as described previously (62).

2.4. Western blotting

Animals were decapitated on Day 7 after the injection of Aβ peptides. The hippocampi were removed on ice-cold glass plate and stored at -80°C. The hippocampal tissues were homogenized as described previously (3). Briefly, the hippocampal tissues were homogenized in ice-cold extraction buffer (150 µl of 20 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl, 2 mM EDTA · 2Na, 50 mM sodium fluoride, 1 mM sodium vanadate, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate (SDS), 1 mg/ml pepstatin, 1 mg/ml aprotinin, and 1 mg/ml leupeptin). Equal amounts of protein, 20µg/lane, were resolved by a 10 % SDS-polyacrylamide gel electrophoresis, and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). Membranes were incubated in 3 % skim milk in a washing buffer (Tris-buffered saline containing 0.05 % (v/v) Tween-20) for 2 h at room temperature. Then the membranes were incubated at 4°C overnight with a diluted (1:1000) anti-nitrotyrosine mouse clone1A6 (Upstate cell signaling, Lake Placid, USA) or with goat anti-actin primary antibody (1:100) (Santa Cruz Biotechnology Inc., Santa Crutz, CA). After a wash, membranes were incubated with horseradish peroxidase-labeled anti-mouse IgG (1:2000) (Kirkegaard & Perry Laboratories, Baltimore, MD) or with donkey anti-goat IgG secondary antibody (1:2000) (Santa Cruz Biotechnology Inc., Santa Crutz, CA). Immunoreactive complexes on the membrane were

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detected using Western blotting detection reagents (Amersham Biosciences Inc., Piscataway,

NJ) according to the manufacturer's instructions, and exposed to X-ray film. The intensity of

each protein band on the film, analyzed with the Atto Densitograph 4.1 system (Atto, Tokyo,

Japan), and was corrected with the corresponding β-actin level. The results were expressed as

the percentage of that of the control.

2.5. Novel object recognition task

This task, based on the spontaneous tendency of rodents to explore a novel object more

often than a familiar one (16), was performed during Day 8 - Day 10 after the $i.c.v.$ injection

of Aβ peptides as described previously (3). A plastic chamber (35× 35× 35 cm) was used in

low light condition during the light phase of the light/dark cycle. The general procedure

consisted of three different phases: a habituation phase, an acquisition phase, and a retention

phase. On the 1st day (habituation phase), mice were individually subjected to a single

familiarization session of 10 min, during which they were introduced in the empty arena, in

order to become familiar with the apparatus. On the 2nd day (acquisition phase) animals were

subjected to a single 10-min session, during which floor-fixed two objects (A and B) were

placed in a symmetric position from the centre of the arena, 15 cm from each and 8 cm from

the nearest wall. The two objects, made of the same wooden material with the similar color

and smell, were different in shape but identical in size. Mice were allowed to explore the

objects in the open field. A preference index for each mouse was expressed as a ratio of the

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amount of time spent exploring object A (TA ×100)/ (TA + TB), where TA and TB are the

time spent on exploring object A and object B, respectively. On the 3rd day (retention phase),

mice were allowed to explore the open field in the presence of two objects: the familiar object

A and a novel object C in different shape but in similar color and size (A and C). A

recognition index, calculated for each mouse, was expressed as the ratio (TC ×100)/ (TA +

TC), where TA and TC are the time spent during retention phase on object A and object C,

respectively. The time spent exploring the object (nose pointing toward the object at a

distance ≤ 1 cm) was recorded by hand.

2.6. Statistical analyses

The results are expressed as the mean ± S.E.. Statistical significance was determined with

one-way ANOVA followed by the Bonferroni multiple comparisons test. $p < 0.05$ was taken as

a significant level of difference.

3. Results

3.1. TNF-α was involved in $A\beta_{25-35}$-induced impairment of recognition memory

The expression of TNF-α and iNOS mRNA in the hippocampus of mice was investigated at

different time points after the i.c.v.-injection of $A\beta_{25-35}$. At two-hour time point, $A\beta_{25-35}$

increased the mRNA expressions of TNF-α and iNOS, the expression of the former was

stronger than that of the latter (Fig. 2 A). $A\beta_{25-35}$ did not increase the expression of iNOS

mRNA in TNF-α (-/-) mouse at the two-hour time point (Fig.2 B). The selective inhibition of

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iNOS activity by aminoguanidine (14) or TNF-α (-/-) prevented Aβ_{25-35} induced nitration of

proteins in the hippocampus or the impairment of recognition memory in mice (Fig.2 C, D).

The Western blott analysis of protein nitration revealed only a single band for nitrated proteins

at the size of 70 kDa. Through extensive studies with different anti-nitrotyrosine antibodies

from different origin, we found that the detection of this single band is owing to the selectivity

of the anti-nitrotyrosine mouse antibody (3). These results, together with previous reports

(2,13,36,65), confirmed that TNF-α is essential for the neurotoxicity of Aβ.

3.2. Restraining TNF-α by thalidomide prevented Aβ-induced impairment of recognition memory

The consistent involvement of TNF-α in the neurotoxicity of Aβ prompted us to examine

an available candidate for anti-TNF-α remedy. Thalidomide, an immunomodulatory drug, has

recently been suggested for the treatment of neurodegenerative disease as a selective inhibitor

of TNF-α (21,60). Thalidomide inhibits the synthesis of TNF-α through the degradation of

mRNA (37). We reexamined the selectivity of thalidomide-induced degradation of TNF-α

mRNA. Thalidomide suppressed the increase of both TNF-α and iNOS mRNA induced by

Aβ_{1-40} (Fig. 3 A,B). The decrease of iNOS mRNA might be due to the decrease of TNF-α

(32). The mRNA expressions of brain-derived neurotrophic factor (BDNF) and glial cell-

derived neurotrophic factor (GDNF), however, were not affected either by Aβ_{1-40} injection or

thalidomide treatment (Fig. 3 C, D). Either the daily treatment with thalidomide or the *i.c.v.*-

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injection of an anti-TNF-α antibody prevented the nitration of proteins in the hippocampus and the impairment of recognition memory induced by Aβ_{25-35} or Aβ_{1-40} (Fig. 4 A, B). The preventive effects of the anti-TNF-α antibody was consistent with previous reports (36,65), and supported the validity of targeting TNF-α by thalidomide to prevent the neurotoxicity of Aβ.

4. Discussion

Providing effective medication or practical strategy for the treatment of the cognitive deficits in AD can have a dramatic effect on the quality of life of a patient.

As a strategy to prevent the cognitive decline in AD, anti-oxidants are well recommended based on the association of the increased cerebral oxidative damage with the progress of the disease (7,22,45). The oxidative damage in the brain of AD is mostly ascribed to the increased level of Aβ that induces the peroxynitrite-mediated nitration of proteins and the resultant irreversible loss of function (9,10,46,53,54,67). It is therefore thus suggested that preventing the formation of peroxynitrite should be the early strategy for interrupting the cognitive failure in AD. Peroxynitrite is the product of the inter-action of nitric oxide (NO) and superoxide (6,46). Although the half-life of NO is extremely short, peroxynitrite could be formed at a rate more than three times faster than the scavenging of superoxide by superoxide dismutase, implying criticality of the over-produced NO (5). The overproduction of NO by Aβ is ascribed to the overexpression of iNOS (1,23,25,40,47,59,64). The deletion of iNOS

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or inhibition of the activity prevents peroxynitrite-mediated damage or the impairment of memory induced by $A\beta_{1-40}$ (25,40,58). Observation of iNOS overexpression in the brain of AD (33,61), supports an anti-iNOS strategy (40,59). However, the involvement of iNOS in the important physiology of life such as sexuality and sleep in the recent reports makes the strategy less favorable (20,26). Therefore, restraining the overproduction of iNOS without affecting its normal expression and function is desired. The involvement of the TNF-α in iNOS-mediated neurotoxicity of $A\beta_{1-40}$ (2,13,36,65), points out a better strategy of targeting TNF-α to prevent the iNOS-driven-peroxynitrite-mediated impairment of memory in the neurotoxicity of $A\beta$.

In the present study, we examined the feasibility of a strategy of targeting TNF-α for the prevention of $A\beta$-induced impairment of memory. To confirm the involvement of TNF-α in the $A\beta$-induced impairment of recognition memory, $A\beta_{25-35}$, the most toxic $A\beta$ species detected in AD brain, and TNF-α (-/-) mouse were utilized. $A\beta_{25-35}$ did not induce the impairment of recognition memory in TNF-α (-/-) mouse. The serious involvement of TNF-α was consistent with the previous reports (2,13,36,65), and boosted the prospect of an anti-TNF-α strategy to prevent the neurotoxicity of $A\beta$. Considering the current availability for practice, thalidomide was selected due to its preferential degradative effect on the mRNA of TNF-α (32,37). Thalidomide preferentially suppressed the increase of TNF-α mRNA and prevented the $A\beta$-induced impairment of recognition memory. An anti-TNF-α antibody also

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provide prevention against the impairment of recognition memory induced by Aβ, validating

the usefulness of the strategy of targeting TNF-α.

A variety of inhibitory agents of the synthesis or the activity of TNF-α is widely

investigated and currently used as remedy in practice (4,35). However, the penetrating ability

of the blood-brain-barrier (BBB) of these agents is one of the factors to challenge their

application for the ailments in the central nervous system. With the ability of penetrating of

BBB, thalidomide is recently emerged as good candidate for the treatment for

neurodegenerative diseases due to its potent inhibitory property for the synthesis of TNF-α

(21,24,60). The drug has recently been reintroduced for treating leprosy, although once was

withdrawn from the general market because of its catastrophic adverse effect of teratogenesis

(31). The treatment with thalidomide in AD may have very limited chance, in the elderly, of

causing teratogenesis in the progeny. Since thalidomide has a neglectable weak inhibitory

effect for NOS including iNOS (52), the attenuation of iNOS-related pathology could most

likely be contributed by its inhibition of the synthesis of TNF-α (32). These reports qualify

thalidomide as an ideal agent for reducing the iNOS/peroxynitrite related pathology via

restraining the increase of TNF-α without evidently harm the physiological function of iNOS.

The neuroprotective effects of thalidomide in an animal model of inflamed brain pathology of

AD in a recent study also recommend and support the use of the drug (48).

In conclusion, the usefulness of restraining TNF-α by thalidomide to prevent Aβ-induced

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impairment of memory in mice would provide a practical fresh strategy for the management

of cognitive deficits in AD.

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