厚生労働科学研究費補助金

認知症対策総合研究事業

『認知症治療を目的とした変異型オリゴマーアミロイドペプチドを抗原とする神経免疫療法の開発』に関する研究

平成２１年度　総括･分担研究報告書

研究代表者　森　　啓
大阪市立大学・大学院医学研究科・教授

平成２２（２０１０）年　３月

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Ⅰ．総括･分担研究報告

厚生労働科学研究費補助金（認知症対策総合研究事業）
（総括・分担）研究報告書

認知症治療を目的とした変異型オリゴマーアミロイドペプチドを抗原とする神経免疫療法の開発に関する研究

研究代表者　　森　啓　大阪市立大学・大学院医学研究科・教授

研究要旨
アルツハイマー病の病因としてAβオリゴマーが重要になってきている。我々の発見したAPP E693Δ変異は、Aβオリゴマー形成を促進する性質がある。この特異な性質を利用してAβオリゴマーモデルとして新しくトランスジェニックマウスを作成した。同モデルは24カ月齢まで老人斑を形成しないことは言うに及ばず、すでに8カ月齢頃からニューロン内にAβオリゴマーを蓄積し始めると、シナプス機能低下と記憶障害が現れることが明らかになった。さらに同モデルは異常リン酸化タウ、活性化ミクログリアとアストロサイト、神経細胞死を観察することができたことは、老人斑がなくともAβオリゴマー単独でアルツハイマー病脳病変が誘導されることを示唆した。

A．研究目的

認知症とりわけ大部分を占めるアルツハイマー病（AD）の対策には、より確かで先進性のある「予防」、「診断」、「治療」面の証拠が必要とされている。このためは個別研究を推進することではなく、国際的な議論の中で高い評価を得る内容でなければならない。すなわち根本治療薬開発のための確実な研究成果によって、国民の福祉医療の大きな課題である高齢者の認知症対策が顕著に改善されることとなる。本研究は国際的に高い競争力と評価を備えた実施内容を推進することで、真摯な国民の期待に応えようとするものであり、最終ゴールを外すことを排除した方向性の上にある。具体的には、本研究は、わが国内で発見された遺伝変異を基に極めて独創性の高い内容を出発点としていることを強調しておきたい。

現在、世界的なアルツハイマー病研究の主流は、治療薬開発の標的のための病因解明に集中しつつあるが、その中でも、Ａβオリゴマーと呼ばれるアミロイド集合体が病因分子として中心的な議論の標的となっている。つまり、Ａβオリゴマーを正確に捉えることが、直接的な診断法と治療薬開発につながると考えられている。問題点は、Ａβオリゴマーとは何かについての国際的な共通認識が結論されていないことにある。

このような問題点があるにもかかわらず、Ａβオリゴマーについての知識については整理されつつある。まず、1998 年にノースウェスタン大学の Klein 博士により ADDLs と呼ばれる可溶性のＡβオリゴマーが発見された。これは、分子量 17kDa および 27kDa と発表された。Ａβ一分子の分子量が約 4.5kDa として３分子あるいは６分子からなる会合体と推測できる。さらに 2002 年には、ハーバード大学の Selkoe 博士によって dimer あるいは low-nＡβオリゴマーが同定されている。2003 年には、Klein 博士が ADDLs には分子量 56kDa の高分子成分を同定しているが、同じ分子量をもつＡβオリゴマーは、2006 年にミネソタ大学の Ashe 博士によってＡβ*56 の別称で議論されたことでＡβオリゴマーの意義がほぼ定着したと云える。

ここでは、発症・進行過程におけるＡβオリゴマーの病理学的役割を、我々が家族性 AD 患者から同定したアミロイド前駆体蛋白質（APP）の新しい変異 E693Δを発現するトランスジェニックマウス（Tg マウス）を用いて調べることである。まず、このマウスでの老人斑形成やＡβオリゴマーの蓄積、シナプス機能や学習記憶機能の変化を生化学、組織化学、電気生理学、行動試験などの手法を用いて調べ、Ａβオリゴマーモデルマウスとしての妥当性を検証する。次に、Ａβオリゴマーの蓄積によって、老人斑の形成なしに、シナプスの消失やタウの異常リン酸化、グリア細胞の活性化、ニューロンの消失などの AD の病理変化が起こるかどうかを組織化学的に調べる。

Ｂ．研究方法

＜トランスジェニックマウスの作成＞

大阪市立大学医学部附属病院にて見出された家族性 AD の APP の新変異（E693Δ）は、老人斑の形成なしに、Ａβのオリゴマー化を促進することで病気を発症させていると考えられた。この変異型遺伝子を発現するモデルマウスは、可溶性Ａβオリゴマーによるシナプス機能障害で始まると考えられ、現在もっとも議論の集中している可溶性Ａβオリゴマー解析に有用と考えられたために、トランスジェニック（Tg）マウスを作成することにした。プリオンプロモーター下に新変異（E693Δ）を組み込んだヒトＡＰＰｃＤＮＡ遺伝子コンストラクトのベクター部分を排除した後、顕微鏡下で受精卵前核へ DNA をマイクロインジェクションした。数時間培養後、DNA を注入した受精卵は偽妊娠状態にした雌マウスの卵管に移植し自然分娩で出産した仔マウスを C57BL/6 にて少なくとも、10 代以上戻し交配したものを確立した。

＜生化学的解析＞

Ａβの単量体および多量体形成はウェスタンブロットにより、β001 抗体、4G8 抗体、6E10 抗体により検出を試みた。

＜免疫組織学的解析＞

マウス脳組織におけるＡβはβ001 抗体、4G8 抗体、6E10 抗体、GFAP 抗体、Iba1 抗体、リン酸化タウ抗体（PHF1）、NeuN 抗体により検出を試みた。

＜電気生理学的解析＞

シナプス機能を検討するために LTP 測定を実施した。

＜行動科学的解析＞

変異型APPを発現するTgマウスの表現型を調べることで、Ａβオリゴマーの病理学的役割を探ることにした。

（倫理面への配慮）

臨床研究ではない、組み換え動物実験を使用した基礎医学的研究であるために、倫理面での配慮は不必要である。ただし、DNA 組み換え、動物実験についての承認は取得済みである

Ｃ．研究結果

マウスプリオンプロモーター下で E693Δ変異 APP を発現する Tg マウスを作製した。対照として、同じプロモーター下で野生型 APP を発現する Tg マウスおよび non-Tg 兄弟マウスを用いた。変異型 APP の発現量は、野生型 APP の約 1/2 であった。

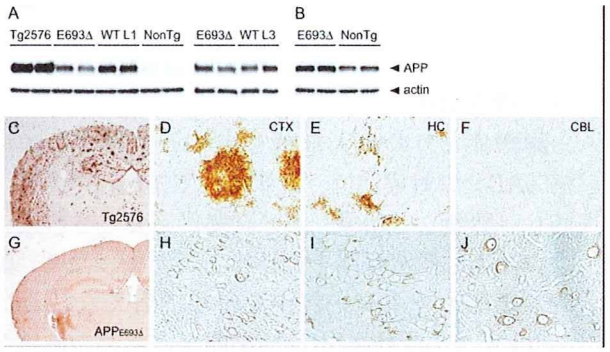

図1. 新しいＡβオリゴマーモデル（APPE693Δ）マウスの脳病理組織像
（A,B）既存老人斑（Tg2576）モデルマウス、野生型 APP モデルマウス、新しいＡβオリゴマー（APPE693Δ）モデルマウス、非 Tg マウスのウェスタンブロット分析。（C-F）Tg2576 マウス（G－Ｊ）APPE693Δマウス。CTX、HC、CBL は各々脳皮質、海馬、小脳をしめす。

様々な月齢でマウスの脳切片を作製し、ＡβやＡβオリゴマーに対する抗体（β001 および NU-1 抗体）で免疫染色を行ったところ、この変異型 APP-Tg マウスは、8 カ月齢頃より大脳皮質や海馬のニューロン

内にAβオリゴマーが蓄積し始めることがわかった（図１）。

しかし、細胞外の老人斑は、24 カ月齢においても検出されなかった[3)]。前シナプスのマーカーであるシナプトフィジンに対する抗体で染色したところ、この変異型 APP-Tg マウスは、8 カ月齢頃より海馬のシナプスが消失し始めることが確認された。そこで、8 カ月齢でこのマウスのシナプス機能および学習記憶機能を電気生理学および行動試験により調べた。その結果、海馬シナプスの長期増強の抑制およびモリス水迷路での空間参照記憶の低下が認められた（図２）。

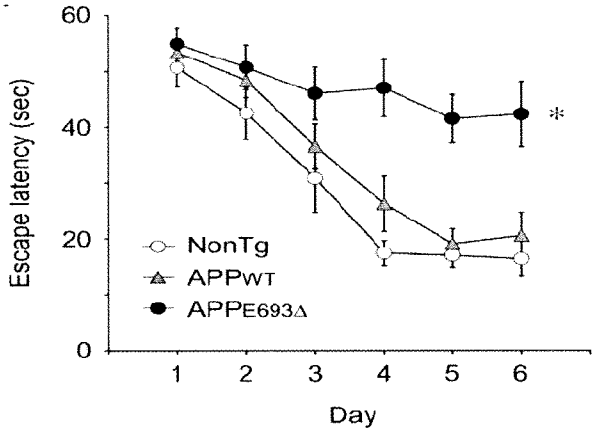

図2. モリス水迷路での空間参照記憶試験
Aβオリゴマーモデル（APPE693Δ：黒丸）、野生型ヒト APP マウス（黒三角）、非 Tg マウス（白丸）。

このマウスがAβオリゴマーのモデルマウスとして妥当であることが示された。

次に、病理タウに対する抗体（PHF-1 および MC1 抗体）、アストロサイトのマーカーGFAP に対する抗体、ミクログリアのマーカーIba-1 に対する抗体、成熟ニューロンのマーカーNeuN に対する抗体などを用いて、このマウスの脳に AD の病理変化が起こっているかどうかを免疫組織化学により調べた。その結果、このマウスは、8 カ月齢でタウの異常リン酸化、12 カ月齢でミクログリアの活性化、18 カ月齢でアストロサイトの活性化、そして 24 カ月齢で海馬ニューロンの消失を示した。

以上の結果より、Aβオリゴマーだけで AD の主な病理変化が引き起こされること、すなわち、Aβオリゴマーだけで AD が発症・進行し得ることが示唆された。

D．考察

Aβオリゴマーのみを蓄積し、フィブリルや老人斑を形成しない AD のモデルマウスはこれが初めてであり、現時点でオリゴマー仮説の問題点を解明することのできる唯一の動物モデルであると考えている。すなわち、従来の AD モデルマウスについても、Aβオリゴマーが証明されたり、議論されたりした例はあるが、老人斑の再現を視野においたモデルであることから、Aβオリゴマーについての議論は不十分であり、さらにはAβオリゴマーと老人斑の共存であるために評価を絞る事ができなかった。

さらに、これまで提唱されてきたAβオリゴマーの役割、作用については主としてシナプス障害が注目されてきており、発症初期に特化した病因として理解されてきた。しかしながら、本研究により脳病態後期反応であるグリア反応、異常リン酸化反応、さらにはニューロンの脱落をも本モデルで観察することができたことは、Aβオリゴマーが単独で、老人斑の存在から独立して作用することを直接的に証明するもので老人斑を対象とした研究の意義については大きな再考を促すこととなると考えられる。また、Aβオリゴマー蓄積、シナプス機能障害、シナプス変性、グリア反応、タウの異常リン酸化、神経細胞死という一連の病理変化が、加齢変化にともなって現れることも AD が老化に伴って増加する疫学調査とも矛盾しないことは強調するに値する。

本モデルマウスは、いわゆるアルツハイマー神経原線維変化（NFT: neurofibrillary tangles）を認めなかった。異常リン酸化タウから NFT へいこうする過程に老人斑が必要なのか、あるいはヒト型タウ分子の発言が必要なのかは不明である。さらに、マウス寿命が約 2 年であるが、NFT 形成に必要な歳月がさらに長いことも考えられる。この点については今後の課題として残されている。

最近、アミロイド老人斑を消去させる治療薬候補のヒト臨床治験が失敗に終わっているが、これはAβオリゴマーを標的としていないことが原因ではないかと推察することができる。本モデルマウスは、Aβオリゴマーをターゲットとする AD の新しい診断・治療薬の開発のための有用なツールとなると期待される。

本研究により、ADの発症・進行過程におけるＡβオリゴマーの病理学的役割を明らかにすることができた。また同時に、本研究により、老人斑はADの進行に必ずしも必要ではない可能性が強く示唆された。このことは、老人斑の病理学的意義やADの定義を考える上で重要な情報になると考えられる。

Ｅ．結論

AD 発症原因分子として注目されているＡβオリゴマーに特化した実験的モデルマウスを作成した。本モデルは、診断および治療薬開発の標的モデルマウスとして今後の研究に有用である。

Ｆ．健康危険情報

該当する内容はない。

Ｇ．研究発表

1.　論文発表

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2.　学会発表

①　招待講演

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② 口頭講演

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H. 知的財産権の出願・登録状況 （予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

テレビ・新聞報道

4月8日 NHK「おはよう日本」（午前6時）
4月8日 NHK「おはよう関西」（午前7時45分）
4月8日 朝日新聞（朝刊）
4月8日 読売新聞（朝刊）
4月8日 日本経済新聞（朝刊）
4月8日 日刊工業新聞（朝刊）
4月8日 日経産業新聞（朝刊）
4月13日 毎日新聞（夕刊）

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tomiyama T, Matsuyama S, Iso H, Umeda T, Takuma T, Ohnishi K, Ishibashi K, Teraoka R, Sakama N, Yamashita T, Nishitsuji K, ItoK, Shimada H, Mary Lambert, William Klein, Mori H	A mouse model of amyloid b oligomers: Their contribution to synaptic alteration, abnorm-al tau phosphorylation, glial activation, and neuronal loss in vivo"	Journal of Neuroscience	30	4845-4856	2010
Nagata T, Tomiyama T, Mori H, Yaguchi T, Nishizaki T	DCP-LA neutralizes mutant amyloid beta peptide-induced impairment of long-term potenti-ation and spatial learning.	Behavioural Brain Research	206	151-154	2010
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研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

III. 研究成果の刊行物・別刷

Neurobiology of Disease

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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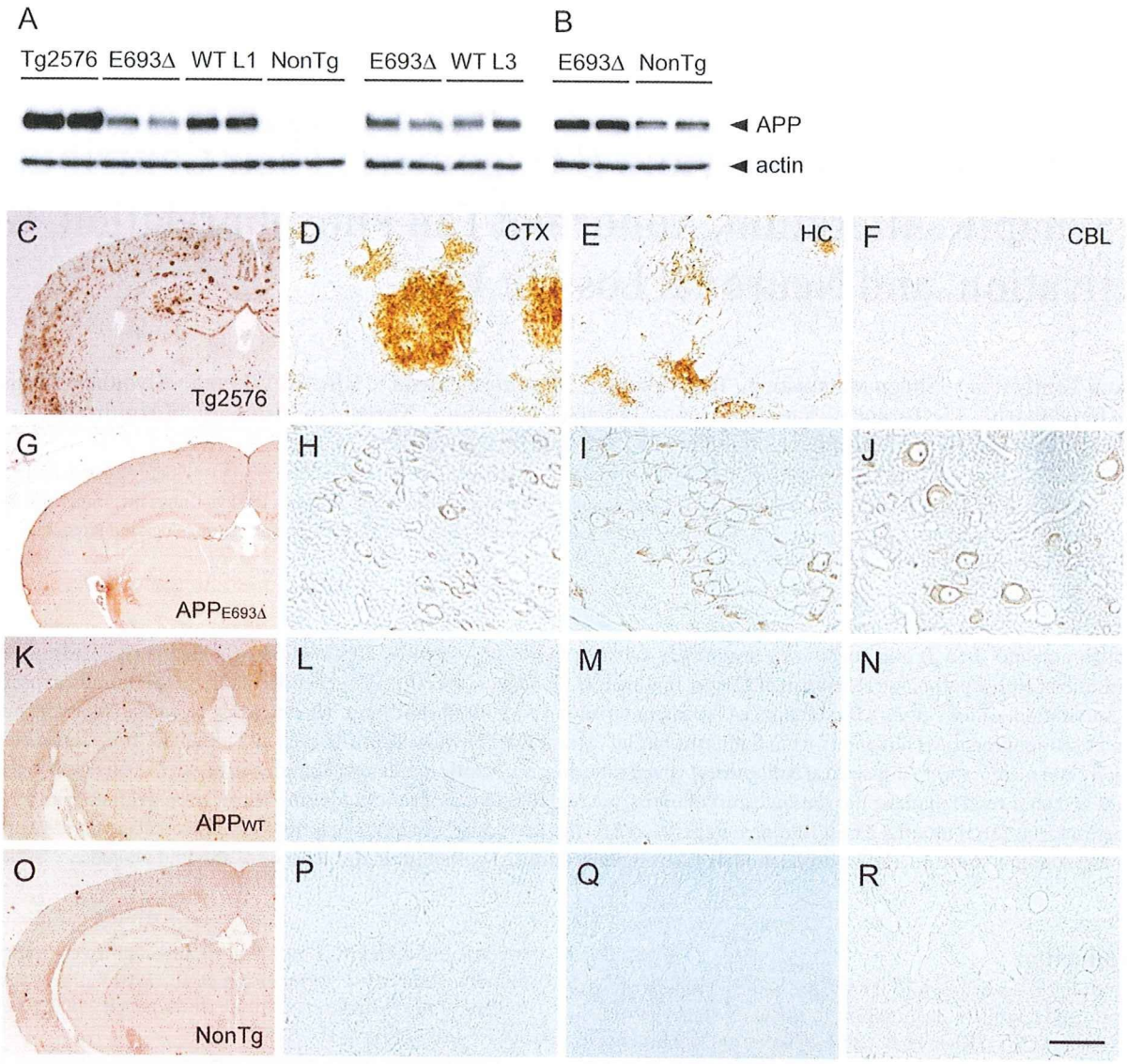

Figure 1. $APP_{E693\Delta}$-Tg mice do not exhibit extracellular amyloid deposits but do display intraneuronal Aβ accumulation. ***A***, ***B***, Levels of expression of human APP in $APP_{E693\Delta}$-Tg mice. Brain homogenates of Tg mice were subjected to Western blotting to examine levels of APP 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.

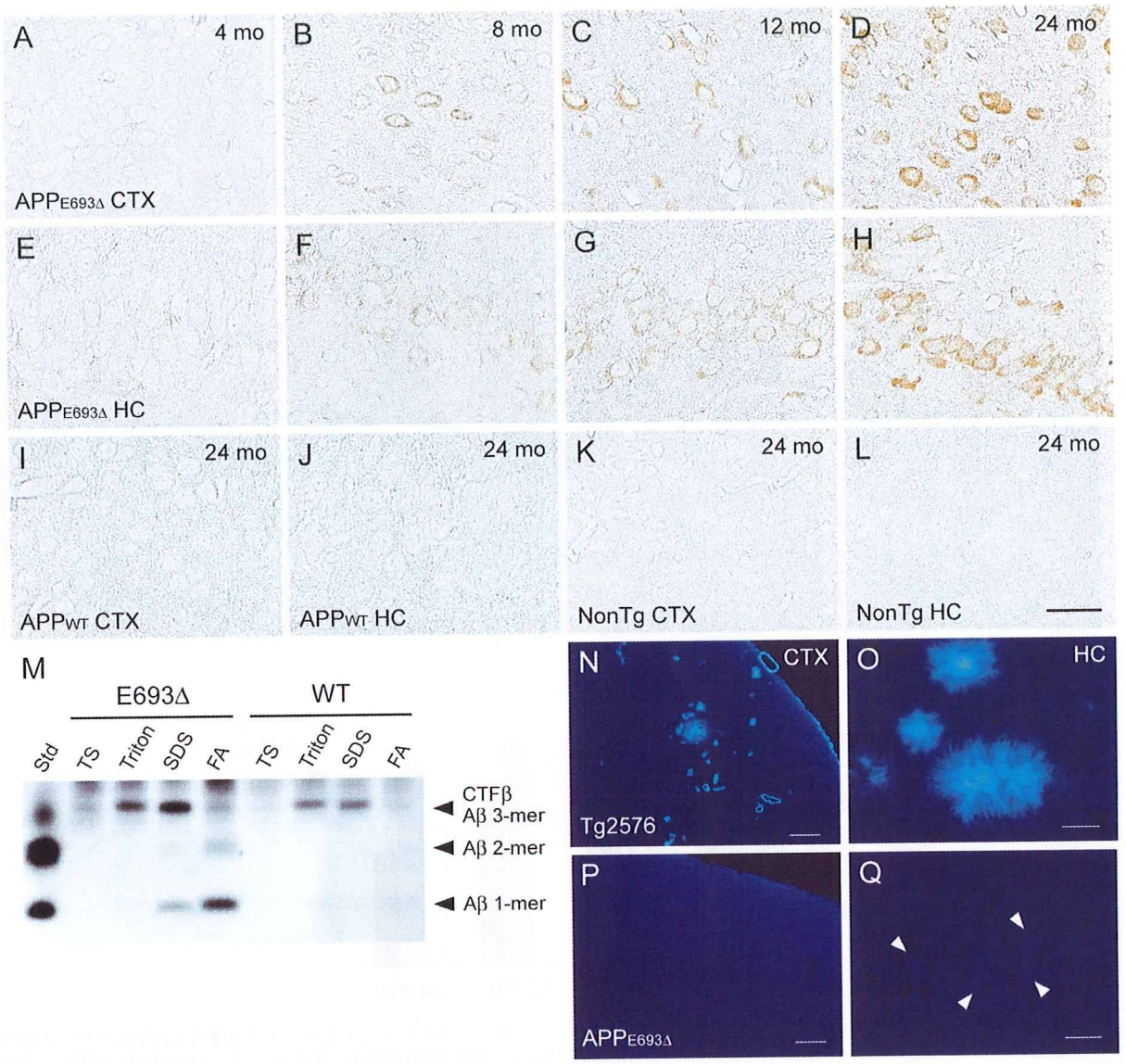

Figure 2. Age-dependent accumulation of intraneuronal Aβ oligomers in $APP_{E693\Delta}$-Tg mice. ***A–L***, Brain sections of 4 (***A***, ***E***)-, 8 (***B***, ***F***)-, 12 (***C***, ***G***)-, and 24 (***D***, ***H***)-month-old $APP_{E693\Delta}$-Tg mice were stained with Aβ oligomer-selective antibody 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

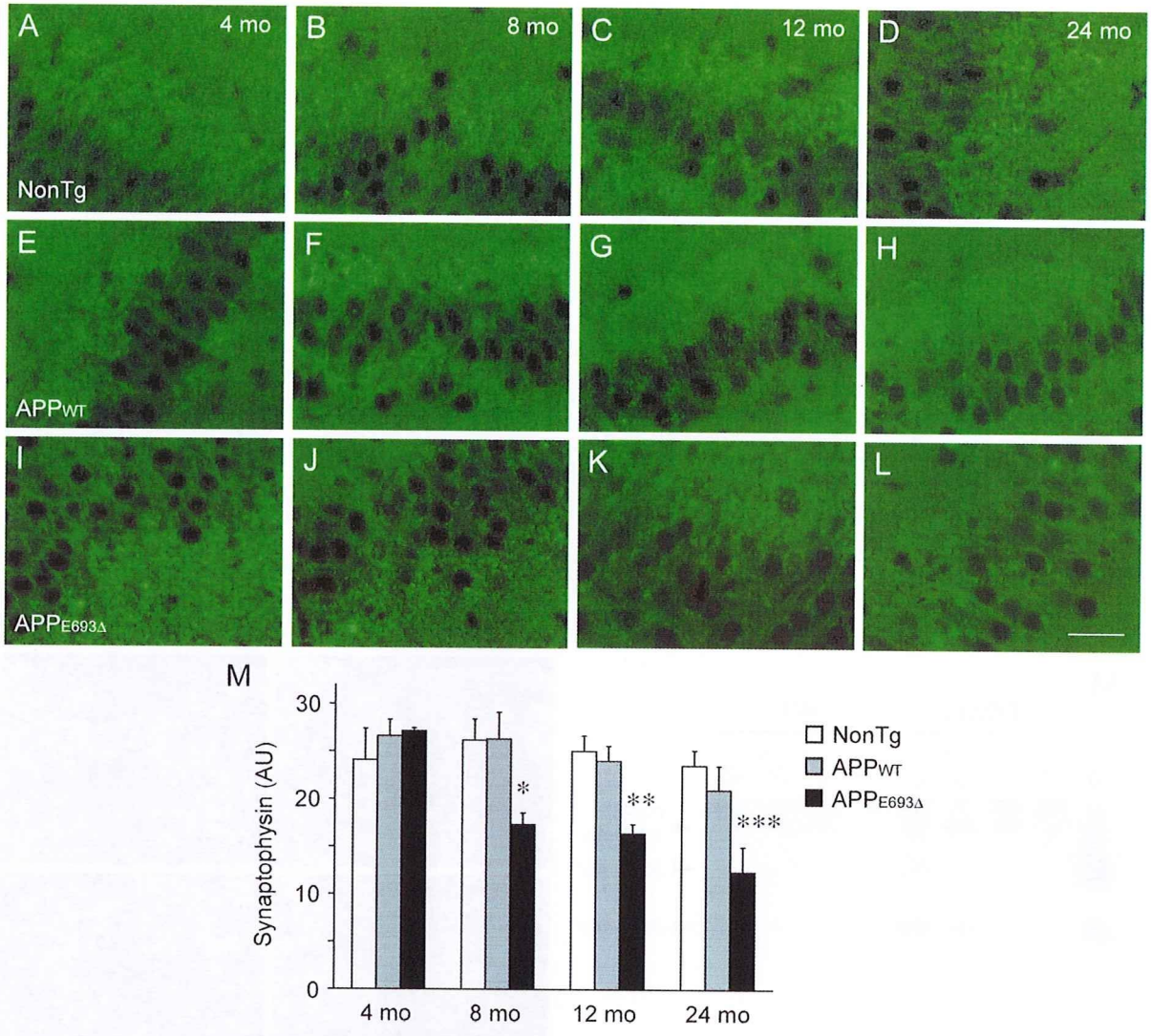

Figure 3. Age-dependent decrease in synaptophysin in $APP_{E693\Delta}$-Tg mice. ***A–L***, Brain sections of 4 (***A***, ***E***, ***I***)-, 8 (***B***, ***F***, ***J***)-, 12 (***C***, ***G***, ***K***)-, and 24 (***D***, ***H***, ***L***)-month-old Tg mice were stained with antibody to the presynaptic 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

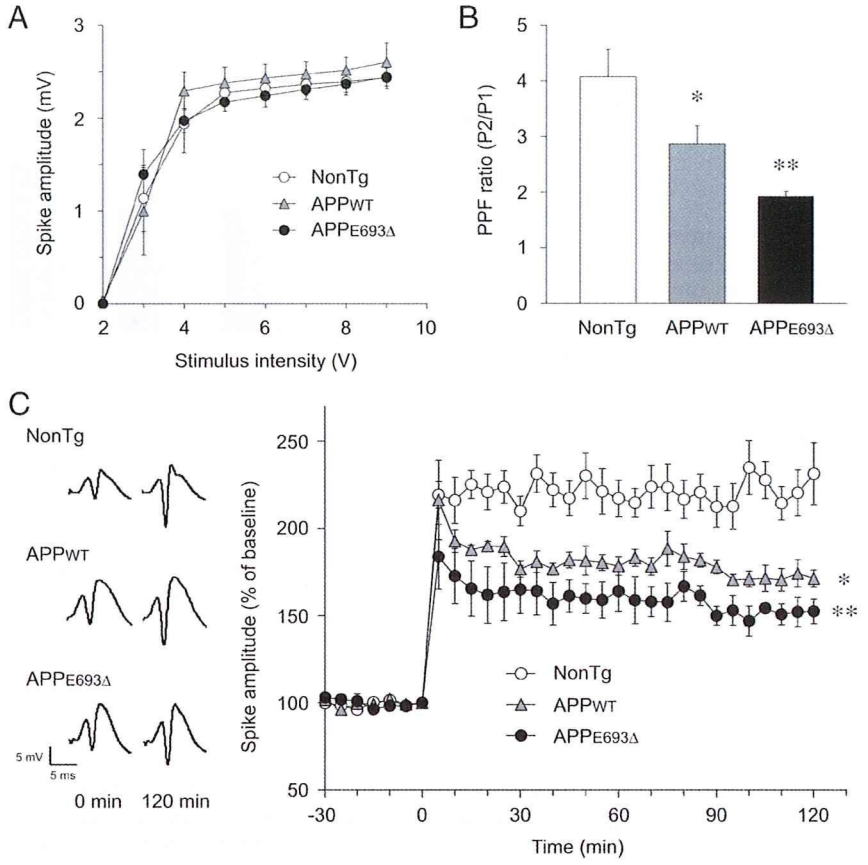

Figure 4. Impairment of synaptic plasticity in $APP_{E693\Delta}$-Tg mice. Synaptic functions of Tg mice were examined by *in vivo* electrophysiology at 8 months. Population spikes were recorded in the granular cell body layer of the dentate gyrus in response to stimulation of the perforant path. ***A***, 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.

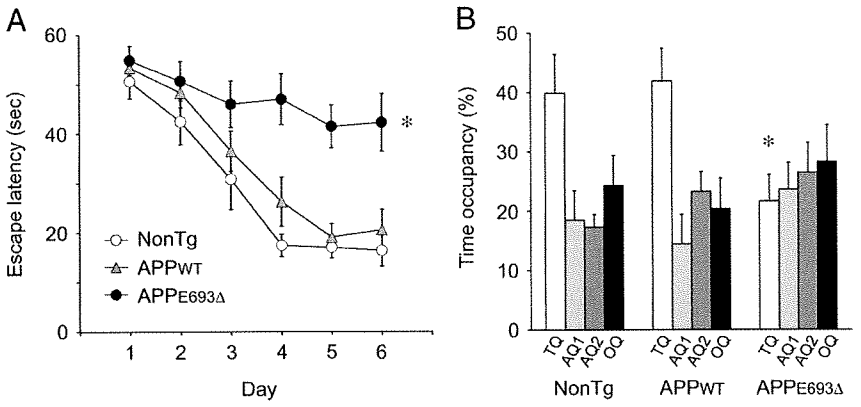

Figure 5. Impairment of memory in $APP_{E693\Delta}$-Tg mice. Spatial reference memory of Tg mice was assessed by the Morris water maze at 8 months. *A*, Mice were trained to swim to the hidden platform for 6 consecutive days. Each point represents the mean latency of five trials per day ± SEM ($n = 9$ 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.

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).

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

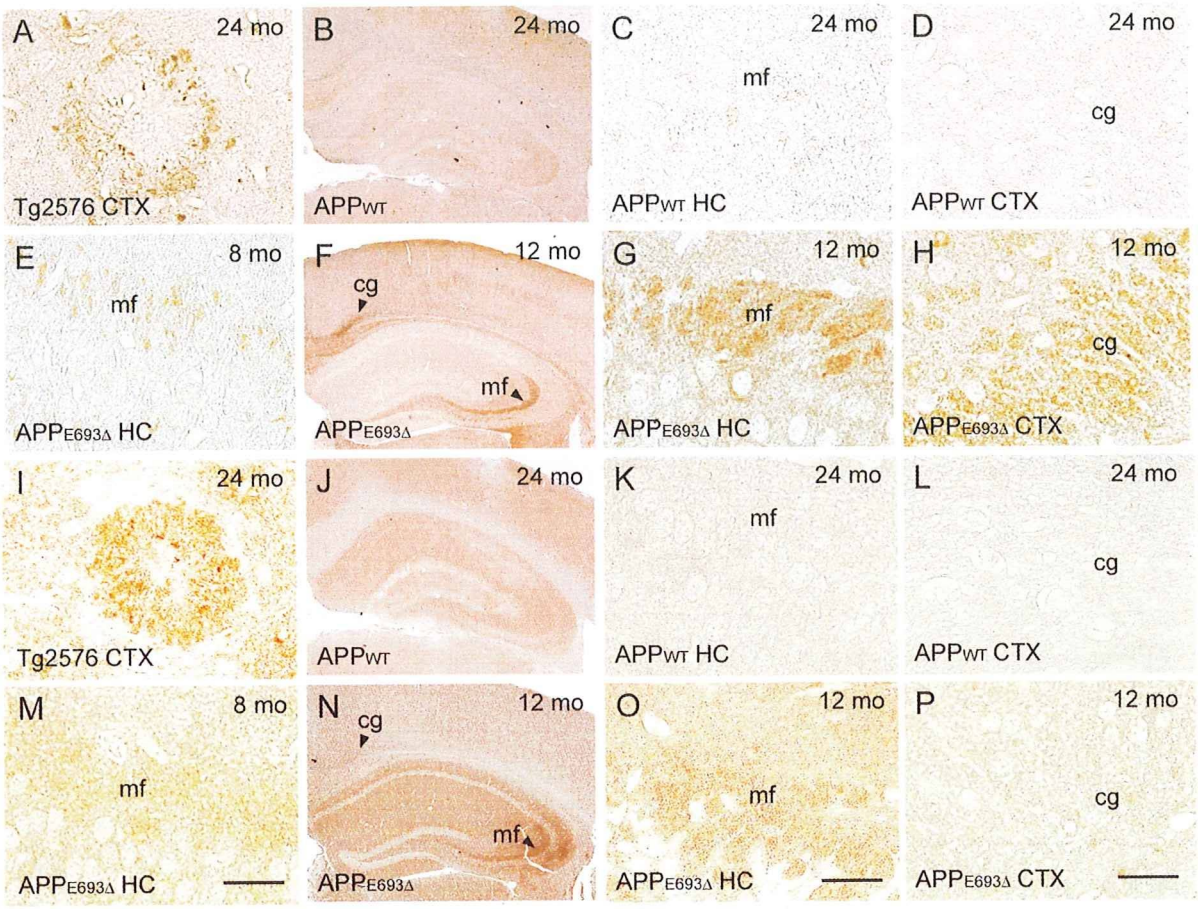

Figure 6. Abnormal tau phosphorylation in $APP_{E693\Delta}$-Tg mice. ***A–P***, Brain sections of 8 (***E***, ***M***)-, 12 (***F–H***, ***N–P***)-, and 24 (***A–D***, ***I–L***)-month-old Tg mice were stained with antibodies reactive to pathological tau, PHF-1 (***A–H***), and MC1 (***I–P***). These 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

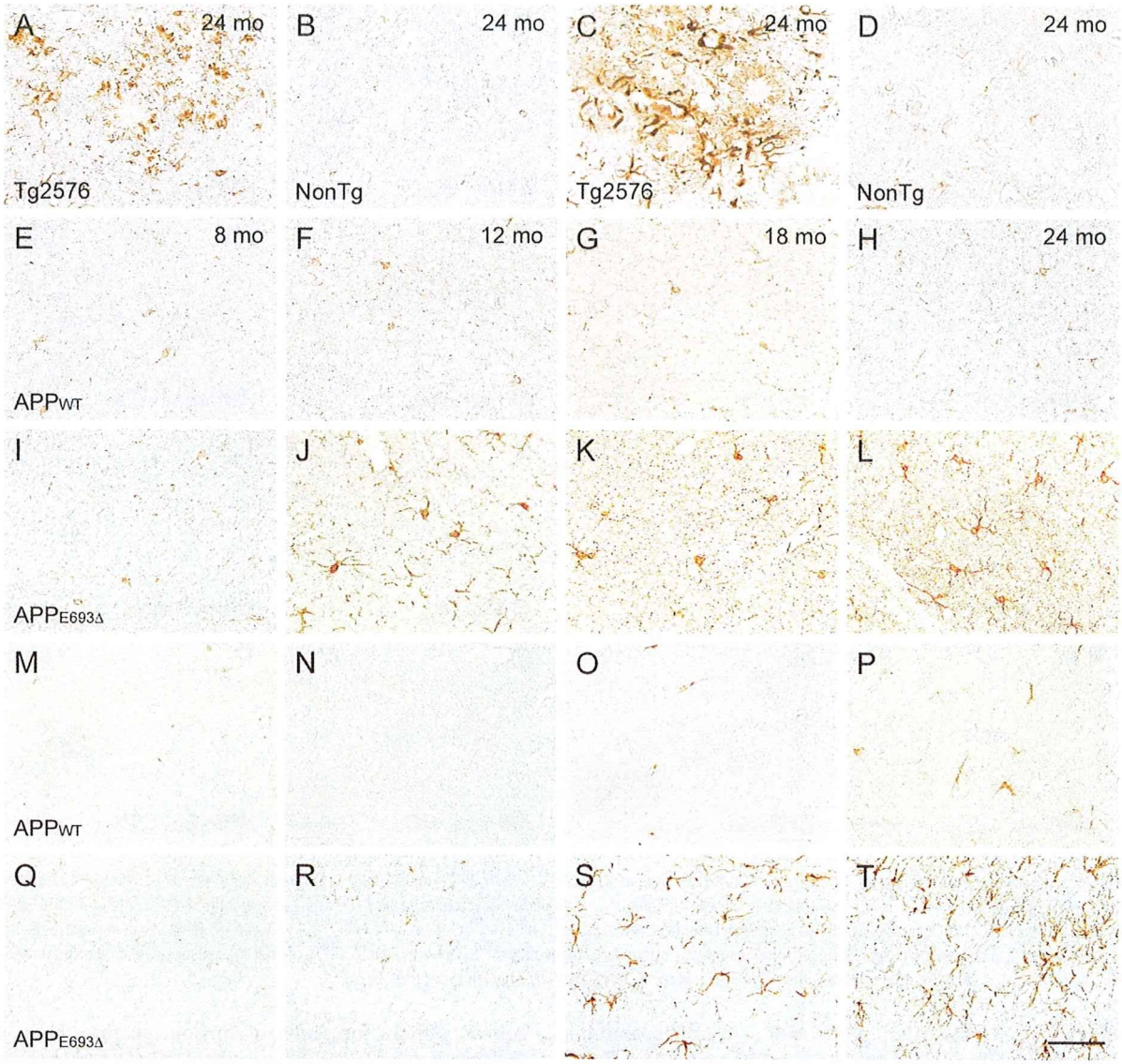

Figure 7. Glial activation in $APP_{E693\Delta}$-Tg mice. ***A–T***, Brain sections of 8 (***E, I, M, Q***)-, 12 (***F, J, N, R***)-, 18 (***G, K, O, S***)-, and 24 (***A–D, H, L, P, T***)-month-old Tg mice were stained with antibodies to Iba-1 (***A, B, E–L***) and GFAP (***C, D, M–T***), which are 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.