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A Mouse Model of Amyloid β Oligomers: Their Contribution to Synaptic Alteration, Abnormal Tau Phosphorylation, Glial Activation, and Neuronal Loss *In Vivo*

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Although amyloid β (Aβ) oligomers are presumed to cause synaptic and cognitive dysfunction in Alzheimer's disease (AD), their contribution to other pathological features of AD remains unclear. To address the latter, we generated APP transgenic mice expressing the E693Δ mutation, which causes AD by enhanced Aβ oligomerization without fibrillization. The mice displayed age-dependent accumulation of intraneuronal Aβ oligomers from 8 months but no extracellular amyloid deposits even at 24 months. Hippocampal synaptic plasticity and memory were impaired at 8 months, at which time the presynaptic marker synaptophysin began to decrease. Furthermore, we detected abnormal tau phosphorylation from 8 months, microglial activation from 12 months, astrocyte activation from 18 months, and neuronal loss at 24 months. These findings suggest that Aβ oligomers cause not only synaptic alteration but also other features of AD pathology and that these mice are a useful model of Aβ oligomer-induced pathology in the absence of amyloid plaques.

Introduction

Soluble oligomers of amyloid β (Aβ) are believed to be a cause of synaptic and cognitive dysfunction in the early stages of Alzheimer's disease (AD) (Klein et al., 2001; Selkoe, 2002). This conclusion is based primarily on experimental evidence that natural and synthetic Aβ oligomers impair synaptic plasticity (Lambert et al., 1998; Walsh et al., 2002; Shankar et al., 2008) and memory (Cleary et al., 2005; Lesné et al., 2006; Shankar et al., 2008) and cause loss of synapses (Lacor et al., 2007; Shankar et al., 2007) when applied exogenously into rat cerebral ventricle, cultured brain slices, or dissociated neurons. In addition, many studies have supported this conclusion by indicating a correlation between soluble Aβ levels and synaptic and cognitive impairment in humans (Lue et al., 1999; Gong et al., 2003) as well as animal models of AD (Mucke et al., 2000; Dodart et al., 2002; Cheng et al. 2007; Matsuyama et al., 2007).

On the other hand, whether Aβ oligomers contribute to other pathological features of AD, such as abnormal tau phosphorylation, glial activation, and neuronal loss, remains unclear. Several studies have demonstrated that exogenously applied Aβ oligomers induce tau hyperphosphorylation (De Felice et al., 2008), activate astrocytes (Hu et al., 1998) and microglia (Jimenez et al., 2008), and cause neuronal death (Lambert et al., 1998; Kayed et al., 2003) *in vitro*. However, in animal models, these findings have never been observed before amyloid plaque deposition (for review, see Duyckaerts et al., 2008). Once Aβ deposits develop, it is difficult to distinguish which pathological features were induced by soluble Aβ oligomers or by insoluble Aβ fibrils.

We recently identified the E693Δ mutation in amyloid precursor protein (APP) in patients with AD (Tomiyama et al., 2008). This mutation produces variant Aβ lacking glutamate-22 (E22Δ). The mutant Aβ peptide does not form amyloid fibrils *in vitro* and patients with the mutation lack deposits of amyloid plaques (Tomiyama et al., 2008). The mutant peptide, however, readily forms abundant oligomers *in vitro* (Tomiyama et al., 2008) and accumulates in oligomeric forms within transfected cells (Nishitsuji et al., 2009). When injected into rat cerebral ventricle, synthetic mutant Aβ E22Δ peptide inhibits hippocampal long-term potentiation (LTP) more potently than wild-type (WT) peptide *in vivo* (Tomiyama et al., 2008). Exogenously ap-

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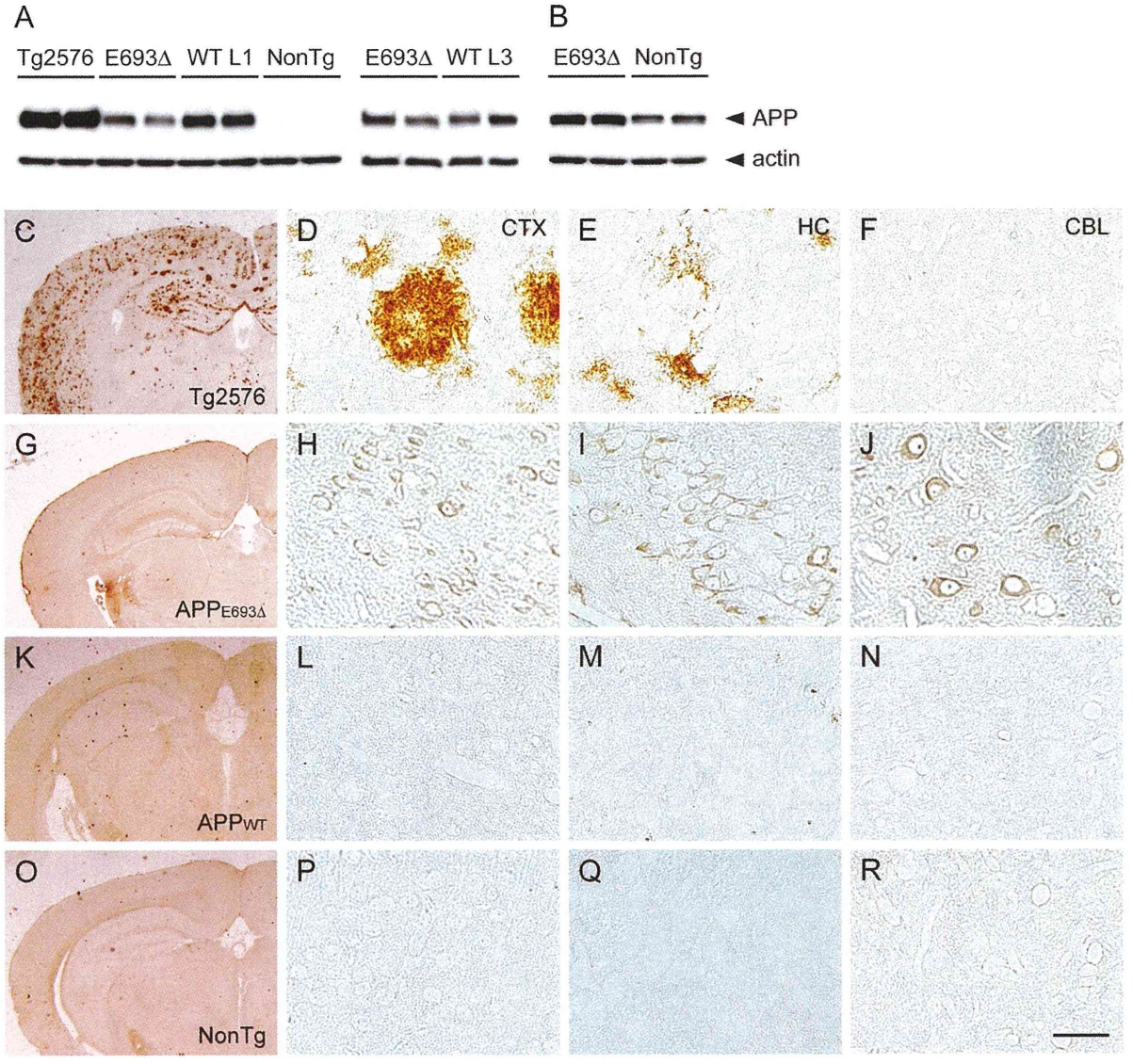

Figure 1. $APP_{E693\Delta}$-Tg mice do not exhibit extracellular amyloid deposits but do display intraneuronal Aβ accumulation. ***A***, ***B***, Levels of expression of human APP in $APP_{E693\Delta}$-Tg mice. Brain homogenates of Tg mice were subjected to Western blotting to examine levels of APP expression. ***A***, Human APP was probed with 6E10 antibody specific to human APP/Aβ. Comparison among the Tg2576 mice, $APP_{E693\Delta}$-Tg mice, APP_{WT}-Tg line 1, and non-Tg littermates was performed at 12 months, while that between the $APP_{E693\Delta}$-Tg mice and APP_{WT}-Tg line 3 was performed at 8 months. ***B***, Human and mouse APP in $APP_{E693\Delta}$-Tg mice and non-Tg littermates were stained at 12 months with C40 antibody recognizing the C-terminal region of human and mouse APP. ***C–R***, Brain Aβ burden in $APP_{E693\Delta}$-Tg mice. Brain sections of 24-month-old Tg mice were stained with β001 antibody to the N-terminal region of Aβ. Tg2576 mice (***C–F***) displayed abundant amyloid plaques in cerebral cortex (***D***) and hippocampus (***E***, CA3 region) but not in cerebellum (***F***), whereas $APP_{E693\Delta}$-Tg mice (***G–J***), APP_{WT}-Tg mice (***K–N***), and non-Tg littermates (***O–R***) exhibited no extracellular amyloid deposits in any regions examined; cerebral cortex (***H***, ***L***, ***P***), hippocampal CA3 region (***I***, ***M***, ***Q***), and cerebellum (***J***, ***N***, ***R***). The $APP_{E693\Delta}$-Tg mice did, however, exhibit intraneuronal staining of Aβ in these regions. CTX, Cerebral cortex; HC, hippocampus; CBL, cerebellum. Scale bar, 30 μm.

plied mutant Aβ E22Δ peptide induces dose-dependent loss of synapses in mouse hippocampal slices (Takuma et al., 2008). These synaptotoxic effects of the mutant Aβ appear to reflect its propensity to undergo oligomerization. These findings suggest that the E693Δ mutation is suitable for production of an animal model of Aβ oligomers in the absence of amyloid plaques. Such a model could provide findings of critical importance in determining whether Aβ oligomers contribute to features of the pathology of AD other than synaptic alteration.

In the present study, we therefore generated transgenic (Tg) mice expressing the mutant $APP_{E693\Delta}$ and compared their pathological features with those of WT APP (APP_{WT})-Tg mice. The findings presented here indicate that Aβ oligomers play pivotal roles in the pathogenesis of AD.

Materials and Methods

Antibodies. Rabbit polyclonal antibodies to Aβ (β001) (Lippa et al., 1999) and APP (C40) (Suga et al., 2004) were prepared in our laboratory. For detection of Aβ oligomers, mouse monoclonal antibody NU-1 (Lambert et al., 2007) was used. Mouse monoclonal antibodies to tau, PHF-1 (Greenberg et al., 1992), and MC1 (Jicha et al., 1997), were kindly gifted by Dr. Peter Davies (Department of Pathology, Albert Einstein College of Medicine, Bronx, NY). Mouse monoclonal antibodies to Aβ (6E10; Signet Laboratories), synaptophysin (SVP-38; Sigma), NeuN, a marker of mature neurons (Millipore Bioscience Research Reagents), GFAP, a marker of astrocytes (Cappel, ICN Pharmaceuticals), and a rabbit polyclonal antibody to Iba-1, a marker of microglia (Wako Pure Chemical Industries), were purchased. Both β001 and 6E10 antibodies recognize full-length APP and its C-terminal fragment generated by β-cleavage (CTFβ) as well as Aβ.

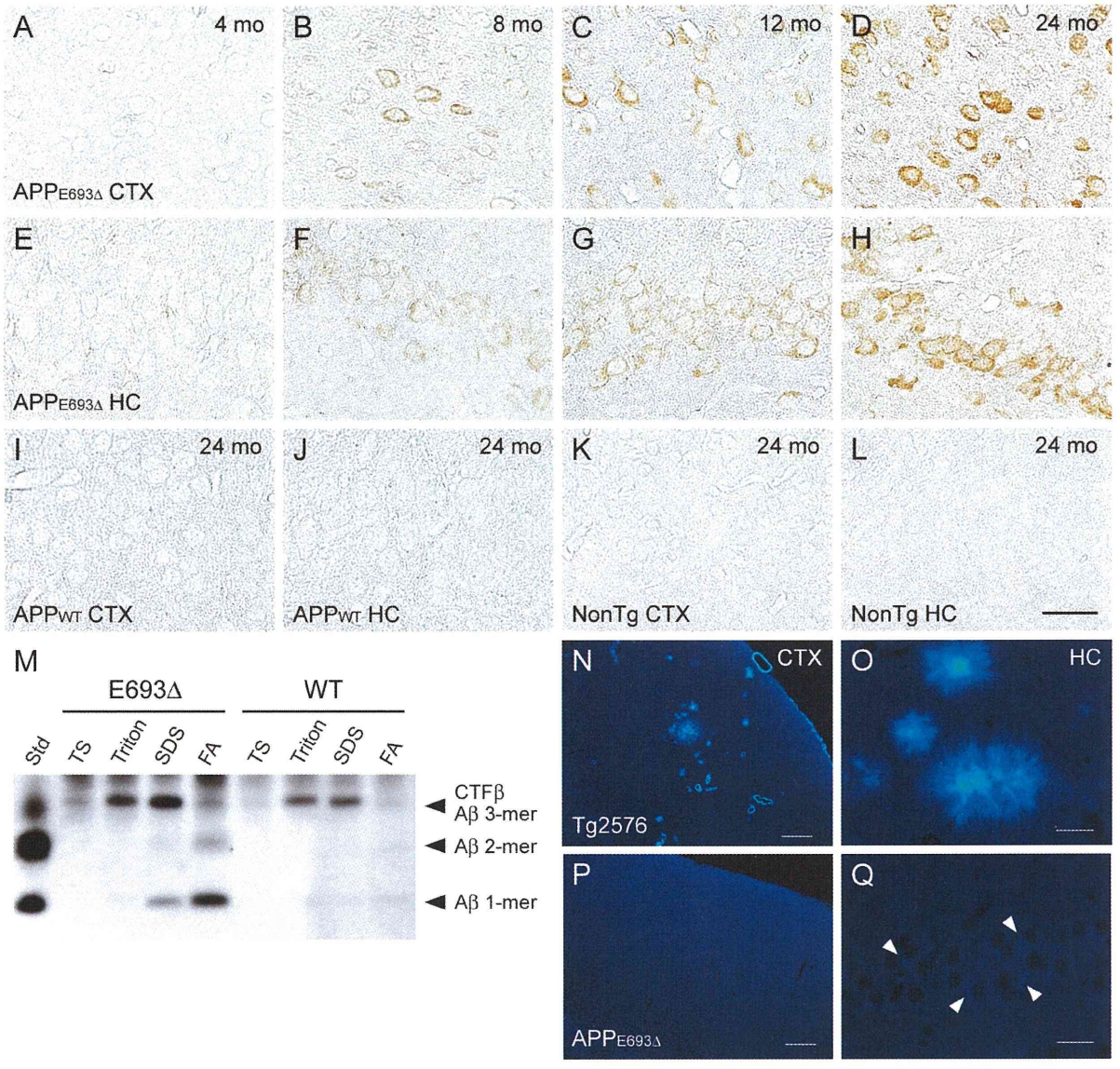

Figure 2. Age-dependent accumulation of intraneuronal Aβ oligomers in $APP_{E693\Delta}$-Tg mice. ***A–L***, Brain sections of 4 (***A***, ***E***)-, 8 (***B***, ***F***)-, 12 (***C***, ***G***)-, and 24 (***D***, ***H***)-month-old $APP_{E693\Delta}$-Tg mice were stained with Aβ oligomer-selective antibody NU-1. Intraneuronal Aβ oligomers first appeared at 8 months in the cerebral cortex (***A–D***) and hippocampus (***E–H***, the CA3 region) and accumulated in age-dependent fashion. No NU-1 staining was observed in APP_{WT}-Tg mice (***I***, ***J***) or non-Tg littermates (***K***, ***L***) even at 24 months; cerebral cortex (***I***, ***K***) and hippocampal CA3 region (***J***, ***L***). Scale bars, 30 μm. ***M***, Oligomer formation of Aβ in $APP_{E693\Delta}$-Tg mice was confirmed by immunoprecipitation/Western blotting analysis. Brain homogenates of 24-month-old Tg mice were fractionated by 4-step ultracentrifugation into TBS-, Triton X-100-, SDS-, and FA-soluble fractions. Aβ in each fraction was immunoprecipitated with 6E10 and stained with β001. Aβ dimers, and possibly trimers, were detected in the FA-extracted fraction from $APP_{E693\Delta}$-Tg mice but only slightly in APP_{WT}-Tg mice. Std, Standard; TS, TBS. ***N–Q***, Brain sections of 24-month-old Tg mice were stained with the amyloid-binding dye thioflavin S. Tg2576 mice exhibited abundant extracellular staining due to parenchymal and vascular amyloid deposits in cerebral cortex (***N***) and hippocampus (***O***), whereas $APP_{E693\Delta}$-Tg mice exhibited no thioflavin S staining in these regions; cerebral cortex (***P***) and hippocampal CA3 region (***Q***). However, $APP_{E693\Delta}$-Tg mice exhibited very weak and somewhat diffuse staining within neurons (***Q***, arrowhead). CTX, Cerebral cortex; HC, hippocampus. Scale bars: ***N***, ***P***, 100 μm; ***O***, ***Q***, 20 μm.

Generation of Tg mice. Tg mice expressing human APP_{695} with the E693Δ mutation were generated using the MoPrP.Xho vector (Borchelt et al., 1996) by the same method as described previously (Matsuyama et al., 2007). MoPrP-APP constructs were injected into B6C3F1 (C57BL/6N × C3H/HeN) embryos. The mice were backcrossed with C57BL/6 mice at least 10 generations. To elucidate the pathological effects of the E693Δ mutation, phenotypes of the $APP_{E693\Delta}$-Tg mice were compared with those of APP_{WT}-Tg mice with the same mouse prion promoter (Matsuyama et al., 2007). As a positive control for immunohistochemistry, Tg2576 mice, a well known model of AD exhibiting massive amyloid deposition (Hsiao et al., 1996), were purchased from Taconic. All mice used were heterozygous for the transgene of interest. Levels of expression of human APP were determined with 6E10 antibody as described previously (Matsuyama et al., 2007). All animal experiments were approved by the committee of Osaka City University and were performed in accordance with the Guide for Animal Experimentation, Osaka City University. Every effort was made to minimize the number of animals used and their suffering.

Immunohistochemistry. Mouse brains were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and deparaffinized with xylene and ethanol. Only for Aβ staining, the sections were pretreated by boiling in 0.01N HCl (pH 2) for 10 min to expose epitopes. We found that GFAP and Iba-1 can be stained well when sections are pretreated with acidic solution, but such a treatment makes the difference between the $APP_{E693\Delta}$-Tg mice and control mice unclear. Therefore, we used untreated sections in staining for these markers. After being washed with 100 mM Tris-HCl, pH 7.6, 150 mM NaCl [Tris-buffered saline (TBS)], the sections to be stained with horseradish peroxidase (HRP) were treated

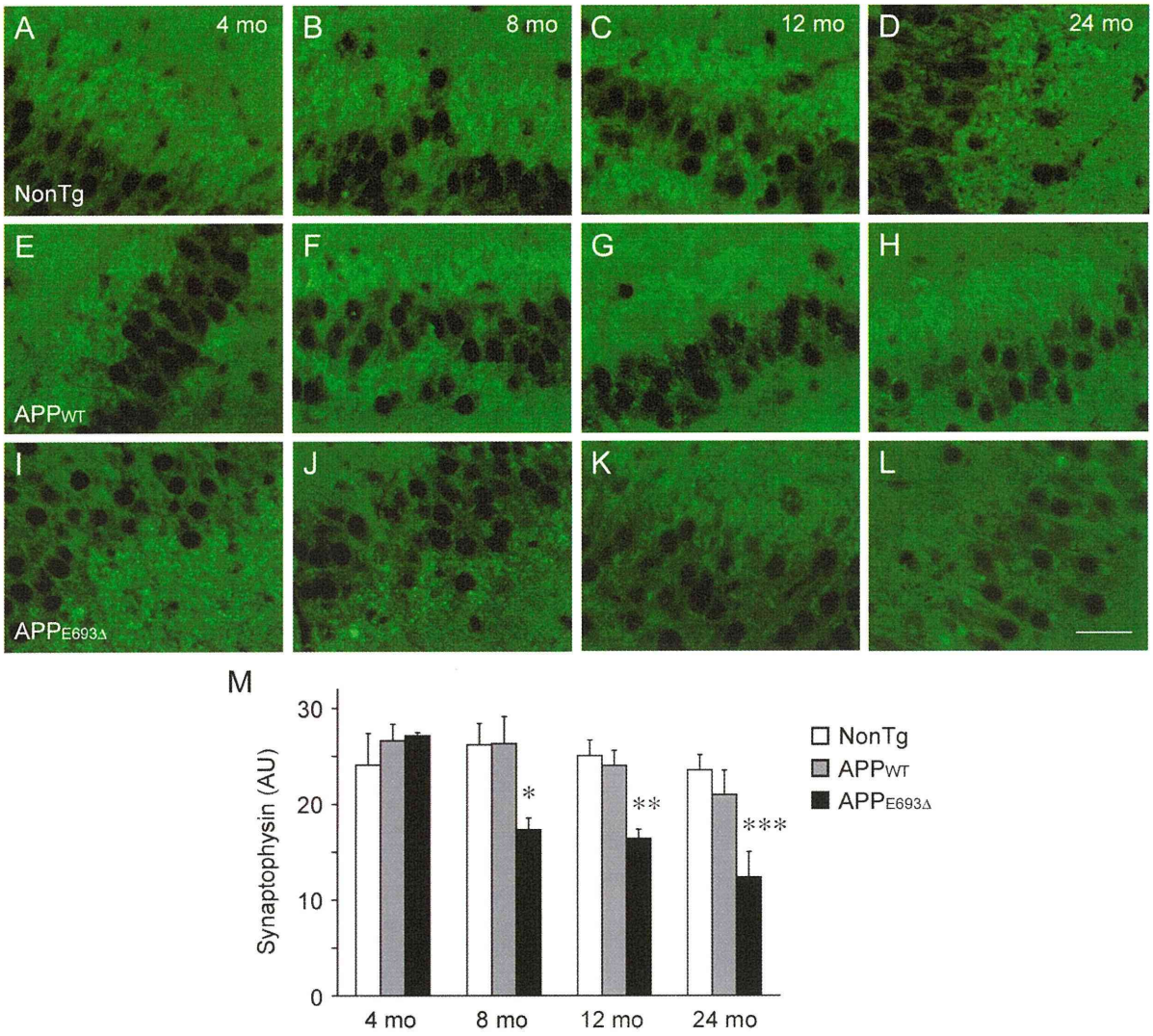

Figure 3. Age-dependent decrease in synaptophysin in $APP_{E693\Delta}$-Tg mice. ***A–L***, Brain sections of 4 (***A***, ***E***, ***I***)-, 8 (***B***, ***F***, ***J***)-, 12 (***C***, ***G***, ***K***)-, and 24 (***D***, ***H***, ***L***)-month-old Tg mice were stained with antibody to the presynaptic marker synaptophysin. All images were taken from the hippocampal CA3 region. Unlike non-Tg littermates (***A–D***) and APP_{WT}-Tg mice (***E–H***), the $APP_{E693\Delta}$-Tg mice (***I–L***) exhibited age-dependent decrease in synaptophysin in the hippocampus from 8 months. Scale bars, 30 μm. ***M***, Synaptophysin fluorescence intensity in 30 μm × 60 μm area of the hippocampal CA3 region was quantified using the NIH ImageJ software and shown in arbitrary units (AU). Each bar represents the mean ± SEM ($n = 3$). *$p = 0.0258$ versus NonTg; $p = 0.0244$ versus APP_{WT}-Tg, **$p = 0.0052$ versus NonTg; $p = 0.0092$ versus APP_{WT}-Tg, ***$p = 0.0140$ versus NonTg; $p = 0.0387$ versus APP_{WT}-Tg.

with 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidases. The sections were then blocked with 20% calf serum in TBS for 1 h. Aβ, tau, and neuronal and glial markers were stained with corresponding antibodies followed by biotin-labeled second antibodies (Vector Laboratories), HRP-labeled avidin-biotin complex (Vector Laboratories), and the substrate DAB (Dojindo). Synaptophysin was stained with SVP-38 antibody followed by FITC-labeled second antibody (Jackson ImmunoResearch Laboratories). Thioflavine S staining to visualize amyloid fibrils was performed as described previously (Oakley et al., 2006). The specimens were observed under a BZ-8000 fluorescence microscope (Keyence). Synaptic density in the hippocampal CA3 region was estimated by quantifying synaptophysin fluorescence intensity in 30 μm × 60 μm area using the NIH ImageJ software obtained from a public website (National Institutes of Health; http://rsb.info.nih.gov/nih-image/). Neuronal loss was evaluated by counting NeuN-positive cells remaining in the pyramidal cell layer of the hippocampal CA3 region within 900 μm from its end toward the dentate gyrus.

Immunoprecipitation/Western blotting of Aβ. Mouse brains, not including the hindbrain, were homogenized by sonication in 4 volumes of TBS containing protease inhibitor mixture (P8340; Sigma), and fractionated by four-step ultracentrifugation including TBS, Triton X-100, SDS, and formic acid (FA) extraction (Kawarabayashi et al., 2001). In brief, the homogenates were centrifuged at 100,000 × *g* at 4°C for 1 h, and the supernatants were harvested. The precipitates were dissolved by sonication in the same volume (4 times tissue weight) of 1% Triton X-100/TBS containing P8340 and centrifuged again. The supernatants were harvested, and the precipitates were then dissolved in 2% SDS/TBS containing P8340 and centrifuged at 100,000 × *g* at room temperature for 1 h. The supernatants were harvested, and the precipitates were finally dissolved in 70% FA. After being centrifuged again, the supernatants were harvested. Then, 100 μl portions of the TBS-, Triton X-100-, and SDS-extracted fractions were diluted 10-, 10-, and 20-fold, respectively, in TBS containing P8340, while 100 μl portions of FA-extracted fractions were diluted tenfold in 1 M Tris solution (pH 11). Aβ in the samples was immunoprecipitated with 1 μg of 6E10 antibody and 10 μl of 50% protein A Sepharose (Pharmacia) at 4°C overnight. After centrifugation, the precipitates were washed three times with 1% Triton X-100/TBS and once with TBS and then boiled for 5 min in SDS sample buffer. The eluates were subjected to SDS-PAGE with 12% NuPage Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were boiled in PBS for 10 min to enhance signals, and Aβ was probed with β001 antibody followed by HRP-labeled

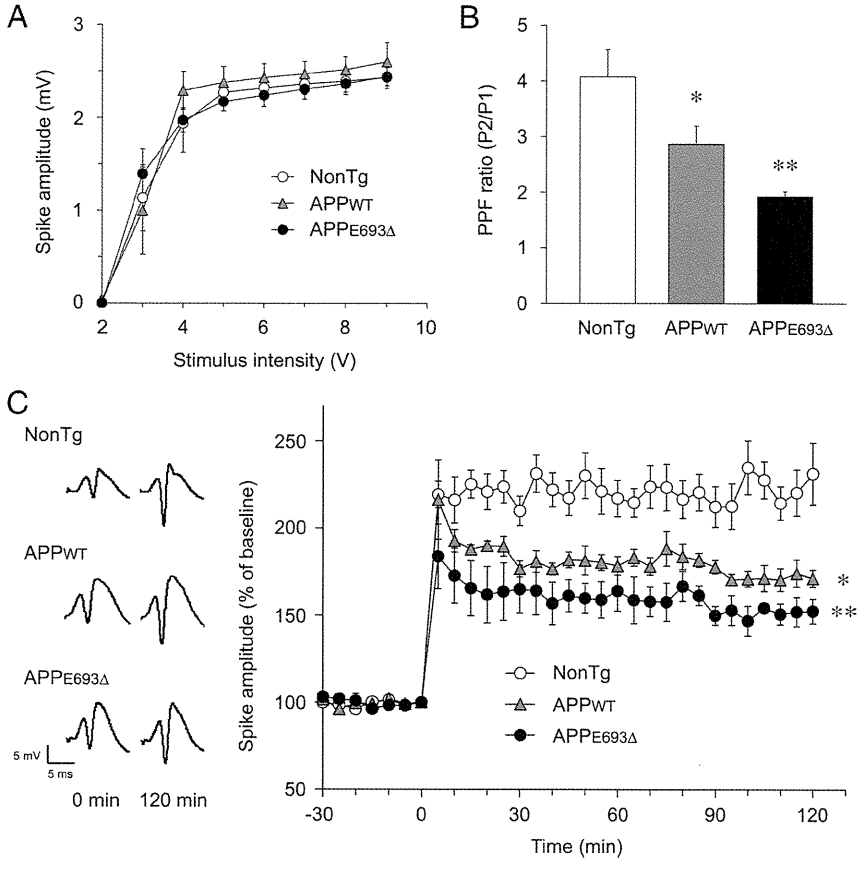

Figure 4. Impairment of synaptic plasticity in $APP_{E693\Delta}$-Tg mice. Synaptic functions of Tg mice were examined by *in vivo* electrophysiology at 8 months. Population spikes were recorded in the granular cell body layer of the dentate gyrus in response to stimulation of the perforant path. ***A***, Basal synaptic transmission was examined by preparing I/O curves with increasing stimulus intensities. No significant difference was observed among the non-Tg littermates, APP_{WT}-Tg, and $APP_{E693\Delta}$-Tg mice ($n = 4$ for each group). ***B***, Short-term synaptic plasticity was studied by testing PPF. Compared with non-Tg littermates, both $APP_{E693\Delta}$-Tg and APP_{WT}-Tg mice exhibited significantly reduced PPF; the reduction was larger in $APP_{E693\Delta}$-Tg mice than in APP_{WT}-Tg mice. $^*p = 0.0275$ versus NonTg, $^{**}p = 0.0002$ versus NonTg; but not significant versus APP_{WT}-Tg ($n = 7$ for $APP_{E693\Delta}$-Tg and NonTg; $n = 5$ for APP_{WT}-Tg). ***C***, Long-term synaptic plasticity was investigated by measuring LTP, which was elicited by delivering HFS to the perforant path. Typical population spikes at 0 and 120 min after HFS are shown. Compared with non-Tg littermates, both $APP_{E693\Delta}$-Tg and APP_{WT}-Tg mice exhibited significant impairment of LTP, which was more severe in the $APP_{E693\Delta}$-Tg mice than the APP_{WT}-Tg mice. $^*p = 0.0093$ versus NonTg, $^{**}p = 0.0003$ versus NonTg; but not significant versus APP_{WT}-Tg, when compared 5–120 min after HFS ($n = 5$ for $APP_{E693\Delta}$-Tg and NonTg; $n = 4$ for APP_{WT}-Tg). All values are the mean ± SEM.

second antibody and the chemiluminescent substrate Immobilon Western (Millipore). Signals were visualized using a LAS-3000 luminescent image analyzer (Fujifilm).

In vivo *electrophysiology.* Experiments were performed with mice at 8 months of age as described previously (Matsuyama et al., 2007). Synaptic functions, including basal synaptic transmission and short-term and long-term synaptic plasticity, were examined *in vivo* by recording of population spikes from the granular cell body layer of the dentate gyrus in response to stimulation of the perforant path.

Behavioral tests. Spatial reference memory in mice was assessed at 8 months of age using the Morris water maze, essentially as described previously (Iso et al., 2007). Male mice were trained to swim to the platform in a pool with a diameter of 96 cm for 6 consecutive days. Training consisted of five trials per day with intertrial intervals of 30 s. At day 7, retention of spatial memory was assessed by a probe trial consisting of a 30 s free swim in the pool without the platform. Locomotor activities of the mice were examined by an open-field test, as described previously (Iso et al., 2007).

Statistical analysis. All data in animal experiments were expressed as the mean ± SEM. Comparisons of means among the three groups were performed with ANOVA followed by Fisher's protected least significant difference test. Differences with a p value of <0.05 were considered significant.

Results

Generation of $APP_{E693\Delta}$-Tg mice

Tg mice expressing the mutant $APP_{E693\Delta}$ under the mouse prion promoter were generated using the MoPrP.Xho vector (Borchelt et al., 1996). Three lines of $APP_{E693\Delta}$-Tg mice were established. Western blotting of brain homogenates with the monoclonal antibody 6E10, which is specific to human APP/Aβ, revealed that lines 1, 2, and 3 possessed the highest, the lowest, and intermediate expression of the transgene, respectively. In this study, line 1, with the highest APP expression was examined. For comparison, we used two lines (lines 1 and 3) of APP_{WT}-Tg mice which had been previously established in our laboratory using the same promoter (Matsuyama et al., 2007). Line 1 was used as a control for immunohistochemistry and behavioral tests, whereas line 3 was used for electrophysiology. We also used the well known Tg2576 mice as another control for immunohistochemistry; these mice overexpress APP harboring the Swedish mutation (K670N/M671L) (APP_{SW}) under the hamster prion promoter (Hsiao et al., 1996).

The levels of expression of human APP in these mice were compared at 8 or 12 months of age by Western blotting with 6E10 antibody. The $APP_{E693\Delta}$-Tg mice expressed human APP to only half the extent of APP_{WT}-Tg line 1 but at levels similar to those in line 3, and only 1/10 that in Tg2576 mice (Fig. 1*A*). We previously reported that both lines 1 and 3 of the APP_{WT}-Tg mice exhibit impaired synaptic plasticity at 8 months and that synaptic plasticity in Tg mice is closely related to their level of expression of APP (Matsuyama et al., 2007). In the present study, we therefore used APP_{WT}-Tg line 3 as a control for electrophysiology. In Western blotting with an antibody to the C-terminal region of APP (C40) (Suga et al., 2004), which recognizes both human and mouse APP, the $APP_{E693\Delta}$-Tg mice exhibited twice the amount of APP as their non-Tg littermates (Fig. 1*B*), indicating that the levels of human APP expressed in $APP_{E693\Delta}$-Tg mice were similar to those of endogenous mouse APP.

$APP_{E693\Delta}$-Tg mice exhibit accumulation of Aβ oligomers within neurons but no extracellular amyloid deposits

We initially examined brain amyloid pathology in our Tg mice by immunohistochemistry with a polyclonal antibody to the N-terminal region of Aβ (β001) (Lippa et al., 1999). At 24 months, the Tg2576 mice displayed abundant extracellular Aβ deposits in the cerebral cortex and hippocampus (Fig. 1*C–F*), as previously reported (Hsiao et al., 1996). On the other hand, neither the $APP_{E693\Delta}$-Tg (Fig. 1*G–J*) nor APP $_{WT}$-Tg mice (Fig.

1*K–N*) exhibited amyloid plaque in any regions we examined at the same age, similar to the non-Tg littermates (Fig. 1*O–R*). We noted, however, that the $APP_{E693\Delta}$-Tg mice exhibited abundant intraneuronal staining in the cerebral cortex, hippocampus (particularly the CA3 region), and cerebellum. The APP_{WT}-Tg mice did not exhibit such intracellular staining despite having higher expression of APP than the $APP_{E693\Delta}$-Tg mice. This finding is consistent with our previous observation that, in transfected cells, mutant Aβ E22Δ accumulated within cells more abundantly than WT Aβ (Nishitsuji et al., 2009).

Since the mutant Aβ E22Δ that accumulated in transfected cells tended to form oligomers (Nishitsuji et al., 2009), it is likely that intraneuronal Aβ in the $APP_{E693\Delta}$-Tg mice also forms oligomers. To test whether this is the case, brain sections from mice at various ages were stained with a monoclonal antibody NU-1 selective to Aβ oligomers (Lambert et al., 2007). As we expected, intraneuronal Aβ in the cerebral cortex and hippocampus of the $APP_{E693\Delta}$-Tg mice was stained by NU-1 (Fig. 2*A–H*). Aβ oligomers first appeared at 8 months in both brain regions and accumulated in age-dependent fashion. The APP_{WT}-Tg mice (Fig. 2*I,J*) and non-Tg littermates (Fig. 2*K,L*) exhibited no NU-1 staining even at 24 months.

Oligomer formation of Aβ in the $APP_{E693\Delta}$-Tg mice was confirmed by immunoprecipitation/Western blotting analysis. Brain tissues at 24 months were homogenized and fractionated by 4-step ultracentrifugation (Kawarabayashi et al., 2001). Initially, extracellular and intracellular soluble Aβ was extracted with TBS, and TBS-insoluble Aβ was sequentially extracted with two types of detergent, Triton X-100 and SDS, and finally with FA, which is commonly used to extract Aβ from amyloid plaque cores. The Aβ in TBS-, Triton X-100-, SDS-, and FA-extracted fractions was immunoprecipitated with 6E10 antibody and stained with β001 antibody. Aβ dimers and possibly trimers were detected in the $APP_{E693\Delta}$-Tg mice, but only slightly in the APP_{WT}-Tg mice (Fig. 2*M*). Notably, Aβ oligomers were fractionated predominantly into insoluble fractions, particularly the FA-extracted fraction. This result appears inconsistent with the prevalent view that Aβ oligomers are soluble, although the presence in AD brains of additional oligomers that could be detected only by detergent extraction has been reported (Gong et al., 2003).

The finding that Aβ oligomers were largely recovered in insoluble fractions suggests the possibility that intraneuronal Aβ in the $APP_{E693\Delta}$-Tg mice may form fibrils, as reported in other APP-Tg mice (Casas et al., 2004; Oakley et al., 2006). To test whether this is the case, brain sections at 24 months were stained with an amyloid-binding dye, thioflavin S. Tg2576 mice displayed abundant, strong staining due to amyloid plaques in the cerebral cortex and hippocampus (Fig. 2*N,O*), whereas $APP_{E693\Delta}$-Tg mice exhibited no staining (Fig. 2*P,Q*), except for very weak, somewhat diffuse staining within neurons in the cerebral cortex and hippocampus. Although the origin of these faint signals is unclear, we suspect that it reflects the binding of thioflavin S to Aβ oligomers rather than to Aβ fibrils, since several amyloid-binding dyes, such as Congo red, thioflavin T, and PIB, have been shown to react with Aβ oligomers as well as Aβ fibrils (Maezawa et al., 2008).

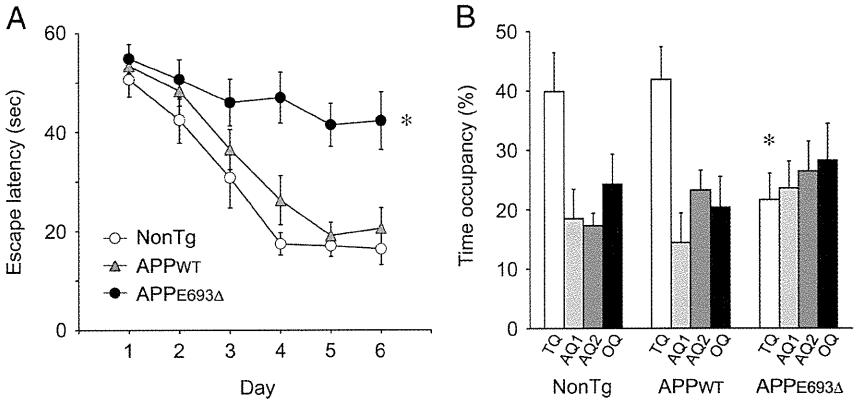

Figure 5. Impairment of memory in $APP_{E693\Delta}$-Tg mice. Spatial reference memory of Tg mice was assessed by the Morris water maze at 8 months. ***A***, Mice were trained to swim to the hidden platform for 6 consecutive days. Each point represents the mean latency of five trials per day ± SEM ($n = 9$ for $APP_{E693\Delta}$-Tg; $n = 8$ for NonTg and APP_{WT}-Tg). The $APP_{E693\Delta}$-Tg mice exhibited significantly longer escape latencies than the APP_{WT}-Tg mice and non-Tg littermates. The APP_{WT}-Tg mice exhibited slightly, but not significantly, longer escape latencies than the non-Tg littermates. $^*p < 0.0001$ versus NonTg; $p = 0.0010$ versus APP_{WT}-Tg. ***B***, At day 7, retention of memory was assessed by a probe trial for 30 s with the platform removed. Each bar represents the mean time occupancy ± SEM in the target quadrant (TQ), adjacent quadrants (AQ1, AQ2), or opposite quadrant (OQ). The $APP_{E693\Delta}$-Tg mice spent significantly shorter time in the target quadrant than the APP_{WT}-Tg mice and non-Tg littermates did. $^*p = 0.0265$ versus NonTg; $p = 0.0149$ versus APP_{WT}-Tg.

Synaptic and cognitive dysfunction in $APP_{E693\Delta}$-Tg mice

Aβ oligomers have been demonstrated to cause synaptic and cognitive dysfunction (Lambert et al., 1998; Walsh et al., 2002; Cleary et al., 2005; Lesné et al., 2006; Shankar et al., 2008; Tomiyama et al., 2008) and loss of synapses (Lacor et al., 2007; Shankar et al., 2007; Takuma et al., 2008) when applied exogenously. To examine whether synaptic alteration occurs in the $APP_{E693\Delta}$-Tg mice, we first examined their synaptic density. Brain sections from mice at various ages were stained with an antibody to the presynaptic marker synaptophysin. Compared with non-Tg littermates (Fig. 3*A–D*) and APP_{WT}-Tg mice (Fig. 3*E–H*), $APP_{E693\Delta}$-Tg mice (Fig. 3*I–L*) exhibited significant decrease in synaptophysin in the hippocampus, particularly in the CA3 region, in an age-dependent fashion from 8 months (Fig. 3*M*). This timing coincides with that of intraneuronal accumulation of Aβ oligomers.

We next examined synaptic function by *in vivo* electrophysiology at 8 months, at which age Aβ oligomers have begun to accumulate in $APP_{E693\Delta}$-Tg mice. In this experiment, we used line 3 of the APP_{WT}-Tg mice as a control, as described above. Population spikes were recorded in the granular cell body layer of the dentate gyrus in response to stimulation of the perforant path. Basal synaptic transmission was tested by preparing input/output (I/O) curves with increasing stimulus intensities. No significant difference was observed among the non-Tg littermates, APP_{WT}-Tg, and $APP_{E693\Delta}$-Tg mice (Fig. 4*A*). Short-term synaptic plasticity was examined by measuring paired-pulse facilitation (PPF). Compared with the non-Tg littermates, both the APP_{WT}-Tg and $APP_{E693\Delta}$-Tg mice exhibited significantly reduced PPF (Fig. 4*B*). The reduction was larger in the $APP_{E693\Delta}$-Tg mice than in the APP_{WT}-Tg mice, although the difference between them was not significant. Last, long-term synaptic plasticity was examined by measuring LTP, which was elicited by delivering high-frequency stimulation (HFS) to the perforant path. Again, significant impairment of LTP was observed in both the APP_{WT}-Tg and $APP_{E693\Delta}$-Tg mice compared with the non-Tg littermates (Fig. 4*C*). The initial potentiation of population spikes was suppressed more profoundly

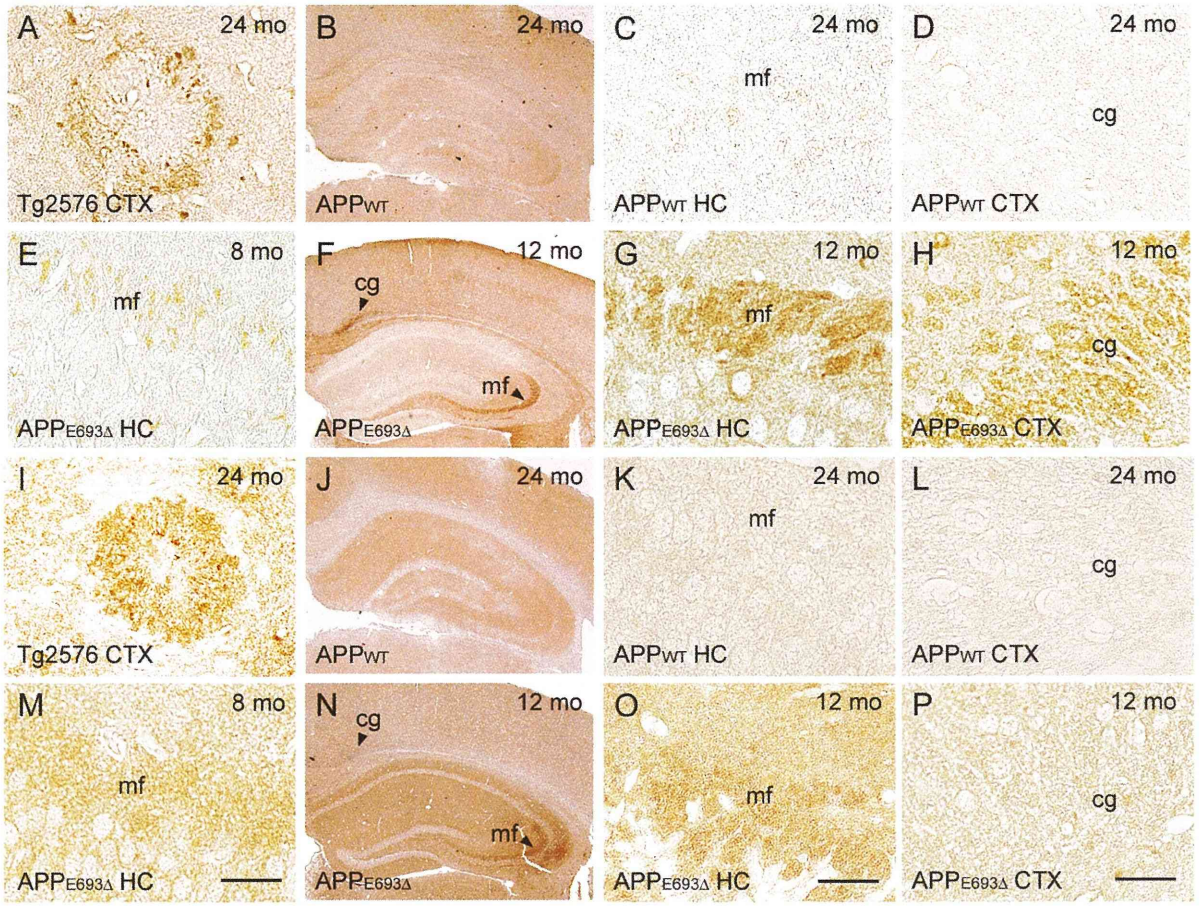

Figure 6. Abnormal tau phosphorylation in $APP_{E693\Delta}$-Tg mice. ***A–P***, Brain sections of 8 (***E***, ***M***)-, 12 (***F–H***, ***N–P***)-, and 24 (***A–D***, ***I–L***)-month-old Tg mice were stained with antibodies reactive to pathological tau, PHF-1 (***A–H***), and MC1 (***I–P***). These antibodies stained dystrophic neurites around amyloid plaques in the Tg2576 mice at 24 months (***A***, ***I***). The APP_{WT}-Tg mice (***B–D***, ***J–L***; ***C***, ***K***, hippocampal CA3 region; ***D***, ***L***, cerebral cortex) exhibited no staining with these antibodies even at 24 months. In contrast, $APP_{E693\Delta}$-Tg mice (***E–H***, ***M–P***) began to display PHF-1-positive and MC1-positive hippocampal mossy fibers (***E***, ***M***, CA3 region) from 8 months. At 12 months, immunoreactivity was more evident (***G***, ***O***, the CA3 region), and the cingulum was also stained with PHF-1 but not MC1 (***H***, ***P***, the cerebral cortex). CTX, Cerebral cortex; HC, hippocampus; mf, mossy fibers; cg, cingulum. Scale bars, 30 μm.

in the $APP_{E693\Delta}$-Tg mice than in the APP_{WT}-Tg mice, and this stronger suppression lasted for at least 120 min, though the difference between them was not significant. Thus, synaptic plasticity was more strongly impaired in the $APP_{E693\Delta}$-Tg mice than in the APP_{WT}-Tg mice.

We also examined cognitive function at the same age (8 months) using the Morris water maze. In this experiment, line 1 of the APP_{WT}-Tg mice was used as a control. Before the trials, spontaneous locomotor activity of mice was measured by the open-field test and confirmed not to differ among the non-Tg littermates, APP_{WT}-Tg, and $APP_{E693\Delta}$-Tg mice (data not shown). The mice were trained for 6 d to remember the location of a hidden platform in a swimming pool, and the time required to reach the platform was measured in every trial. The $APP_{E693\Delta}$-Tg mice exhibited significant deficits in performance, with longer escape latencies than the APP_{WT}-Tg mice and non-Tg littermates (Fig. 5*A*). The APP_{WT}-Tg mice exhibited slightly, though not significantly, longer escape latencies than the non-Tg littermates. In a probe trial at day 7, the APP_{WT}-Tg mice and non-Tg littermates spent ~40% of their time in the target quadrant, whereas the $APP_{E693\Delta}$-Tg mice swam in that area only ~20% of the time (Fig. 5*B*). Thus, spatial reference memory of $APP_{E693\Delta}$-Tg mice was found to be markedly disrupted, whereas that of APP_{WT}-Tg mice was not affected.

In summary, the $APP_{E693\Delta}$-Tg mice exhibited synaptic alteration in parallel with intraneuronal accumulation of Aβ oligomers without formation of amyloid plaques. These features indicate that the Tg mouse is a suitable model for investigation of Aβ oligomer-induced pathology free of the effects of amyloid plaques even at old ages.

Abnormal tau phosphorylation in $APP_{E693\Delta}$-Tg mice

Based on the above findings, we moved to the next study on the contribution of Aβ oligomers to other features of AD pathology, such as abnormal tau phosphorylation, glial activation, and neuronal loss. Aβ oligomers have been demonstrated to cause these pathological changes *in vitro* when applied exogenously to cultured cells and brain slices (Hu et al., 1998; Lambert et al., 1998; Kayed et al., 2003; De Felice et al., 2008; Jimenez et al., 2008). However, it is still unclear whether Aβ oligomers have similar pathological effects *in vivo*. In studies of APP-Tg mice reported thus far, none of these pathological features were detected before amyloid plaque formation (Duyckaerts et al., 2008).

We first examined abnormal tau phosphorylation in $APP_{E693\Delta}$-Tg mice. Brain sections from mice at various ages were stained with two antibodies reactive to pathological tau: PHF-1, which is specific to the phosphorylation at Ser396/Ser404 (Greenberg et al., 1992), and MC1, which recognizes the conformational epitopes of pathological tau (Jicha et al., 1997). In the Tg2576 mice, these antibodies stained dystrophic neurites around amyloid plaques at 24 months (Fig. 6*A*,*I*). The non-Tg

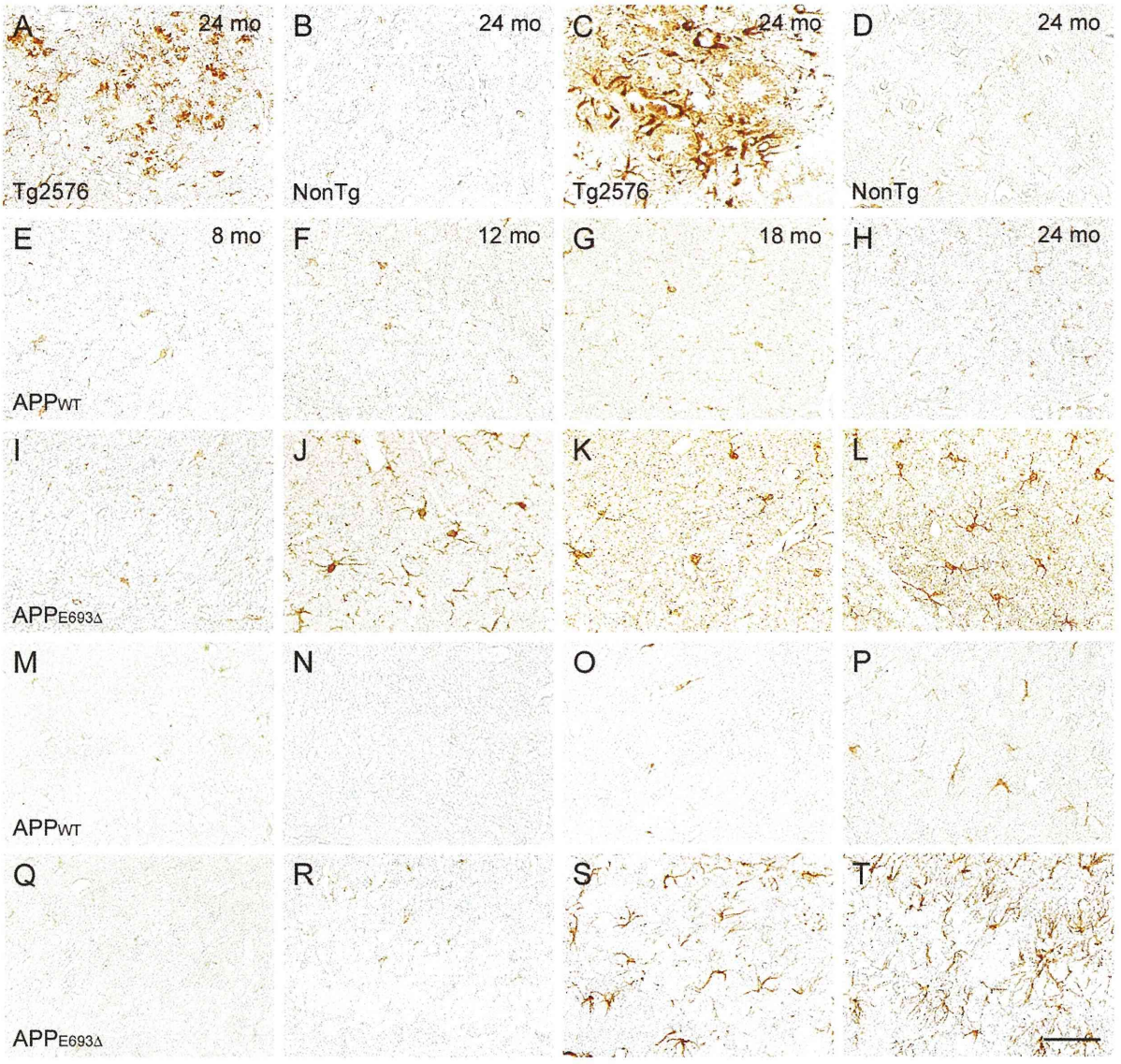

Figure 7. Glial activation in $APP_{E693\Delta}$-Tg mice. ***A–T***, Brain sections of 8 (***E***, ***I***, ***M***, ***Q***)-, 12 (***F***, ***J***, ***N***, ***R***)-, 18 (***G***, ***K***, ***O***, ***S***)-, and 24 (***A–D***, ***H***, ***L***, ***P***, ***T***)-month-old Tg mice were stained with antibodies to Iba-1 (***A***, ***B***, ***E–L***) and GFAP (***C***, ***D***, ***M–T***), which are markers of microglia and astrocytes, respectively. All images were taken from the hippocampal CA3 region, except those of the Tg2576 mice, which were obtained from cerebral cortex. The Tg2576 mice (***A***, ***C***) at 24 months exhibited massive staining with these antibodies around amyloid plaques, while the non-Tg littermates (***B***, ***D***) exhibited no staining at 24 months. The APP_{WT}-Tg mice (***E–H***, ***M–P***) possessed no Iba-1-positive cells and only a few GFAP-positive cells at 24 months. In contrast, the $APP_{E693\Delta}$-Tg mice (***I–L***, ***Q–T***) displayed Iba-1-positive cells from 12 months and GFAP-positive cells from 18 months in both the hippocampus and cerebral cortex. Scale bar, 30 μm.

littermates (data not shown) and APP_{WT}-Tg mice (Fig. 6*B–D*,*J–L*) exhibited no staining with these antibodies in any regions we examined even at 24 months. In contrast, the $APP_{E693\Delta}$-Tg mice began to exhibit PHF-1-positive and MC1-positive staining in hippocampal Mossy fibers from 8 months (Fig. 6*E*,*M*). At 12 months, immunoreactivity was more pronounced, and the cingulum in cerebral cortex was also stained with PHF-1 but not MC1 (Fig. 6*F–H*,*N–P*). These findings demonstrated that Aβ oligomers caused abnormal tau phosphorylation in the absence of amyloid plaques.

Activation of astrocytes and microglia in $APP_{E693\Delta}$-Tg mice

We next examined glial activation. Brain sections from mice at various ages were stained with antibodies to GFAP and Iba-1, which are markers of astrocytes and microglia (Imai et al., 1996), respectively. At 24 months, Tg2576 mice displayed massive staining around amyloid plaques with these antibodies (Fig. 7*A*,*C*). The non-Tg littermates exhibited no staining with these antibodies even at 24 months under our staining conditions (Fig. 7*B*,*D*). The APP_{WT}-Tg mice possessed no Iba-1-positive cells (Fig. 7*E–H*) and only a few GFAP-positive cells (Fig. 7*M–P*) in cerebral cortex and hippocampus at 24 months. In contrast, the $APP_{E693\Delta}$-Tg mice began to display Iba-1-positive cells at 12 months (Fig. 7*I–L*) and GFAP-positive cells at 18 months (Fig. 7*Q–T*) in these regions. The observed staining in the $APP_{E693\Delta}$-Tg mice indicates increased expression of microglial and astrocyte marker proteins, which is believed to reflect activation of these cells.

Neuronal loss in $APP_{E693\Delta}$-Tg mice

Finally, we examined whether neuronal loss occurs in the $APP_{E693\Delta}$-Tg mice. Brain sections from mice at various ages were stained with an antibody to NeuN, a marker of mature neurons (Mullen et al., 1992). Compared with non-Tg littermates (Fig. 8*A*,*E*) and APP_{WT}-Tg mice (Fig. 8*B*,*F*), $APP_{E693\Delta}$-Tg mice (Fig.

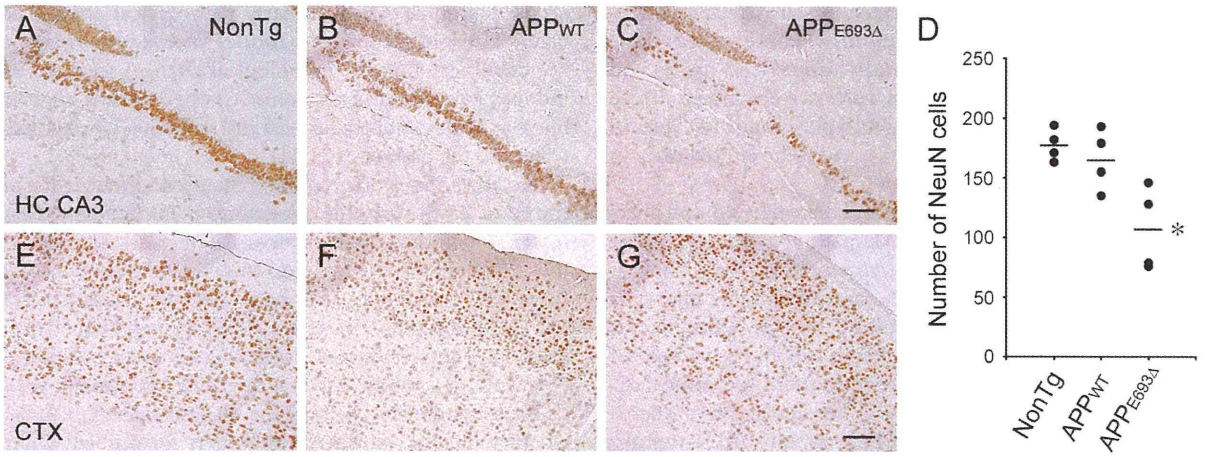

Figure 8. Neuronal loss in $APP_{E693\Delta}$-Tg mice. ***A–C, E–G***, Brain sections of Tg mice were stained with an antibody to NeuN, a marker of mature neurons. Compared with non-Tg littermates (***A***, ***E***) and APP_{WT}-Tg mice (***B***, ***F***), $APP_{E693\Delta}$-Tg mice (***C***, ***G***) exhibited significant decrease in NeuN-positive cells in the hippocampal CA3 region, but no decrease in cerebral cortex at 24 months; hippocampal CA3 region (***A–C***) and cerebral cortex (***E–G***). No significant difference was observed between non-Tg littermates and APP_{WT}-Tg mice at 24 months. ***D***, NeuN-positive cells in the pyramidal cell layer of the hippocampal CA3 region were counted within 900 μm from its end toward the dentate gyrus in the photographs. *$p = 0.0044$ versus NonTg; $p = 0.0121$ versus APP_{WT}-Tg ($n = 4$). CTX, Cerebral cortex; HC, hippocampus. Scale bars, 100 μm.

8*C*,*G*) exhibited a significant decrease in NeuN-positive cells in the hippocampal CA3 region at 24 months (Fig. 8*D*). No apparent decrease in NeuN-positive cells was observed in the cerebral cortex at this age. The APP_{WT}-Tg mice did not exhibit significant neuronal loss compared with the non-Tg littermates. These findings suggest that Aβ oligomers triggered the pathological cascades leading to neuronal death, in which neuronal loss occurred a long period of time after the start of intraneuronal accumulation of Aβ oligomers at 8 months. The hippocampal CA3 region appears to be particularly vulnerable to the toxic effects of Aβ oligomers.

These findings together suggest that Aβ oligomers, which are localized within neurons in $APP_{E693\Delta}$-Tg mice, significantly contribute not only to synaptic alteration but also to other features of AD pathology *in vivo*.

Discussion

In the present study, we generated novel APP-Tg mice expressing the E693Δ mutation to test *in vivo* the ability of Aβ oligomers to produce the synaptic, cognitive, and neuropathological features of AD. On immunohistochemical examination, $APP_{E693\Delta}$-Tg mice exhibited age-dependent accumulation of Aβ oligomers within neurons in the cerebral cortex and hippocampus from 8 months, but no amyloid plaques even at 24 months. Biochemical analysis confirmed the accumulation of Aβ dimers and possibly trimers in their brains. This is consistent with our previous findings that mutant Aβ E22Δ peptides neither formed amyloid fibrils *in vitro* nor were deposited in amyloid plaques in AD patient brains, and instead formed abundant oligomers *in vitro* (Tomiyama et al., 2008) and accumulated in oligomeric forms within transfected cells (Nishitsuji et al., 2009). The enhanced formation of Aβ oligomers and the lack of amyloid plaques in our $APP_{E693\Delta}$-Tg mice indicate that this mouse model is suitable for study of the contribution of Aβ oligomers to the pathogenesis of AD.

We initially tested in our $APP_{E693\Delta}$-Tg mice whether the consensus that Aβ oligomers cause early synaptic pathology in AD is correct. Synaptic and cognitive functions were examined by *in vivo* electrophysiology and behavioral tests using the Morris water maze at 8 months. By this age, Aβ oligomers had begun to accumulate within neurons in the hippocampus and cerebral cortex of the $APP_{E693\Delta}$-Tg mice. Although their basal synaptic transmission was not affected at this age, short-term and long-term synaptic plasticity (PPF and LTP, respectively) and spatial reference memory were significantly impaired. Our control APP_{WT}-Tg mice also exhibited weaker but significant impairment of synaptic plasticity at 8 months, despite little accumulation of Aβ oligomers. One possible explanation for these findings is that *in vivo* electrophysiology is a very sensitive assay, and that therefore even trace amounts of Aβ oligomers in the APP_{WT}-Tg mice could be found to cause synaptic dysfunction. Such small amounts of Aβ oligomers may not be detected by immunohistochemistry or Western blotting, and may be insufficient to cause cognitive impairment in water maze tasks which may be less sensitive than other behavioral tasks. Another possibility is that elevated secretion of Aβ from neurons may lead to synaptic depression (Kamenetz et al., 2003; Ting et al., 2007), in which Aβ monomer, as well as oligomers, may act as a negative regulator of synaptic transmission. Alternatively, overexpression of APP in neurons may itself cause synaptic dysfunction, independent of Aβ production. We also examined whether loss of synapses occurred in our $APP_{E693\Delta}$-Tg mice. Immunohistochemistry for the presynaptic marker synaptophysin demonstrated that synaptic density in the hippocampus of these mice decreased in age-dependent fashion from 8 months, in parallel with accumulation of Aβ oligomers. Thus, Aβ oligomers were confirmed to cause early synaptic pathology in the absence of amyloid plaques in our $APP_{E693\Delta}$-Tg mice.

The next and main objective of the present study was to clarify whether Aβ oligomers contribute *in vivo* to features of AD pathology other than synaptic alteration. Although numerous lines of APP-Tg mice have been shown to exhibit abnormal tau phosphorylation in the brain, this was detected only after amyloid plaque formation and within the dystrophic neurites surrounding plaques (Duyckaerts et al., 2008). We examined our $APP_{E693\Delta}$-Tg mice for tau pathology using immunohistochemistry. In the absence of amyloid plaques, the mice displayed abnormal tau phosphorylation in the hippocampus and cerebral cortex from 8 months, in parallel with intraneuronal accumulation of

Aβ oligomers. Tau hyperphosphorylation was detected in hippocampal mossy fibers and the cingulum, which connect granule cells of the dentate gyrus and the hippocampal CA3 region and the cingulate gyrus and entorhinal cortex, respectively. These neural connections have been thought to play important roles in learning, memory, and consciousness, and to be altered early in AD (Arendt, 2004; Villain et al., 2008).

Clustering of activated astrocytes and microglia around amyloid plaques is another feature of AD pathology thought to be involved in neurodegeneration via cytokine and chemokine release from these cells. Activation of astrocytes and/or microglia has been observed in many APP-Tg mice, though again this was only after amyloid plaque formation and was in the vicinity of plaques (Duyckaerts et al., 2008). On immunohistochemical examination, the $APP_{E693\Delta}$-Tg mice exhibited activation of microglia from 12 months and activation of astrocytes from 18 months in the cerebral cortex and hippocampus. Although we did not confirm the presence of extracellular Aβ oligomers in $APP_{E693\Delta}$-Tg mouse brain, the observed glial activation and recruitment may be caused by overflow of extracellular diffusible Aβ oligomers from neurons (Oddo et al., 2006). Alternatively, glial cells might have been activated and recruited by cytokines or chemokines released from glial cells which neighbored and were in contact with aberrant neurons containing Aβ oligomers.

The most striking feature of AD pathology is neuronal loss. In APP-Tg mice, the occurrence of neuronal loss has in several lines been reported only after intense development of amyloid plaques (Duyckaerts et al., 2008). On immunohistochemical examination, we found that our $APP_{E693\Delta}$-Tg mice, despite their lack of amyloid plaques, exhibited significant neuronal loss in the hippocampal CA3 region at 24 months. To our knowledge, this is the first report that neuronal loss was induced by Aβ oligomers alone *in vivo* in the absence of amyloid plaques. No neuronal loss was observed at younger ages or in other brain regions, suggesting that Aβ oligomer-induced neuronal death requires long exposure of cells to Aβ oligomers *in vivo* and occurs in cells vulnerable to the toxic effects of Aβ oligomers. This is consistent with our observation that, in patients with the E693Δ mutation, mild atrophy began to occur initially in the hippocampus several years after the onset of AD (H. Shimada, S. Ataka, T. Tomiyama, J. Takeuchi, H. Takechi, H. Mori, and T. Miki, unpublished observations).

Our findings imply that intracellular Aβ plays important roles in synaptic alteration and subsequent neuropathology. A similar relationship between intracellular Aβ and synaptic pathology has been reported in other Tg mice. In the triple transgenic 3xTg-AD mice, synaptic and cognitive dysfunction were found to be correlated with the accumulation of intraneuronal Aβ in the hippocampus (4 months) before amyloid plaque formation (12 months) (Oddo et al., 2003; Billings et al., 2005). In arcAβ mice, cognitive dysfunction was observed at 6 months, after the intraneuronal accumulation of Aβ (3 months) but before amyloid plaque formation (7 months) (Knobloch et al., 2007). Furthermore, in AD and Tg2576 mouse brains, morphological alterations of synapses occurred in association with intraneuronal accumulation of Aβ (Takahashi et al., 2002), and some of the intraneuronal Aβ formed oligomers (Takahashi et al., 2004). We also previously observed that in AD brains, synaptophysin was decreased around neurons containing Aβ oligomers (Ishibashi et al., 2006). Many other studies of patients with AD (Gouras et al., 2000; Fernández-Vizarra et al., 2004) and Down syndrome (Mori et al., 2002) and of APP-Tg mice (Casas et al., 2004; Lord et al., 2006; Oakley et al., 2006) have demonstrated that intraneuronal accumulation of Aβ is an early pathological change observed before amyloid plaque formation. However, we cannot exclude the possibility that the pathological changes we observed were induced by extracellular soluble Aβ oligomers. It appears possible that both extracellular and intracellular Aβ oligomers contribute to the pathology of AD.

Notably, Aβ oligomers predominantly accumulated in insoluble fractions, particularly the FA-extracted fraction, in $APP_{E693\Delta}$-Tg mice. This finding appears inconsistent with the prevalent view that Aβ oligomers are soluble. However, detergent-insoluble features of intracellular Aβ have also been demonstrated in 3xTg-AD mice (Billings et al., 2005). It may be that the soluble fraction of intracellular Aβ oligomers is in an equilibrium with the insoluble fraction, and that the E22Δ mutation in Aβ tends to shift the equilibrium toward the insoluble fraction. It is unclear whether these insoluble Aβ oligomers form fibrils within cells. While very weak thioflavin S staining was observed in neurons in the $APP_{E693\Delta}$-Tg mice, this staining may have reflected its binding to Aβ oligomers rather than Aβ fibrils. Alternatively, intraneuronal thioflavin S staining may reflect the presence of fibrillar aggregates of hyperphosphorylated tau. In our previous study, Aβ was found to localize largely at endosomes, and to a lesser extent in the ER, Golgi, lysosomes, and autophagosomes in cultured cells (Nishitsuji et al., 2009). In AD and Tg2576 mouse brains, Aβ was found to accumulate in multivesicular bodies, which are a type of endosome (Takahashi et al., 2002). Thus, endosomes may be the main site of accumulation of insoluble Aβ. A study to determine the subcellular localization of Aβ oligomers in our $APP_{E693\Delta}$-Tg mice is ongoing.

In summary, we found that Aβ oligomers caused not only synaptic alteration but also abnormal tau phosphorylation, microglial activation, astrocyte activation, and neuronal loss *in vivo* in the absence of amyloid plaques. Our findings provide a new insight into the pathogenesis of AD, that amyloid plaque formation is not an absolute requirement for the onset and development of AD. Instead, Aβ oligomers play pivotal roles throughout the progression of AD. It is true that we have not yet succeeded in forming neurofibrillary tangles in these mice and thus need to refine this model. Nevertheless, our Tg mice are, at present, the only animal model of Aβ oligomer-induced pathology avoiding the effects of amyloid plaques even at old ages, and are thus a valuable means of investigation of the pathological and physiological roles of Aβ oligomers and for evaluation of strategies for treatment of AD that specifically target Aβ oligomers.

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Short communication

DCP-LA neutralizes mutant amyloid β peptide-induced impairment of long-term potentiation and spatial learning

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ABSTRACT

Long-term potentiation (LTP) was monitored from the CA1 region of the intact rat hippocampus by delivering high frequency stimulation (HFS) to the Schaffer collateral commissural pathway. Intraventricular injection with mutant amyloid β_{1-42} peptide lacking glutamate-22 ($A\beta_{1-42}E22\Delta$), favoring oligomerization, 10 min prior to HFS, inhibited expression of LTP, with the potency more than wild-type amyloid β_{1-42} peptide. Intraperitoneal injection with the linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) 70 min prior to HFS neutralized mutant $A\beta_{1-42}E22\Delta$ peptide-induced LTP inhibition. In the water maze test, continuous intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide for 14 days prolonged the acquisition latency as compared with that for control, with the potency similar to wild-type $A\beta_{1-42}$ peptide, and intraperitoneal injection with DCP-LA shortened the prolonged latency to control levels. The results of the present study indicate that DCP-LA neutralizes mutant $A\beta_{1-42}E22\Delta$ peptide-induced impairment of LTP and spatial learning.

1. Introduction

Accumulating studies have pointed to the implication of amyloid β (Aβ) peptide in the pathogenesis of Alzheimer disease. Lines of evidence have suggested that soluble Aβ oligomers, but not Aβ fibrils, play a critical role in synaptic and cognitive disorders for Alzheimer disease [1,3,7,13]. Indeed, Aβ oligomers decrease synaptic density [8,14], inhibit long-term potentiation (LTP), a cellular model of learning and memory, in the hippocampus [9,17,18], and impair memory function [2,10]. We have found a novel amyloid precursor protein mutation (E693Δ) to produce variant $A\beta_{1-42}$ lacking glutamate-22 ($A\beta_{1-42}E22\Delta$) in Japanese pedigrees showing Alzheimer-type dementia and Alzheimer disease [16]. Notably, $A\beta_{1-42}E22\Delta$ peptide, favoring oligomerization, inhibited expression of the *in vivo* hippocampal LTP [16].

In our earlier study, 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a newly synthesized linoleic acid derivative, stimulated presynaptic glutamate release by enhancing activity of presynaptic α7 nicotinic acetylcholine (ACh) receptors through direct and selective activation of protein kinase C-ε (PKC-ε), thereby inducing an 'LTP-like' long-lasting facilitation of the *in vitro* and the *in vivo* hippocampal synaptic transmission [6,15,20]. In addition, DCP-LA ameliorated disorder of spatial learning and memory induced by intraperitoneal injection with scopolamine or continuous intraventricular injection with $A\beta_{1-40}$ peptide for rats [12] and improves age-related learning impairment for accelerated-senescence-prone mice 8 (SAMP8) [19].

The present study examined the effect of DCP-LA on LTP inhibition and spatial learning disorders induced by $A\beta_{1-42}E22\Delta$ peptide. The results show that DCP-LA neutralizes mutant $A\beta_{1-42}E22\Delta$ peptide-induced impairment of LTP and spatial learning.

2. Materials and methods

2.1. Animal care

All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Aβ peptides

Wild-type $A\beta_{1-42}$ peptide was purchased from American Peptide Company (Sunnyvale, CA, USA) and $A\beta_{1-42}E22\Delta$ peptide was synthesized based upon the results of mass spectrometry (American Peptide Company).

2.3. LTP monitoring

Under urethane (1.5 g/kg, intraperitoneal injection) anesthesia, field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 region of the hippocampus of Wistar rats (male, 6w) by electrically stimulating the Schaffer collateral (0.033 Hz, 0.1 ms in duration). The parameters for high frequency stimulation (HFS) to induce LTP were four trains with an inter-train interval of 200 ms and each train consisted of ten 30-s 200-Hz pulses. Phosphate-buffered saline (PBS)

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or DCP-LA (1 mg/kg weight) was intraperitoneally injected 70 min before HFS and subsequently, PBS (2 μl), wild-type $A\beta_{1-42}$ peptide (10 ng in 2 μl PBS), or mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng in 2 μl PBS) was injected in the right-sided lateral ventricle for 30 s using a Hamilton microsyringe 10 min before HFS.

2.4. Water maze test

PBS, wild-type $A\beta_{1-42}$ peptide (10 ng/day), or mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng/day) was continuously injected in the right-sided lateral ventricle of Wistar rats (male, 6w) for 14 days using an osmotic pressure pump, and then PBS or DCP-LA (1 mg/kg weight) was intraperitoneally injected 30 min prior to water maze task.

A circular plastic water tank with 180 cm in diameter and 45 cm in deep was used for a water maze test. The entire inside of the pool was painted black, and the pool was filled up to 25 cm from the bottom with muddy water containing India ink at 22 °C. A platform (11 cm in diameter) painted black was placed into water, the top sinking 1 cm below water surface. The pool was put in a test room, where there were several marks those rats are able to see from the pool. The position of the marks remained unchanged throughout testing. A platform was located in the constant position, i.e., in the middle of one quadrant, equidistant from the center and edge of the pool. Rats facing the wall of the pool were placed into water at one of 5 positions selected at random, and time from start to escape onto the platform (acquisition latency) was measured. When succeeded, rats were allowed to stay on the platform for 10 s. Rats received the task for consecutive 16 days and the acquisition latency (time form the start to arrival onto the plate) was measured.

2.5. Statistical analysis

Statistical analysis was carried out using Fisher's Protected Least Significant Difference (PLSD) test.

3. Results

3.1. DCP-LA neutralizes mutant $A\beta_{1-42}E22\Delta$ peptide-induced LTP inhibition

We monitored fEPSPs from the CA1 region of the intact rat hippocampus by electrically stimulating the Schaffer collateral commissural pathway. For control, HFS enhanced fEPSP slopes to 170–250% of basal levels, being evident at 120 min after HFS (LTP) (Fig. 1A and B). Intraventricular injection with wild-type $A\beta_{1-42}$ peptide inhibited expression of LTP ($P<0.001$ as compared with control LTP, Fisher's PLSD test), and DCP-LA (1 mg/kg, i.p.) significantly recovered the LTP inhibition to a level similar to control LTP ($P<0.0001$ as compared with LTP for wild-type $A\beta_{1-42}$ peptide treatment, Fisher's PLSD test) (Fig. 1A). More marked inhibition of LTP was obtained with mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng) ($P<0.0001$ as compared with LTP for wild-type $A\beta_{1-42}$ peptide treatment, Fisher's PLSD test), and DCP-LA (1 mg/kg, i.p.) significantly improved the LTP disruption ($P<0.0001$ as compared with LTP for mutant $A\beta_{1-42}E22\Delta$ peptide treatment, Fisher's PLSD test); conversely, DCP-LA enhanced LTP more than control LTP (Fig. 1B).

3.2. DCP-LA ameliorates mutant $A\beta_{1-42}E22\Delta$ peptide-induced spatial learning impairment

In the water maze test, intraventricular injection with wild-type $A\beta_{1-42}$ peptide prolonged the acquisition latency (Fig. 2A). A similar effect was obtained with mutant $A\beta_{1-42}E22\Delta$ peptide, with no significant difference in the latency between wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide (Fig. 2B). Intraperitoneal injection with DCP-LA (1 mg/kg, i.p.) significantly shortened the prolonged latency for both the treatment with wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide (each $P<0.001$ as compared with the latency for wild-type $A\beta_{1-42}$ or mutant $A\beta_{1-42}E22\Delta$ peptide treatment without DCP-LA, Fisher's PLSD test), reaching the levels for control (Fig. 2A and B). This indicates that DCP-LA ameliorates spatial learning disorders induced not only by wild-type $A\beta_{1-42}$ peptide but mutant $A\beta_{1-42}E22\Delta$ peptide.

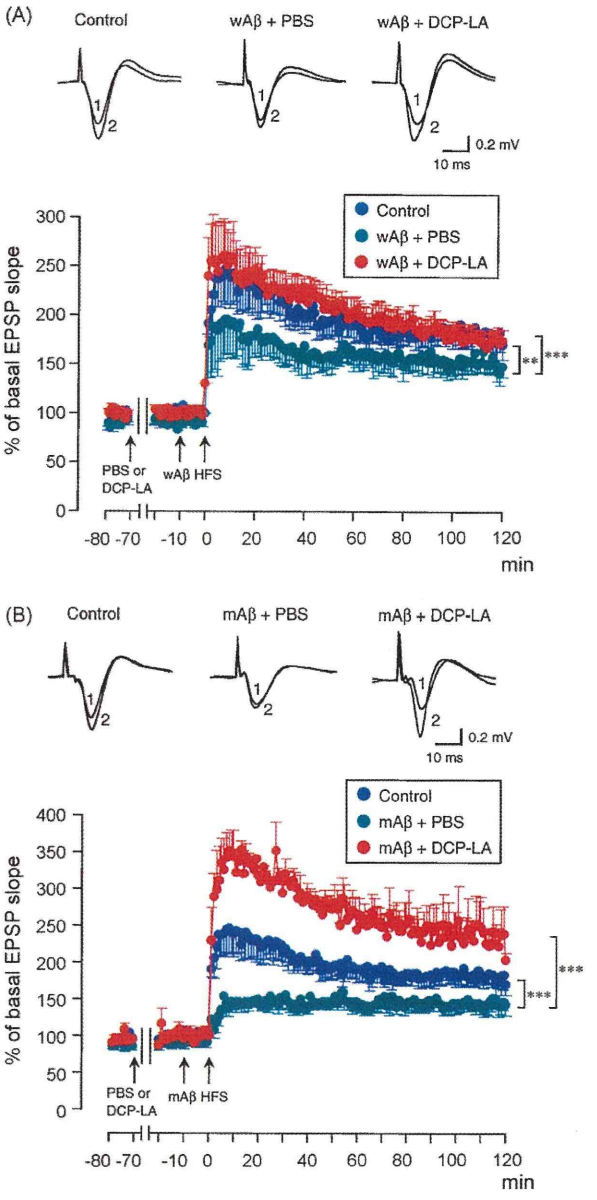

Fig. 1. Effect of DCP-LA on LTP inhibition induced by wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide. fEPSPs were monitored from the CA1 region of the intact rat hippocampus. DCP-LA (1 mg/kg) or PBS was intraperitoneally injected 70 min prior to HFS, to induce LTP, followed by intraventricular injection with wild-type $A\beta_{1-42}$ peptide (wAβ) (10 ng) (A) or mutant $A\beta_{1-42}E22\Delta$ peptide (mAβ) (10 ng) (B) 10 min prior to HFS. fEPSPs recorded at 0 (1) and 120 min (2) are superimposed. Control, intraventricular injection with PBS and intraperitoneal injection with PBS; wAβ + PBS, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with PBS; wAβ + DCP-LA, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with DCP-LA; mAβ + PBS, intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide and intraperitoneal injection with PBS; mAβ + DCP-LA, intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide and intraperitoneal injection with DCP-LA. In the graphs, each point represents the mean (±SEM) percentage of basal fEPSP slope (0 min) ($n=5$). $^{**}P<0.001$; $^{***}P<0.0001$, Fisher's PLSD test.

4. Discussion

One might think that the effects of the linoleic acid derivative DCP-LA on synaptic transmission are similar to the effects of nicotine, an agonist of nicotinic ACh receptors including α7 receptors. DCP-LA, however, is not an agonist of nicotinic ACh receptors, distinct from nicotine, and therefore, DCP-LA does not

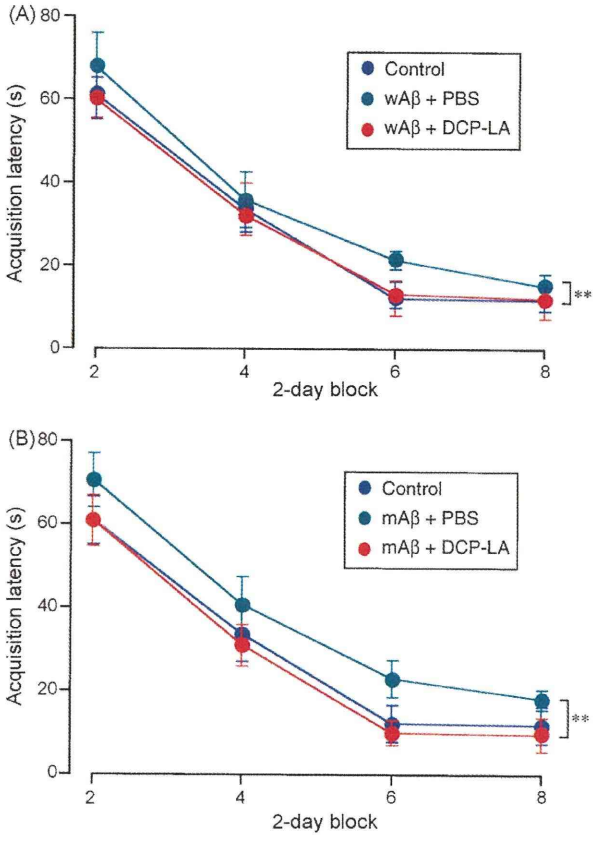

Fig. 2. Effect of DCP-LA on spatial learning impairment induced by wild-type $A\beta_{1-42}$ and mutant $A\beta_{1-42}E22\Delta$ peptide. PBS, wild-type $A\beta_{1-42}$ peptide (10 ng/day) (A), or mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng/day) (B) was continuously injected in the ventricle for 14 days, and then PBS or DCP-LA (1 mg/kg weight) was intraperitoneally injected 30 min prior to water maze task. Water maze task was performed two trials per day and the acquisition latency was measured. Control, intraventricular injection with PBS and intraperitoneal injection with PBS; wAβ + PBS, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with PBS; wAβ + DCP-LA, intraventricular injection with wild-type $A\beta_{1-42}$ peptide and intraperitoneal injection with DCP-LA; mAβ + PBS, intraventricular injection with mutant $A\beta_{1-42}E22\Delta$ peptide and intraperitoneal injection with PBS; mAβ + DCP-LA, intraventricular injection with mutant $A\beta_{1-42}$ E22Δ peptide and intraperitoneal injection with DCP-LA. In the graphs, each point represents the mean (±SEM) acquisition latency from consecutive 2 days ($n = 10$). $^{**}P < 0.001$, Fisher's PLSD test.

affect the receptor inactivation. DCP-LA serves as a potentiator of nicotinic ACh receptor responses under the control of PKC [6,15,20]. With regard to the presynaptic action, DCP-LA directly and selectively activated PKC-ε, that is enriched in presynaptic terminals, thereby enhancing activity of presynaptic α7 ACh receptors, causing an increase in presynaptic glutamate release responsible for long-lasting facilitation of hippocampal synaptic transmission [6,15,20]. With regard to the postsynaptic action, DCP-LA stimulated exocytosis of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunits, GluR1 and GluR2, by activating Ca^{2+}/calmodulin-dependent protein kinase II through protein phosphatase-1 inhibition in rat hippocampal slices [5], which also participates in DCP-LA-induced facilitation of hippocampal synaptic transmission. The long-lasting facilitation of hippocampal synaptic transmission would account for the beneficial action of DCP-LA on learning and memory deficits induced by scopolamine and $A\beta_{1-40}$ peptide or in association with aging [12,19].

We have confirmed that mutant $A\beta_{1-42}E22\Delta$ peptide tends to favor soluble oligomers rather than fibrils [16]. Much attractive finding was that a considerably low dose of mutant $A\beta_{1-42}E22\Delta$ peptide (10 ng) is capable of inhibiting LTP. Like mutant $A\beta_{1-42}E22\Delta$ peptide, wild-type $A\beta_{1-42}$ peptide still inhibited LTP, but to a lesser extent. These results may support the note that Aβ oligomers, but not Aβ fibrils, are a critical factor for Alzheimer-type dementia. Soluble Aβ oligomers, accordingly, could be a target of Alzheimer disease [4]. In the present study, DCP-LA recovered LTP inhibition induced by wild-type $A\beta_{1-42}$ peptide. Surprisingly, DCP-LA abolished mutant $A\beta_{1-42}E22\Delta$ peptide-induced LTP inhibition and furthermore, enhanced the LTP greater than control LTP. The underlying mechanism, however, is presently unknown. A study shows that docosahexaenoic acid, a *cis*-unsaturated free fatty acid, suppresses τ phosphorylation induced by Aβ oligomers [11]. This may account for the protective effect of the linoleic acid derivative DCP-LA against synaptotoxicity induced by Aβ oligomers.

In the water maze test, intraventricular injection with a low dose (total 140 ng) of mutant $A\beta_{1-42}E22\Delta$ peptide prolonged the acquisition latency as compared with that for control, with the potency similar to wild-type $A\beta_{1-42}$ peptide, indicating that soluble wild-type $A\beta_{1-42}$ or mutant $A\beta_{1-42}E22\Delta$ peptide might cause Alzheimer-type dementia. DCP-LA shortened each prolonged latency to control levels. DCP-LA, thus, appears to exert its beneficial action on spatial learning disorders induced by mutant $A\beta_{1-42}E22\Delta$ oligomers as well as wild-type $A\beta_{1-42}$ peptide bearing synaptotoxicity.

In conclusion, the results of the present study show that mutant $A\beta_{1-42}E22\Delta$ peptide inhibits LTP, with the potency more than wild-type $A\beta_{1-42}$ peptide, and impairs spatial learning. DCP-LA neutralizes the LTP inhibition and improves spatial learning disorders. This represents that DCP-LA could be developed as a promising drug for treatment of Alzheimer-type dementia and Alzheimer disease.

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1. Alzheimer病
2）病因・病態
②新しいAPP変異の同定による Aβオリゴマー仮説の検証*

梅田知宙**／富山貴美**／森　啓**

Key Words : amyloid β, oligomer, mutation

キーセンテンス
- 家族性AD患者においてAPPでは初めての欠失型変異（E693Δ）を同定した．
- 本変異APPより産生される欠失型変異（E22Δ）Aβは，フィブリルを形成せず，多くのオリゴマーを形成した．
- 本変異Aβはそのオリゴマー形成によりシナプス障害をひき起こすと考えられる．

はじめに

Alzheimer病（AD）では脳内にアミロイドβ（Aβ）が蓄積している．このAβが脳内において神経毒性をもつことがADの原因であると考えられている．Aβはその凝集状態により可溶性分子種（Aβモノマー，オリゴマー，プロトフィブリルなど）と不溶性分子種（Aβフィブリル）に分けられる．以前は，Aβフィブリルに神経毒性がありADをひき起こすと考えられていたが，現在では，Aβオリゴマーがその発症を担うとの見方（オリゴマー仮説）が強まっている．たとえば，Aβオリゴマーを海馬スライス培養に添加した際には長期増強（LTP）が抑制され，ラット脳にインジェクションすると学習記憶障害がひき起こされた[1)2)]．これらAβオリゴマーの添加によってシナプス数自体が減少することも培養系において示されている[3)4)]．実際にADでは脳内のAβオリゴマーが増加しており[5)6)]，認知障害の程度と相関するシナプスの変性[7)8)]はAβフィブリルよりもむしろAβオリゴマーの量と相関[9)10)]している，などの報告がなされている．しかし，ADの脳内において，実際にAβオリゴマーがその発症に寄与しているという直接的証拠は存在していなかった．ADの脳内にはAβオリゴマー同様にAβフィブリルも常に存在することから，そのどちらがADの発症にもっとも寄与するものであるのかを検証することは非常に困難であった．最近われわれは，この検証において非常に重要な知見を与える新しいAPP変異を家族性AD患者に発見したので紹介する[11)]．

臨床像および変異の同定

本変異の発端者は受診時57歳の日本人女性で，もの忘れの自覚症状があったが，MMSEスコアが健常レベルであったことからmild cognitive impairment（MCI）として診断された．この時点でMRIやPETによる皮質の萎縮やグルコース代謝に異常は認められず，SPECTによる側頭葉での軽度の脳血流低下が示されたのみであった．しかし，患者はこの後，進行性の認知機能の低下を示し，DSM-IIIRとNINCDS-ADRDAの基準に基

* 1. Alzheimer's disease. 2) Etiology and pathogenesis. ②Verification of the Aβ oligomer hypothesis with a novel APP mutation.

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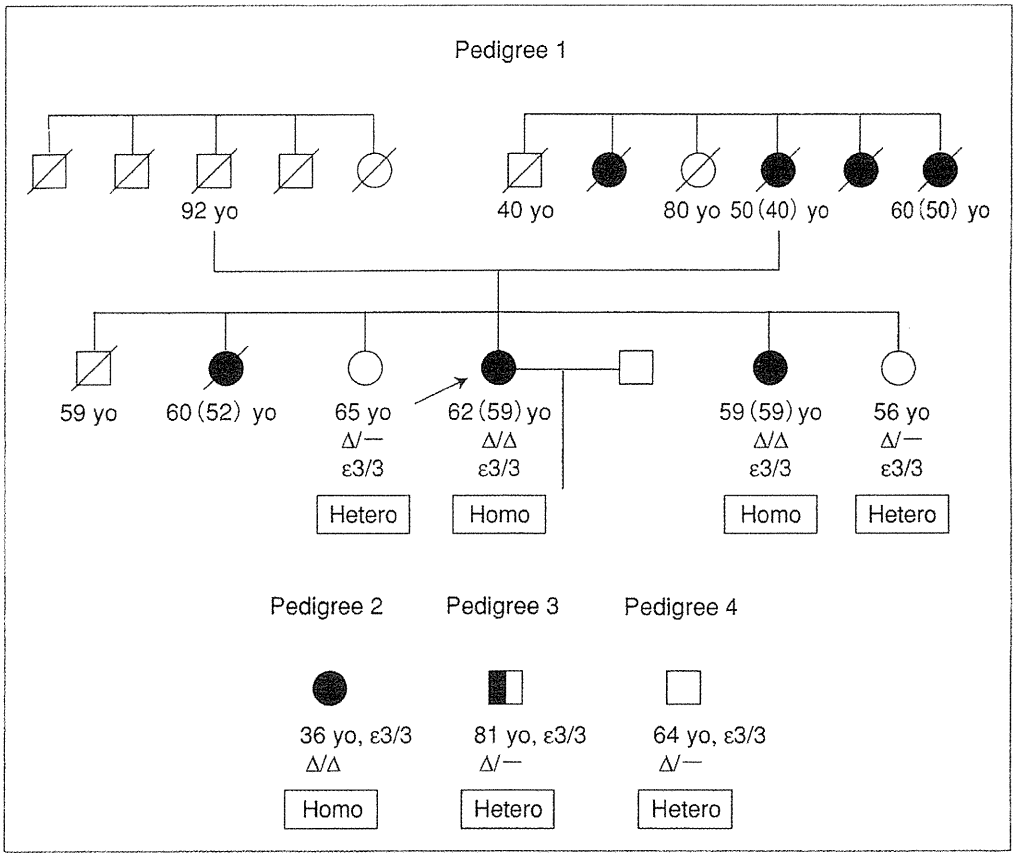

図1 変異の同定された家系

本変異は，認知症を発症している患者においてはホモで保持されている一方，認知症を発症していない者ではヘテロでの保持であった．□男性，○女性．塗り潰しが発症者．半塗りがMCI．矢印で発端者を示している．

づき，2年の後にADと診断された．その2年後にはMMSEスコアがさらに18まで低下し，この頃から小脳性運動失調，歩行障害，観念運動性失行，錐体路障害がみられるようになった．これらのことからわれわれは，この患者が典型的なADではなく，AD様の認知症であると診断した．この患者はさらに2年後にはMMSEスコアが5まで低下する重度の認知障害を呈するようになったが，MRIにおいては軽度の頭頂葉の萎縮が観察されたのみであった．この患者がMCIと診断された当初から，その家族歴から家族性認知症の疑いがあったため，当研究室においてこの患者の遺伝子診断を行った．

遺伝子診断の結果，われわれはこの患者とその家族から*APP*遺伝子の新しい変異を発見した．その変異はAPPの693番目のコドンGAAがまるまる抜け落ちることで，コードするアミノ酸のグルタミン酸が欠失するという，*APP*遺伝子では初めての欠失型となる変異であった．この欠失部位はAβ内部(Aβの22番目のグルタミン酸)に存在し，この部位はこれまでにもアミノ酸置換をひき起こす変異が数多く報告されている遺伝子変異のいわばホットスポットである．この変異は，この患者および同様に認知症を発症している妹においてホモで保持されている一方，認知症を発症していない他の2人の姉妹においてはヘテロでの保持であった(図1)．

われわれはさらに，AD，非AD型認知症，健常者からなる日本人5,310例の血液サンプルより本変異のスクリーニングを行った(図1)．その結果，同様の変異をさらに三つの別々の家系より発見した．三つのうちの一つについてはホモで本変異を保持しており，ADを発症していた．残り二つについてはヘテロでの保持であり，一つは健常者であったが，もう一つはMCIと診断されているものであった．

これらの結果より，本変異によるADの発症は家族性ADでは初めての劣性遺伝で生じている可能性が示唆された．これまでに知られている家族性ADの変異がいずれも，脳内でのAβ蓄積を増加させるメカニズムによって優性遺伝での発症をひき起こしていたのに対して，本変異はまったく別のメカニズムで発症に寄与している可能性が考えられた．

変異の効果

本変異をもつAPPから産生されるAβについて調べるために培養細胞に変異*APP*遺伝子を導入し，細胞から分泌されたAβの質量分析を行った．その結果，本変異APPより産生されるAβは，推測されたとおり22番目のグルタミン酸を欠失した変異Aβであった．

われわれはさらに，本変異によるAβ産生への影響について解析を行った．先ほどと同様に変異*APP*遺伝子を導入された培養細胞から分泌されるAβ量を野生型APPの場合と比較した．発現しているAPP量に差はない状態で，Aβ40の分泌量もAβ42の分泌量も本変異によって低下していた．さらにその際のAβ42/Aβ40比には野生型と変異型との間で違いはなかった．この変異によるAβ分泌の低下は，実際に本変異によるAD患者のCSF中のAβ量が低下していることからも確かめられた．

Aβの22番目およびその前後のアミノ酸に生じるミスセンス変異は，ネプリライシンやIDE (insulin-degrading enzyme)などのAβ分解酵素に対して耐性となることが知られている[12][13]．そこで，本変異Aβをネプリライシンや IDEとともにインキュベートした後，ウエスタンブロットによって分解されずに残ったAβ量を測定した．その結果，本変異Aβは野生型Aβと比べて半分程度しか分解されておらず，本変異によりAβの分解酵素耐性が上昇することが確認された．

次に，本変異のAβ凝集性への影響について検討を行った．フィブリル中のβシート構造を検出するチオフラビンアッセイにおいて，本変異Aβのフィブリル形成は1週間のインキュベートにおいても観察されなかった．このことは電子顕微鏡による観察によっても確認された(図2-A)．さらにウエスタンブロットにおいて，分子サイズの違いによって多量体を直接みることで本変異Aβのオリゴマー化についても検討した(図2-B)．野生型Aβは1週間のインキュベートによって急速にモノマーが減少し，オリゴマー形成もわずかに認められるのみであった．おそらくこれは，これらの低分子量会合体がフィブリル形成に使われたためであると考えられた．これに対し本変異Aβは，溶解直後からダイマー，トリマー，テトラマーなどの低分子オリゴマーを多量に形成し，これらのオリゴマーは1週間のインキュベート中安定に存在し続けた．これらの結果より本変異によってAβは素早いオリゴマー形成を行い，さらにフィブリル凝集は起こさずに，むしろオリゴマーの状態で安定化することが示された．このオリゴマー形成の促進は，実際の本変異によるAD患者のCSF中に多くのオリゴマーが存在したことによっても確認された．

アミロイドイメージング

AD患者の脳にはAβフィブリルの沈着により形成される老人斑が多量に存在することが知られている．本変異によりAβが本当にフィブリルを形成しないのならば，本変異を有する患者の脳内では老人斑はどうなっているのであろうか．剖検が不可能な本症例において脳内のアミロイド沈着を調べるため，われわれは，Aβフィブリルに特異的に結合するPittsburgh compound-B (PIB)をトレーサーとするPIB-PETを行った．その結果，*in vitro*での検討と同様に，本症例患者の脳では，通常のADでみられるような強いシグナルは検出できず，わずかなシグナルが小脳，側頭葉，後頭葉から得られたのみであった．つまり，本変異は脳へのAβフィブリルの沈着なしに，おそらくAβオリゴマーのみでADの臨床症状をひき起こしていると考えられた．

シナプス毒性

次にわれわれは，本変異Aβの毒性について検討した[14]．まず，神経系培養細胞へのAβ添加による細胞毒性をMTTアッセイで測定した．その結果，野生型Aβが濃度依存的に細胞毒性を発揮したのに対し，本変異Aβは高濃度においてわずかな細胞毒性を示しただけであった．フィブリ

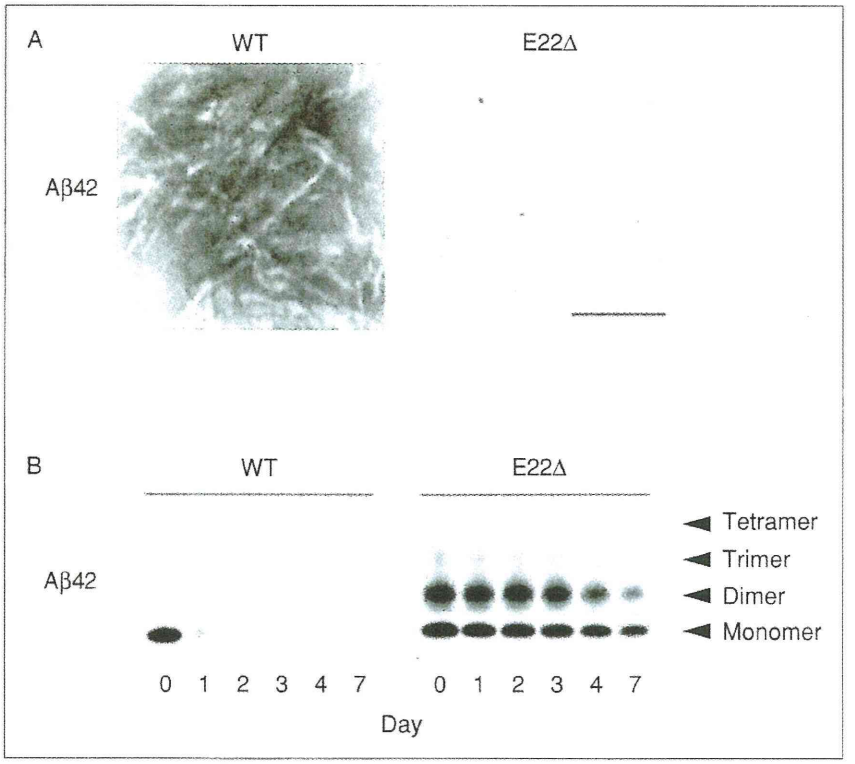

図2 変異Aβの凝集

A：合成ペプチドをPBSで溶解し，100μMの濃度で1週間インキュベートしたものを電子顕微鏡で観察した．野生型Aβ42はフィブリルを形成したが，変異型Aβ42はまったく形成しなかった．Scale bar：100nm．B：合成ペプチドをPBSで溶解し，100μMの濃度でインキュベートして経時的にサンプリングしウエスタンブロットを行った．野生型Aβ42よりも変異型Aβ42でより多くのオリゴマーが安定して検出された．

ルを形成しない本変異Aβによっては細胞毒性は観察されないというこの結果は，細胞死を誘導するような毒性はフィブリルによってひき起こされるとするこれまでの考え方と一致している．一方，オリゴマーについてはシナプス障害をひき起こすことが報告されている[3)4)]．そこで次に，本変異Aβのシナプスへの影響を調べるためにマウス海馬のスライス培養に対してAβの添加を行った．すると，本変異Aβは野生型のものよりも強く前シナプスマーカー蛋白であるシナプトフィジンを減少させることが観察された．添加Aβ濃度が低濃度の場合，野生型Aβは神経細胞に対し栄養因子的に働いてシナプトフィジンを上昇させたが，本変異Aβについてはこのような効果は観察されなかった．さらにわれわれは，ラットの脳室にこれらAβを注入し海馬LTPを*in vivo*で測定した．やはりここでも本変異Aβが強くLTPを阻害しており，シナプス障害が生じていることが確認された．これらの結果は，変異によってオリゴマー化したAβが脳内においてシナプス障害をひき起こすことが，本変異によるAD発症のメカニズムの一つであることを示している．

本変異APPを発現する細胞からのAβの分泌量は野生型APPに比べ低下していたが，本変異はβ-およびγ-セクレターゼによるAPPの切断を阻害しなかった[15)]．本変異APPを発現させた培養細胞におけるAβの細胞内局在を免疫細胞化学的に観察したところ，本変異Aβは細胞内オルガネラに蓄積しており，それによりERストレスを介したアポトーシスシグナルが誘導されていた．本変異によるAβの過剰なオリゴマー形成が，その細胞内輸送に影響を与え，その結果，分泌が低下したのかもしれない．細胞外におけるAβオリゴマーの毒性だけではなく，Aβの細胞内蓄積による毒性も，AD発症に重要である可能性が示唆された．