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ORIGINAL PAPER

Selegiline Reverses $A\beta_{25–35}$-Induced Cognitive Deficit in Male Mice

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Abstract Alzheimer's disease (AD) is biochemically characterized by the occurrence of extracellular deposits of amyloid beta peptide (Aβ) and intracellular deposits of the hyperphosphorylated tau protein, which are causally related to the pathological hallmarks senile plaques and neurofibrillary tangles. Monoamine oxidase B (MAO-B) activity, involved in the oxidation of biogenic monoamines, is particularly high around the senile plaques and increased in AD patients in middle to late clinical stages of the disease. Selegiline is a selective and irreversible MAO-B inhibitor and, although clinical trials already shown the beneficial effect of selegiline on cognition of AD patients, its mechanism of action remains to be elucidated. Therefore, we first investigated whether selegiline reverses the impairment of object recognition memory induced by $A\beta_{25–35}$ in mice, an established model of AD. In addition, we investigated whether selegiline alters MAO-B and MAO-A activities in the hippocampus, perirhinal and remaining cerebral cortices of $A\beta_{25–35}$-injected male mice. Acute (1 and 10 mg/kg, p.o., immediately post-training) and subchronic (10 mg/kg, p.o., seven days after $A\beta_{25–35}$ injection and immediately post-training) administration of selegiline reversed the cognitive impairment induced by $A\beta_{25–35}$ (3 nmol, i.c.v.). Acute administration of selegiline (1 mg/kg, p.o.) in combination with $A\beta_{25–35}$ (3 nmol) decreased MAO-B activity in the perirhinal and remaining cerebral cortices. Acute administration of selegiline (10 mg/kg, p.o.) decreased MAO-B activity in hippocampus, perirhinal and remaining cerebral cortices, regardless of $A\beta_{25–35}$ or $A\beta_{35–25}$ treatment. MAO-A activity was not altered by selegiline or $A\beta_{25–35}$. In summary, the current findings further support a role for cortical monoaminergic transmission in the cognitive deficits observed in AD.

Keywords Alzheimer's disease · MAO inhibitors · MAO-B · Perirhinal cortex · Memory · Object recognition

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Introduction

It has been estimated that nearly 36 million people lived with Alzheimer's disease (AD) and other dementias worldwide in 2010, and that this number may increase to 66 million in 2030 and 115 million in 2050 [1]. The increase in life expectancy and the high treatment costs of AD make this disease a major burden for modern society [2]. These data, combined with the current view that the available therapy, at best, confers partial and temporary relief of cognitive deficit [3, 4] and does not alter the progress of the disease [5], has boosted preclinical studies in the field.

Selegiline is a selective and irreversible inhibitor of monoamine oxidase B (MAO-B) activity, clinically employed to slow down the progression of Parkinson's disease [6–8]. This enzyme is responsible for the catabolism of biogenic amines, such as dopamine, benzylamine and phenylethylamine, increasing dopamine levels at the synaptic cleft [9]. In this context, the use of selegiline for the treatment of neurodegenerative diseases like AD is plausible, since decreased levels of dopamine and norepinephrine were found in the brain of AD patients, when compared with age-matched controls [10], and patients in the middle to late clinical stages of AD present increased MAO-B activity [11, 12]. In fact, selegiline has already been tested in pre-clinical and clinical trials for the treatment of cognitive decline related with AD [13–15]. However, little is known about its mechanism of action and brain regions involved in the ameliorative effects of selegiline on AD-related cognitive decline.

Therefore, in this study we investigated whether selegiline reverses the cognitive impairment induced by $A\beta_{25–35}$ injection in mice, a suitable animal model of AD, and whether it alters MAO-B activity in two cerebral structures known to be involved in the memory of the object recognition task, the hippocampus and perirhinal cortex.

Materials and Methods

Animals

The experiments were conducted using male Swiss mice (2–3 months old). The animals had free access to water and food (Guabi, Santa Maria, Rio Grande do Sul, Brazil), and were maintained in a humidity and temperature-controlled room (22 ± 2 °C) under a 12-h light–dark cycle. Behavioral experiments were conducted in a sound-attenuated and air-regulated room, where the animals were habituated 1 h prior to experiments. Behavioral tests were conducted during the light phase of the cycle (between 9:00 a.m. and 5:00 p.m.) using independent experimental groups of mice. All animal experimentation reported in this study was approved by the Ethics Committee of the Federal University of Santa Maria (process number 67/2011) and conducted in accordance with the Policies on the Use of Animals and Humans in Neuroscience Research, revised and approved by the Society for Neuroscience Research in January 1995. All experimental protocols were designed aiming to keep the number of animals used to a minimum, as well as their suffering.

Drugs

$A\beta_{35–25}$, $A\beta_{25–35}$, selegiline hydrochloride (*R*-(–)-deprenyl hydrochloride), clorgyline hydrochloride and kynuramine dihydrobromide were purchased from Sigma (St. Louis, MO, USA). Selegiline hydrochloride was dissolved in saline (0.9 % NaCl) for in vivo experiments. For ex vivo experiments clorgyline hydrochloride, kynuramine dihydrobromide and selegiline hydrochloride were dissolved in assay buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, 3.6 mM KCl, pH 7.4). All the other reagents used were of analytical grade and were purchased from local suppliers.

$A\beta_{25–35}$, and $A\beta_{35–25}$ (inverted sequence used as control), were dissolved in 50 mM phosphate buffer saline (PBS; pH 7.4) at a concentration of 3 mM and stored at −20 °C. For Aβ aggregation 3 mM of $A\beta_{35–25}$ and $A\beta_{25–35}$ peptide were incubated at 37 °C for 4 days.

$A\beta_{25–35}$ Administration: Mouse Model of AD

Intracerebroventricular injections of $A\beta_{25–35}$ and, $A\beta_{35–25}$ (3 nmol/3.2 μL) were performed as described previously [16]. Briefly, mice were anesthetized with isoflurane and the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. The microinjections were performed using a Hamilton syringe connected to a 28-gauge stainless-steel needle with 3 mm in length.

Novel Object Recognition Task

The novel object recognition task was performed in a 30 × 30 × 30 cm wooden chamber, with walls painted black and the front wall made of Plexiglas and the floor covered with ethyl vinyl acetate sheet. A light bulb, hanging 60 cm above the behavioral apparatus, provided constant illumination of about 40 lux, and an air-conditioner provided constant background sound isolation. The objects used were pairs of plastic mounting bricks, each pair with different shapes (rectangular, pyramid and stair-like shapes) and colors (white, red and blue), but same size. Throughout the experiments objects were used in a counterbalanced manner and animals did not previously display preference for any of the objects [17]. Chambers and objects were cleaned after each subject was tested with 30 % ethanol.

Six days after Aβ peptide injection, the novel object recognition task was performed [18–20]. The task consisted of habituation, training and testing sessions, each of them with the duration of 10 min. In the first session, mice were habituated to the behavioral apparatus and then returned to their home cage. Twenty-four hours later, training session took place, where animals were exposed to two of the same objects (object A), and the exploration time was recorded with two stopwatches. Exploration was recorded when the animal touched or reached the object

with the nose at a distance of less than 2 cm. Climbing or sitting on the object was not consider exploration. The test session was carried out 24 h after training. Mice were placed back in the behavioral chamber and one of the familiar objects (i.e. object A) was replaced by a novel object (i.e. object B). The time spent exploring the familiar and the novel objects were recorded. The discrimination index was then calculated, taking into account the difference of time spent exploring the novel (B) and the familiar (A) object × 100 divided by the sum of time spent exploring the novel (B) and the familiar (A), and used as a cognitive parameter ($[(T_{novel} - T_{familiar})/(T_{novel} + T_{familiar})] \times 100$) [21].

Saline or selegiline (1 or 10 mg/kg, p.o.) were administered immediately after training of the novel object recognition task (acute model), or sub chronically (10 mg/kg, p.o., once a day for 7 days) with the last dose administered immediately after the training session. The doses of selegiline were chosen based on a pilot experiment.

Monoamine Oxidase Assay

Immediately after the training session of the novel object recognition task, one group of animals received saline (0.9 % NaCl, 10 mL/kg, p.o.) or selegiline (1 or 10 mg/kg, p.o.). One hour after drug administration the animals were killed and the hippocampi, perirhinal and remaining cerebral cortices (cerebral cortex without the perirhinal cortex) were dissected and homogenized in assay buffer (16.8 mM Na_2HPO_4, 10.6 mM KH_2PO_4, 3.6 mM KCl, pH 7.4). MAO-A and MAO-B activities were measured by detecting the formation of the fluorescent product 4-hydroxyquinoline (4-HQ) using kynuramine as substrate, as previously described [22–24]. Briefly, assays were performed in duplicate in a final volume of 500 µL containing 0.25 mg of protein and incubated at 37 °C for 30 min. MAO-A and MAO-B activities were isolated pharmacologically by incorporating 250 nM selegiline (selective MAO-B inhibitor) or 250 nM clorgyline (selective MAO-A inhibitor) into the reaction mixture. The reaction mixture was pre-incubated at 37 °C for 5 min and the reaction was started by the addition of 60 µM kynuramine. Results were expressed as nmol of 4-HQ/mg of protein/min.

Statistical Analysis

Data were analyzed by two-way analysis of variance followed by Bonferroni's post hoc test, when appropriate, presented as mean ± SEM. Differences were considered significant when $p < 0.05$. F values are presented only if $p < 0.05$.

Results

Effects of Selegiline on $A\beta_{25–35}$-Induced Cognitive Impairment

No significant difference between groups in the time spent exploring both objects in the training session was found, indicating no biased exploration of the objects (data not shown). $A\beta_{25–35}$ impaired object recognition performance at testing, an effect that was reverted by the subchronic [$F(_{1,16}) = 6.41$, $p < 0.05$; Fig. 1] and the acute administration of 1 mg/kg selegiline [$F(_{1,29}) = 5.77$, $p < 0.05$; Fig. 2a] and 10 mg/kg selegiline [$F(_{1,29}) = 8.52$, $p < 0.05$; Fig. 2b].

Effect of Selegiline on MAO Activity Ex Vivo

In order to address whether the ameliorative effects of selegiline on memory of $A\beta_{25–35}$-treated male mice were due to an inhibition of MAO-B activity, we performed MAO activity assay in memory-relevant brain areas, such as hippocampus, cerebral cortex and perirhinal cortex.

Figures 3 and 4 show that i.c.v. injection of $A\beta_{25–35}$ peptide (3 nmol) did not alter MAO-B and MAO-A activities of saline-treated mice in any brain structure examined compared with $A\beta_{35–25}$ control mice. The acute administration of selegiline (1 mg/kg, p.o.) did not alter MAO-B activity in the hippocampus, regardless whether the animals received $A\beta_{25–35}$ or not (Fig. 3a). On the other hand, acute selegiline decreased MAO-B activity in the cerebral cortex of both $A\beta_{25–35}$- and $A\beta_{35–25}$-treated mice

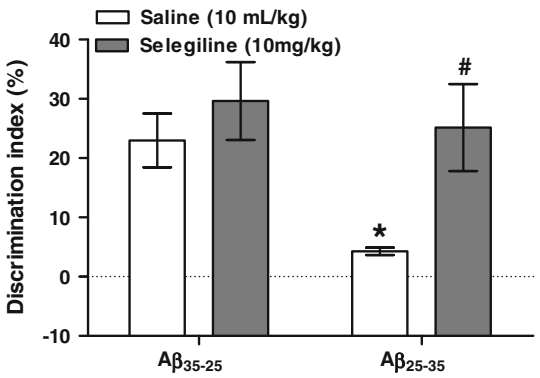

Fig. 1 Subchronic oral administration of selegiline (10 mg/kg, once a day for seven days after $A\beta_{25–35}$ injection and immediately post-training) reverses the memory impairment induced by $A\beta_{25–35}$ peptide (3 nmol, i.c.v.) on the discrimination index in the object recognition task in mice. $A\beta_{35–25}$ and saline were used as vehicle. Data are the mean ± SEM for 5 animals per group. $^{*}p < 0.05$ compared with saline-treated $A\beta_{35–25}$ group and $^{\#}p < 0.05$ compared with saline-treated $A\beta_{25–35}$ group, two-way ANOVA followed by Bonferroni's test

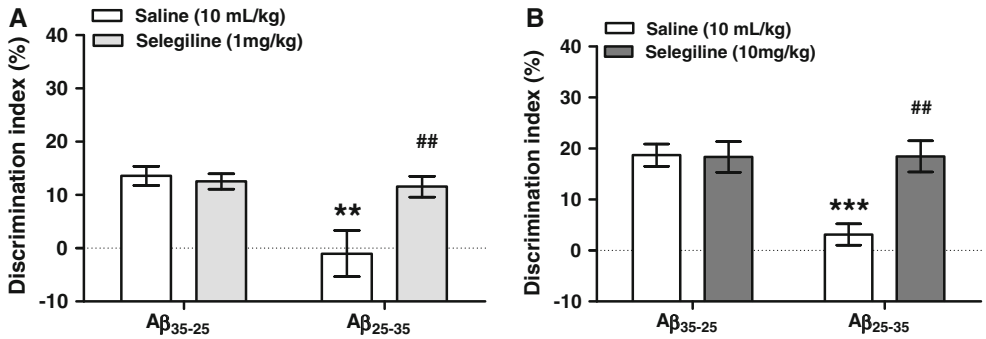

Fig. 2 Acute oral administration of selegiline (1 mg/kg **a** or 10 mg/kg **b** immediately post-training) reverses the memory impairment induced by $A\beta_{25–35}$ peptide (3 nmol, i.c.v.) on the discrimination index in the object recognition task in mice. $A\beta_{35–25}$ and saline were used as vehicle. Data are the mean ± SEM for 8–9 animals per group. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with saline-treated $A\beta_{35–25}$ group and $^{\#\#}p < 0.01$ compared with saline-treated $A\beta_{25–35}$ group, two-way ANOVA followed by Bonferroni's test

$[F_{(1,25)} = 6.18;\ p < 0.05,$ Fig. 3c]. Interestingly, acute selegiline (1 mg/kg, p.o.) decreased MAO-B activity in the perirhinal cortex only in those animals that were injected with $A\beta_{25–35}$ $[F_{(1,13)} = 25.16;\ p < 0.05,$ Fig. 3e]. Moreover, as expected, the acute administration of selegiline (1 mg/kg, p.o.) did not alter MAO-A activity in any brain region, regardless whether the animals received $A\beta_{25–35}$ or not (Fig. 3b, d, f).

Selegiline (10 mg/kg, p.o., immediately after training) significantly inhibited MAO-B activity in hippocampus $[F_{(1,12)} = 25.25;\ p < 0.01,$ Fig. 4a], cerebral cortex $[F_{(1,12)} = 29.44;\ p < 0.01,$ Fig. 4c], and perirhinal cortex $[F_{(1,11)} = 32.28;\ p < 0.01,$ Fig. 4e] of both $A\beta_{35–25}$ and $A\beta_{25–35}$ treated mice. Moreover, selegiline (10 mg/kg, p.o) or $A\beta_{25–35}$ did not alter MAO-A activity in any brain structures studied (Fig. 4b, d, f).

Discussion

In this study we showed that both acute and subchronic selegiline administration reverted $A\beta_{25–35}$ peptide-induced cognitive impairment in the object recognition task in male mice. While the effects of selegiline, a selective and irreversible inhibitor of MAO-B, on memory have already been shown in both pre-clinical and clinical studies [25–28], it remains to be addressed whether these effects are dependent of MAO-B inhibition and which brain areas are involved in this effect.

Our results are consistent with those obtained by Tsunekawa et al.[25], who have shown that subcutaneously administered selegiline (3 mg/kg) improves the cognitive impairment induced by $A\beta_{25–35}$ using the Y-maze and conditioned fear learning tasks in mice. Our results are also in agreement with de Lima et al. [27], who have shown that selegiline (1 mg/kg, subcutaneously, for 21 days) reverses the memory impairment of aged male Wistar rats, in the object recognition task. Moreover, the intraperitoneal co-administration of selegiline (1 or 2.5 mg/kg) and donepezil (0.3 or 3 mg/kg) significantly ameliorates scopolamine plus *p*-chlorophenylalanine-induced memory deficits of rats, in the Morris water maze [26]. Accordingly, selegiline (10 mg/day, for 24 weeks) has a long-term beneficial effect on the memory of patients with criteria for mild to moderate AD [28].

In this study we also showed that acutely administered selegiline (1 mg/kg, p.o.) decreases MAO-B activity in the perirhinal and cerebral cortices of $A\beta_{25–35}$ peptide-injected rats and that selegiline (10 mg/kg, p.o.) decreases MAO-B activity in hippocampus, cerebral cortex and perirhinal cortex regardless of $A\beta_{25–35}$ treatment. In this context, it has been shown that neurofibrillary tangles, a major neuropathological finding in AD, initially appear in a subregion of the perirhinal cortex and in the entorhinal cortex, before spreading to the hippocampus [29, 30]. These data suggest that MAO-B activity of the cerebral cortex (including the perirhinal area) of $A\beta_{25–35}$ peptide-injected mice is more sensitive to the inhibitory effect of selegiline than that of the hippocampus. MAO-B inhibition may increase the availability of dopamine and norepinephrine in the synaptic cleft of selected cerebral structures. In this regard, the stimulation of beta-adrenergic and D1/D5 dopaminergic receptors by norepinephrine or dopamine might enhance memory consolidation through activation of the cyclic AMP/protein kinase A signaling pathway in the hippocampus [31, 32]. Since acutely administered selegiline (1 mg/kg) reversed the deleterious effect of $A\beta_{25–35}$ on cognition and inhibited MAO-B activity only in the cerebral cortex, one might reasonably argue that these effects may be due to MAO-B inhibition in this cerebral structure.

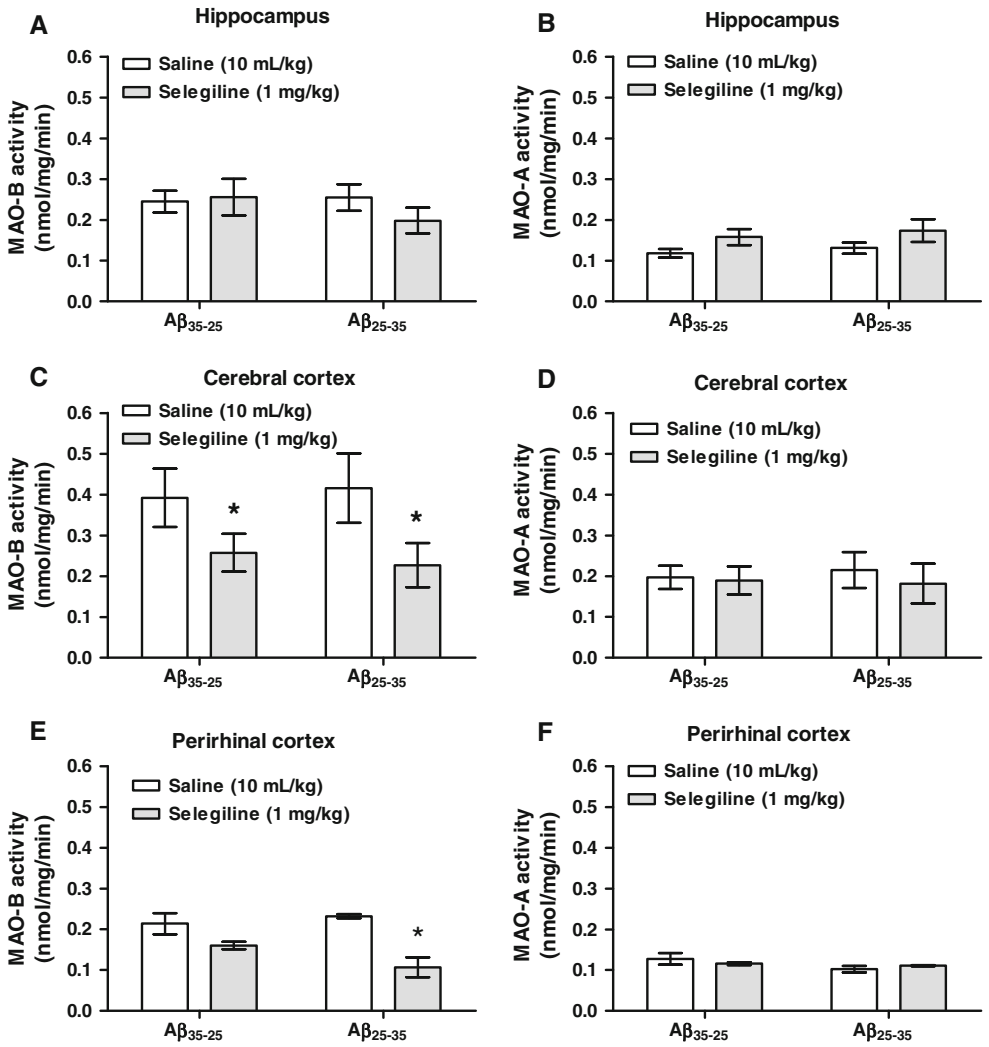

Fig. 3 Effect of acute administration of selegiline (1 mg/kg, p.o, immediately after training) and $A\beta_{25-35}$ peptide (3 nmol, i.c.v.) on monoamine oxidase (MAO) A and B activity in mice. MAO-B and MAO-A activities were determined in hippocampus (**a, b**) cerebral cortex (**c, d**) and perirhinal cortex (**e, f**) respectively. $A\beta_{35-25}$ and saline were used as vehicle. Data are the mean ± SEM of 6–8 (A–D) and 4–5 (E, F) animals per group. $^{*}p < 0.05$ compared with respective saline-treated group, two-way ANOVA followed by Bonferroni's test

Despite the already discussed effects of selegiline on MAO-B activity, this compound displays a myriad of effects that cannot be explained exclusively by its MAO-B inhibitory action. It has been shown that selegiline have a trophic-like action, protecting neurons from damage in a similar fashion as brain-derived neurotrophic factor and ciliary neurotrophic factor [33]. It is also postulated that selegiline enhances dopamine release and block its reuptake, after been metabolized to amphetamine [34]. This compound also displays antiapoptotic action [35] and antioxidant activity, increases nitric oxide (NO) production with consequent dilation of cerebral blood vessels [14, 36–39]. NO facilitates synaptic plasticity and memory formation in rats [14, 40–42]. Therefore, it is possible that mechanisms other than inhibition of MAO-B activity underlies the currently described facilitatory effect of selegiline on cognition, including increased blood perfusion of the brain with consequent improvement of the energetic status and activation of putative neuroprotective and trophic mechanisms, that facilitate synaptic plasticity.

In summary, this study shows that acute and subchronic oral administration of selegiline reverses the memory impairment induced by i.c.v. administration of $A\beta_{25-35}$ peptide. This study also shows that selegiline (1 mg/kg) decreases MAO-B activity in the cerebral cortex of $A\beta_{25-35}$-treated mice, and that selegiline (10 mg/kg) decreases MAO-B activity in hippocampus, perirhinal and remaining cerebral cortices. The fact that the perirhinal

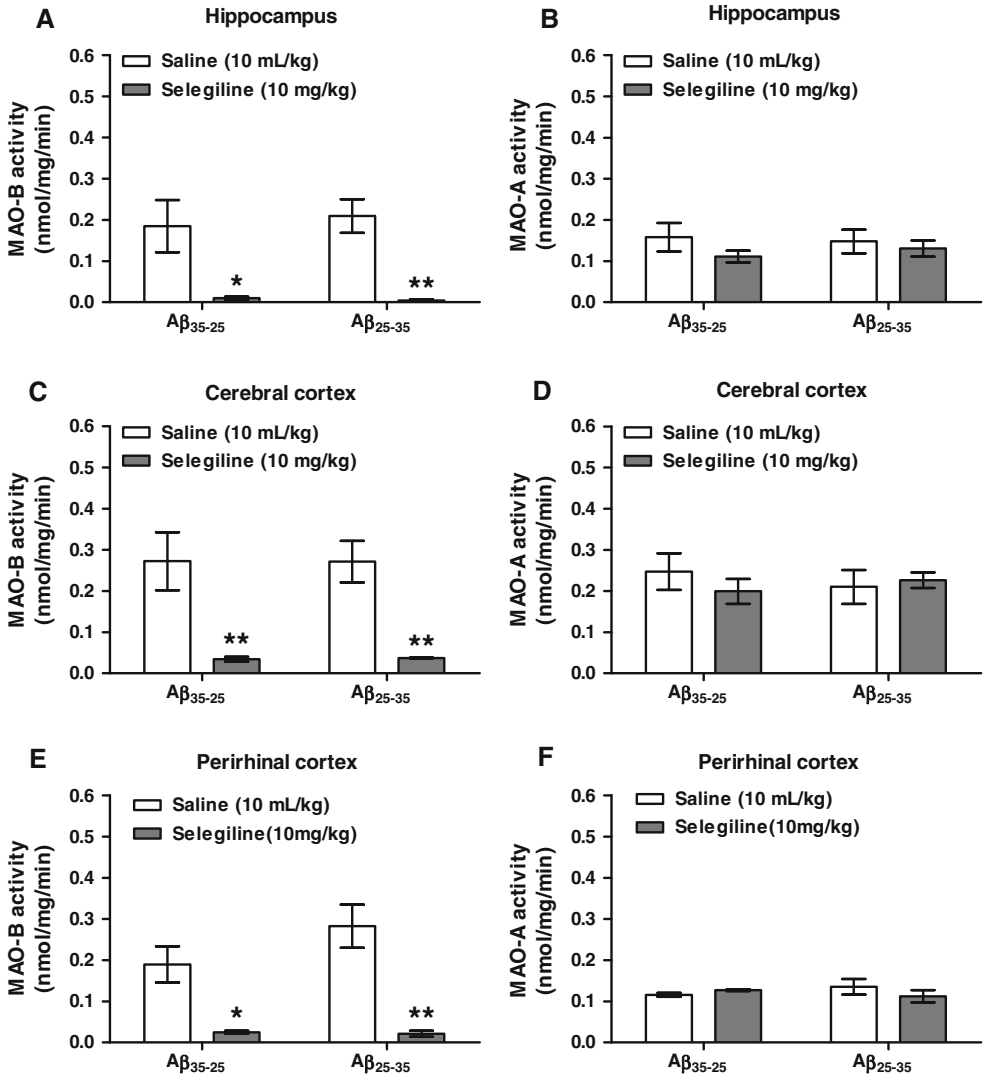

Fig. 4 Effect of acute administration of selegiline (10 mg/kg, p.o, immediately after training) and $A\beta_{25-35}$ peptide (3 nmol, i.c.v.) on monoamine oxidase (MAO) A and B activity in mice. MAO-B and MAO-A activities were determined in hippocampus (**a**, **b**) cerebral cortex (**c, d**) and perirhinal cortex (**e, f**) respectively. $A\beta_{35-25}$ and saline were used as vehicle. Data are the mean ± SEM of 3–4 animals per group. $*p < 0.05$ and $**p < 0.01$ compared with respective saline-treated group, two-way ANOVA followed by Bonferroni's test

cortex is one of the first structures affected in AD supports the use of selegiline in the early stages of the disease.

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Conflict of interest The authors have declared that there is no conflict of interest.

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