by the Institute for Laboratory Animal Research of Nagoya University and to the European Communities Council Directive of 24 November 1986 (86-609/EEC).

On the day of surgery, a cannula attached to a mini-osmotic pump was implanted in the rat right cerebral ventricle (A 20.3 mm, L 1.2 mm, V 4.5 mm) as previously described (Nitta et al., 1994) and β-amyloid-(1–40) (Feinchemikalien, Switzerland) was continuously infused at a dose of 0.3 nmol/12 μl/day for 14 days. Control animals received 0.3 nmol/12 μl/day of β-amyloid-(40–1), the reverse sequence of (1–40). Both peptides (1–40) and (40–1) were dissolved in 35% acetonitrile–0.1% trifluoroacetic acid (vehicle). We have previously confirmed that β-amyloid-(40–1) or vehicle itself has no effect on learning behaviour at this flow rate (Yamada et al., 1999). On day 14 after the start of β-amyloid infusion, rats were submitted to the conditioned fear stress procedure. After the first behavioural session, some animals were anaesthetized with pentobarbital 6%, transcardiacally perfused with 200 ml of saline solution and their brains were quickly removed from the skull. The cerebral cortex, hippocampus and cerebellum were immediately dissected out, and subsequently stored at −80 °C until assayed.

2.2. Drugs

(+)-*N*-cyclopropylmethyl-*N*-methyl-1,4-diphenyl-1-ethyl-but-3-en-1-ylamine hydrochloride (igmesine, JO-1784) was synthesized at Institut de Recherche Jouveinal/Parke-Davis. Progesterone (4-pregnene-3,20-dione) was from Sigma/Aldrich (St. Louis, MO, USA). Dehydroepiandrosterone sulfate (5-androsten-3β-ol-17-one sulfate) and (+)-SKF-10,047 were from Research Biochemicals (Natick, MA, USA). *N*,*N*-Dipropyl-2-(4-methoxy-3-(2-phenylethoxy)phenyl)ethylamine (NE-100) was provided by Taisho Pharmaceuticals (Tokyo, Japan). [1,2,6,7-^{3}H(*N*)]Progesterone (3589 GBq/mmol, 37 MBq/ml), [7-^{3}H(*N*)]pregnenolone ([^{3}H]pregnenolone, 777 GBq/mmol, 37 MBq/ml), [1,2,6,7-^{3}H(*N*)]dehydroepiandrosterone ([^{3}H]dehydroepiandrosterone, 2220 GBq/mmol, 37 MBq/ml) and [7-^{3}H(*N*)]dehydroepiandrosterone sulfate ([^{3}H]dehydroepiandrosterone sulfate, 592 GBq/mmol, 37 MBq/ml) were from New England Nuclear (Boston, MA, USA). The pregnenolone antibody was from AbCys (Paris, France); progesterone and dehydroepiandrosterone antibodies were from Biovalley (Marne-la-Vallée, France). Progesterone was suspended in sesame oil; other drugs were solubilised in distilled water or saline solution. Drugs were injected subcutaneously (s.c.) or intraperitoneally (i.p.), in a volume of 100 μl/20 g of body weight.

2.3. Conditioned fear stress procedure

The apparatus was a transparent acrylic rectangular cage (25 × 30 × 47 high cm) equipped with a metal wire floor. The cage was inserted in a sound-attenuated box and was illuminated with a 20-W bulb. Each rat was placed into the test cage and received intermittent electric shocks (0.1 Hz, 200 ms, 100 V DC) for 10 min through an isolated pulse stimulator (Nihon Koden, Tokyo, Japan). Each animal received electric footshocks in the range of 0.2–0.5 mA, because the current resistance of the animal varied between 200 and 500 kΩ (Kamei et al., 1997). The test session was performed 24 h after the first session. Animals were placed again into the test cage, but no footshock was delivered. The spontaneous motility of rats was measured using an infrared beams activity device (Scanet SV-10, Neuroscience, Osaka, Japan or Opto-varimex, Columbus Instruments, Columbus, OH, USA), in which the cage was inserted. The non-shocked control group was operated similarly, except for the absence of shock treatment. The σ_1 receptor ligands, or appropriate vehicle solutions, were administered 30 min before the test session.

2.4. Extraction and purification of neurosteroids

Brain samples were thawed, weighed and homogenized in ice-cold 10 mM phosphate buffer saline, pH 7.4. Recovery tracers ([^{3}H]progesterone, [^{3}H]pregnenolone, [^{3}H]dehydroepiandrosterone, [^{3}H]dehydroepiandrosterone sulfate, 50 Bq each) were added. Then, 10 ml of ethylacetate/isooctane, 1/1 vol/vol, was added and the tubes were vigorously stirred for 8 min. After centrifugation at 4000 × *g* for 5 min, the organic phase was removed and the extraction step was repeated twice. This organic phase was then defatted with a MeOH 90%/isooctane separation. The aqueous extracts containing unconjugated steroids were further purified by reverse-phase chromatography on C_{18} cartridges (Amersham, Les Ulis, France). The isooctane phases containing lipoidal derivatives were thrown away. Sulfate esters were hydrolysed. The aqueous phase from the first separation was

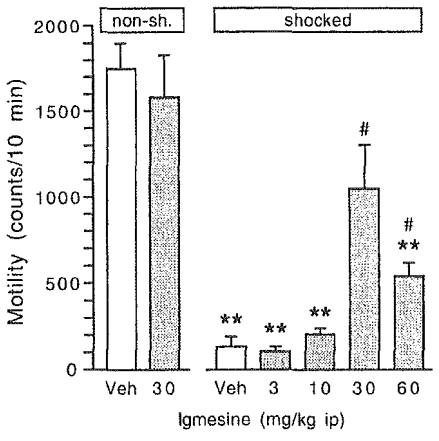

Fig. 1. Effect of the σ_1 receptor agonist igmesine in naive rats submitted to the conditioned fear stress. Igmesine was administered i.p. 30 min before the motility measurement. The number of animals per experimental group was $n=4–6$. $^*P<0.05$, $^{**}P<0.01$ compared to the vehicle (Veh)-treated non-shocked (non-sh.) group; $^{\#}P<0.05$ compared to the Veh-treated shocked group (Dunn's test).

brought to pH = 1.0 with a few drops of sulfuric acid and to a NaCl concentration of 20% by adding 2/1 vol/vol of a 30% NaCl solution. Extraction with ethylacetate was again performed as described above, and this extract, which contained steroid sulfates, was hydrolysed at 37 °C for 16 h. Ethylacetate extracts were washed once with 1 N NaOH (0.25 vol.) and twice with water (0.25 vol.). The extracts were finally dried.

The different steroids were separated using partition chromatography on Celite[545] (Prolabo, Fontenay-sous-Bois, France) microcolumns, with propanediol, 1 g, as the stationary phase. Impregnated celite was settled in 5-ml disposable glass pipettes. Extracts were taken up in 1 ml of isooctane saturated with propanediol, and deposited onto the columns. Progesterone was eluted with 19-ml isooctane, pregnenolone with 15 ml of isooctane/benzene (7/3 vol/vol) and dehydroepiandrosterone with 20 ml of isooctane/benzene (1/1 vol/vol). The recovery of the different steroids added as tracers was routinely 60–80%.

After separation, each steroid was quantified by radioimmunoassay using specific antibodies presenting minimal cross-reactions. Measurements were performed in triplicate of four dilutions of each purified sample. Results are expressed as ng/g of tissue.

2.5. Statistical analysis

Results are expressed as mean ± S.E.M. Behavioural data were analyzed using the Dunn's multiple comparisons test after a non-parametric Kruskal–Wallis analysis of variance (ANOVA, KW values). Neurosteroid measurements were analyzed using a two-way ANOVA (*F*-values),

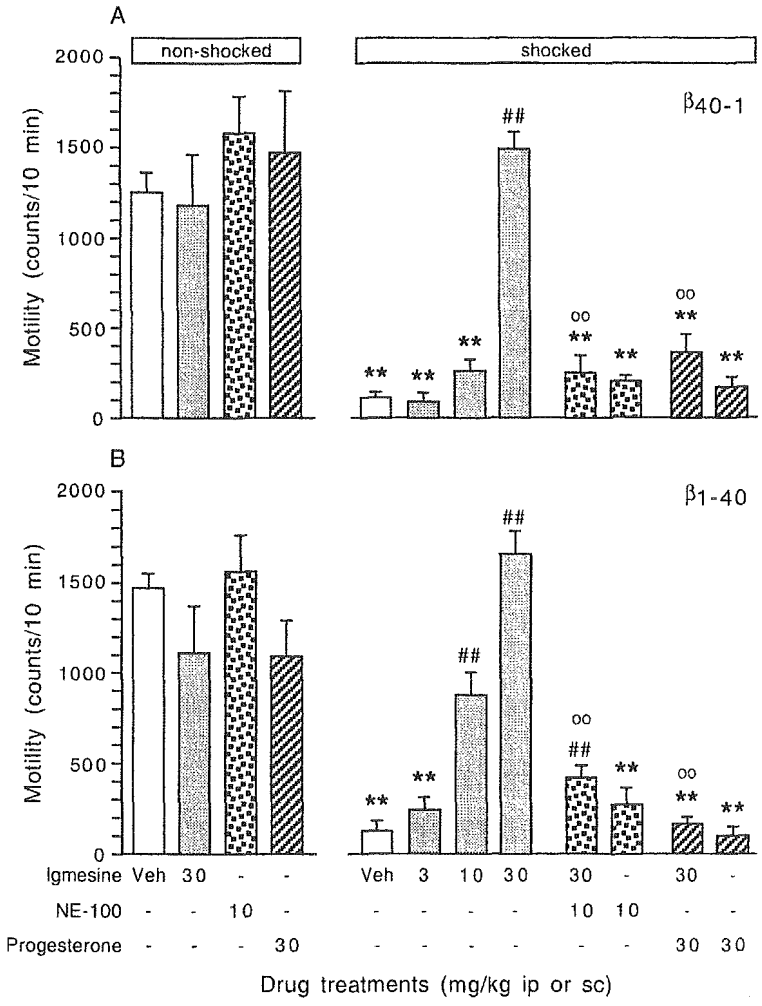

Fig. 2. Effect of igmesine on the conditioned fear stress response in rats infused during 14 days with: (A) β-amyloid-(40–1) or (B) β-amyloid-(1–40) protein. The σ_1 receptor antagonist NE-100 or the neuroactive steroid progesterone was injected i.p. or s.c., respectively, 15 min before igmesine, administered i.p. 30 min before the motility measurement. The number of animals per experimental group was $n = 4–6$. $^{**}P < 0.01$ compared to the vehicle (Veh)-treated non-shocked group; $^{\#\#}P < 0.01$ compared to the Veh-treated shocked group; $^{oo}P < 0.01$ compared to the igmesine (30)-treated shocked group (Dunn's test).

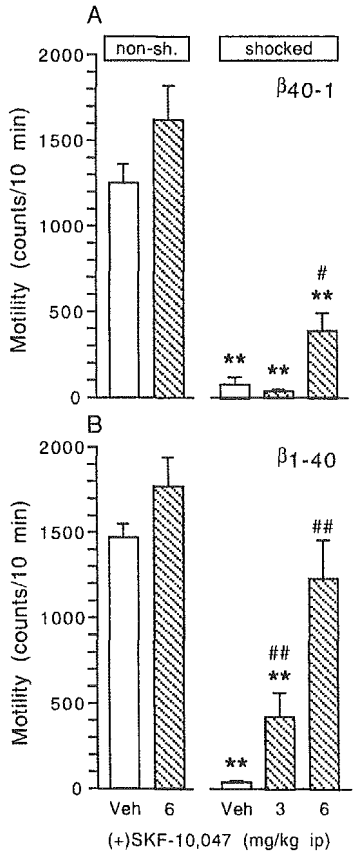

Fig. 3. Effect of the σ_1 receptor agonist (+)-SKF-10,047 on the conditioned fear stress response in rats infused during 14 days with: (A) β-amyloid-(40–1) or (B) β-amyloid-(1–40) protein. (+)-SKF-10,047 was administered i.p. 30 min before the motility measurement. The number of animals per experimental group was $n=5$. $^{**}P<0.01$ compared to the vehicle (Veh)-treated non-shocked (non-sh.) group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared to the Veh-treated shocked group (Dunn's test).

with the infusion treatment and exposure to shock as independent parameters, post-hoc comparisons being made using the Welch's test. The criteria for statistical significance was $P<0.05$.

3. Results

3.1. Effects of the σ_1 receptor agonists on conditioned fear stress in β-amyloid-infused rats

Treatment with the σ_1 receptor agonist igmesine, tested in the 3–60 mg/kg i.p. dose range, resulted in an attenuation of the highly significant decrease in motility observed in rats that experienced the unavoidable electric footshock (KW = 26.84, $P<0.001$; Fig. 1). At 30 and 60 mg/kg, igmesine induced a significant ($P<0.05$), but bell-shaped effect.

Rats infused chronically with either β-amyloid-(40–1) or β-amyloid-(1–40) protein exhibited, similarly as intact animals, a highly significant decrease of motility after shock (Fig. 2A and B). In control, β-amyloid-(40–1)-treated animals, the igmesine treatment attenuated the decrease of motility, in a similar dose–response effect as compared to non-infused animals (KW = 51.07, $P<0.0001$; Fig. 2A). The σ_1 receptor agonist induced a highly significant effect at 30 mg/kg. This effect was blocked by the selective σ_1 receptor antagonist NE-100 (10 mg/kg i.p.) or the neuroactive steroid progesterone (30 mg/kg s.c.) (Fig. 2A). In β-amyloid-(1–40)-treated animals, igmesine also attenuated the decrease of motility (KW = 54.64, $P<0.0001$; Fig. 2B). The effect was even potentiated, since the compound induced a highly significant effect at a lower dose, 10 mg/kg, as well as at 30 mg/kg (Fig. 2B). The maximal effect was blocked by NE-100 or progester-

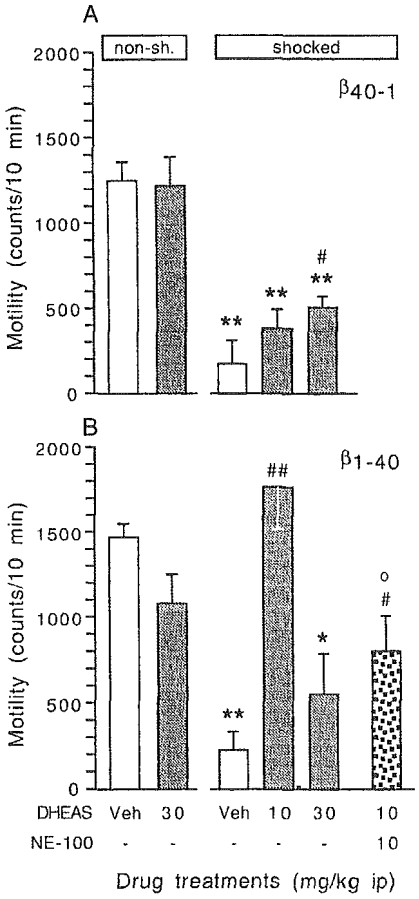

Fig. 4. Effect of the neuroactive steroid dehydroepiandrosterone sulfate on the conditioned fear stress response in rats infused during 14 days with: (A) β-amyloid-(40–1) or (B) β-amyloid-(1–40) protein. NE-100 was injected i.p. 15 min before dehydroepiandrosterone sulfate (DHEAS), administered s.c. 30 min before the motility measurement. The number of animals per experimental group was $n=5$–6. $^{*}P<0.05$, $^{**}P<0.01$ compared to the vehicle (Veh)-treated non-shocked group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared to the Veh-treated shocked group; $^{o}P<0.05$ compared to the dehydroepiandrosterone sulfate (30)-treated shocked group (Dunn's test).

one (Fig. 2B). None of the compounds, tested at their highest active dose, affected the motility in non-shocked groups (Fig. 2A and B).

The anti-stress effect of the prototypic σ_1 receptor agonist (+)-SKF-10,047 was also tested in rats infused with either β-amyloid-(40–1) or β-amyloid-(1–40) protein. In β-amyloid-(40–1)-treated animals, (+)-SKF-10,047 attenuated the decrease of motility (KW = 20.55, $P < 0.001$; Fig. 3A). The σ_1 receptor agonist induced a significant but limited attenuation at 6 mg/kg. In β-amyloid-(1–40)-treated animals, the efficacy of (+)-SKF-10,047 to increase the shocked rats motility was potentiated, since highly significant effects were measured at 3 and 6 mg/kg (KW = 19.15, $P < 0.001$). At the latter dose, a complete reversion was measured (Fig. 3B).

3.2. Effect of the neuroactive steroid dehydroepiandrosterone sulfate on conditioned fear stress in β-amyloid-infused rats

The anti-stress effect of the σ_1 receptor-related neuroactive steroid dehydroepiandrosterone sulfate was examined in rats infused with either β-amyloid-(40–1) or β-amyloid-(1–40) protein. In β-amyloid-(40–1)-treated animals, dehydroepiandrosterone sulfate attenuated the decrease of motility (KW = 19.79, $P < 0.001$; Fig. 4A). The steroid induced a significant but limited attenuation at 30 mg/kg. In β-amyloid-(1–40)-treated animals, the dehydroepiandrosterone sulfate efficacy to increase the shocked rats motility was potentiated, since a highly significant reversion was observed at the lower dose of 10 mg/kg (KW = 17.70, $P < 0.001$; Fig. 4B). This motility increase was significantly, but not fully, blocked by NE-100, confirming the involvement of the σ_1 receptor in this effect (Fig. 4B).

3.3. Neurosteroid levels in β-amyloid-infused rats

Progesterone levels were measured in several brain regions, the hippocampus, cortex and cerebellum, of rats infused with β-amyloid-(40–1) or (1–40) protein (Fig. 5). In the hippocampus, the two-way ANOVA resulted in a highly significant effect of the β-amyloid treatment [$F(1,12) = 67.63$, $P < 0.001$], but not of stress [$F(1,12) = 3.28$, $P = 0.10$]. However, the treatment × stress interaction was significant [$F(1,12) = 6.58$, $P < 0.05$]. Indeed, in control β-amyloid-(40–1)-treated rats, the stress significantly increased hippocampal progesterone level (Fig. 5A). In β-amyloid-(1–40)-treated animals, the non-shocked group showed a decreased basal progesterone level, that remained unchanged in the shocked group (Fig. 5A). In turn, shocked β-amyloid-(1–40)-treated animals presented almost one third the progesterone contents of shocked β-amyloid-(40–1)-treated ones. In the cortex, highly significant effects were measured for the β-amyloid treatment [$F(1,11) = 55.80$, $P < 0.001$] and stress [$F(1,11) = 10.13$, $P < 0.01$], but not for the treatment × stress interaction. β-Amyloid-(1–40)-treated animals exhibited significantly less progesterone levels (Fig. 5B), in basal as well as stress conditions. For both β-amyloid-(40–1)- and (1–40)-treated groups, the stress only moderately increased progesterone levels. Progesterone levels in the cerebellum appeared unchanged among experimental groups (Fig. 5C).

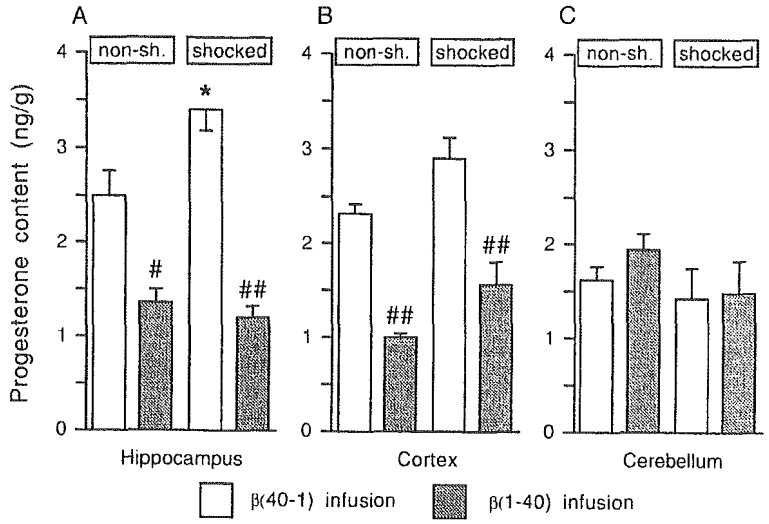

Fig. 5. Brain contents in progesterone in the hippocampus (A), cortex (B) or cerebellum (C) of rats infused during 14 days with β-amyloid-(40–1) or β-amyloid-(1–40) protein. Progesterone levels were measured in non-shocked or shocked animals, sacrificed 30 min after the test session in the conditioned fear procedure. The number of samples was $n = 3–4$ per condition. $^{*}P < 0.05$ compared to the respective non-shocked group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the respective β-amyloid-(40–1)-treated group (Welch's test).

Pregnenolone and dehydroepiandrosterone levels were measured in the different conditions in the hippocampus, cortex and cerebellum, while the levels in sulfate ester of each steroid was measured in the hippocampus (Figs. 6 and 7). In the hippocampus, significant effects for pregnenolone levels were measured for stress [$F(1,12)=11.57$, $P<0.01$] and the β-amyloid treatment × stress interaction [$F(1,12)=10.46$, $P<0.01$]. Indeed, in control β-amyloid-(40–1)-treated rats but not in β-amyloid-(1–40)-treated animals, stress significantly increased hippocampal pregnenolone level (Fig. 6A). In the cortex, a highly significant effect was measured only for stress [$F(1,11)=19.98$, $P<0.001$], indicating no change induced by the β-amyloid-(1–40)-treatment (Fig. 6B). Pregnenolone levels in the cerebellum appeared unchanged among experimental groups (Fig. 6C). In the hippocampus,

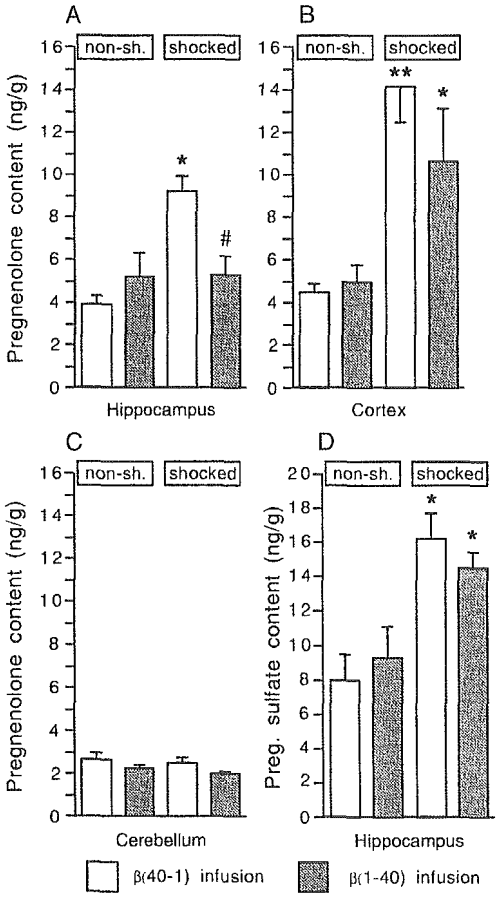

Fig. 6. Brain contents in pregnenolone in the hippocampus (A), cortex (B) or cerebellum (C) of rats infused with β-amyloid-(40–1) or β-amyloid-(1–40) protein. (D) Hippocampal content in pregnenolone sulfate in rats with β-amyloid-(40–1) or β-amyloid-(1–40) protein. Neurosteroid levels were measured in non-shocked or shocked animals, sacrificed 30 min after the test session in the conditioned fear procedure. The number of samples was $n=3$–4 per condition. $^{*}P<0.05$, $^{**}P<0.01$ compared to the respective non-shocked group; $^{\#}P<0.05$ compared to the respective β-amyloid-(40–1)-treated group (Welch's test).

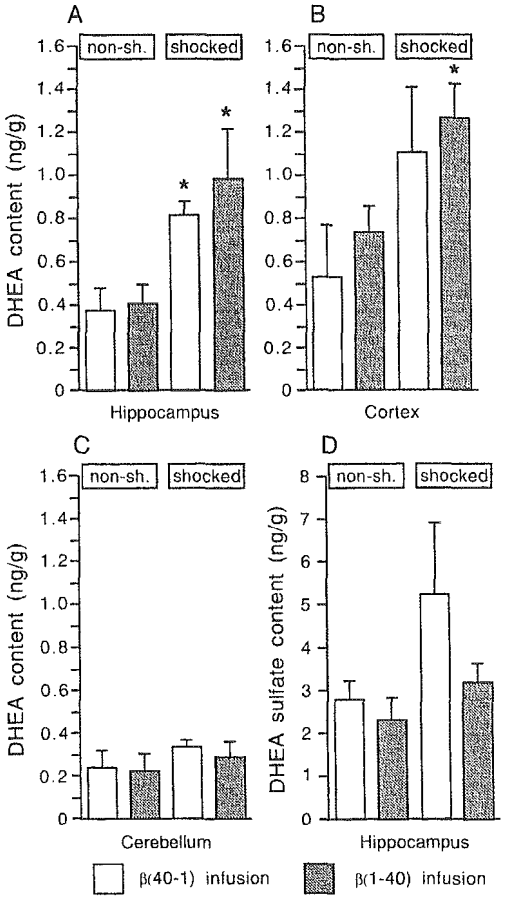

Fig. 7. Brain contents in dehydroepiandrosterone (DHEA) in the hippocampus (A), cortex (B) or cerebellum (C) of rats infused with β-amyloid-(40–1) or β-amyloid-(1–40) protein. (D) Hippocampal content in dehydroepiandrosterone sulfate (DHEAS) in rats with β-amyloid-(40–1) or β-amyloid-(1–40) protein. Neurosteroid levels were measured in non-shocked or shocked animals, sacrificed 30 min after the test session in the conditioned fear procedure. The number of samples was $n=3$–4 per condition. $^{*}P<0.05$ compared to the respective non-shocked group (Welch's test).

pregnenolone sulfate levels varied significant after stress [$F(1,11)=18.19$, $P<0.01$], but no difference was measured between β-amyloid-(40–1)- and (1–40)-treated rats (Fig. 6D).

Dehydroepiandrosterone levels were much lower in all brain structures, and significant variations were only observed after stress in the hippocampus [$F(1,12)=13.48$, $P<0.01$; Fig. 7A] and cortex [$F(1,11)=6.57$, $P<0.05$; Fig. 7B]. No difference was measured between β-amyloid-(40–1)- and (1–40)-treated rats and in the cerebellum among all experimental groups (Fig. 7C). In the hippocampus, dehydroepiandrosterone sulfate levels failed to change significantly after stress [$F(1,11)=1.78$, $P=0.21$], or according to the β-amyloid-treatment [$F(1,11)=0.27$, $P=0.61$; Fig. 7D].

4. Discussion

Major depression affects between 30% and 50% of the patients who develop Alzheimer's disease and has serious consequences not only for the evolution of the patient, in terms of increased disabilities, but also for caregivers. Results of current antidepressant therapies have produced unsatisfactory findings without fully addressing the benefits of depression reduction (Boland, 2000; Espiritu et al., 2001; Lyketsos et al., 2003). Preclinical studies are thus requested to propose alternative strategies based on novel antidepressants that may present a preserved efficacy in the demented subject. We report here, using a non-transgenic rat model of Alzheimer's disease, induced by the chronic intracerebroventricular infusion of β-amyloid-(1–40) peptide, that the effect of the selective σ_1 receptor agonists, igmesine, (+)-SKF-10,047 or dehydroepiandrosterone sulfate, was enhanced in this model compared to control animals, in the conditioned fear stress test. As compared to β-amyloid-(40–1) peptide-infused control animals, the active dose of each compound was lower by two-to three-fold in β-amyloid-(1–40)-treated rats, with an increase of the intensity of the effect observed for (+)-SKF-10,047 or dehydroepidrosterone sulfate.

Selective σ_1 receptor agonists showed antidepressant efficacy in several animal models of behavioural despair, particularly the tail suspension test or the forced swim test (Matsuno et al., 1996; Ukai et al., 1998; Urani et al., 2001), suggesting that depressive symptoms may be alleviated by the drugs acting through this receptor. Indeed, a preliminary report outlined the potential clinical efficacy of igmesine in depression (Pande et al., 1998). This antidepressant efficacy may involve the wide-range neuromodulatory action, affecting both intracellular Ca^{2+} mobilisations and responses to neurotransmitters, including glutamatergic, cholinergic and monoaminergic systems, known to be involved in the physiopathological changes sustaining depressive states (Maurice et al., 1999). The conditioned fear stress paradigm has been originally reported by Fanselow (1980). Rats exhibit a marked suppression of motility when they are replaced in the same environment in which they previously experienced an aversive electric footshock. This motor suppression is regarded as a conditioned emotional response to the environment associated with the previous footshock. Indeed, when animals returned into the same environment in which they received the aversive shock, they exhibited a marked suppression of motility. However, when they were placed in a different environment, the difference in motility between shocked and non-shocked mice was not observed (Kameyama et al., 1985).

The conditioned fear stress-induced motor suppression could be attenuated by treatments with antidepressants acting as selective serotonin reuptake inhibitors, citalopram or fluvoxamine (Hashimoto et al., 1996; Inoue et al., 1996; Li et al., 2001), suggesting that the freezing behavior is mediated by serotonergic receptor inactivation (Inoue et al., 1996). However, the conditioned fear stress-induced motor suppression was poorly sensitive to anxiolytics, such as diazepam and chlordiazepoxide (Kameyama and Nagasaka, 1982; Nagasaka and Kameyama, 1983) but attenuated by the benzodiazepine antagonist flumazenil (Izumi et al., 1999). As a result, the conditioned fear stress model may be useful for investigating the pathogenesis of mood disorders, particularly those considered to be treatment resistant, and for developing novel therapeutic drugs. It has been shown that σ_1 receptors play an important role in conditioned fear stress response (for reviews, see Kamei et al., 1998; Maurice et al., 1999). Several σ_1 receptor agonists such as (+)-SKF-10,047 and dextromethorphan attenuated the conditioned fear stress-induced motor suppression in rodents, the effects being antagonized by the σ_1 receptor antagonist NE-100 (Kamei et al., 1996). In addition, dehydroepiandrosterone sulfate and pregnenolone sulfate attenuated the conditioned fear stress-induced motor suppression in mice, via their interaction with the σ_1 receptor (Noda et al., 2000). Progesterone behaved as a potent σ_1 receptor antagonist, since it antagonized the attenuating effects of (+)-SKF-10,047, dehydroepiandrosterone sulfate and pregnenolone sulfate, similarly to what was observed with NE-100 (Noda et al., 2000). In the present study, we confirmed that (+)-SKF-10,047 or dehydroepiandrosterone sulfate are active in the conditioned fear stress in control rats. In addition, the efficacy of igmesine was demonstrated.

The chronic infusion of β-amyloid-(1–40) at a dose of 300 pmol/day provoked numerous physiopathological changes and behavioural impairments reminiscent of Alzheimer's disease (Yamada and Nabeshima, 2000). Accumulation of β-amyloid-(1–40) in the hippocampus and cerebral cortex was evident immunohistochemically following a 14-day period of infusion (Nitta et al., 1997). An interesting observation in our study is that fear conditioning is not affected by β-amyloid peptide treatment-since the stress-induced motor suppression is the same in treated animals as in intact rats-unlike other kinds of more complex memory tests. Indeed, a significant impairment of spatial reference memory formation in a water maze and a deficit of passive avoidance performance was observed in β-amyloid-(1–40)-infused animals, which was accompanied by a mild, but significant, reduction of choline acetyltransferase activity in the hippocampus (Nitta et al., 1997). Impairment of long-term potentiation (Itoh et al., 1999) and reduced activation of protein kinase C (Olariu et al., 2002) were also observed. The chronic infusion of β-amyloid-(1–40), even at very low concentrations, directly inhibited various cholinergic neuronal functions independently of apparent neurotoxicity, suggesting a possible link between chronic infusion of β-amyloid-(1–40) burden and cholinergic dysfunction in Alzheimer's disease. Using an in vivo brain microdialysis technique, we observed that KCl-and nicotine-induced increase in acetylcholine and dopamine release in the hippocampus/cerebral cortex and the striatum, respectively, is markedly impaired by the chronic infusion of β-amyloid-(1–40), although the basal levels of these neurotransmitters

in the β-amyloid-(1–40)-infused rats did not differ from those in vehicle-infused control animals (Itoh et al., 1996). The reduction of nicotine-induced ACh release may be partly due to a decrease in affinity of nicotinic ACh receptors (Olariu et al., 2001, 2002). In addition, the chronic infusion of β-amyloid-(1–40) resulted in changes in the ciliary neurotrophic factor protein levels (Yamada et al., 1995) and in the mRNA expression of BDNF in the hippocampus (Tang et al., 2000). The latter playing a major role in both the etiology of major depression and Alzheimer's disease (Tsai, 2003). All these physiological disturbances may contribute to the onset of learning and memory deficits and support the idea that infusion of β-amyloid-(1–40) in rats provoked neurotoxic changes relevant to the physiopathology of Alzheimer's disease (Yamada and Nabeshima, 2000).

The importance of neurosteroids in mood disorders and depression has been demonstrated (for review, see Van Broekhoven and Verkes, 2003). Recent reports focused on the physiopathological significance of neurosteroids in Alzheimer's disease and related dementia (Wolkowitz et al., 1997, 2003; De Bruin et al., 2002; Brown et al., 2003; Weill-Engerer et al., 2002). On one hand, neurosteroids, and particularly dehydroepiandrosterone sulfate, are expected to elicit a marked neuroprotection in the brain. On the second hand, brain structural abnormalities related to Alzheimer's disease, both β-amyloid deposits and neurofibrillary tangles, which result from the aggregation of pathologic tau proteins, affect brain neurosteroid expression. Brown et al. (2000) reported that β-amyloid-(1–42) protein increased dehydroepiandrosterone levels in human glia-derived cell lines, after a 24-h application. The authors suggested that glial cells might be able to resist to the β-amyloid-induced toxicity because of their ability to produce dehydroepiandrosterone. Direct measurements of pregnenolone, pregnenolone sulfate, dehydroepiandrosterone, dehydroepiandrosterone sulfate, progesterone and allopregnanolone were performed in individual brain regions of Alzheimer's disease patients and aged non-demented controls, including hippocampus, amygdala, frontal cortex, striatum, hypothalamus and cerebellum (Weill-Engerer et al., 2002). A general trend towards decreased levels of all steroids was observed in brain regions of Alzheimer's disease patients compared to controls. Pregnenolone sulfate levels were significantly lower in the striatum and cerebellum; dehydroepiandrosterone sulfate levels were significantly reduced in the hypothalamus, striatum and cerebellum; and progesterone and allopregnanolone levels were markedly but non-significantly reduced in several brain structures, including the hypothalamus, striatum, frontal cortex, or amygdala. A significant negative correlation was found between the levels of cortical β-amyloid peptides and those of pregnenolone sulfate in the striatum and cerebellum and between the levels of phosphorylated tau proteins and dehydroepiandrosterone sulfate in the hypothalamus (Weill-Engerer et al., 2002). Since high levels of key proteins implicated in the formation of plaques and neurofibrillary tangles were correlated with decreased brain levels of pregnenolone sulfate and dehydroepiandrosterone sulfate, the authors supported the concept of a possible neuroprotective role of these neurosteroids in Alzheimer's disease.

In the present study, we chose to measure neurosteroid levels in the hippocampus and cortex, because of: (i) the demonstrated importance of these forebrain structures in depression (Reid and Stewart, 2001); (ii) the importance of the neurosteroid/σ_1 receptor interaction within these structures (Maurice et al., 1999); and (ii) their particular vulnerability to the β-amyloid-(1–40) infusion (Nitta et al., 1997). It must be however outlined that σ_1 receptor agonists ameliorated the conditioned fear stress response through the involvement of mesolimbic dopaminergic systems (Kamei et al., 1997), and thus basal ganglia structures may also be of interest.

We observed significant decreases in brain neurosteroid levels in β-amyloid-(1–40)-infused rats, either in basal conditions or after the fear stress. Pregnenolone and dehydroepiandrosterone sulfate levels failed to increase after stress in β-amyloid-(1–40)-infused rats. Most significantly, continuous β-amyloid-(1–40) infusion caused a marked decrease of progesterone levels in the hippocampus and cortex of rats, and these alterations were not affected by the conditioned fear stress. Interestingly, the β-amyloid-mediated neurotoxic process seems to differentially affect the activity of the different enzymes involved in the steroid biosyntheses. In particular, the 3β-hydroxysteroid dehydrogenase enzyme activity is likely to be mainly affected, consistently with the important decrease in progesterone measured in β-amyloid-(1–40) rats. Since progesterone, released in stressful situations, acts as an endogenous antagonist at the σ_1 receptor, an enhanced behavioural efficacy of the σ_1 receptor agonists was observed. This mechanism has recently been demonstrated through pharmacological manipulations of the progesterone levels (adrenalectomy/castration and administration of inhibitors of the enzymes involved in progesterone synthesis and metabolism) for both the memory and behavioural despair responses (Phan et al., 1999; Urani et al., 2002). However, although this proposed mechanism might serve as an interesting basis to design new, efficient antidepressants for indications such as the Alzheimer's disease-related depression, several points must be further examined. In particular, dehydroepiandrosterone sulfate efficacy was increased in β-amyloid-(1–40)-infused rats. This increase could be due to an interaction with σ_1 receptor since it was partially blocked by NE-100, but may also originate from parallel pathways to be elucidated. At the clinical level, Wolkowitz et al. (1997, 2003) reported that dehydroepiandrosterone, administered to the patients with treatment-resistant depression for 6 months, provoked a marked improvement in depression ratings (Wolkowitz et al., 1997), but more recently, in a randomized, double-bind, placebo-

controlled study, that it allowed only a transient effect on cognitive performances, narrowly missing significance (Wolkowitz et al., 2003). The lack of effect of dehydroepiandrosterone itself in Alzheimer's disease patients encourages the use of more selective synthetic compounds, such as σ_1 receptor agonists.

In summary, the present study showed an increased antidepressant efficacy of σ_1 receptor agonists in a non-transgenic model of Alzheimer's disease, induced by the chronic infusion of β-amyloid-(1–40) in rats. This effect was coherent with decreased brain level in neurosteroids, and particularly progesterone, as previously demonstrated in mice (Phan et al., 1999, 2002; Urani et al., 2001) and in humans (Wolkowitz et al., 2003). The present results confirmed previous observations in mice injected acutely into the lateral ventricle with aggregated β-amyloid-(25–35) peptide (Urani et al., 2002). Targeting the σ_1 receptor thus appears as a promising alternative for alleviating the depressive symptoms in Alzheimer's disease patients.

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References

Boland, R.J., 2000. Depression in Alzheimer's disease and other dementias. Curr. Psychiatry Rep. 2, 427–433.

Brown, R.C., Cascio, C., Papadopoulos, V., 2000. Pathways of neurosteroid biosynthesis in cell lines from human brain: regulation of dehydroepiandrosterone formation by oxidative stress and β-amyloid peptide. J. Neurochem. 74, 847–859.

Brown, R.C., Han, Z., Cascio, C., Papadopoulos, V., 2003. Oxidative stress-mediated DHEA formation in Alzheimer's disease pathology. Neurobiol. Aging 24, 57–65.

De Bruin, V.M.S., Vieira, M.C.M., Rocha, M.N.M., Viana, G.S.B., 2002. Cortisol and dehydroepiandrosterone sulfate plasma levels and their relationship to aging, cognitive function, and dementia. Brain Cogn. 50, 316–323.

Espiritu, D.A.V., Rashid, H., Mast, B.T., Fitzgerald, J., Steinberg, J., Lichtenberg, P.A., 2001. Depression, cognitive impairment and function in Alzheimer's disease. Int. J. Geriatr. Psychiatry 16, 1098–1103.

Evans, D.A., Funkenstein, H.H., Albert, M.S., Scherr, P.A., Cook, N.R., Chown, M.J., Hebert, L.E., Hennekens, C.H., Taylor, J.O., 1989. Prevalence of Alzheimer's disease in a community population of older persons, higher than previously reported. J. Am. Med. Assoc. 262, 2551–2556.

Fanselow, M.S., 1980. Conditioned and unconditional components of post-shock freezing. Pavlovian J. Biol. Sci. 15, 177–182.

Forstl, H., Burns, A., Luthert, P., Cairns, N., Lantos, P., Levy, R., 1992. Clinical and neuropathological correlates of depression in Alzheimer's disease. Psychol. Med. 22, 877–884.

Hanner, M., Moebius, F.F., Flandorfer, A., Knaus, H.G., Striessnig, J., Kempner, E., Glossmann, H., 1996. Purification, molecular cloning, and expression of the mammalian σ_1 binding site. Proc. Natl. Acad. Sci. U. S. A. 93, 8072–8077.

Hashimoto, S., Inoue, T., Koyama, T., 1996. Serotonin reuptake inhibitors reduce conditioned fear stress-induced freezing behavior in rats. Psychopharmacology (Berl.) 123, 182–186.

Inoue, T., Hashimoto, S., Tsuchiya, K., Izumi, T., Ohmori, T., Koyama, T., 1996. Effect of citalopram, a selective serotonin reuptake inhibitor, on the acquisition of conditioned freezing. Eur. J. Pharmacol. 311, 1–6.

Itoh, A., Nitta, A., Nadai, M., Nishimura, K., Hirose, M., Hasegawa, T., Nabeshima, T., 1996. Dysfunction of cholinergic and dopaminergic neuronal systems in beta-amyloid protein-infused rats. J. Neurochem. 66, 1113–1117.

Itoh, A., Akaike, T., Sokabe, M., Nitta, A., Iida, R., Olariu, A., Yamada, K., Nabeshima, T., 1999. Impairments of long-term potentiation in hippocampal slices of beta-amyloid-infused rats. Eur. J. Pharmacol. 382, 167–175.

Izumi, T., Inoue, T., Tsuchiya, K., Hashimoto, S., Ohmori, T., Koyama, T., 1999. Effects of the benzodiazepine antagonist flumazenil on conditioned fear stress in rats. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 23, 1247–1258.

Kamei, H., Kameyama, T., Nabeshima, T., 1996. (+)-SKF-10,047 and dextromethorphan ameliorate conditioned fear stress through the activation of phenytoin-regulated σ_1 sites. Eur. J. Pharmacol. 299, 21–28.

Kamei, H., Noda, Y., Kameyama, T., Nabeshima, T., 1997. Role of (+)-SKF-10,047-sensitive sub-population of σ_1 receptors in amelioration of conditioned fear stress in rats: association with mesolimbic dopaminergic systems. Eur. J. Pharmacol. 319, 165–172.

Kamei, H., Kameyama, T., Nabeshima, T., 1998. Effects of sigma receptor ligands on conditioned fear stress. Methods Find. Exp. Clin. Pharmacol. 20, 613–618.

Kameyama, T., Nagasaka, M., 1982. Effects of apomorphine and diazepam on a quickly learned conditioned suppression in rats. Pharmacol. Biochem. Behav. 17, 59–63.

Kameyama, T., Nabeshima, T., Yamada, K., 1985. Differences of alteration in opioid systems induced by conditioned suppression and electric footshock in mice. Pharmacol. Biochem. Behav. 22, 249–254.

Kekuda, R., Prasad, P.D., Fei, Y.J., Leibach, F.H., Ganapathy, V., 1996. Cloning and functional expression of the human type 1 sigma receptor (hSigmaR1). Biochem. Biophys. Res. Commun. 229, 553–558.

Kim, H.C., Yamada, K., Nitta, A., Olariu, A., Tran, M.H., Mizuno, M., Nakajima, A., Nagai, T., Kamei, H., Jhoo, W.K., Im, D.H., Shin, E.J., Hjelle, O.P., Ottersen, O.P., Park, S.C., Kato, K., Mirault, M.E., Nabeshima, T., 2003. Immunocytochemical evidence that amyloid β(1–42) impairs endogenous antioxidant systems in vivo. Neuroscience 119, 399–419.

Li, X.B., Inoue, T., Hashimoto, S., Koyama, T., 2001. Effect of chronic administration of flesinoxan and fluvoxamine on freezing behavior induced by conditioned fear. Eur. J. Pharmacol. 425, 43–50.

Lyketsos, C.G., Del Campo, L., Steinberg, M., Miles, Q., Steele, C.D., Munro, C., Baker, A.S., Sheppard, J.M., Frangakis, C., Brandt, J., Rabins, P.V., 2003. Treating depression in Alzheimer disease, efficacy and safety of sertraline therapy, and the benefits of depression reduction. The DIADS. Arch. Gen. Psychiatry 60, 737–746.

Matsuno, K., Kobayashi, T., Tanaka, M.K., Mita, S., 1996. σ_1 receptor subtype is involved in the relief of behavioural despair in the mouse forced swimming test. Eur. J. Pharmacol. 312, 267–271.

Maurice, T., 2002. Improving Alzheimer's disease-related cognitive deficits with sigma1 (σ_1) receptor agonists. Drug News Perspect. 15, 1–9.

Maurice, T., Phan, V.L., Urani, A., Kamei, H., Noda, Y., Nabeshima, T., 1999. Neuroactive steroids as endogenous effectors for the sigma1 (σ_1) receptor, pharmacological evidence and therapeutic opportunities. Jpn. J. Pharmacol. 81, 125–155.

Nabeshima, T., Matsuno, K., Kamei, H., Noda, Y., Kameyama, T., 1985. Electric footshock-induced changes in behavior and opioid receptor function. Pharmacol. Biochem. Behav. 23, 769–775.

Nagasaka, M., Kameyama, T., 1983. Effects of diazepam, meprobamate,

chlorpromazine and apomorphine on a quickly learned conditioned suppression in mice. J. Pharmacobio-Dyn. 6, 523–526.
Nitta, A., Itoh, A., Hasegawa, T., Nabeshima, T., 1994. β-Amyloid protein-induced Alzheimer's disease animal model. Neurosci. Lett. 170, 63–66.
Nitta, A., Fukuta, T., Hasegawa, T., Nabeshima, T., 1997. Continuous infusion of β-amyloid protein into cerebral ventricle induces learning impairment and neuronal and morphological degeneration. Jpn. J. Pharmacol. 73, 51–57.
Noda, Y., Kamei, H., Kamei, Y., Nagai, T., Nishida, M., Nabeshima, T., 2000. Neurosteroids ameliorate conditioned fear stress: an association with sigma$_1$ receptors. Neuropsychopharmacology 23, 276–284.
Olariu, A., Tran, M.H., Yamada, K., Mizuno, M., Hefco, V., Nabeshima, T., 2001. Memory deficits and increased emotionality induced by β-amyloid-(25–35) are correlated with the reduced acetylcholine release and altered phorbol dibutyrate binding in the hippocampus. J. Neural Transm. 108, 1065–1079.
Olariu, A., Yamada, K., Mamiya, T., Hefco, V., Nabeshima, T., 2002. Memory impairment induced by intracerebroventricular infusion of β-amyloid-(1–40) involves down-regulation of protein kinase C. Brain Res. 957, 278–286.
Olin, J.T., Schneider, L.S., Katz, I.R., Meyers, B.S., Alexopoulos, G.S., Breitner, J.C., Bruce, M.L., Caine, E.D., Cummings, J.L., Devanand, D.P., Krishnan, K.R., Lyketsos, C.G., Lyness, J.M., Rabins, P.V., Reynolds III, C.F., Rovner, B.W., Steffens, D.C., Tariot, P.N., Lebowitz, B.D., 2002. Provisional diagnostic criteria for depression of Alzheimer disease. Am. J. Geriatr. Psychiatry 10, 125–128.
Pande, A.C., Genève, J., Scherrer, B., 1998. Igmesine, pharmacology and clinical update (abstracts). XXIst CINP Congress, Glasgow, Scotland, July 12–16, 1998, p. 30S.
Phan, V.L., Su, T.P., Privat, A., Maurice, T., 1999. Modulation of steroidal levels by adrenalectomy/castration and inhibition of neurosteroid synthesis enzymes affect σ_1 receptor-mediated behaviour in mice. Eur. J. Neurosci. 11, 2385–2396.
Phan, V.L., Urani, A., Romieu, P., Maurice, T., 2002. Strain differences in σ_1 receptor-mediated behaviours are related to neurosteroid levels. Eur. J. Neurosci. 15, 1523–1534.
Purandare, N., Burns, A., Craig, S., Faragher, B., Scott, K., 2001. Depressive symptoms in patients with Alzheimer's disease. Int. J. Geriatr. Psychiatry 16, 960–964.
Reid, I.C., Stewart, C.A., 2001. How antidepressants work: new perspectives on the pathophysiology of depressive disorder. Br. J. Psychiatry 178, 230–299.
Romieu, P., Martin-Fardon, R., Bowen, W.D., Maurice, T., 2003. Sigma1 (σ_1) (σ_1) receptor-related neuroactive steroids modulate cocaine-induced reward. J. Neurosci. 23, 3572–3576.
Selkoe, D.J., 1991. The molecular pathology of Alzheimer's disease. Neuron 6, 487–498.
Sheline, Y.I., Wang, P.W., Gado, M.H., Csernansky, J.G., Vannier, M.W., 1996. Hippocampal atrophy in recurrent major depression. Proc. Natl. Acad. Sci. U. S. A. 93, 3908–3913.
Tang, Y., Yamada, K., Kanou, Y., Miyazaki, T., Xiong, X., Kambe, F., Murata, Y., Seo, H., Nabeshima, T., 2000. Spatiotemporal expression of BDNF in the hippocampus induced by the continuous intracerebroventricular infusion of β-amyloid in rats. Mol. Brain Res. 80, 188–197.
Tran, M.H., Yamada, K., Olariu, A., Mizuno, M., Ren, X.H., Nabeshima, T., 2001. Amyloid β-peptide induces nitric oxide production in rat hippocampus: association with cholinergic dysfunction and amelioration by inducible nitric oxide synthase inhibitors. FASEB J. 15, 1407–1409.
Tran, M.H., Yamada, K., Nabeshima, T., 2002. Amyloid β-peptide induces cholinergic dysfunction and cognitive deficits: a minireview. Peptides 23, 1271–1283.
Tran, M.H., Yamada, K., Nakajima, A., Mizuno, M., He, J., Kamei, H., Nabeshima, T., 2003. Tyrosine nitration of a synaptic protein synaptophysin contributes to amyloid β-peptide-induced cholinergic dysfunction. Mol. Psychiatry 8, 407–412.
Tsai, S.J., 2003. Brain-derived neurotrophic factor: a bridge between major depression and Alzheimer's disease? Med. Hypotheses 61, 110–113.
Ukai, M., Maeda, H., Nanya, Y., Kameyama, T., Matsuno, K., 1998. Beneficial effects of acute and repeated administrations of σ receptor agonists on behavioural despair in mice exposed to tail suspension. Pharmacol. Biochem. Behav. 61, 247–252.
Urani, A., Roman, F.J., Phan, V.L., Su, T.P., Maurice, T., 2001. The antidepressant-like effect induced by sigma1 (σ_1) receptor agonists and neuroactive steroids in mice submitted to the forced swimming test. J. Pharmacol. Exp. Ther. 298, 1269–1279.
Urani, A., Romieu, P., Roman, F.J., Maurice, T., 2002. Enhanced antidepressant effect of sigma1 agonists in β_{25-35}-amyloid peptide-treated mice. Behav. Brain Res. 134, 239–247.
Van Broekhoven, F., Verkes, R.J., 2003. Neurosteroids in depression: a review. Psychopharmacology 165, 97–110.
Weill-Engerer, S., David, J.P., Sazdovitch, V., Liere, P., Eychenne, B., Pianos, A., Schumacher, M., Delacourte, A., Baulieu, E.E., Akwa, Y., 2002. Neurosteroid quantification in human brain regions: comparison between Alzheimer's and nondemented patients. J. Clin. Endocrinol. Metab. 87, 5138–5143.
Wolkowitz, O.M., Reus, V.I., Roberts, E., Manfredi, F., Chan, T., Raum, W.J., Ormiston, S., Johnson, R., Canick, J., Brizendine, L., Weingartner, H., 1997. Dehydroepiandrosterone (DHEA) treatment of depression. Biol. Psychiatry 41, 311–318.
Wolkowitz, O.M., Kramer, J.H., Reus, V.I., Costa, M.M., Yaffe, K., Walton, P., Raskind, M., Peskind, E., Newhouse, P., Sack, D., De Souza, C., Sadowsky, C., Roberts, E., DHEA-Alzheimer's Disease Collaborative Research, 2003. DHEA treatment of Alzheimer's disease: a randomized, double-blind, placebo-controlled study. Neurology 60, 1071–1076.
Wragg, R.E., Jeste, D.V., 1989. Overview of depression and psychosis in Alzheimer's disease. Am. J. Psychiatry 146, 577–587.
Yamada, K., Nabeshima, T., 2000. Animal models of Alzheimer's disease and evaluation of anti-dementia drugs. Pharmacol. Ther. 88, 93–113.
Yamada, K., Nitta, A., Saito, T., Hu, J., Nabeshima, T., 1995. Changes in ciliary neurotrophic factor content in the rat brain after continuous intracerebroventricular infusion of β-amyloid-(1–40) protein. Neurosci. Lett. 201, 155–158.
Yamada, K., Tanaka, T., Mamiya, T., Shiotani, T., Kameyama, T., Nabeshima, T., 1999. Improvement by nefiracetam of β-amyloid-(1–42)-induced learning and memory impairments in rats. Br. J. Pharmacol. 126, 235–244.
Zubenko, G.S., 2000. Neurobiology of major depression in Alzheimer's disease. Int. Psychogeriatr. 12, 217–230.
Zubenko, G.S., Moossy, J., 1988. Major depression in primary dementia: clinical and neuropathological correlates. Arch. Neurol. 45, 1182–1186.
Zubenko, G.S., Zubenko, W.N., McPherson, S., Spoor, E., Marin, D.B., Farlow, M.R., Smith, G.E., Geda, Y.E., Cummings, J.L., Petersen, R.C., Sunderland, T., 2003. A collaborative study of the emergence and clinical features of the major depressive syndrome of Alzheimer's disease. Am. J. Psychiatry 160, 857–866.

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Research report

β-Amyloid (1–42)-induced learning and memory deficits in mice: involvement of oxidative burdens in the hippocampus and cerebral cortex

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Abstract

We have demonstrated that oxidative stress is involved, at least in part, in β-amyloid protein (Aβ)-induced neurotoxicity in vivo [Eur. J. Neurosci. 1999;11:83–90; Neuroscience 2003;119:399–419]. However, mechanistic links between oxidative stress and memory loss in response to Aβ remain elusive. In the present study, we examined whether oxidative stress contributes to the memory deficits induced by intracerebroventricular injection of Aβ (1–42) in mice. Aβ (1–42)-induced memory impairments were observed, as measured by the water maze and passive avoidance tests, although these impairments were not found in Aβ (40–1)-treated mice. Treatment with antioxidant α-tocopherol significantly prevented memory impairment induced by Aβ (1–42). Increased activities of the cytosolic Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and mitochondrial Mn-superoxide dismutase (Mn-SOD) were observed in the hippocampus and cerebral cortex of Aβ (1–42)-treated animals, as compared with Aβ (40–1)-treated mice. The induction of Cu,Zn-SOD was more pronounced than that of Mn-SOD after Aβ (1–42) insult. However, the concomitant induction of glutathione peroxidase (GPX) in response to significant increases in SOD activity was not seen in animals treated with Aβ (1–42). Furthermore, glutathione reductase (GRX) activity was only increased at 2 h after Aβ (1–42) injection. Production of malondialdehyde (lipid peroxidation) and protein carbonyl (protein oxidation) remained elevated at 10 days post-Aβ (1–42), but the antioxidant α-tocopherol significantly prevented these oxidative stresses. Therefore, our results suggest that the oxidative stress contributes to the Aβ (1–42)-induced learning and memory deficits in mice.

Keywords: Alzheimer's disease; Amyloid beta protein; Superoxide dismutase; Glutathione peroxidase; Oxidative stress; Hippocampus; Memory impairments

1. Introduction

Alzheimer's disease (AD) is the most common cause of progressive cognitive impairment in the elderly [16,29,48]. The characteristic neuropathology of AD is the accumulation of senile plaques and neurofibrillary tangles in vulnerable brain regions. The senile plaques are primarily composed of amyloid beta peptide (Aβ), which is a 40–42 amino acid peptide fragment of the amyloid protein precursor that plays an important role in the development of AD. However, the mechanism by which Aβ causes neuronal injury and cognitive impairment is not yet clearly understood.

Nabeshima and his colleagues demonstrated that a continuous infusion of Aβ (1–40) into the cerebral ventricle in rats results in learning and memory deficits that were accompanied by a reduction of choline acetyltransferase activity, suggesting that accumulation of Aβ is related to cognitive

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impairments in AD [39,40]. Memory impairment induced by Aβ (1–42) is potentiated by the long-term deprivation of estrogens in female rats [50]. In rats treated with Aβ (1–40), dysfunction of cholinergic and dopaminergic neuronal systems are observed, as evidenced by the decrease in the nicotine- and KCl-induced stimulation of acetylcholine and dopamine release in vivo [18]. Furthermore, long-term potentiation is impaired in the CA1 field of the hippocampal slices prepared from the rat brain after continuous i.c.v. infusion of Aβ (1–40) [19].

In addition, they demonstrated that the continuous infusion of Aβ (1–40) into the cerebral ventricle induced a time-dependent expression of inducible nitric oxide synthase and an overproduction of nitric oxide in the hippocampus although Aβ (40–1) had no effect [46]. The Aβ-induced overproduction of nitric oxide which reacts rapidly with superoxide radical to yield highly reactive peroxynitrite caused an increase in tyrosine nitration of a synaptic protein synaptophysin in the hippocampus [47]. We have also demonstrated that the prolonged infusion of Aβ (1–42) results in a significant reduction of the immunoreactivity of antioxidant enzymes in the rat brain areas, although the same treatment with Aβ (40–1) had little effect [25].

Evidence suggests that oxidative stresses are involved in the mechanism of Aβ-induced neurotoxicity [4–6], and AD pathogenesis [34,53]. For example, exposure to Aβ increases lipid peroxidation, protein oxidation, and the formation of hydrogen peroxide in cultured cells [2]. Similarly, increases in lipid peroxidation, protein carbonyl and oxidation of mitochondrial DNA have been observed in the brains of AD patients [31]. Yamada et al. [51] demonstrated that treatment with antioxidants, such as idebenone and α-tocopherol prevents the learning and memory deficits induced by Aβ (1–42). However, they did not find increased lipid peroxidation in the brains of the Aβ (1–42)-infused rats [51].

To examine the hypothesis that oxidative stress is involved in the learning and memory deficits evoked by Aβ, we used different experimental conditions to those of Yamada et al. [51]. We investigated the time course (2 h, 2, 4, 10, and 20 days after Aβ injection), and changed the administration method [a single i.c.v. injection of Aβ (1–42)], and used a different animal model (mice) in the present study. It has not yet been demonstrated whether Aβ (1–42)-induced changes in the activities of endogenous antioxidant enzymes contribute to oxidative stress and memory function changes in the animal models. Thus, in this study, we examined the effects of the potent antioxidant α-tocopherol on memory function, antioxidant enzyme activity, lipid peroxidation, and protein oxidation, after the administration of Aβ (1–42) or Aβ (40–1) in mice. In young mice, Aβ is initially deposited in the cingulate cortex carrying the mutant amyloid precursor protein (APP), but it rapidly encroaches on the entire cerebral cortex and hippocampus [35]. In this study, we focused on these brain regions using old mice.

2. Materials and methods

2.1. Animals and amyloid β-peptide (Aβ) administration

All animals were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals. Fifteen months old male C57BL/6 mice (Bio Genomics, Inc., Charles River Technology, Gapyung-Gun, Gyeonggi-Do, Korea) weighing about 45 ± 2 g were maintained on a 12:12 h light:dark cycle and fed ad libitum. They were adapted for 2 weeks to the above conditions before experiment.

Aβ (1–42; US Peptide, CA, USA) and Aβ (40–1; Bachem, Torrance, CA, USA) were dissolved in 35% acetonitrile containing 0.1% trifluoroacetic acid. The Aβ (1–42) or Aβ (40–1) administration [400 pmol, intracerebroventricular injection (i.c.v.)] was performed according to the procedure established by Laursen and Belknap [26]. The dose of Aβ is comparable to that of Yan et al. [52]. Briefly, each mouse was injected (without anesthesia) at bregma with a 50 μl Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. The injection volume was 5 μl. The injection placement or needle track was visible and was verified at the time of dissection.

2.2. Experimental design

α-Tocopherol (150 mg/kg, p.o.) was dissolved in soybean oil, and administered orally in a volume of 1 ml/kg, to Aβ (1–42)-treated mice for 27 consecutive days. The experimental schedule is shown in Fig. 1. α-Tocopherol administration began 7 days before Aβ (1–42) i.c.v. injection, and continued throughout the experimental period. The behavioural study began on day 3 after Aβ (1–42) i.c.v. injection, and carried out sequentially. In the behavioural study, α-tocopherol administration was carried after behavioural test to avoid a direct effect on performance. Mice were sacrificed at 2 h, 2, 4, 10 and 20 days after Aβ (1–42) i.c.v. injection to examine enzyme activities (Cu,Zn-superoxide dismutase, Mn-superoxide dismutase, glutathione peroxidase, and glutathione reductase) and oxidative stresses (malondialdehyde and protein carbonyl) in the brain.

2.3. Water maze test

The apparatus was a circular water, 97 cm in diameter and 60 cm height. During testing, the tank was filled with water (23 ± 2 °C) that was clouded with powered milk. A transparent platform was set inside the tank and its top was submerged 2 cm below the water surface in the center of one among the four quadrants of the maze. The tank was located in a large room with many extramaze cues that were constant throughout the study [37,39]. The movements of the animal in the tank were monitored with a video tracking system (EthoVision, Noldus, The Netherlands).

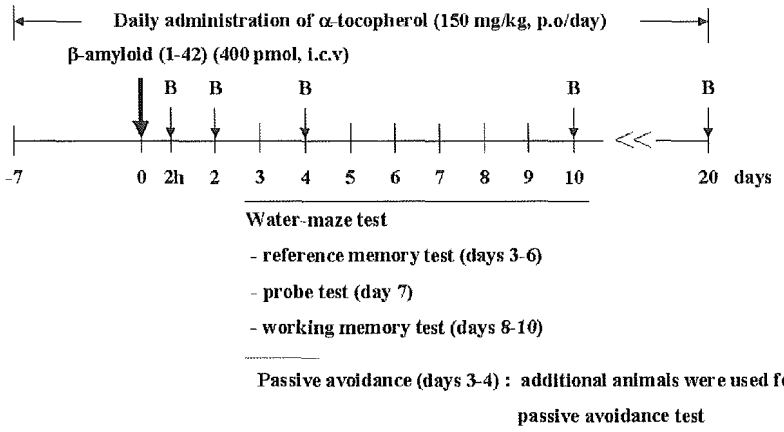

Fig. 1. Experimental schedule. B: biochemical measurements (enzyme activity, lipid peroxidation, and protein oxidation).

2.3.1. Reference memory test

For each training trial, the mouse was put into the pool at one of the five positions, the sequence of the positions being selected randomly. The platform was located a constant position throughout the test period in the middle of one quadrant, equidistant from the center and edge of the pool. In each training session, the latency to escape on to the hidden platform was recorded. If the mouse found the platform, it was allowed to remain there for 10 s and was then returned to its home cage. If the mouse was unable to find the platform within 60 s, the training was terminated and a maximum score of 60 s was assigned. Training was conducted for four consecutive days, four times a day, from day 3 to 6 after the start of Aβ i.c.v. injection.

2.3.2. Probe test

On day 7 after the start of Aβ i.c.v. injection, a single probe trial was conducted. The platform was removed from the pool and each mouse was allowed to swim for 60 s in the maze. The number of times the mouse crossed the annulus where the platform had been located was recorded.

2.3.3. Working memory (repeated acquisition) test

Working memory test was conducted three consecutive days from day 8 to 10 and consisted of five trials per day. The working memory test was procedurally similar to reference memory test except that the platform location was changed daily. The first trial of the day was an informative sample trial in which the mouse was allowed to swim to the platform in its new location. Spatial working memory was regarded as the mean escape latency of the second to fifth trials.

2.4. Passive avoidance test

Passive avoidance was measured using a Gemini Avoidance System (San Diego Instrument, San Diego, CA) which consists of two-compartment shuttle chambers with a constant current shock generator. On an acquisition trial, each mouse was placed into the start chamber, which remained darkened. After 20 s, the chamber light was illuminated and the door was opened for mouse to move into the dark chamber freely. Immediately it entered the dark chamber, the door was closed and an inescapable scrambled electric shock (0.3 mA, 3 s, once) was delivered through the floor grid. Then the mouse was returned to its home cage. Twenty-four hours later, each mouse was again placed in the start chamber again (retention trial). The interval between the placement in the lighted chamber and the entry into the dark chamber was measured as latency in both acquisition and retention trials (maximum 300 s) [51,52].

2.5. Tissue preparation

Mice were anesthetized with pentobarbital (50 mg/kg) and then perfused transcardially with ice-cold 0.9% NaCl (10 ml/10 g body weight) to remove the free radical-scavenging and -generating sources in the brain [23]. The cerebral cortex, hippocampus were carefully excised, and the tissues stored at −70 °C. Homogenates were sonicated for 30 s in a cell disruptor (Bronson Sonic, NY) and centrifuged at 10,000 × *g* for 20 min. The resulting supernatant was used to measure activities of glutathione peroxidase (GPX) and glutathione reductase (GRX).

2.6. Determination of superoxide dismutase (SOD)

Homogenates of hippocampus or cerebral cortex were centrifuged at 25,000 × *g* for 15 min at 4 °C and supernatant dialyzed in 50 mM PBS (pH 7.8) containing 1 mM EDTA. SOD activity was determined based on inhibition of superoxide-dependent reactions. The reaction mixture contained 70 mM potassium phosphate buffer (pH 7.8), 30 μM cytochrome *c*, 150 μM xanthine, and tissue extract in phosphate buffer diluted 10 times with PBS in a final volume of

3 ml. The reaction was initiated by adding 10 μl of 50 units xanthine oxidase, and the change in absorbance at 550 nm recorded. One unit of SOD is defined as the quantity required to inhibit the rate of cytochrome *c* reduction by 50%. For estimating total SOD, 10 μM potassium cyanide (KCN) was added to the medium to inhibit cytochrome oxidase activity [32]. For estimating Mn-SOD activity, 1 mM KCN was added to the incubation mixture to inhibit Cu,Zn-SOD activity [32]. The activity of Cu,Zn-SOD was calculated by the subtraction of the Mn-SOD activity from the total SOD activity.

2.7. Determination of glutathione peroxidase (GPX)

GPX activities of the hippocampus and cortex were analyzed by a spectrophotometric assay described by Lawrence and Burk [27], using 2.0 mM reduced glutathione and 0.25 mM H_2O_2 as substrate. One unit of GPX is defined as the quantity that catalyzes the oxidation of 1 nM NADPH/min at 25 °C. Protein was measured using the BCA protein assay reagent and bovine serum albumin was used as a standard.

2.8. Determination of glutathione reductase (GRX)

GRX activities were measured based on the method discribed by Eklow et al. [13]. The reaction mixture contained 1 mM oxidized glutathione and 100 μl of sample in phosphate buffer (pH, 7.0) containing 1 mM EDTA. The reaction started with adding NADPH (final concentration of 0.11 mM) and the decrease in absorbance of NADPH at 340 nm was measured. One unit of activity is defined as 1 nM of NADPH oxidized.

2.9. Determination of malondialdehyde (MDA)

The amount of MDA in homogenates of hippocampus, cerebral cortex was determined by the methods of Jareno et al. [20] with some modification [22,23]. In brief, 0.1 ml of the homogenate diluted 10 times with phosphate buffered saline (PBS) was mixed with 0.75 ml working solution (thiobarbituric acid 0.37% and perchloric acid 6.4%, 2:1, v/v) and heated to 95 °C for 1 h. After cooling (10 min in ice water bath), the flocculent precipitate was removed by centrifugation at 3200 × *g* for 10 min. The supernatant was neutralized and filtered prior to injection on a octadecylsilane 5 μm column. Mobile phase consisted of 50 mM PBS (pH 6.0):methanol (58:42, v/v). Isocratic separation with 1.0 ml/min flow rate and detection at 532 nm using a UV–vis high-performance liquid chromatography detector were performed.

2.10. Determination of protein carbonyl

The extent of protein oxidation in the cerebral cortex and hippocampus was assessed by measuring the content of protein carbonyl groups, which was determined spectrophotometrically with the 2,4-dinitrophenylhydrazine (DNPH)-labeling procedure as described by Oliver et al. [41]. The results are expressed as nmol of DNPH incorporated/mg protein [23] based on the extinction coefficient for aliphatic hydrazones of 21 mM^{-1} cm^{-1}. Protein was measured using the BCA protein assay reagent (Pierce, Rockford, IL, USA).

2.11. Statistics

Data are expressed as the mean ± S.E.M. The statistical significance in the behavioral and biochemical effects of α-tocopherol was determined by one-way analysis of variance (ANOVA), followed by Bonferroni's test or ANOVA with Duncan's new multiple (DMR) test. Two-way ANOVA was conducted to analyze data from water maze training trials.

3. Results

3.1. Effects of α-tocopherol on performance of the water maze task by Aβ (1–42)-treated mice

The changes in escape latency onto a hidden platform produced by training trials are shown in Fig. 2A. Two-way ANOVA with all treatment groups showed significant effects mainly of group ($F_{3576} = 9.084$, $P = 6.97 \times 10^{-6}$) and training ($F_{15.576} = 13.772$, $P = 4.82 \times 10^{-30}$), but not group by trial interactions ($F_{45.576} = 0.449311$, $P = 1.00$). The escape latencies of Aβ(1–42)-injected mice were significantly delayed, as compared to those of Aβ(40–1)-injected mice ($P < 0.0005$; post-hoc analysis). Prolonged treatment with α-tocopherol significantly improved the Aβ(1–42)-induced impairment of performance ($P < 0.05$; post-hoc analysis).

The results of the 60 s probe test also showed significant memory impairment in the Aβ (1–42)-treated mice. The number of annulus crossings, representing the number of passes over the platform site, was significantly decreased ($F_{118} = 5.40$, $P = 0.032$) in the Aβ (1–42)-treated mice as compared to Aβ (40–1)-treated mice. This was significantly reversed ($F_{118} = 6.08$, $P = 0.024$) by α-tocopherol administration (one-way ANOVA) (Fig. 2B).

Working memory was assessed as the mean escape latency of the second to the fifth trials (test trials), for three days. There were no significant differences among the four groups in the sample trials (the first trial) of three consecutive days (data not shown). However, the mean escape latency during test trials of the Aβ (1–42)-treated mice was significantly longer ($F_{1238} = 5.06$, $P = 0.026$) than that of the Aβ(40–1)-treated mice. Repeated treatment with α-tocopherol significantly ameliorated ($F_{1238} = 4.13$, $P = 0.043$) the Aβ(1–42)-induced impairment of performance in working memory test (one-way ANOVA). There was no significant difference between Aβ(40–1)-treated mice and α-tocopherol- plus Aβ(40–1)-treated mice in the hidden platform test, probe, or working memory tests (Fig. 2C).

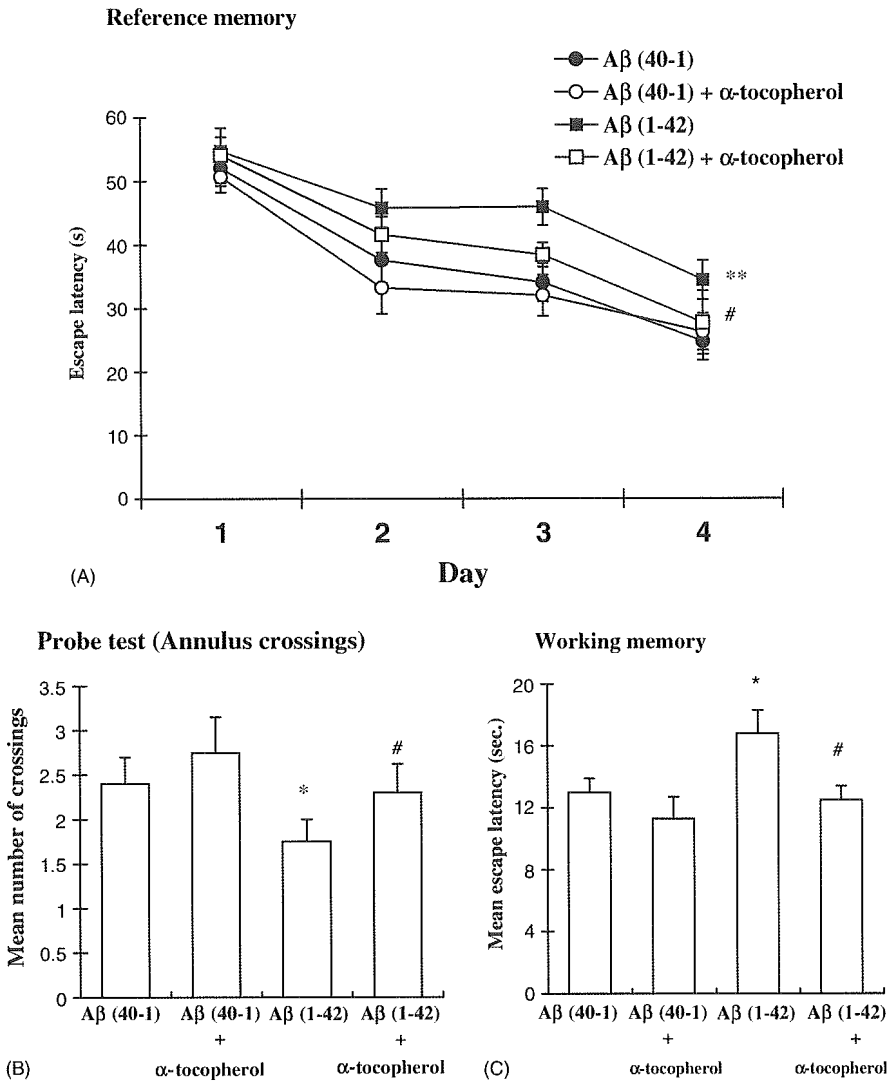

Fig. 2. The effects of α-tocopherol on the performance of Aβ (1–42)-treated mice in the training (A), probe trial (B), and working memory (C) trials of the water maze test. The training trials were carried out on days 3–6 (four per day), the probe trials were performed on day 7, and the working memory trials (five per day) were carried out on days 8–10 after the i.c.v. injection of Aβ. Each value is the mean ± 10 animals. The statistical significance of the training trials was calculated using two-way ANOVA followed by post-hoc analysis, and those of the probe and working memory trials were calculated using one-way ANOVA. $^{*}P < 0.05$ or $^{**}P < 0.0005$ vs. Aβ (40–1)-treated mice, $^{\#}P < 0.05$ vs. Aβ (1–42)-treated mice.

3.2. Effects of α-tocopherol on performance of the passive avoidance task by Aβ(1–42)-treated mice

In the acquisition trial, the step-through latencies did not differ among the four groups. The step-through latency in the retention trial was significantly decreased ($F_{118} = 19.79$, $P = 0.00031$) in the Aβ(1–42)-treated mice, as compared to the Aβ(40–1)-treated mice. The α-tocopherol-plus Aβ(1–42)-treated mice showed a significantly longer step-through latency ($F_{118} = 4.65$, $P = 0.045$) than did the Aβ(1–42)-treated mice. There were no significant differences between the Aβ(40–1)-treated and α-tocopherol- plus Aβ (40–1)-treated mice in the acquisition trial or retention trials (Fig. 3).

3.3. Effects of α-tocopherol on Cu,Zn-SOD activity in the brains of Aβ (1–42)-treated mice

Cu,Zn-SOD activity did not significantly change in the absence of Aβ (1–42). However, Aβ (1–42) treatment resulted in early increases in Cu,Zn-SOD activity in the cerebral cortex and hippocampus [at 2 h and 2 days: $P < 0.01$ versus Aβ (40–1); at 4 days: $P < 0.05$ versus Aβ (40–1)]. The enzyme activity in the cerebral cortex was comparable

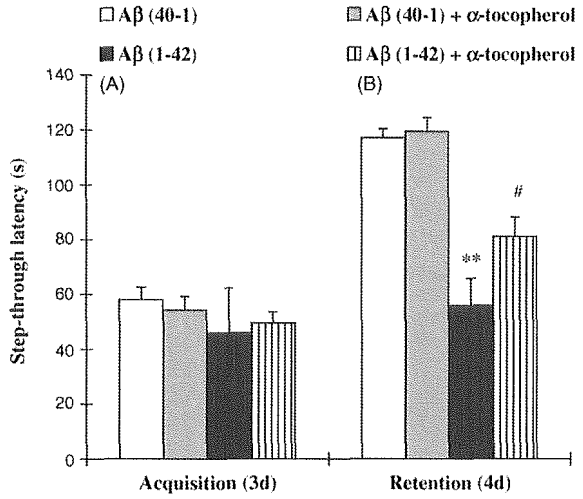

Fig. 3. The effects of α-tocopherol on the performance of Aβ (1–42)-treated mice in the acquisition (A) and retention (B) trials of the passive avoidance task. The task was performed on days 3–4 after the i.c.v. injection of Aβ. Each value is the mean ± 10 animals. $^{**}P < 0.0005$ vs. Aβ (40–1)-treated mice, $^{\#}P < 0.05$ vs. Aβ (1–42)-treated mice (one-way ANOVA).

to that in the hippocampus. Increases in Cu,Zn-SOD activity induced by Aβ (1–42) were significantly attenuated in the presence of α-tocopherol [at 2 h and 2 days: Aβ (1–42) versus Aβ (1–42) plus α-tocopherol, $P < 0.05$]. Increases in Cu,Zn-SOD activity had returned to near vehicle or Aβ (40–1) levels at 10 days (Fig. 4A).

3.4. Effects of α-tocopherol on Mn-SOD activity in the brains of Aβ (1–42)-treated mice

The animals treated with non-toxic Aβ (40–1) or vehicle did not significantly induce Mn-SOD. Similar to Cu,Zn-SOD activity, Mn-SOD activity was significantly induced in the cerebral cortex and hippocampus with Aβ (1–42) treatment [at 2 h and 2 days: $P < 0.05$ versus Aβ (40–1)]. The enzyme activity in the hippocampus appeared to be more susceptible than the cerebral cortex activity to Aβ (1–42). The Aβ (1–42)-caused increases in Mn-SOD activity were significantly prevented by prolonged treatment with α-tocopherol [at 2 h and 2 days (both brain regions): Aβ (1–42) versus Aβ (1–42) plus α-tocopherol, $P < 0.05$]. The increases in Mn-SOD activity had returned to near vehicle or Aβ (40–1) levels at 4 days (Fig. 4 B).

3.5. Effects of α-tocopherol on GPX activity in the brains of Aβ (1–42)-treated mice

The GPX activity did not change without Aβ (1–42) treatment. However, GPX activity was significantly increased in the cerebral cortex and hippocampus at 2 h and 2 days after i.c.v. injection of Aβ (1–42) [at 2 h and 2 days: $P < 0.05$ versus Aβ (40–1)]. Interestingly, GPX activities were also significantly induced in animals treated with Aβ (1–42) plus α-tocopherol [at 2 h and 2 days: Aβ (40–1) plus α-tocopherol versus Aβ (1–42) plus α-tocopherol, $P < 0.05$]. The GPX activity in animals treated with Aβ (1–42) was comparable to that in animals treated with Aβ (1–42) plus α-tocopherol, suggesting that α-tocopherol induced the compensative induction of GPX. No significant changes in GPX activity were observed in the cerebral cortex and hippocampus after 4 days (Fig. 5A).

3.6. Effects of α-tocopherol on GRX activity in the brains of Aβ (1–42)-treated mice

GRX activity was not apparently induced without exposure to Aβ (1–42). However, GRX activity was significantly increased at the very early stage (2 h) post-Aβ(1–42) insult [at 2 h: $P < 0.05$ versus Aβ (40–1)]. Similar to GPX, GRX activity was significantly induced in the animals treated with Aβ (1–42) plus α-tocopherol [at 2 h: Aβ (40–1) plus α-tocopherol versus Aβ (1–42) plus α-tocopherol, $P < 0.05$]. The GRX activity of animals treated with Aβ (1–42) is comparable to that of animals treated with Aβ (1–42) plus α-tocopherol. The increases in GRX activity had almost returned to vehicle or Aβ (40–1) levels in the cerebral cortex and hippocampus at 2 days (Fig. 5B).

3.7. Effects of α-tocopherol on lipid peroxidation levels in the brains of Aβ (1–42)-treated mice

No differences in the lipid peroxidation levels (as MDA) were observed in the cerebral cortex or hippocampus of vehicle- and Aβ(40–1)-treated animals. Aβ(1–42)-induced increases in MDA levels still evident at 10 days [in the cerebral cortex at 2 h, 2, 4, and 10 days; $P < 0.01$, $P < 0.05$, $P < 0.05$, and $P < 0.05$ versus Aβ(40–1); in the hippocampus at 2 h, 2, 4, and 10 days; $P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.05$ versus Aβ(40–1)]. These increases in MDA levels were significantly attenuated by the treatment with α-tocopherol [at 2, 4, and 10 days in the cerebral cortex and hippocampus: Aβ (1–42) versus Aβ (1–42) plus α-tocopherol, $P < 0.05$]. MDA values had almost returned to those of vehicle or Aβ(40–1) levels at 20 days after Aβ(1–42) injection (Fig. 6A).

3.8. Effects of α-tocopherol on protein oxidation levels in the brains of Aβ (1–42)-treated mice

No significant changes in protein carbonyl were found in the vehicle- and Aβ(40–1)-treated animals. Similar to the MDA time course, the Aβ(1–42)-induced increases in the formation of protein carbonyl lasted for at least 10 d [in the cerebral cortex at 2 h, 2, 4, and 10 days; $P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.05$ versus Aβ(40–1); in the hippocampus at 2 h, 2, 4, and 10 days; $P < 0.01$, $P < 0.05$, $P < 0.05$ and $P < 0.05$ versus Aβ(40–1)]. This significant formation of

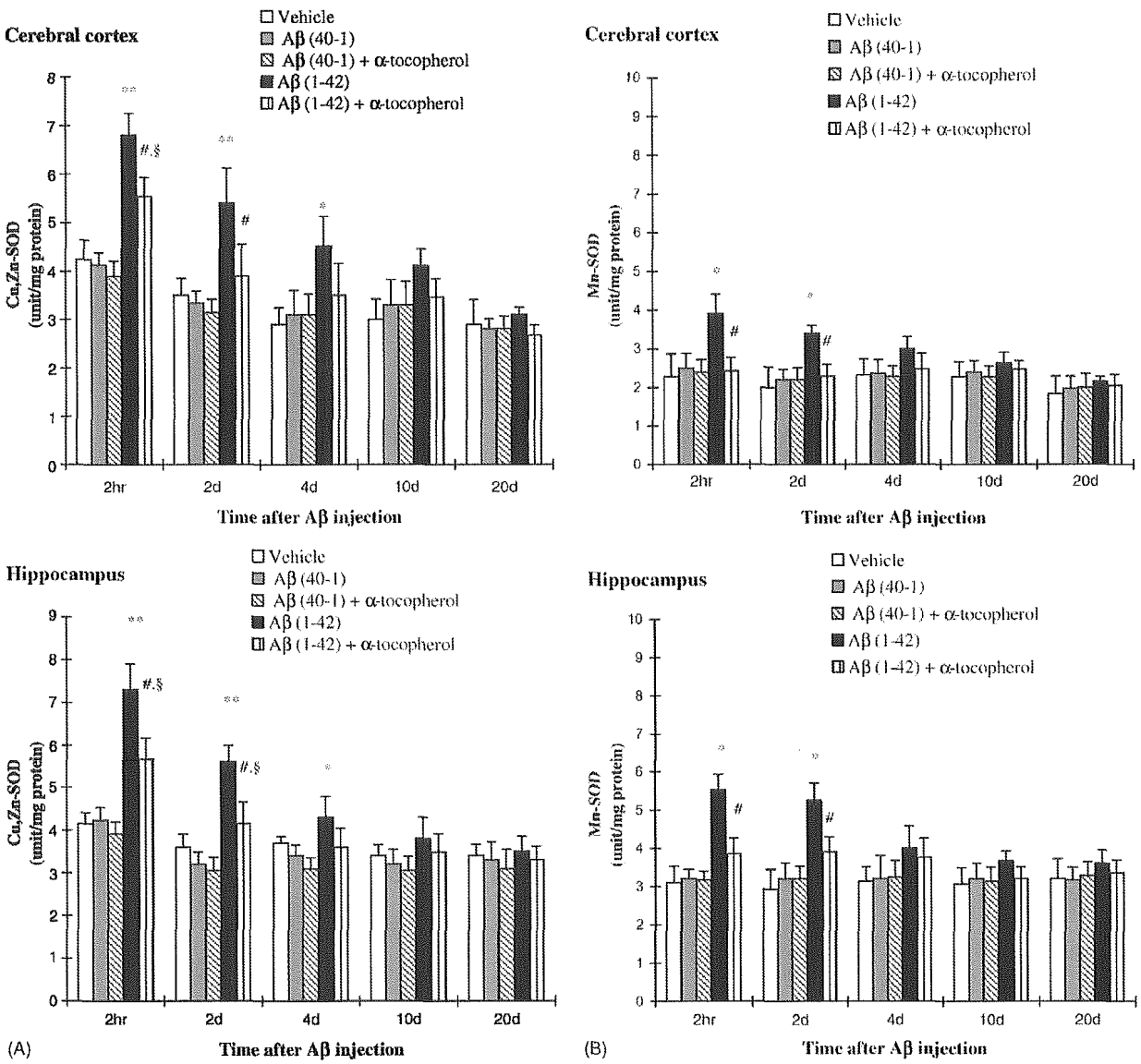

Fig. 4. The effects of α-tocopherol on the activity changes of Cu,Zn-SOD (A) and Mn-SOD (B) induced by Aβ in the cerebral cortex and hippocampus of the mice. Each value is the mean ± S.E.M. of 10 animals. $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. Aβ (40–1), $^{\#}P < 0.05$ vs. Aβ (1–42), $^{\S}P < 0.05$ vs. Aβ(40–1) + α-tocopherol (ANOVA with DMR test).

protein carbonyl was significantly inhibited by the chronic treatment with α-tocopherol [at 2 h, 2, 4, and 10 days in the cerebral cortex and hippocampus: Aβ (1–42) versus Aβ (1–42) plus α-tocopherol, $P < 0.05$]. These protein carbonyl levels had returned to near basal values at 20 days after Aβ(1–42) injection (Fig. 6B).

4. Discussion

In the present study, we demonstrated that a single i.c.v. injection of Aβ (1–42) impaired performance in a water maze task and the retention of long-term memory in a passive avoidance task, in accordance with previous investigations using mice [52] and rats [39,40,51]. Similarly, acute i.c.v. injection of Aβ (25–35) in mice has also been reported to impair spatial reference memory in a water maze task [36]. These results suggest that the accumulation of neurotoxic Aβ fragments, such as Aβ (1–42) and Aβ (25–35), impairs spatial reference memory in mice, although nontoxic Aβ fragment Aβ (40–1) has no effect. Furthermore, the potent antioxidant α-tocopherol also attenuated these memory impairments (resulting in impaired performance in the passive avoidance and water maze tests) induced in mice

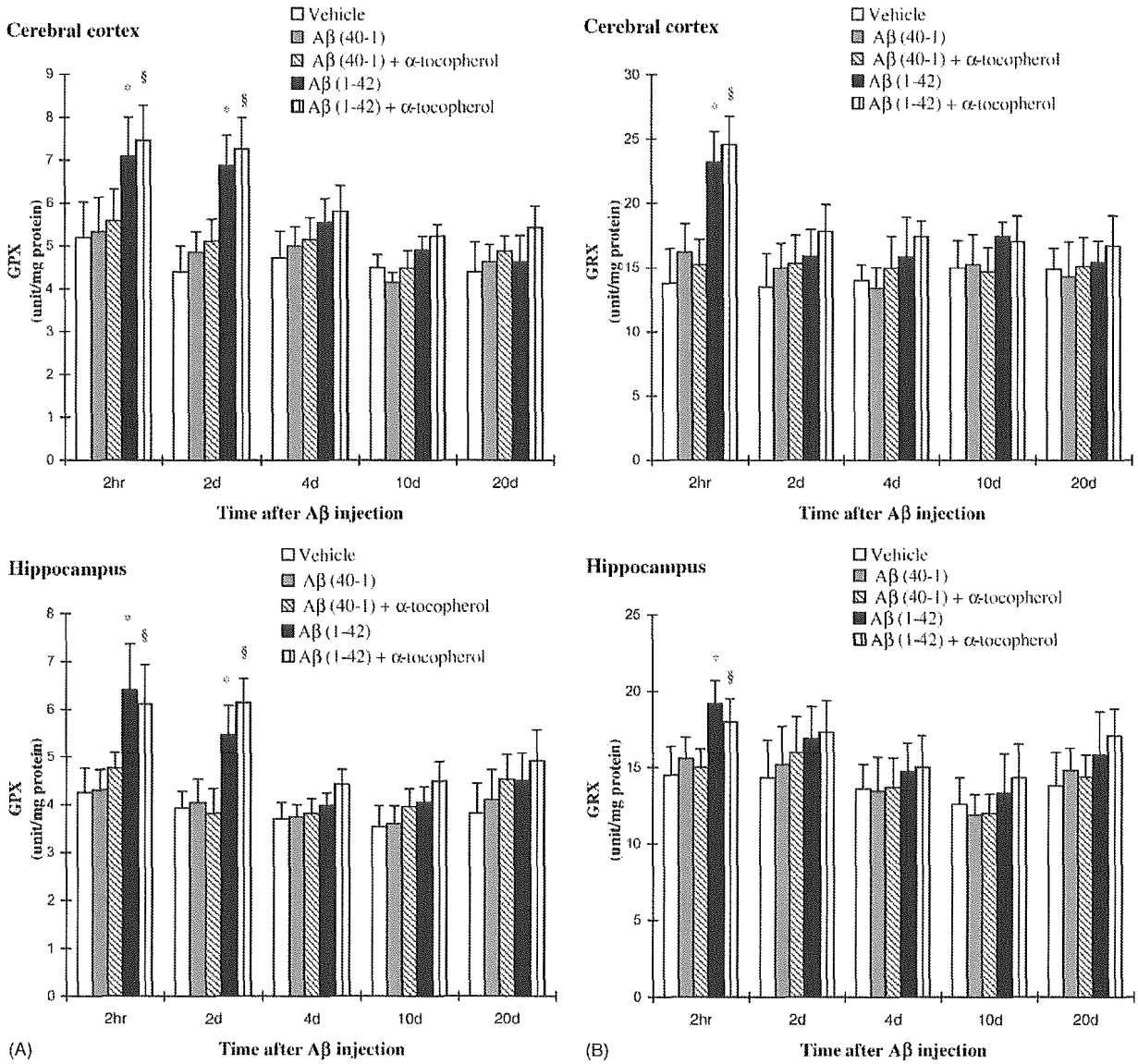

Fig. 5. The effects of α-tocopherol on the activity changes of GPX (A) and GRX (B) induced by Aβ in the cerebral cortex and hippocampus of the mice. Each value is the mean ± S.E.M. of 10 animals. $^{*}P < 0.05$ vs. Aβ (40–1), $^{\S}P < 0.05$ vs. Aβ(40–1) + α-tocopherol (ANOVA with DMR test).

by the single i.c.v. injection of Aβ (1–42). This result supports previous finding, demonstrated by Yamada et al. [51], that α-tocopherol and another antioxidant, idebenone, prevent behavioral deficits in water maze task performance in Aβ (1–42)-infused rats. However, Yamada et al. [51] did not observe increased formation of malondialdehyde (MDA) in the cerebral cortex and hippocampus of the Aβ (1–42)-infused rats at day 19 after the start of Aβ (1–42)-infusion. Similarly, we could not find a significantly increased level of MDA in mice at day 20 after a single injection with Aβ (1–42) in this study, although the levels of MDA and protein carbonyl in the cerebral cortex and hippocampus remained elevated for 10 days.

Recently, we demonstrated that continuous i.c.v. infusion of Aβ (1–42) in rats resulted in a significant decrease of the protein expressions of Mn-SOD, GSH, GPX and glutathione-*S*-transferase-π in the rat brain, suggesting that Aβ (1–42) impairs antioxidant capacity [25]. This finding indicates the cytological effects of oxidative stress induced by Aβ (1–42). In certain antioxidant systems, there might be a time lag between the synthesis of protein and the expression of mRNA following neurotoxicity

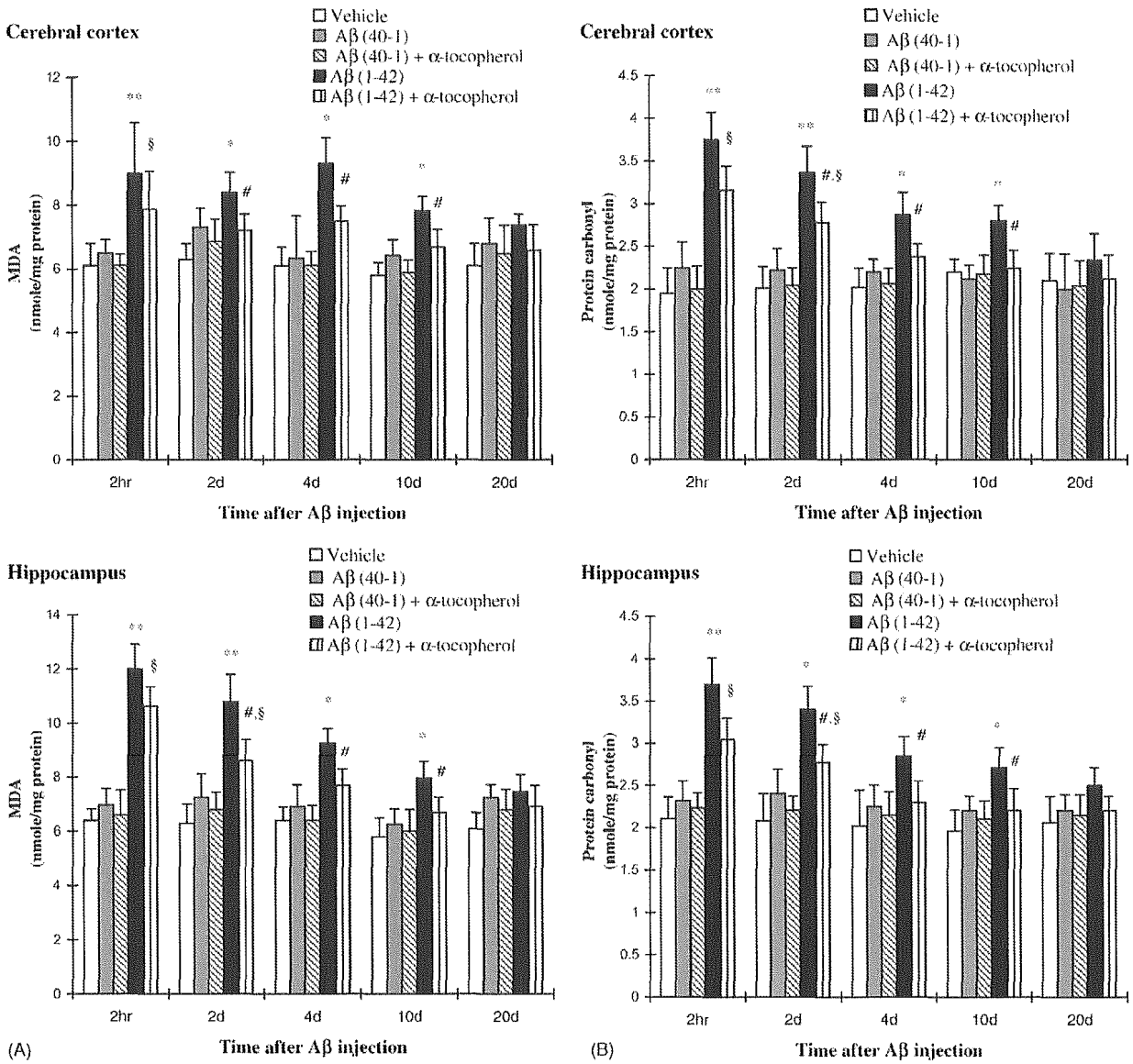

Fig. 6. The effects of α-tocopherol on the formation changes of MDA (A) and protein carbonyl (B) induced by Aβ in the cerebral cortex and hippocampus of the mice. Each value is the mean ± S.E.M. of 10 animals. $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. Aβ (40–1), $^{\#}P < 0.05$ vs. Aβ (1–42), $^{\S}P < 0.05$ vs. Aβ(40–1) + α-tocopherol (ANOVA with DMR test).

[1,24]. Therefore, measurements of the activity time course of antioxidant enzymes are important when comparing the alterations induced by Aβ with those found in AD patients.

We observed significant induction of Cu,Zn-SOD, Mn-SOD, GPX and GRX activities in the cerebral cortex and hippocampus of the Aβ (1–42)-treated mice, although these changes were not observed in Aβ (40–1)-treated mice. To our knowledge, this is the first time that Aβ-induced increases of the activity of overall antioxidant enzymes have been described in the brain of an animal model. The increases in the activity of Cu,Zn-SOD were the most pronounced among these enzymatic antioxidants. The elevation in Cu,Zn-SOD activity appeared to parallel increases in MDA and protein carbonyl in the same areas, suggesting that these events are needed to scavenge superoxide radicals induced by Aβ (1–42).

The present findings support the hypothesis that increased Cu,Zn-SOD activity can lead to an accumulation of H_2O_2, which, in the absence of a simultaneous increase in GPX activity, could increase the Fenton reaction, leading to the stimulation of lipid peroxidation and protein oxidation resulting,

in cellular damage [6,11,22]. Furthermore, an uncompensated excess of Cu,Zn-SOD may contribute to the loss of neurons in selective areas in AD. This idea is supported by the fact that Down's syndrome, which includes a triplication of the Cu, Zn SOD gene and correspondingly increased enzyme activity, invariably involves degenerative changes similar to Alzheimer's disease [8]. Overall, these findings suggest that Cu,Zn SOD may play a role in Alzheimer's pathology.

Extensive evidence exists for lipid peroxidation being an important mechanism of neurodegeneration in the AD brain. Aβ is widely reported to cause lipid peroxidation that is inhibited by antioxidants in brain cell membranes [4,12,52]. In addition, Aβ leads to 4-hydroxy-2-nonenal (HNE) and acrolein formation [33], and these alkenals alter the conformation of membrane proteins [45]. We have previously shown, consistently, an induction of HNE and 8-hydroxyguanosine (a marker of oxidative damage to DNA and RNA) immunoreactivities following infusion of Aβ (1–42) [21]. Protein oxidation is an important factor in aging and age-related neurodegenerative disorders [28,44]. Oxidative modification of proteins can lead to diminished functions for specific proteins [28,44]. Protein oxidation is most often indexed by the presence of protein carbonyls [44], which arise from a direct free radical attack on vulnerable amino acids side chains or from the products of glycation, glycoxidation, and lipid peroxidation reactions with protein (e.g., HNE and acrolein) [5]. The increased protein carbonyl in the AD brain, together with the altered activities of antioxidant enzymes (in some cases due to oxidation), coupled with studies showing that Aβ (1–42)- or Aβ (1–40)-induced neuronal protein oxidation can be inhibited by antioxidants [54], suggest that Aβ-induced protein oxidation may account, in part, for neurodegeneration in the AD brain.

α-Tocopherol is the most prevalent and efficacious lipid-soluble antioxidant in biological systems and inhibits the chain reaction of lipid peroxidation by trapping the chain carrying peroxyl radicals [38]. α-Tocopherol is reported to prevent the spatial learning deficit induced by i.c.v. injection of the cholinotoxin ethylcholine mustard aziridinium ion (AF64A), without affecting memory in control animals [49]. The ability of a number of antioxidants, including idebenone, α-tocopherol, and iron chelators, to abrogate GSH depletion-induced neurotoxicity [42] suggests that exogenously applied antioxidants are balancing the cellular antioxidant debt produced by GSH depletion. Furthermore, investigations in vitro have shown that α-tocopherol inhibits Aβ-induced neuronal cell death and protein oxidation [55], suggesting that oxidative stress is one of the mechanisms of the neurotoxicity of Aβ. Here, we confirmed that the imbalances in enzymatic antioxidants and the increases in lipid peroxidation and protein oxidation induced by Aβ (1–42) are prevented by α-tocopherol, confirming that oxidative stress is involved in at least part of the neurotoxic mechanism of the Aβ (1–42) in vivo.

Because elevated levels of copper, zinc, and iron are found in amyloid deposits of AD-affected brains [30], the oxidative stress observed in AD may be related to the production of reactive oxygen species by metal-bound forms of Aβ. Huang et al. [17] have shown that the binding of Cu^{2+} and Zn^{2+} to Aβ modulates the toxicity of the peptide through the generation of H_2O_2 by electron transfer to O_2. The binding of Cu^{2+} and Zn^{2+} to Aβ also induces the precipitation of the peptide [3,30]. Increased binding of these metals to Aβ is evident in AD [30], and it has been demonstrated that the metal-mediated redox activity and aggregation of Aβ, as well as amyloid deposition in APP 2576 transgenic mice, are inhibited by treatment with a bioavailable zinc/copper selective chelator, clioquinol [10]. Because α-tocopherol possesses metal chelating effects [9], we cannot rule out the possibility that the metal chelating effects of α-tocopherol may play a role in attenuating the imbalance of antioxidant enzymes and oxidative stress induced by Aβ (1–42) in mice.

Although Mn-SOD, in contrast to Cu,Zn-SOD, appeared to be not highly induced in the brain regions we examined, Aβ (1–42)-treated mice showed increased Mn-SOD activities at an early time point (2 days). This may be due to the formation of H_2O_2 in the mitochondria [7]. The relationship between mitochondrial damage, glutathione status/GSH dependent enzymes, oxidative stress, and neuronal dysfunction has been demonstrated by the effects of excessive production of H_2O_2 within mitochondria, which leads to depletion of mitochondrial GSH, in turn, causes the oxidation of protein thiols and the impairment of mitochondrial function [14]. Thus, it is possible that the loss of GSH may result in mitochondrial damage. It is likely that the converse situation is also true, namely, that impairment of mitochondrial function may lead to a decrease in cytosolic GSH. Because approximately 90% of total cellular GSH is localized in the cytosolic fraction [43], a GSH-depleted condition may be a common event leading to the disruption of the cellular activities of mitochondria and cytoplasm. GRX reduces oxidized GSSG back to two GSH molecules to replenish stores of this universal antioxidant. GSH and glutathione reductants, including GRX, have been shown to prevent mitochondrial oxidative damage [7]. However, GRX activity in this study was induced only at the early time-point after Aβ (1–42) treatment, suggesting that Aβ (1–42) might enhance mitochondrial toxicity in the brains of mice.

Cholinergic transmission is crucial to learning and memory, and its alteration is considered one of the main causes of cognitive disorder such as AD. As described in introduction, we have reported that Aβ (1–42 or 1–40) impairs cholinergic neuronal function, learning, and memory [18,39,40,46]. Similarly, a single i.c.v. injection of a picomolar dose of Aβ (1–42) effectively impaired learning and memory behaviour in mice. This behavioural abnormality was accompanied by decrease in cortical acetylcholine levels and increase in hippocampal glial fibrillary acidic protein (GFAP)-like immunoreactivity (GFAP-IR) [52], supporting previous reports in rats that continuous infusion of Aβ (1–40) blunts choline

acetyltransferase activity and increases GFAP-IR [39,40]. Further, Yan et al. [52] demonstrated that Aβ(1–42) injection induced a marked increase in interleukin-1β-like immunoreactivity (IL-1β-IR) in the hippocampus. Thus, in addition to the decreased acetylcholine levels, Aβ(1–42)-induced increase in IL-1β-IR may have contributed to learning and memory deficit in mice.

Interestingly, GSSG inhibits the binding of the specific ligand [^{3}H]quinuclidinyl benzilate to muscarinic cholinergic receptors [15], suggesting that GSH homeostasis is important in maintaining learning/memory functions [25]. We confirmed that α-tocopherol protects against Aβ-induced impaired homeostasis of GPX and GRX activities in this study. Thus, the present finding suggests that GSH-dependent antioxidant enzymes (GPX and GRX) are possible markers for evaluating antioxidant status, as well as learning/memory functions, of Aβ-treated model animals.

This study showed that a single i.c.v. injection of Aβ (1–42) resulted in significant impairment of memory function, imbalances in the activities of antioxidant enzymes, and increases in the oxidative stress (lipid peroxidation and protein oxidation) in mice, and that the potent antioxidant α-tocopherol significantly prevented these toxicities induced by Aβ (1–42). Therefore, our results suggest that oxidative stress contributes to Aβ (1–42)-induced learning and memory deficits in mice.

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References

[1] Aksenov MY, Tucker HM, Nair P, Aksenova MV, Butterfield DA, Estus S, et al. The expression of key oxidative stress-handling genes in different brain regions in Alzheimer's disease. J Mol Neurosci 1998;11:151–64.

[2] Behl C, Davis JB, Lesley R, Schubert D. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 1994;77:817–27.

[3] Bush AI, Pettingell WH, Multhaup G, Paradis MD, Vonsattel JP, Gusella JF, et al. Rapid induction of Alzheimer Aβ amyloid formation by zinc. Science 1994;265:1464–7.

[4] Butterfield DA, Hensley K, Harris M, Mattson M, Carney J. β-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease. Biochem Biophys Res Commun 1994;200:710–5.

[5] Butterfield DA, Stadtman ER. Protein oxidation process in aging brain. Adv Cell Aging Gerontol 1997;2:161–91.

[6] Butterfield DA, Lauderback CM. Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid β-peptide-associated free radical oxidative stress. Free Radic Biol Med 2002;32:1050–60.

[7] Cassarino DS, Bennett Jr JP. An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses, and cell death in neurodegeneration. Brain Res Rev 1999;29:1–25.

[8] Ceballos I, Nicole A, Briand P, Grimber G, Dalacourte A, Falment S, et al. Expression of human Cu–Zn superoxide dismutase gene in transgenic mice: model for gene dosage effect in Downs syndrome. Free Radical Res 1991;12/13:581–9.

[9] Cheng ZJ, Kuo SC, Chan SC, Ko FN, Teng CM. Antioxidant properties of butein isolated from *Dalbergia odorifera*. Biochim Biophys Acta 1998;1392:291–9.

[10] Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, et al. Treatment with a copper–zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer's disease transgenic mice. Neuron 2001;30:641–2.

[11] Coyle JT, Puttfarcken P. Oxidative stress, glutamate, and neurodegenerative disorders. Science 1993;262:689–95.

[12] Daniels WM, van Rensburg SJ, van Zyl JM, Taljaard JJ. Meltonin prevents beta-amyloid-induced lipid peroxidation. J Pineal Res 1998;24:78–82.

[13] Eklow L, Moldeus P, Orrenius S. Oxidation of glutathione during hydroperoxide metabolism. A study using isolated hepatocytes and the glutathione reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea. Eur J Biochem 1984;138:459–63.

[14] Floyd RA, Carney JM. Free radical damage to protein and DNA: mechanisms involved and relevant observations on brain undergoing oxidative stress. Ann Neurol 1992;32:S22–S227.

[15] Frey WH, Najarian MM, Kurma KS, Emory CR, Menning PM, Frank JC, et al. Endogenous Alzheimer's brain factor and oxidized glutathione inhibit antagonist binding to muscarinic receptor. Brain Res 1996;724:87–94.

[16] Hendrie HC. Epidemiology of Alzheimer's disease. Geriatrics 1997;52:S4–8.

[17] Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, et al. The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. Biochemistry 1999;38:7609–16.

[18] Itoh A, Nitta A, Nodai M, Nishimura K, Hirose M, Hasegawa T, et al. Dysfunction of cholinergic and dopaminergic neuronal systems in β-amyloid protein-infused rats. J Neurochem 1996;66:1113–7.

[19] Itoh A, Akaike T, Sokabe M, Nitta A, Iida R, Olariu A, et al. Impairment of long-term potentiation in hippocampal slices of β-amyloid-infused rats. Eur J Pharmacol 1999;382:167–75.

[20] Jareno EJ, Bosch-Morell F, Fernandez-Delgado R, Donat J, Romero FJ. Serum malondialdehyde in HIV seropositive children. Free Radic Biol Med 1998;24:503–6.

[21] Jhoo WK, Kim HC, Yamada K, Im DH, Shin EJ, Park SJ, et al. Prolonged exposure to β-amyloid protein enhances 4-hydroxy-2-nonenal modifies proteins in the rat brain. Soc Neurosci Abstr 2000;26:1020.

[22] Kim HC, Jhoo WK, Choi DY, Im DH, Shin EJ, Suh JH, et al. Protection of methamphetamine nigrostriatal toxicity by dietary selenium. Brain Res 1999;851:76–86.

[23] Kim HC, Jhoo WK, Bing G, Shin EJ, Wie MB, Kim WK, et al. Phenidone prevents kainate-induced neurotoxicity via antioxidant mechanisms. Brain Res 2000;874:15–23.

[24] Kim HC, Bing G, Jhoo WK, Ko KH, Kim WK, Suh JH, et al. Changes of hippocampal Cu/Zn-superoxide dismutase after kainate treatment in the rat. Brain Res 2000;853:215–26.

[25] Kim HC, Yamada K, Nitta A, Olariu A, Tran MH, Mizuno M, et al. Immunocytochemical evidence that amyloid β (1–42) impairs endogenous antioxidant systems in vivo. Neuroscience 2003;119:399–419.

[26] Laursen SE, Belknap JK. Intracerebroventricular injections in mice. Some methodological refinements. J Pharmacol Meth 1986;16:355–7.

[27] Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 1976;71:952–8.

[28] Liu R, Liu IY, Thompson RF, Doctrow SR, Malfroy B, Baudry M. Reversal of age-related learning deficits and brain oxidative stress in