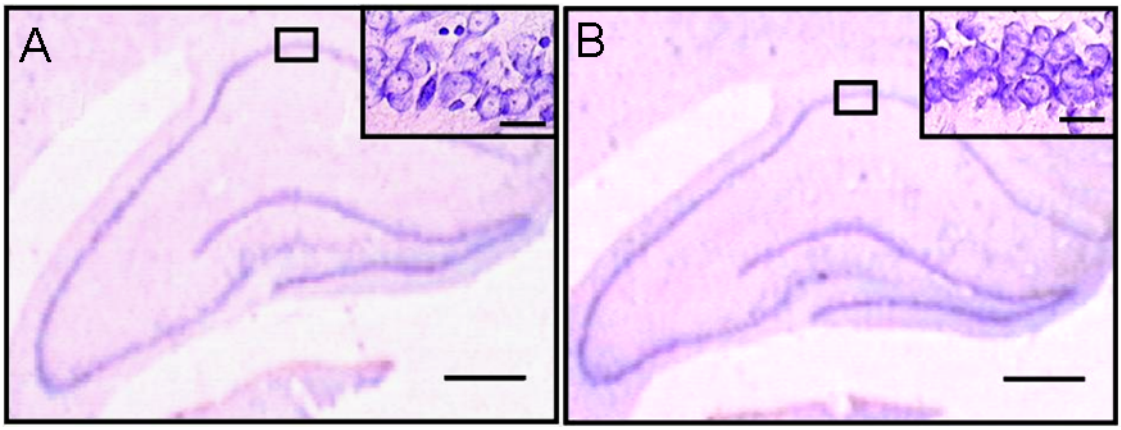

Fig. 4. Representative photomicrographs of 6 µm thick coronal sections of the hippocampus of aged male rats. **A** and **B** show the sections of control and deprenyl-treated groups, respectively (scale bar = 500 µm). The insets show photomicrographs of the CA1 pyramidal cell layers representative of the magnification at which the stereological estimates were made (scale bar = 15 µm). The small rectangles indicate the positions at which the inset photomicrographs were taken.

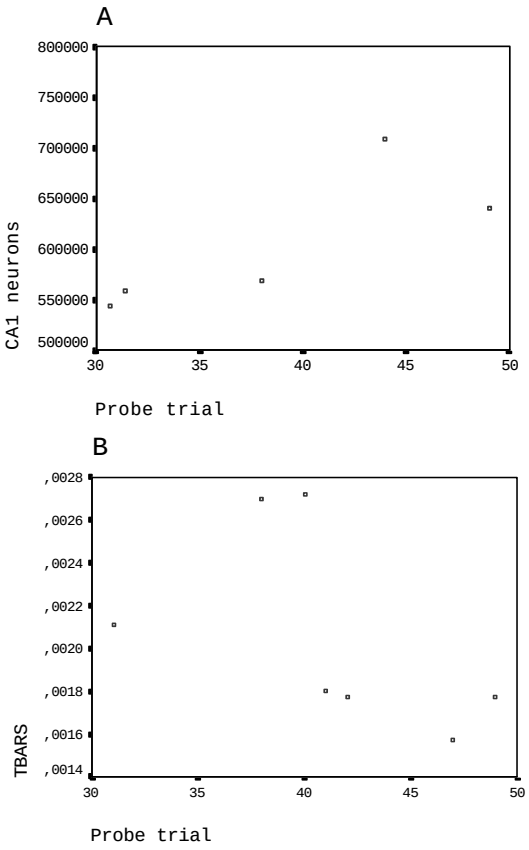

Fig. 5. The scattergrams of probe trials. P-significance for CA1 neurons (A) and TBARS (B), $p<0.05$.

Discussion

The present study examines the effects of chronic deprenyl administration at a dose of 1 mg/kg/day on spatial memory, oxidant stress markers and total neuron count of hippocampus CA1 region of aged male rats. Age-related deficits in learning and memory are associated with hippocampal structural and biochemical changes. Age-related structural alterations in the hippocampus suggest that the hippocampal formation may be especially vulnerable to the effects of aging (D'Hooge and De Deyn 2001, Geinisman *et al.* 2004). Hippocampal volume reductions and a significant neuron loss in the CA1 area and dentate granular neurons have been reported (Uylings and De Brabander 2002, Driscoll *et al.* 2003). There is little knowledge about effects of deprenyl on age-related microanatomical changes in hippocampal neurons. It has been reported that the number of neurons in the brain decreases with aging and deprenyl treatment can counter this alteration (Amenta *et al.* 1994). In this study, we showed the total number of neurons in the CA1 hippocampus region was significantly higher in deprenyl-treated rats. The correlation between spatial memory performance and total number of CA1 neurons was significant. This result indicates that the effect of deprenyl on spatial memory may be related to the prevention of age-dependent neuronal loss. Although apoptosis may play a role in the

normal aging process, both apoptosis and aging were affected by oxidant stress (Zhang and Herman 2002). As deprenyl has antioxidant and anti-apoptotic effects, it may decrease or delay the age-related neuronal death.

Spatial learning and memory declines progressively during aging. Both aged humans and laboratory animals show spatial memory impairments as compared with their younger counterparts (Muir *et al.* 1999, Erickson and Barnes 2003). The hippocampus and other structures, which are critical for spatial memory, exhibit changes in their functional and morphological organization during aging (D'Hooge and De Deyn 2001). The reason for selecting the hippocampus, prefrontal cortex and striatum regions for this study was their forming interconnected neural circuits for spatial memory (Floresco *et al.* 1997, Seamans *et al.* 1998). In this study we demonstrated that spatial memory was better in deprenyl-treated aged rats. In some studies done before, it has been suggested that deprenyl may improve age-related cognitive deficits and increase spatial memory performance in aged rats as in previous studies (Bickford *et al.* 1997, Kiray *et al.* 2004). It is not clear how deprenyl can cause this cognitive enhancing effect. It may be due to several mechanisms including induction of neurotrophins or antioxidant enzymes. Apoptosis is suggested to be considered in normal aging and neurodegenerative disorders (Zhang and Herman 2002). The anti-apoptotic activity of deprenyl may also be responsible for this effect.

The brain tissue is highly vulnerable to oxidative stress because of its oxidative damage potential (Leutner *et al.* 2001, Floyd and Hensley 2002). ROS can react with polyunsaturated fatty acids to form lipid peroxides and the accumulation of end-products of lipid peroxidation with age may contribute to the aging process (Wickens 2001, Montine *et al.* 2002). Lipid peroxidation is one of the determinants of ROS inducing oxidative damage. It has been demonstrated that lipid peroxidation increases with aging (Inal *et al.* 2001, Kasapoglu and Ozben 2001, Leutner *et al.* 2001). Our study showed that deprenyl treatment significantly decreased lipid peroxidation in all of three brain regions. In previous studies it has been demonstrated that deprenyl can decrease lipid peroxidation in the whole brain or in some brain regions (Alper *et al.* 1999, Kaur *et al.* 2003).

The organism has some protective antioxidant defense enzymes and repair systems against oxidative damage. This defense systems include enzymes of SOD and GPx. The activities of these enzymes increase in oxidative stress conditions (Ashok and Ali 1999, Melov 2002, Wickens 2001). It has been suggested that the activities of antioxidant enzymes decline with age and this mechanism may contribute to the aging process (Wickens 2001). In our study we demonstrated a significant increase in prefrontal cortex and hippocampus GPx activities. We showed that deprenyl did not affect SOD activity. In our previous study, we demonstrated the effect of deprenyl on SOD activity in aged female rats (Kiray *et al.* 2004). The results indicated that the effects of deprenyl on antioxidant enzymes may vary according to the sex of animals. In contrast to our study Carrillo *et al.* (1991) reported an increase in SOD activity by deprenyl treatment, but no effect on GPx activity. Bhattacharya and Kumar (2000) reported an increase in GPx activity which is in accordance with our findings. Our data showed that deprenyl suppressed the age-related elevation in lipid peroxidation in three brain regions. In this study, the reason for the increase in GPx enzyme activities of prefrontal cortex and hippocampus may be related to high age-sensitivity of these regions (Kaur *et al.* 2003).

In conclusion, we have demonstrated that deprenyl increases spatial memory performance in aged male rats and this effect may be related to the decrease in lipid peroxidation with deprenyl treatment and can prevent age-related neuronal loss. We have provided support for parameters that examine age-related impairment in spatial memory and regional brain differences caused by aging. The results of such studies will be useful for pharmacological modification of the aging process and development of new drugs.

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