components in lipid raft fractions hint that C_2A domain–PIP_2 interaction may be involved in the distribution of plasmalemmal DAT. In contrast, syntaxin 1A and synaptophysin, the integral membrane proteins, were almost completely recovered in soluble cytosolic fraction, but not in a detergent-resistant fraction. Rim 2, a scaffolding protein with C_2 domain, is known to interact with Piccolo and to regulate presynaptic events. However, its similar subdistribution in the three fractions was different from that of Piccolo C_2A domain. To get an insight into the interplay among DAT, Piccolo C_2A domain and PIP_2, double immunostaining was performed. We found that Piccolo C_2A domain mainly anchored nonuniformly to the inner leaflet of plasma membrane (Figure 5e), which is consistent with its property of targeting membrane PIP_2. Notably, the distribution pattern of C_2A domain resembled that of hDAT, as revealed by the paralleled immunoreactivities at membrane microdomains (Figure 5f).

Internalization of plasmalemmal DAT is PIP_2-dependent

The concept of PIP_2 as a spatially localized regulator of membrane trafficking is clearly illustrated by its key role in clathrin-mediated endocytosis for transporter. If plasmalemmal DAT is triggered to internalize by METH, it should be accompanied by PIP_2 for recruiting endocytic adaptors through PIP_2-binding modules. To test this idea, hDAT and PIP_2 were double-stained in hDAT-PC12 cells after treatment of saline or 1 μM METH for 30 min. Surprisingly, the internalized DAT triggered by METH was found to colocalize with the PIP_2 in the cytosolic compartment (Figure 6, bottom panel), whereas the saline-treated cells only showed the constitutively internalized PIP_2 and DAT (Figure 6, top panel). These results further demonstrated that DAT internalization is also a clathrin-dependent process requiring the assembly of endocytic components like PIP_2.

Interaction of Piccolo C_2A domain and PIP_2

Although Piccolo C_2A domain binding to PIP_2 has been demonstrated using artificial membranes,[15] there is no evidence indicating interaction of the two molecules in living models. We first investigated whether plasmalemmal clusters of Piccolo immunoreactivity coincide with sites of local PIP_2 accumulation using double immunostaining. The clusters of Piccolo immunoreactivities in dendrite profile colocalized precisely with those of PIP_2 in the primary cultured dopaminergic neurons (Figure 7a). Moreover, the localization of transfected C_2A domain in hDAT-PC12 cells was similar with that of PIP_2, which revealed a patchy staining pattern at plasma membrane (Figure 7b). Importantly, the clusters with strong immunoreactivity of C_2A domain also showed substantially larger and stronger labeling macroscopic of PIP_2 clusters, indicating that C_2A domain may sequester PIP_2, thus augmenting the formation of microscopically detectable plasmalemmal PIP_2 clusters.

To better understand the interaction of the two molecules, we generated a PIs binding model of Piccolo C_2A domain with Ca^{2+} docking. As show in Figure 7c, the three-dimensional structure indicated that the predicted PIs binding sites are Ca^{2+}-binding loops at the top of C_2A domain, which shows the similar binding residues for phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and PIP_2. Notably, the crystal packing contacts for PIP_2 were the clusters of basic/aromatic residues including 4668–4670 (DNN), 4697–4698 (QK), 4738–4743 (DYDRFS) and 4746 (D). The potential importance of these residues is highlighted by the fact that they are completely conserved among rat, mouse, human and chicken Piccolo.[22] Calculation of the electrostatic surface potential of C_2A domain showed that PIP_2 binding sites are positively charged (Figure 7d), further indicating that clustering PIs by C_2A domain depends on electrostatic interactions between the

Figure 6 Piccolo C_2A domain attenuates dopamine transporter (DAT) internalization responding to methamphetamine (METH). Double-immunostaining of PIP_2 (red) and hDAT (green) in hDAT-PC12 cells. The internalization of hDAT was promoted by METH, which is accompanied by PIP_2 (bottom panel). The saline-treated cells show strong immunoreactivities of both hDAT and PIP_2 (top panel).

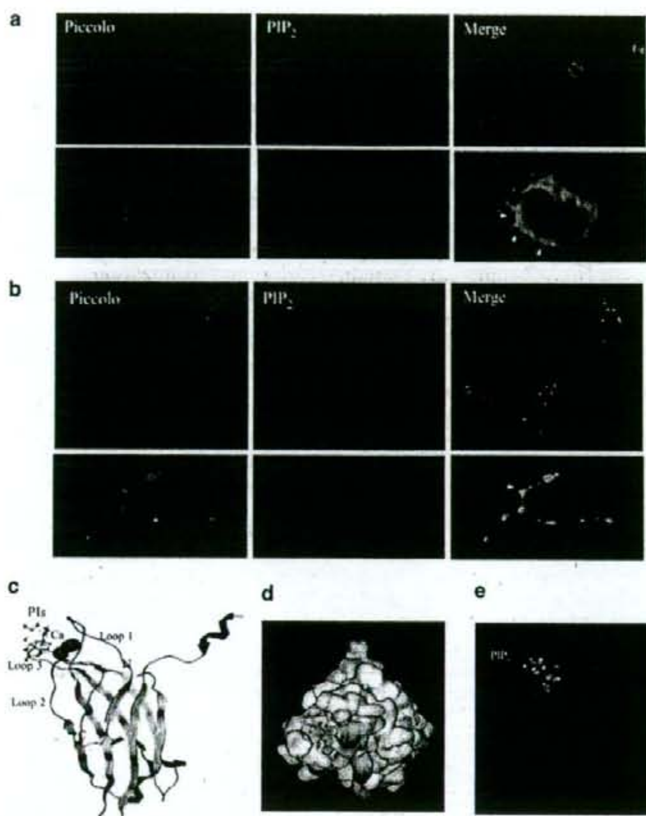

Figure 7 Interaction of Piccolo and PIP_2. (**a**) PIP_2 (red) colocalizes precisely with Piccolo (green) along the presynaptic terminal in primary cultured dopaminergic neurons (arrowed). (**b**) PIP_2 (red) accumulates at plasmalemmal rafts, where it colocalizes with Piccolo (green) in hDAT-PC12 cells. Arrowheads point to regions of intense staining of PIP_2 and Piccolo. (**c**) Model of Piccolo C_2A domain with three bound Ca^{2+} ions on top (green spheres). The top surface of C_2A domain shows the binding sites for the headgroups of PIs. (**d**) Surface plot showing the electrostatic potential of C_2A domain. Blue, positive; red, negative charge; white, neutral. PIP_2 is pointed. (**e**) Space-filling model of PIP_2 is shown on top in pink C_2A domain, which provides a cupped shape of polybasic region to accommodate PIP_2.

positively charged residues in proteins and the negatively charged headgroups of PIs. The lowest binding energies of Piccolo C_2A domain for PI, PIP and PIP_2 with Ca^{2+} docking were −59.491, −93.229 and −102.642 Kcal, respectively, suggesting a specific interaction between PIP_2 and C_2A domain. Furthermore, the space-filling model showed that PIP_2 is tightly packed against the top surface of C_2A domain, which forms a favorable pocket to accommodate the moiety of PIP_2 (Figure 7e).

Piccolo regulated DAT function not through syntaxin 1A
As syntaxin 1A has been demonstrated to regulate the expressions and activities of serotonin transporter (SERT) and γ-aminobutyric acid (GABA) transporters,[23,24] Piccolo might regulate DAT surface expression through interaction with syntaxin 1A. We first investigated whether syntaxin 1A could bind to Piccolo, though syntaxin is identified to bind to Piccolo.[25] The lysates from hDAT-PC12 cells were immunoprecipitated with anti-syntaxin 1A, followed by hDAT immunoblotting. As shown in Supplementary Figure 1a, hDAT were present in the lysate. As expected, we also detected co-immunoprecipitation of hDAT and syntaxin 1A in following immunoprecipitation with anti-hDAT (Supplementary Figure 1b). These results showed an apparent association of these two molecules, which was supported by previous reports.[26] We then investigated whether syntaxin 1A could regulate DAT activity. The hDAT-PC12 cells were pretreated with Bont/C1, a toxin that specifically cleaves syntaxin 1A, followed by

[^{3}H]DA uptake assay; moreover, Bont/B that specifically cleaves the vesicle *N*-ethylmaleimide-sensitive factor attachment receptor protein synaptobrevin was used as a control. As shown in Supplementary Figure 1c, Bont/C1 (0.5–5 nM) failed to alter the [^{3}H]DA uptake in the cells treated with saline. Although Bont/C1 slightly elevated [^{3}H]DA uptake in the cells exposed to METH compared with Bont/B, the difference was not significant. To exclude that such incapability of Bont/C1 in modulating DA uptake was a result of the low concentration or short exposure time, we treated the cells with Bont/C1 at 0.25 μM for 6 h. However, [^{3}H]DA uptake was also not altered (data not shown). Additionally, exposure of METH at the concentration ranging from 0.5–20 μM for 30 min did not alter the expression level of syntaxin 1A in hDAT-PC12 cells (data not shown). Taken together, these data suggest that DAT and syntaxin 1A may mechanically, but not functionally, interact. Given the incapability of syntaxin 1A itself in modulating DAT, it unlikely mediates the role of Piccolo in regulating DAT expression at plasma membrane.

Discussion

The contribution of dopaminergic transmission to behavioral sensitization has been well recognized. Expression of certain proteins appears to be compensatory adaptation to the excessive DA signaling, which could be biologically adaptive mechanisms contributing to addiction. Nevertheless, some proteins likely function in a reverse manner. For example, we have previously found that the expression of tissue plasminogen activator plays a positive role in morphine-induced synaptic plasticity,[19] whereas tumor necrosis factor-α expression in NAc inhibits METH-induced dependence.[18] Piccolo expression was upregulated by repeated METH administration and partial knockdown of Piccolo expression by antisense technique led to elevated synaptic DA concentration in the NAc and two major behavioral manifestations in mice: heightened hyperlocomotor activity and rewarding effect. These findings strongly show that Piccolo overexpression elicited by METH may serve as a homeostatic mechanism that prevents behavioral sensitization by maintaining the expression and activity of the plasmalemmal DAT.

The human Piccolo gene contains more than 25 exons spanning over 350 kb of genomic DNA maps to 7q11.23-q21.3, a region of chromosome 7 implicated as a linkage site for autism and Williams Syndrome.[22] Therefore, dysfunction of Piccolo may be involved in cognitive impairment and mental retardation.[27] The mechanism underlying Piccolo upregulation caused by METH remains to be elucidated. Nevertheless, inhibitory feedback to the excessive DA signaling would be a plausible candidate.

Piccolo has been reported to localize at the GABAergic and glycinergic presynaptic terminal,[10] and our findings in immunostaining demonstrated that it is also expressed at dopaminergic presynaptic terminal. DAT can be internalized from the plasma membrane at a relatively rapid rate, which provides a mechanism by which the turnover rate and density of the plasmalemmal DAT can be quickly and finely modulated.[6,8] Signaling molecules, glycosylation and DAT substrates have been shown to regulate DAT membrane trafficking. Given those findings *in vivo* behaviors tests and the properties of Piccolo, we assumed that Piccolo may play a role in modulating DA flux and DAT distribution at dopaminergic terminals. To address this issue, we investigated DA uptake and membrane DAT expression in hDAT-PC12 cells expressing different functional domain of Piccolo. METH caused DA uptake inhibition in parallel with decreased DAT surface expression, which was well consistent with those works defining the dynamically internalized DAT in hDAT-PC12 cells triggered by amphetamine. These results further support the notion that redistribution of surface DAT caused by METH-like drugs may present an important mechanism underlying the consequently reduced DAT activity. Our data showed that Piccolo C_2A, but not PDZ domain, attenuated METH-induced DA uptake inhibition by retaining DAT expression at cell surface. Because DAT can be internalized and/or recycled, we speculated that the decreased loss of membrane DAT could be resulted from attenuated DAT internalization. Such hypothesis was demonstrated by reversible biotinylation, which revealed the decreased DAT internalization in C_2A domain-transfected cells responding to METH.

It is well established that PIP_2 functions in regulating cytoskeleton, channels and transporters, and membrane trafficking at presynaptic terminal.[16,28,29] Especially, PIP_2 is essential at several stages of endocytosis for the sequential recruitment of adaptor and accessory proteins to endocytic sites.[30,31] METH rapidly causes both DAT internalization and conformational rearrangement to an intracellularly oriented transporter from which DA is released. Such process is proposed to be a drastic membrane movement and requires PIP_2 to assemble various molecules to form endocytic compartment. Significantly, we found that PIP_2 exhibits a similar distribution pattern with DAT at membrane microdomains. Furthermore, internalized DTA triggered by METH is accompanied with PIP_2 in endocytic compartments. These results indicate that PIP_2 is an important regulator in the process of DAT internalization.

A couple of scaffolding proteins such as GAP43, CAP23 and Dap160 have shown their ability to sequester membrane PIP_2, thus potentially modulating the endocytic process.[32,33] In this study we obtained several evidences further supporting the notion that Piccolo can electrostatically sequester PIP_2. Firstly, Piccolo C_2A domain may laterally bind membrane PIP_2, and augment PIP_2 clusters in hDAT-PC12 cells. In principle, the augmented clusters could represent the sequestration of phospholipids like PIP_2 at the plasma membrane.[34] Secondly, the crystal

packing contacts for PIP_2 were the clusters of basic/aromatic residues, which exhibit a universal capability of sequestering membrane PIP_2.[35] Thirdly, the space-filling model showed that Piccolo C_2A domain may pocket PIP_2 by a cupped shape of polybasic region, where the local positive potential electrostatically attracts the negatively charged PIP_2. Finally, C_2A domain shows stronger interacting potential with PIP_2 than PI or PIP. Our results are consistent with previous investigations indicating that PIs binding with Piccolo C_2A domain is largely driven by electrostatic interaction.[15]

Based on these findings, we speculated that Piccolo C_2A domain may regulate METH-triggered DAT internalization through sequestering PIP_2, and the findings in immunostaining strongly support this prediction. Piccolo C_2A domain mainly anchors nonuniformly to the inner leaflet, which is accompanied with the retention of DAT and PIP_2 at membrane microdomains; moreover, it clearly attenuated METH-triggered DAT and PIP_2 internalization in cytosol. These results show that Piccolo may sequester or 'control' locally PIP_2 by C_2A domain in membrane raft and suppress PIP_2-dependent endocytic process, thus leading to the attenuated DAT internalization.

How does the Piccolo C_2A domain–PIP_2 interaction fulfill a function in modulating DAT internalization and psychostimulant responsiveness? An explanation could be that the endocytic process for DAT internalization is inhibited directly through PIP_2 sequestration. Given the strong dependence of the endocytic machinery on PIP_2, more membrane PIP_2 is considerably mobilized for the accelerated DAT internalization triggered by METH. This situation would place the endocytic machinery of dopaminergic presynaptic terminal in a compromised position of insufficient availability of PIP_2, and thus slowing down the DAT internalization. Similarly, a dominant-negative mutant of dynamin I, a component of endocytic machinery, inhibits both PKC- and amphetamine-dependent DAT internalization;[7,36] interruption of adaptor proteins present in clathrin-coated pits like epsin interferes with DAT endocytosis.[37] Another explanation could be that Piccolo C_2A domain may retain DAT at cell surface by promoting membrane stability. METH causes both DAT internalization and conformational rearrangement to an intracellularly oriented transporter from which DA is released. In this process PIP_2 acts as a positive regulator in modulating actin filament assembly and membrane movement by creating membrane microdomains and binding proteins with lipid-specific interaction.[38,39] Therefore, overexpressed Piccolo elicited by METH may enhance the association with membrane PIP_2 or other PIs through C_2A domain and disturb PIP_2-dependent actin assembly, thereby strengthening membrane stability and weakening DAT internalization. In this case, Piccolo may function as a general stabilizer for plasma membrane and DAT. It is worth noting that protein interacting with C kinase 1 (PICK1), a skeletal component, may also stabilize and maintain DAT at plasma membrane.[40]

Piccolo likely binds to syntaxin 1A through its C_2A domain, because synaptotagmin C_2A domain which shares a great structural similarity with Piccolo C_2A domain interacts with syntaxin 1A.[15,40] Syntaxin 1A directly regulates the expressions and activities of SERT and GABA transporter.[23,24] Interestingly, a recent work has identified that syntaxin 1A also binds to DAT.[26] However, Piccolo C_2A domain appears not to regulate METH-induced DAT internalization through syntaxin 1A, because DA uptake is not affected when syntaxin 1A is inhibited.

Our findings reveal that Piccolo is capable of regulating METH-induced DAT internalization, leading to the change of DA signaling and synaptic strength. The precise mechanism underlying the role of C_2A domain–PIP_2 interplay in DAT internalization remains to be determined. No matter which mechanism could be more reasonable, sequestration of PIP_2 in lateral domains through C_2A domain appears to be important for Piccolo to regulate DAT internalization. Therefore, a greater understanding of the molecular regulators for PIP_2, which governs DAT trafficking, would shed light on the modulation of DAT surface presentation. Further investigation measuring membrane fluorescence resonance energy transfer and PIP_2 turnover/mobilization will help interpret the contribution of the proposed mechanisms.

The present investigation illustrates a paradigm that Piccolo, a presynaptic scaffolding protein, targets membrane PIP_2 by its C_2A domain, contributing to the regulation of DAT internalization. Piccolo upregulation may represent a homeostatic response of dopaminergic neurons in the NAc to excessive dopaminergic transmission, dampening hypersensitivity and rewarding effect.

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Manuscript

Restraining tumor necrosis factor-alpha by thalidomide prevents the Abeta-induced impairment of recognition memory in mice

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Abstract

No effective remedy has currently been realized to prevent the cognitive impairments of Alzheimer's disease (AD). The interruption of the toxic pathways of amyloid beta peptide (Aβ) still remains promising for the treatment. The involvement of tumor necrosis factor-alpha (TNF-α) in the toxicity of $A\beta_{1-40}$ in recent reports provide a fresh target for the interruption. In the current study, we evaluated the feasibility of a strategy that target TNF-α to prevent the impairment of memory induced by Aβ. The *i.c.v.*-injection of $A\beta_{25-35}$ increased the hippocampal mRNA expression of both TNF-α and inducible nitric oxide synthase (iNOS), of which the former was stronger. The knock-out of TNF-α (TNF-α (-/-)) in mouse prevented the increase of iNOS mRNA induced by $A\beta_{25-35}$. Not only the inhibition of iNOS activity but also TNF-α (-/-) prevented the nitration of proteins in the hippocampus and the impairment of recognition memory in mice induced by $A\beta_{25-35}$. Daily treatment with thalidomide (20mg/kg), a preferential degrader of TNF-α mRNA, or *i.c.v.*-injection of an anti-TNF-α antibody (10ng/mouse) prevented the nitration of proteins in the hippocampus and the impairment of recognition memory induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ in mice. These results suggested the practicability of targeting TNF-α as a preventive strategy against Aβ-mediated cognitive impairments.

Key words: Amyloid beta (25-35), tumor necrosis factor alpha, protein nitration, recognition memory, thalidomide

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Introduction

Treating the cognitive impairments of Alzheimer's disease (AD) is a complex practice due to its multifactoral pathology that including the increase of toxic amyloid beta peptide (Aβ), neuroinflammation, oxidative damage, and neurodegeneration in critical brain regions involved in memory and cognition (18,42,49,53,63,66). Although there have been many recent advances in the understandings of the pathological process of AD, therapeutic approaches still remain limited to targeting the toxic pathways of Aβ, the assumed central player in the pathogenesis of the disease (15,19,30,38,39,41,50).

The neurotoxicity of Aβ has recently been implicated by the involvement of tumor necrosis factor - alpha (TNF-α), a multifunctional cytokine that triggers a wide range of cellular responses (35). TNF-α is found upregulated in AD patients (8,17,43,56), and involved in $A\beta_{1-40}$-induced inhibition of long-term potentiation in hippocampal slices and the impairment of spatial memory in mice (36,65). $A\beta_{1-40}$ induces toxicity by a mechanism of TNF-α dependent overexpression of inducible nitric oxide synthase (iNOS) (2,13,69). In general, TNF-α is regarded as an inducer of the expression of iNOS (11,23,51). The induced iNOS produces high amount of NO (25,59) which interacts rapidly with superoxide to form proxynitrite (6,46). Proxynitrite mediates the extensive nitration of proteins in the brain of AD and evidently correlated with the increased level of cerebral Aβ and the severity of cognitive impairment (27,28,54,57). The impairments of memory induced by Aβ in mice are prevented

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by the genetic deletion of TNF-α receptor or iNOS as well as by scavenging of peroxynitrite (3,36,40). The implications of the involvement of TNF-α in iNOS-driven peroxynitrite and memory failure prompt a fresh strategy of targeting the synthesis of TNF-α for the prevention of Aβ-induced cognitive impairment. In this study, we have reconfirmed the critical involvement of TNF-α in the neurotoxicity of $A\beta_{25-35}$, the most toxic Aβ fragment that detected in the brain of AD patient (29,44,68), by utilizing TNF-α (-/-) mouse and evaluated the usefulness of targeting TNF-α by using thalidomide to prevent the Aβ-induced impairment of memory in mice.

2. Material and methods

2.1. Animals

Male C57BL/6 (wild type, WT) mice, male TNF-α knock out (TNF-α (-/-)) mice (55), and male ICR mice (Nihon SLC Co., Shizuoka, Japan), were used. The animals were housed in a controlled environment (23±1°C, 50±5% humidity) and allowed food and water ad lib. The room lights were kept on between 8:00a.m and 8:00p.m. All experiments were performed in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in "Principles of Laboratory Animal Care".

2.2. Treatment and experimental design

$A\beta_{25-35}$ and $A\beta_{35-25}$ (Bachem, Bubendorf, Switzerland) were dissolved in sterile double-

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distilled water in a concentration of 1mg/ml and stored at -20 °C before use and incubated for aggregation at 37 °C for 4 days before the injection. $A\beta_{1-40}$ and $A\beta_{40-1}$ (Bachem, Bubendorf, Switzerland) were dissolved in 35 % acetonitrile / 0.1 % trifluoroacetic acid to a stock concentration of 1mg/ml and stored at -20 °C before use. Peptides including $A\beta_{25-35}$ and $A\beta_{35-25}$ were *i.c.v.*- injected at a volume of 3ul. Peptides including $A\beta_{40-1}$ and $A\beta_{1-40}$ were i.c.v.-injected at a volume of 5ul. $A\beta_{35-25}$ and $A\beta_{40-1}$ were injected as the control. All peptides were injected as described previously (3,34). Aminoguanidine (Wako, Saitama, Japan) was dissolved in saline and *i.p.*-injected at the dose of 100mg/kg/day as described previously (36,59). Thalidomide (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and *p.o.*-administrated at the dose of 20mg/kg/day that was selected according to the previous literature on its pharmacodynamic study in mice (12). Anti-TNF-α antibody (α-TNF-α) (R&D Systems, Minneapolis, MN) was *i.c.v.*-injected at the dose of 10ng that followed by the injection of Aβ peptides with 15 minutes interval as described previously (36). The schedule of administration of peptides and drugs as well as biochemical and behavioral investigations are shown in Fig. 1.

2.3. Real-time reverse transcription-polymerase chain reaction

Mice were decapitated at the indicated time-points after the i.c.v.-injection of $A\beta_{25-35}$ (Day 0). The hippocampi were removed on ice-cold glass plate and stored at -80°C. The hippocampal tissue was homogenized and total RNA was extracted using an RNeasy total

RNA isolation kit, following the supplier's protocol (Qiagen, Valencia, CA). cDNA was synthesized by using a Superscript™ reverse transcriptase kit (Invitrogen, Carlsbad, CA). The primer sequences were given below: for iNOS (Gene Bank access: NM_010927), forward primer: 5'-GGGCAGCCTGTGAGACCTT-3'; reverse primer: 5'-GCATTGGAAGTGAAGCGTTTC-3'; TaqMan probe: TGTCCGAAGCAAACATCACATTCAGATCC; For TNF-α (Gene Bank access: NM_023517), forward primer: 5'-CTTTCGGTTGCTCTTTGGTTGAG-3'; reverse primer: 5'-GCAGCTCTGTCTGTTGGATCAG-3'; TaqMan probe: TGCGACAGCACAAGTCACAGCCCC; for the brain-derived neurotrophic factor (BDNF) (Gene Bank access: BC034862), forward primer: 5'-GCAAACATGTCTATGAGGGTTCG-3'; reverse primer: 5'-ACTCGCTAATACTGTCACACACG-3'; TaqMan probe: ACTCCGACCCTGCCCGCCGT; for glial cell-derived neurotrophic factor (GDNF) (Gene Bank access: NM_010275), forward primer: 5'- GAAGAGAGAGGAATCGGCAGG-3'; reverse primer: 5'-TGGCCTCTGCGACCTTTC-3'; TaqMan probe : AGCTGCCAGCCCAGAGAATTCCAGAG; For all, the experimental amplification protocol consisted of a first round at 95°C for 3 min and then 30 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 1 min, with a final extension reaction carried out at 72°C for 10 min. PCR was carried out on Bio-Rad iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The signal was detected according to the manufacturer's instructions. The expression levels were calculated

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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

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 $A\beta_{25\text{-}35}$-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 $A\beta_{25\text{-}35}$. At two-hour time point, $A\beta_{25\text{-}35}$ increased the mRNA expressions of TNF-α and iNOS, the expression of the former was stronger than that of the latter (Fig. 2 A). $A\beta_{25\text{-}35}$ 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 $A\beta_{25-35}$ 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 $A\beta_{1-40}$ (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 $A\beta_{1-40}$ 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 $A\beta_{25-35}$ or $A\beta_{1-40}$ (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 overpexpression 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 Aβ.

In the present study, we examined the feasibility of a strategy of targeting TNF-α for the prevention of Aβ-induced impairment of memory. To confirm the involvement of TNF-α in the Aβ-induced impairment of recognition memory, $A\beta_{25-35}$, the most toxic Aβ 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 Aβ. 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 Aβ-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

impairment of memory in mice would provide a practical fresh strategy for the management of cognitive deficits in AD.

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Legends for figures:

Fig. 1 The experimental schedule.

Fig. 2 TNF-α is required for the iNOS-mediated nitration of proteins and impairment of memory induced by $A\beta_{25-35}$. At different time points after the *i.c.v.*-injection of $A\beta_{25-35}$ (Day 0) in mice, the mRNA expression levels of TNF-α and iNOS in the hippocampus were investigated by using real-time RT-PCR. A: $A\beta_{25-35}$ induced a sharp increase of TNF-α and iNOS mRNA in two hours after injection, the expression of the former was stronger than that of the latter. Data were presented as the mean ± S.E. (n=4). * $p < 0.05$ vs. control. B: TNF-α (-/-) prevented the increase of iNOS mRNA which was induced by $A\beta_{25-35}$ in WT mice. Data were presented as the mean ± S.E. (n=4). * $p < 0.05$ vs. control. C-D: Either the selective inhibition of iNOS activity by AG or TNF-α (-/-) prevented the $A\beta_{25-35}$-induced nitration of proteins in the hippocampus (Day 7) and the impairment of novel object recognition memory (Day 8-10). Data were presented as the mean ± S.E., (n=4 for C, n=10 for D), *$p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. $A\beta_{25-35}$; TNF-α: tumor necrosis factor-alpha; iNOS: inducible nitric oxide synthase; WT: wild type; TNF-α (-/-): TNF-α knock out; AG: aminoguanidine; $A\beta_{25-35}$: Amyloid beta peptide (25-35).

Fig. 3 Thalidomide suppressed the increase of TNF-α mRNA induced by $A\beta_{1-40}$. Thalidomide (20mg/kg) was administrated *p.o.* one hour before the *i.c.v.*-injection of $A\beta_{1-40}$ in mice. A-D: Two hours after the injection of $A\beta_{1-40}$, the mRNA levels of TNF-α, iNOS, BDNF,

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and GDNF in the hippocampus were investigated by using real-time RT-PCR. Thalidomide suppressed the increase of TNF-α and iNOS mRNA induced by $A\beta_{1-40}$. The mRNA levels of BDNF and GDNF in the hippocampus were not changed either by $A\beta_{1-40}$ injection or thalidomide treatment. Data were presented as the mean ± S.E., n=4, *p <0.05 vs. control, $^{\#}p$ <0.05 vs. $A\beta_{1-40}$; TNF-α: tumor necrosis factor-alpha; iNOS: inducible nitric oxide synthase; GDNF: glial cell-derived neurotrophic factor; BDNF: brain-derived neurotrophic factor; $A\beta_{1-40}$: Amyloid beta peptide (1-40). DMSO: dimethyl sulfoxide.

Fig. 4 Thalidomide prevented the nitration of proteins and the impairment of memory induced by Aβ. A-B: Thalidomide (20mg/kg) was administrated *p.o.* daily until Day 3 after the *i.c.v.*-injection of $A\beta_{25-35}$ or $A\beta_{1-40}$ (Day 0). An anti-TNF-α antibody (α-TNF-α) (10ng/mouse) was *i.c.v.*-injected 15 minutes prior to the injection of $A\beta_{25-35}$ or $A\beta_{1-40}$. Either the daily treatment with thalidomide or the *i.c.v.*-injection of α-TNF-α antibody prevented the nitration of proteins in the hippocampus (Day 7) and the impairment of novel object recognition memory (Day 8-10) induced by $A\beta_{25-35}$ or $A\beta_{1-40}$. Data were presented as the mean ± S.E., (n=4 for E, n=10 for F), *p <0.05 vs. control; $^{\#}p$ <0.05 vs. $A\beta_{25-35}$ or $A\beta_{1-40}$; α-TNF-α: anti-TNF-α antibody; $A\beta_{35-25}$: Amyloid beta peptide (35-25). $A\beta_{1-40}$: Amyloid beta peptide (1-40).

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Figure(s)

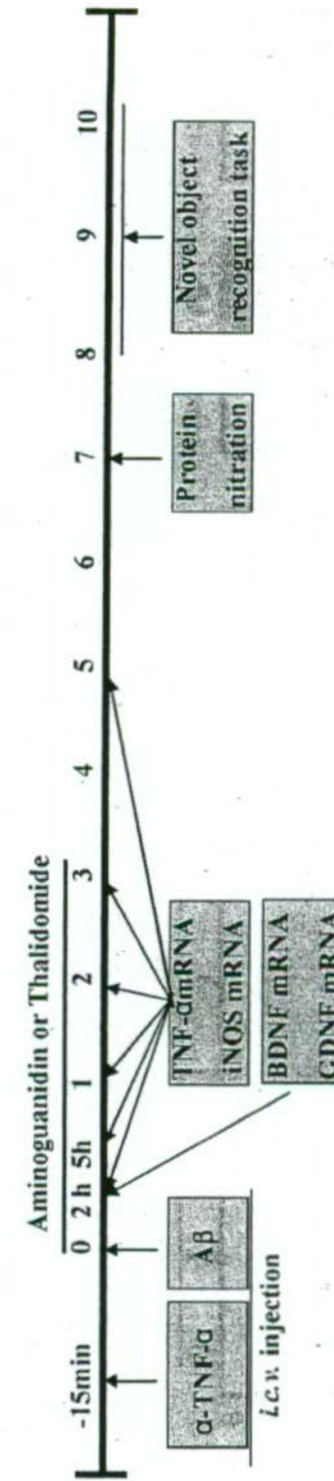

Fig. 1