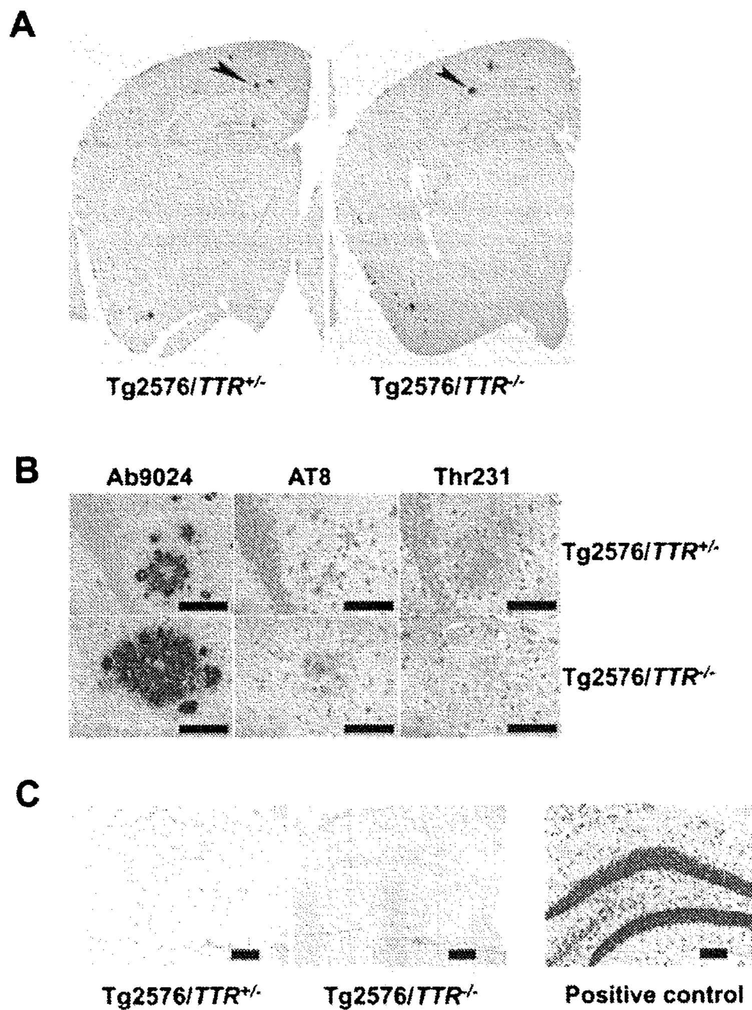

Figure 3. *Immunohistochemistry of Tg2576/TTR$^{+/-}$ and Tg2576/TTR$^{-/-}$ brains.* Immuno-labeling of left hemi-brain sections of 18-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice with Ab9204. **A.** The higher magnification of the hippocampal Aβ plaque with giant cores indicated by an arrowhead in **A** (**B**, *left panels*). Serial sections (5 μm) were labeled with AT8, and anti-phosphorylated tau (Thr231). AT8 and Thr-231 labeled punctate dystrophic neurites in and around Aβ plaques (**B**, *middle and right panels, respectively*). Scale bar; 50 μm. The hippocampal dentate gyrus areas of 18-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice stained with transferase-mediated dUTP nick end labeling. **C.** No apoptotic cells were found in the hippocampus. A DNaseI-treated sample was stained in parallel with the samples as a positive control. Scale bar; 100 μm. TTR = transthyretin.

Transthyretin deficiency does not affect Aβ deposition in the hippocampus of Tg2576 mice

The hippocampus is highly susceptible area to Aβ deposition in both humans (5) and Tg2576 mice (15). To investigate the effect of TTR deficiency on Aβ deposition in the hippocampus, we measured the total Aβ burden in the hippocampus of Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice. The Aβ deposits were first detected in the hippocampus of both the mice at 13 months of age, and showed an age-related increase (Figure 2D). Although the total Aβ burden in Tg2576/*TTR*$^{+/-}$ mice was consistently greater than that in Tg2576/*TTR*$^{-/-}$ mice, the difference was not statistically significant. Thus, the TTR deficiency does not affect Aβ deposition in the hippocampus of Tg2576 mice.

Transthyretin deficiency does not increase but decreases the level of Aβ40 in the brain of Tg2576 mice

Different forms of Aβ, biochemically distinguishable by their solubility properties, are present in varying amounts during the lifetime of Tg2576 mice. Detergent-soluble Aβ (SDS fraction) is present throughout life; however, detergent-insoluble Aβ (FA fraction) is absent up to age 6 months (18). It had been reported in AD that the predominant Aβ peptide present in CAA is Aβ40; however, in brain parenchymal plaques, it is Aβ42 (1, 7, 17, 29, 44). To evaluate whether or not TTR affects the level of different forms of Aβ, we quantified the Aβ40 and Aβ42 in SDS and FA fractions of brain homogenates from Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice by sandwich ELISA, as described under *Methods*. The number and age of 13–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice examined were shown in Table 2. Aβ40 and Aβ42 levels in SDS and FA fractions increased with age in both the mice. There was no significant difference in the levels of Aβ40 and Aβ42 in both the fractions between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice up to 17 months of age. In 18–20-month-old Tg2576/*TTR*$^{-/-}$ mice, however, the levels of Aβ40 in both SDS and FA fractions were significantly reduced by 35.2% and by 41.6%, respectively, relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 4A,B). The level of Aβ42 in SDS fraction was also significantly reduced by 57.8% in 18–20-month-old

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Table 2. The number and age of mice examined by sandwich enzyme-linked immunosorbent assay. Abbreviation: n = number of mice.

Age (months)	Tg2576/*TTR*$^{+/-}$ (n)	Tg2576/*TTR*$^{-/-}$ (n)
13	2	2
14	3	3
15	2	2
16	3	3
17	2	2
18	5	5
20	2	2
Total	19	19

Tg2576/*TTR*$^{-/-}$ mice relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.01$) (Figure 4C). On the other hand, there was no significant difference in the levels of Aβ42 in FA fraction between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice (Figure 4D). The mean level of Aβ42 in FA fraction is much higher than that in SDS fraction. Thus, there was no significant difference in the sum of Aβ42 levels in both the fractions between aged Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice. Thus, TTR deficiency does not increase but rather decreases the level of Aβ40 in the brain of aged Tg2576 mice, a result, which is in good agreement with the immunohistochemistry data, suggesting that TTR increases the vascular Aβ burdens in the brain of aged mice (Figure 2).

Transthyretin deficiency does not affect the distribution and degree of tau phosphorylation in the brain of Tg2576 mice

In contrast to human AD, Tg2576 mice lack NFT, and develop the phosphorylated tau-immunoreactive aberrant structures that are exclusively associated with congophilic Aβ plaques (27, 48, 49). Stein *et al* reported that chronic infusion of an antibody against TTR into the hippocampus of Tg2576 led to an increase of tau phosphorylation within the CA1 neuronal field (42). To investigate whether or not TTR deficiency affected the distribution and degree of tau phosphorylation, the brain slices of 16–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice were stained with either

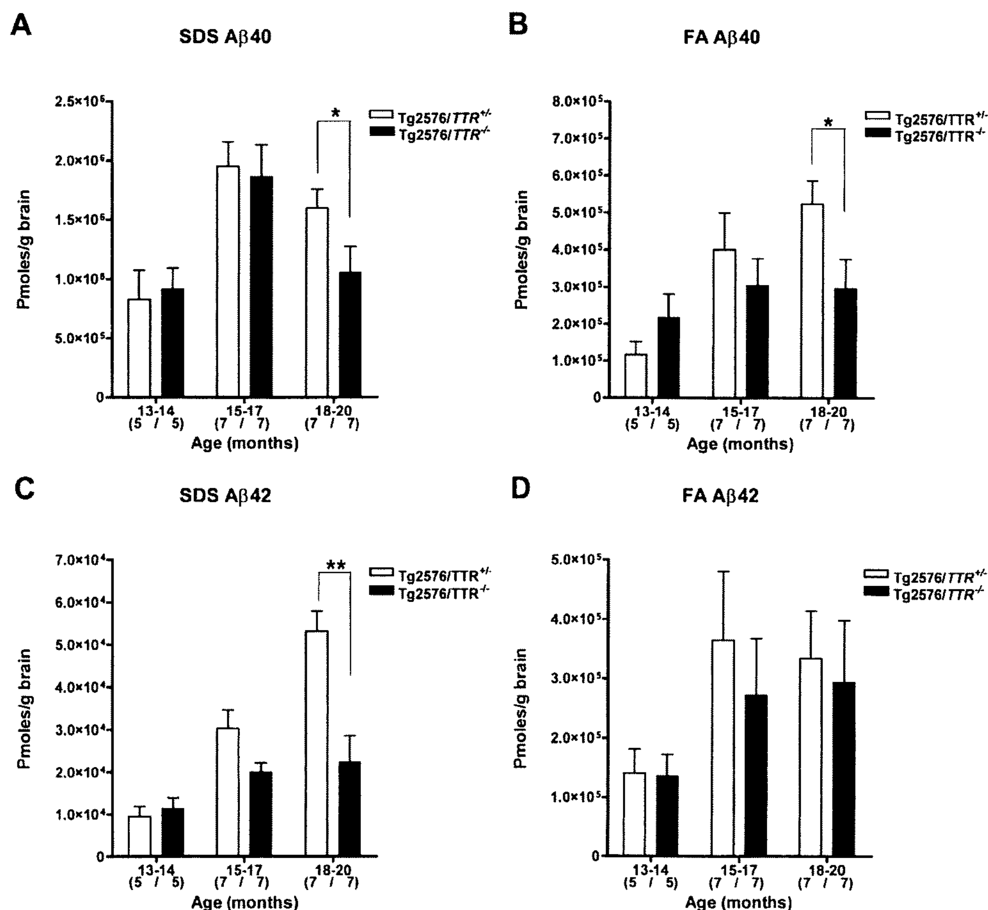

Figure 4. *The Aβ level in the brain of Tg2576/TTR$^{+/-}$ and Tg2576/TTR$^{-/-}$ mice.* The Aβ40 (**A,B**) and Aβ42 (**C,D**) in Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ brains were quantified by sandwich enzyme-linked immunosorbent assay. The samples were sequentially extracted in 2% sodium dodecylsulfate (SDS) (**A,C**) and 70% FA (**B,D**). All data are expressed as mean ± standard error of the mean. Numbers in parentheses denote numbers of mice examined. $*P<0.05$, $**P<0.01$. TTR = transthyretin.

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AT8 or Thr231 antibody, as described under *Methods*. Both the antibodies reacted only with the punctate dystrophic neurites (DNs) within the Aβ plaques in hippocampus and cerebral cortex in both the mice (Figure 3B). The abundance of the DNs immunopositive with the antibodies in Tg2576/*TTR*$^{-/-}$ mice was much the same as that in Tg2576/*TTR*$^{+/-}$ mice (Figure 3B). No NFT was detected in any of the mice examined. Thus, TTR deficiency does not affect tau phosphorylation in the brain of Tg2576 mice.

No apoptotic cells are detected in the hippocampus of Tg2576/TTR$^{+/-}$ and Tg2576/TTR$^{-/-}$ mice

Tg2576 mice do not develop severe neuronal loss observed in AD (15). Stein and Johnson suggested that high level of TTR in the hippocampus of Tg2576 mice might protect the mice from severe neuronal loss (43). Furthermore, the same group reported that chronic infusion of an antibody against TTR into the hippocampus of Tg2576 mice led to an increase of neuronal loss and apoptosis within the CA1 neuronal field (42). To determine whether or not TTR deficiency induces apoptosis in the hippocampus of Tg2576 mice, the brain sections from 18–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice were subjected to TUNEL immunohistochemistry, as described under *Methods*. Apoptotic cells were never detected in the hippocampus or other parts of brain of any of the mice examined (Figure 3C). These results indicate that TTR deficiency does not induce apoptosis in the brain of Tg2576 mice.

DISCUSSION

To investigate the role of TTR in the Aβ deposition *in vivo*, we generated a mouse line carrying a null mutation at the endogenous *TTR* locus and the human mutant APP cDNA with the Swedish mutation (Tg2576/*TTR*$^{-/-}$ mouse) by crossing Tg2576 mice with TTR-deficient mice generated through gene targeting. We then asked whether Aβ deposition was accelerated in Tg2576/*TTR*$^{-/-}$ mice relative to the heterozygous mutant Tg2576 (Tg2576/*TTR*$^{+/-}$) mice. Contrary to our expectations, the degree of total Aβ deposition, tau phosphorylation and apoptosis in the brain was not increased by eliminating TTR in Tg2576 mice. Moreover, the degree of vascular Aβ burden in the aged Tg2576/*TTR*$^{-/-}$ mice was significantly reduced relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice. Our experiments present, for the first time, compelling evidence that TTR does not suppress but rather accelerates vascular Aβ deposition in the mouse model of AD.

We confirmed that there was no significant difference in the onset, progression and distribution of total Aβ deposition between Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice up to age 17 months by immunohistochemistry (Figure 2A). However, total Aβ burden in 18–20-month-old Tg2576/*TTR*$^{-/-}$ mice was significantly reduced relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 2A). The result suggested that TTR does not suppress but rather accelerates Aβ deposition in the brain of Tg2576 mice. Although both Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice are smaller than non-transgenic littermates, both of them display no obvious phenotypic abnormalities, and their fertility is normal up to age 10 months. This observation is consistent with the immunohistochemistry data.

We then separately assessed vascular amyloid and plaque burdens in the brain of Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice. Although Aβ plaque burden was much the same between 7–20-month-old Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice (Figure 2C), vascular amyloid burden in the aged (18–20-month-old) Tg2576/*TTR*$^{-/-}$ mice was significantly reduced relative to the age-matched Tg2576/*TTR*$^{+/-}$ mice ($P < 0.05$) (Figure 2B). The quantification of Aβ40 and Aβ42 in the brain homogenates from Tg2576/*TTR*$^{+/-}$ and Tg2576/*TTR*$^{-/-}$ mice by sandwich ELISA demonstrated that TTR deficiency does not increase, but rather decreases the level of Aβ40 in the aged Tg2576 mice (Figure 4). Because the predominant Aβ peptide present in vascular amyloid deposits is reportedly Aβ40 (1, 7, 44), the result is also in good agreement with our immunohistochemistry data (Figure 2), suggesting that TTR increases the vascular Aβ burden in the brain of aged Tg2576 mice.

The reason why vascular amyloid burden is increased by TTR is not clear. Amyloid deposits of all types, including Aβ deposits, contain glycosaminoglycans (GAGs) and serum amyloid P component (SAP). A role for GAGs in amyloidosis is inferred from the observation that small molecules that interfere with GAG/amyloid interactions reduce murine experimental amyloid A (AA) amyloid progression (19). An amyloid-binding protein SAP protects amyloid fibrils from proteolysis *in vitro* (46), and the induction of AA amyloidosis is significantly retarded in the SAP-deficient mice relative to wild-type mice (4, 47). On the other hand, recent evidence indicates that Aβ is mainly cleared out of the brain to blood via transport through the blood-brain barrier, and via the interstitial fluid (ISF) bulk flow along periarterial drainage pathways into the CSF, and from there into the blood (26, 33, 52, 56). It is the CSF and perhaps the ISF and not the brain parenchyma (41) that is enriched in TTR. Thus we think it likely that when Aβ drains from the brain parenchyma along periarterial drainage pathways, it may come into contact with TTR which may protect Aβ deposits from proteolysis like GAG and SAP, thereby, slightly increases vascular amyloid burden over the ages.

Schwarzman *et al* reported that TTR in the CSF binds Aβ, and prevents Aβ fibril formation *in vitro*. They, however, also reported that apoE prevents Aβ fibril formation too (36, 37). It has been well established that apoE promotes assembly of Aβ fibril (23, 32). Thus, TTR may promote the fibrillization of Aβ too. Moreover, Holtzman *et al* found that a transgenic mouse model of AD on an apoE$^{-/-}$ background had significantly reduced Aβ deposition relative to the same mouse model expressing wild-type murine apoE (apoE$^{+/+}$), human apoE3 (apoE3$^{+/-}$) or human apoE4 (apoE4$^{+/-}$) (12). Therefore, TTR null Tg2576 (Tg2576/*TTR*$^{-/-}$) mice may represent mice that are unable to form Aβ fibrils, and the Aβ detected in the brain of the mice could be due in part to apoE.

Stein *et al* reported that chronic infusion of anti-TTR antibody into the hippocampus of Tg2576 mice increased Aβ burden, and led to tau hyperphosphorylation, neuronal loss and apoptosis in the CA1 neuronal field (42). These observations suggest the importance of TTR in inhibition of Aβ fibril formation and toxicity. However, contrary to these reports, our experiments suggested that TTR does not suppress but rather enhances Aβ deposition in Tg2576 mice. The reason for the discrepancy between data of other authors and our data is not clear. TTR is complexed with retinol-binding protein (RBP) and thyroid hormone *in vivo*. In the *in vitro* Aβ aggregation assay, however, recombinant TTR alone, not complexed with RBP or thyroid hormone, was used to examine its ability to inhibit Aβ fibril formation (36, 37). Association of TTR with RBP and thyroid hormone may affect its binding capacity

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with Aβ *in vivo*. Tau phosphorylation and apoptosis were induced by Aβ in hippocampal cultures (42). Thus, as suggested by Stein *et al*, high intrahippocampal concentration of Aβ, induced temporarily by the disruption of TTR binding of Aβ by the antibody, might have caused localized neurodegeneration in the CA1 field. The neurodegeneration reportedly detected in the antibody-infused limited area of hippocampus of Tg2576 mice (42), however, was not detected in the entire brain of TTR-deficient Tg2576 mice.

Stein and Johnson reported that the lack of neurodegeneration was associated with increased level of TTR synthesized in the hippocampus of Tg2576 mice (43). However, Lazarov *et al* reported that the individual levels of TTR mRNA in the hippocampi of a transgenic mouse model of AD, which co-expresses familial AD-linked mutant APP, and presenilin 1 (PS1) cDNAs were considerably variable (20). Furthermore, it had been reported that choroid plexus is the sole site of TTR synthesis within the brain; in this regard, Sousa *et al* recently confirmed that TTR is not produced in the brain parenchyma of either wild-type or Tg2576 mice, using laser dissection microscopy (41). The finding suggests that contamination by choroid plexus might lead to misinterpretation of the role of TTR in Aβ deposition in the brain.

In the present study, we compared the onset and progression of Aβ pathology in TTR null Tg2576 (Tg2576/$TTR^{-/-}$) mice with those in heterozygous mutant Tg2576 mice (Tg2576$TTR^{+/-}$). Thus, one factor which causes the discrepancy between our data and other authors' data obtained by examining only Tg2576 mice homozygous for the wild-type *TTR* gene (Tg2576/$TTR^{+/+}$) for comparison might be the difference in the levels of TTR. However, the onset and progression of Aβ pathology in our Tg2576/$TTR^{+/-}$ mice are rather delayed than accelerated relative to those in Tg2576/$TTR^{+/+}$ mice previously reported by other authors (6, 18, 45, 55). Thus, the possibility that homozygous levels of TTR would be required to prevent Aβ pathology appears to be remote.

On the other hand, Nunes *et al* reported that peptidylglycine α-amidating monooxygenase, the rate-limiting enzyme in neuropeptide maturation, is over-expressed in the peripheral, and central nervous systems of $TTR^{-/-}$ mice that, consequently, display increased neuropeptide Y (NPY) levels relative to wild-type mice (28). NPY is known to be a substrate of neprilysin, which is an Aβ-degrading protease (50). Another Aβ-degrading enzyme, insulin-degrading enzyme (IDE) is also known as insulin and amylin protease (30, 50). Hyperinsulinaemia is known to increase the risk of developing AD. Thus, it is suggested that hyperinsulinaemia may elevate Aβ level through insulin's competition with Aβ for IDE (30). Analogous to the competition, the increase in NPY levels in $TTR^{-/-}$ mice might competitively reduce neprilysin clearance of Aβ. Thus, Tg2576/$TTR^{-/-}$ mice might display enhanced Aβ deposition relative to Tg2576/$TTR^{+/-}$ mice through NPY's competition with Aβ for neprilysin. Contrary to the expectation, Tg2576/$TTR^{-/-}$ mice display rather suppressed Aβ deposition relative to Tg2576/$TTR^{+/-}$ mice (Figures 2 and 4). The results say that TTR does not suppress but rather accelerates Aβ deposition in Tg2576 mice.

Contrary to our findings, Choi *et al* recently reported that in a different transgenic mouse model of AD heterozygous for the disrupted *TTR* gene ($TTR^{+/-}$), brain Aβ deposition is significantly accelerated relative to the age-matched model homozygous for the wild-type *TTR* gene ($TTR^{+/+}$) (8). Their observation, which suggests that TTR suppresses Aβ deposition, contradicts ours. It is important to note that there are several critical differences in the experimental designs which might have caused the contradiction between their data and ours: (i) the AD model mouse we examined (Tg2576) is distinct from that Choi *et al* examined. They used *ceAPPswe/PS1ΔE9* mice that harbor not only the human mutant APP cDNA with the double mutation K670N and M671L linked to a Swedish familial AD but also the human mutant PS1 cDNA with the exon 9 deletion linked to a familial AD (16, 21). In contrast to Tg2576 mice, their control singly transgenic mice that express the human mutant APP cDNA alone are free of brain Aβ deposits up to age 14 months and co-expression of human mutant APP, and PS1 accelerates the amyloid deposition (3, 21). Furthermore, comparative analysis of cortical gene expression patterns between Tg2576 mice homozygous for the PS1 knock-in mutation (Tg2576/$PS1^{264L/264L}$) and control Tg2576 mice heterozygous for the PS1 mutation (Tg2576/PS1$P^{264L/+}$) by DNA micro-array analysis revealed that the patterns are distinct, although there were some common regulated genes (54). All these observations suggest that the molecular pathogenesis of Aβ deposition in the two mouse models is different; (ii) the level of human variant APP in the brain of Tg2576 mice is more than fourfold higher than that of endogenous brain APP (14). On the other hand, although the level of human variant APP in the brain of *ceAPPswe/PS1ΔE9* mice is not described (8), variant PS1ΔE9 reportedly elevates Aβ42/Aβ40 ratio (3). Thus the contradiction between their results and ours might be caused by the significant difference in the levels of Aβ42 and/or Aβ40 between Tg2576 and *ceAPPswe/PS1ΔE9* mice; and (iii) Choi *et al* compared the degree of Aβ deposition between the brains of *ceAPPswe/PS1ΔE9* mice heterozygous for the disrupted *TTR* gene ($TTR^{+/-}$) and the mice homozygous for the wild-type *TTR* gene ($TTR^{+/+}$) (8), in contrast to the TTR null ($TTR^{-/-}$) and $TTR^{+/-}$ Tg2576 mice we examined for comparison. Thus in their study, in contrast to our study, the individual differences in the levels of brain TTR among the $TTR^{+/-}$ and control $TTR^{+/+}$ mice should critically affect the results, and hence, the elucidation of the relationship between TTR and Aβ deposition. They described that the levels of immunoreactive TTR in the extracts from the brains of *ceAPPswe/PS1ΔE9/*$TTR^{+/-}$ mice are clearly lower relative to the age-matched *ceAPPswe/PS1ΔE9/*$TTR^{+/+}$ mice at all ages examined (8). However, the report is lacking in important details about the levels of TTR in the individual animals that would make the data more compelling. For example, given only the pictorial data with sample number of 1, it is not clear that the differences in the brain levels of TTR between *ceAPPswe/PS1ΔE9/*$TTR^{+/+}$ and *ceAPPswe/PS1ΔE9/*$TTR^{+/-}$ mice are really significant. On the other hand, it is possible that TTR, as a peripheral Aβ binding protein, may have the ability to act as a peripheral Aβ 'sink'; whereby, it pulls Aβ from the brain into the periphery, hence decreasing the amount of Aβ in the brain (26, 33, 52, 56). Thus, if we had examined Tg2576$TTR^{+/+}$ mice, we, too, might have detected a decrease in Aβ deposition as Choi *et al* did.

All the above differences may cause the contradiction between their data and ours. Moreover, they described that the levels of brain TTR were significantly lower in human AD patients compared with age-matched controls and negatively correlated with the abundance of amyloid plaques. However, the references they cited didn't refer to the brain TTR levels but to the CSF TTR levels in the patients (8) and, to our knowledge, the comparison of the brain TTR levels between AD patients and control disease-free individuals has not yet been reported.

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In conclusion, our results indicated, for the first time, TTR does not suppress but accelerates vascular Aβ burden in the brain of Tg2576 mice. However, the mechanism(s) by which TTR affects the Aβ deposition *in vivo* are not yet elucidated. Taken together with the Choi et al's contradictory finding (8), our finding suggests that the role of TTR in the pathogenesis of AD remains to be understood.

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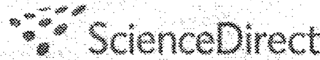

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Research Report

Motor impairment and aberrant production of neurochemicals in human α-synuclein A30P+A53T transgenic mice with α-synuclein pathology☆

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ABSTRACT

Missense point mutations, duplication and triplication in the α-synuclein (αSYN) gene have been identified in familial Parkinson's disease (PD). Familial and sporadic PD show common pathological features of αSYN pathologies, e.g., Lewy bodies (LBs) and Lewy neurites (LNs), and a loss of dopaminergic neurons in the substantia nigra that leads to motor disturbances. To elucidate the mechanism of αSYN pathologies, we generated TgαSYN transgenic mice overexpressing human αSYN with double mutations in A30P and A53T. Human αSYN accumulated widely in neurons, processes and aberrant neuronal inclusion bodies. Sarcosyl-insoluble αSYN, as well as phosphorylated, ubiquitinated and nitrated αSYN, was accumulated in the brains. Significantly decreased levels of dopamine (DA) were recognized in the striatum. Motor impairment was revealed in a rotarod test. Thus, TgαSYN is a useful model for analyzing the pathological cascade from aggregated αSYN to motor disturbance, and may be useful for drug trials.

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1. Introduction

αSYN was originally isolated from senile plaques in Alzheimer's disease as a protein of 35 highly hydrophobic amino acid metabolites, known as the non-amyloid component (NAC), derived from a 140 amino-acid precursor encoded by a gene on chromosome 4 (Ueda et al., 1992; Chen et al., 1995), which has homology to rat and Torpedo αSYN and songbird synelfin (George et al., 1995). αSYN is highly abundant in presynaptic terminals (Iwai et al., 1995) and has potential roles in synaptic function and neural plasticity (George et al., 1995; Clayton and George, 1998).αSYN binds to phospholipid vesicles and inhibits PLD2, a regulator of vesicle membrane budding (Liscovitch et al., 2000; Lotharius and Brundin, 2000; Payton et al., 2000), and also plays modulatory roles in the release of dopamine vesicles (Abeliovich et al., 2000).

A few cases of familial Parkinson's disease (FPD) have been linked to missense point mutations in αSYN with A53T (Polymeropoulos et al., 1997), A30P (Kruger et al., 1998) and E46K (PARK1) (Zarranz et al., 2004). Soon after the first A53T missense mutation of αSYN was discovered, the main component of Lewy bodies (LBs) was identified as insoluble aggregates of αSYN (Baba et al., 1998). αSYN and phosphorylated-Ser129 αSYN accumulated in LBs and Lewy neurites (LNs) in PD and Dementia with Lewy bodies (DLB) (Fujiwara et al., 2002; Hasegawa et al., 2002). Then, a second causative gene known as *parkin* (Kitada et al., 1998) was found in familial autosomal recessive juvenile Parkinson's disease (PARK2). *Parkin* ubiquitinates αSYN normally and this process is aberrantly altered in PD (Shimura et al., 2001). Acceleration of oligomerization or protofibrillization is a common property of mutant αSYN (Conway et al., 2001; Choi et al., 2004). Recently, triplication of the αSYN locus (PARK4) was identified in an "Iowanian kindred" with autosomal dominant Lewy body disease (Singleton et al., 2002). Subsequently, duplication of the αSYN gene locus was also reported as a cause of familial PD (Chartier-Harlin et al., 2004). These findings suggest that overexpression of wild type αSYN also leads to facilitation of insoluble aggregation of αSYN. α-synucleinopathy is a disease entity which shares common pathological accumulation of insoluble aggregates of αSYN in the neurons and processes of PD, DLB, Hallervorden–Spatz disease, pure autonomic failure and in the glial cells of multiple system atrophy (MSA) (Goedert, 2000; Hardy and Gwinn-Hardy, 1998; Spillantini et al., 1997; Tu et al., 1998; Galvin et al., 2000; Shoji et al., 2000; Arai et al., 2000).

To elucidate the pathological mechanism of LBs and LNs associated with the decrease in dopamine (DA) production, it is necessary to investigate the aberrant mechanism of mutant αSYN, which is an essential molecule consisting of LBs and LNs (Baba et al., 1998). Here, we generated transgenic (Tg) mice expressing human mutant αSYN A30P+A53T under a human Thy-1 promoter, named as TgαSYN. Overexpression of double mutant human αSYN was expected to lead to further synergistic effects and induce severe α-synucleinopathies and neurodegeneration (Citron et al., 1998; Chishti et al., 2001). TgαSYN showed significant motor impairment in rotarod test, accumulation of insoluble αSYN, aberrant inclusions and decreased dopamine levels. These findings indicate that TgαSYN is a useful animal model to investigate the crucial pathogenesis of α-synucleinopathies, and it may help to develop therapeutic agents.

2. Results

2.1. *Expression of αSYN in transgenic mice and analyses of RT-PCR*

We used the transgene construct hThy1-αSYN A30P+A53T to generate transgenic (Tg) mice, TgαSYN (Fig. 1a). PCR analysis of tail-derived DNA revealed 18 positive Tg mice for human αSYN and EGFP among 129 F0 mice. Five of the 18 Tg mice showed the strongest green fluorescence under irradiation at 365 nm ultraviolet (Fig. 1b). These selected independent lines (#8707, #8713, #8718, #8812, #8819) were mated with BDF1 mice and raised for examination. The following Tg mice were analyzed: 18 positive Tg progenies, 60 F1 Tg (#8707: 2, #8713: 31, #8718: 5, #8812: 10, #8819: 12) and 135 F2 Tg (#8707: 0, #8713: 101, #8718: 2, #8812: 29, #8819: 3). The mRNA expressions of human αSYN A30P+A53T and EGFP in TgαSYN brains were confirmed by RT-PCR, showing the same expression levels of human αSYN A30P+A53T and EGFP at three, eight and 17 months old, respectively (Figs. 1c and d). Western blot using LB509 recognized a 16 kD band corresponding to human αSYN only in Tg mice. AB5038 recognized a 16 kD band corresponding to both human and mouse αSYN. The expression level of human αSYN was 130% of that of endogenous mouse αSYN (Fig. 1e).

2.2. *Histological studies*

Immunocytochemistry of sagittal sections of a seven-month-old #8707 TgαSYN brain by LB509 revealed extensive human αSYN immunostaining in the brainstem, hippocampus, thalamus, cerebral cortex and cerebellum (Fig. 2a, arrow indicates the substantia nigra), but no staining in the non-Tg mouse (Fig. 2b). The TgαSYN brain showed atrophy of the cerebral cortex and cerebellum (Fig. 2a). The HE stain showed eosinophilic inclusion bodies and vacuoles in the cytoplasm of neurons in the substantia nigra (Fig. 2c, arrow), and in the dentate nucleus of TgαSYN (Fig. 2h, arrow). These cytoplasmic inclusions were stained with human-αSYN specific antibody, LB509 (Fig. 2d, arrow, and Fig. 2i, arrow), and anti-αSYN antibody, 42/α-Synuclein (Fig. 2j, arrows). Nitrated α/β synuclein was also stained in the cytoplasmic inclusions (Fig. 2e, arrow,). Ubiquitin-positive inclusions were observed in neurons at brainstem (Fig. 2f, arrow), and dystrophic neurites in the dentate nucleus of TgαSYN (Fig. 2g). Staining of phosphorylated synuclein showed diffuse staining in somatodendrites of TgαSYN neurons (Fig. 2k). Gallyas–Braak staining revealed dystrophic neurites in the dentate nucleus of TgαSYN (Fig. 2l) in ubiquitin-positive structures in the same region (Fig. 2g). Anti-tyrosine hydroxylase (TH) immuno-positive neurons in the locus ceruleus showed weak immunostaining intensity in TgαSYN (Fig. 2m), compared with those of non-Tg mice brains (Fig. 2n). The intensity of substance P immunopositive synapses in the striata of TgαSYN brains (Fig. 2o) was weaker than that of non-Tg mice brains (Fig. 2p). Severe astrocytosis

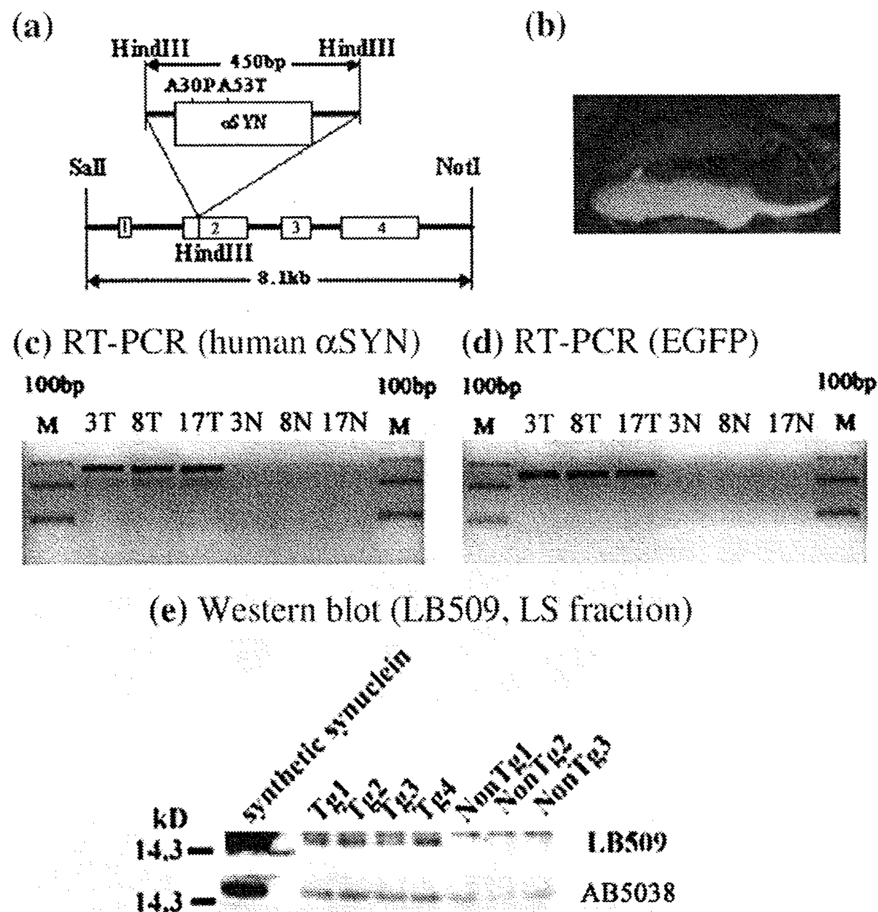

Fig. 1 – The mutant αSYN A30P+A53T construct and the expression of EGFP. (a) The structure of the construct: hThy1-αSYN A30P+A53T. (b) TgαSYN (#8713) showed fluorescence by EGFP (enhanced green fluorescence protein) under 365 nm long wave UV (EGFP-negative non-Tg mouse in the upper location, EGFP-positive Tg mouse in the lower location). (c) Analyses of RT-PCR transcripts: Human αSYN mRNA transcripts (exons 2–4) were detected as 280 bp in TgαSYN brains, but not in non-Tg mice brains, and the intensity of PCR products was the same level as three-, eight-, and 17-month-old Tg mice brains. (d) EGFP mRNA transcripts were detected in TgαSYN brains at the same level at three, eight and 17 months of age, but not in non-Tg mice brains (N showed non-Tg mice brains, T showed Tg mice brains). (e) The expression of human αSYN was detected as a 16 kD band at the same size as recombinant synthetic αSYN by Western blot using LB509 in LS-soluble fractions of TgαSYN #8713 (Tg1–4) mice brains, but not in three 14-month-old non-Tg mice brains (upper lane). AB5038 recognized a 16 kD band corresponding to human and mouse αSYN (lower lane). The expression level of human αSYN was about 130% of that of endogenous mouse αSYN. Synthetic αSYN (SS) was used as a marker of 16 kD αSYN (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA).

was observed in the cerebellum of a 16-month-old TgαSYN (Fig. 2q). An EM study demonstrated cytoplasmic inclusions (Fig. 2r, arrow) and intranuclear inclusions (Fig. 2s, arrows) in the neurons of the brainstem. These inclusion bodies lacked the typical halo and fibrillar structure of LBs.

2.3. *Western blot analysis*

Fourteen-month-old TgαSYN showed a 16 kD band corresponding to αSYN in the LS-soluble fraction (L), Triton-soluble fraction (T), sarcosyl-soluble (S) and sarcosyl-insoluble fractions (I) (Fig. 3a: arrow). In sarcosyl-insoluble fraction, smear pattern was detected in TgαSYN#8812(T3), which accumulated much synuclein histologically. The anti-phosphorylated αSYN antibody pSyn#64 labeled the same size band as αSYN, 16 kD (Fig. 3b: arrow). These findings presented that sarcosyl-insoluble human αSYN and phosphorylated αSYN was accumulated in TgαSYN brains as reported in DLB brains (Hasegawa et al., 2002).

2.4. *Rotarod test for motor function of TgαSYN*

The rotarod test demonstrated that significant motor impairment appeared after a shorter time in TgαSYN; they dropped from the rotating rod faster than non-Tg littermates. The motor impairment was found at three months of age ($p<0.01$) and thereafter deteriorated with age ($p<0.001$, Fig. 4).

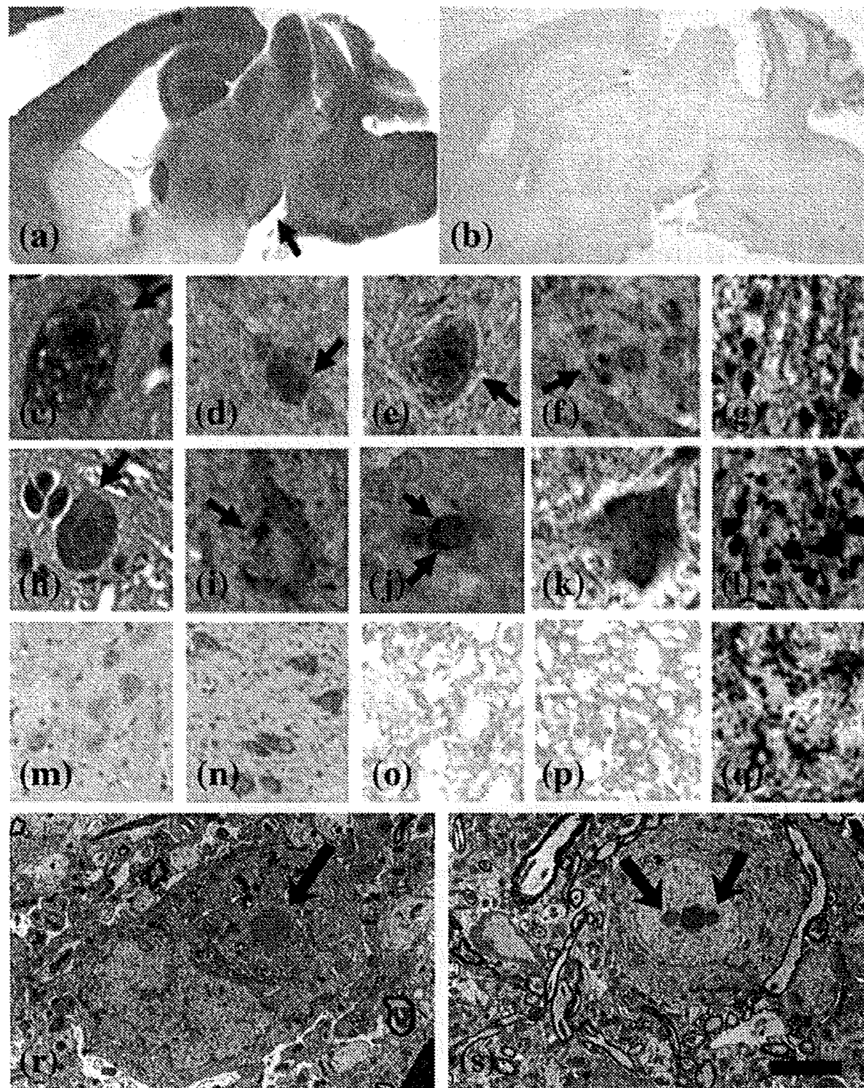

Fig. 2 – The pathological features of TgαSYN at seven months of age and age-matched non-Tg mice. (a) A sagittal section of a seven-month-old #8707 TgαSYN brain labeled by LB509 showed extensive αSYN accumulation prominently in the brainstem, hippocampus, thalamus, cerebellum and cerebral cortex. The substantia nigra of the midbrain is also labeled (arrow). The cerebral cortex and cerebellum showed atrophy. (b) No staining in the non-Tg mice brain by LB509. (c) Hematoxylin–eosin stain showed eosinophilic inclusion bodies and vacuoles in the cytoplasm of neurons in the substantia nigra of #8707 TgαSYN (arrow). (d) LB509 detected cytoplasmic inclusions in the substantia nigra of #8707 TgαSYN (arrow). (e) Anti-nitrated α/β SYN monoclonal antibody (Syn12) immunostained cytoplasmic inclusions in the substantia nigra (arrow), as well as in 14-month-old #8713 TgαSYN. (f) Ubiquitin-positive inclusions are shown in the substantia nigra of #8707 TgαSYN (arrow). (g) Ubiquitin-positive dystrophic neurites in the cerebellum dentate of 16-month-old #8713 TgαSYN. (h) Eosinophilic cytoplasmic inclusions (arrow) in the dentate nucleus of #8707 TgαSYN. (i) LB509-positive inclusion in the dentate nucleus of #8707 TgαSYN (arrow). (j) Cytoplasmic inclusions immunostained with a mouse monoclonal antibody 42/α-Synuclein in the brainstem of #8812 TgαSYN (arrow). (k) The PSer129 αSYN antibody immunostained the cytoplasm of neurons in the substantia nigra of #8707 TgαSYN. (l) Gallyas–Braak stain of dystrophic neurites in the dentate nucleus of a 16-month-old #8713 TgαSYN. (m) Tyrosine hydroxylase (TH) immuno-positive neurons in the locus ceruleus showed less immunostaining in the #8707 TgαSYN brain, than the non-Tg mouse brain (n). (o) The intensity of substance P immuno-positive synapses in the striatum of #8707 TgαSYN brain was lower than non-Tg mice brain (p). (q) Astrocytosis in the cerebellum of 16-month-old #8713 TgαSYN. (r) Electron-dense inclusions were found in the cytoplasm of neurons in the brainstems of 8-month-old #8718 TgαSYN by an EM study (arrow). (s) In the brainstem of the same mouse, intracellular inclusions (arrows) were also detected. Scale bar = 1 mm in a, b, 12.5 μm in c–f, h–k, 50 μm in g, l, m, n, 25 μm in o, p, and 0.38 μm in r, s.

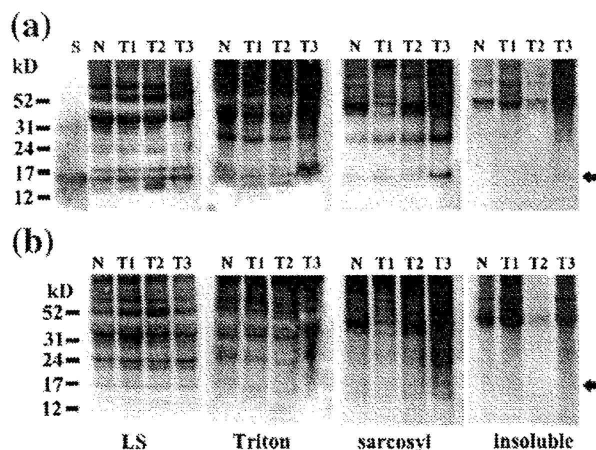

Fig. 3 – Western blot analysis. The expression of human αSYN was 16 kD (lane S; corresponded to recombinant human αSYN) by Western blot using antibody LB509 (a) and pSyn#64 (b) in LS-soluble (L), Triton-soluble (T), sarcosyl-soluble (S) and sarcosyl-insoluble (I) fractions of non-Tg (N), TgαSYN#8713 (T1), TgαSYN#8819 (T2), and TgαSYN#8812 (T3), 14-month-old mice brains.

2.5. *Measurement of neurochemicals*

There was no significant difference in brain weight among Tg and non-Tg mice at 10 and 17 months of age. Compared with age-matched non-Tg control mice, the levels of DA in the striatum were significantly decreased in 10 month-old ($p=0.0159$) and 17 month-old mice brains ($p=0.0286$). DA decreased approximately 17% to 24% in the striatum of TgαSYN brains (Fig. 5a). A significant decrease in DA was also detected in the hypothalamuses of 17-month-old TgαSYN brains ($p=0.0079$. Fig. 5b). NE was not decreased in any areas of 10-month-old TgαSYN brains (Fig. 5c). Serotonin was decreased in the hypothalamuses of 10- and 17-month-old TgαSYN brains ($p=0.0079$, $p=0.0286$, respectively. Fig. 5d). ACh decreased in the striatum in 17-month-old TgαSYN ($p=0.0286$. Fig. 5e). There was no significant alteration in DOPAC, HVA, MHPG, 5-HIAA and Ch levels in any areas of TgαSYN (data not shown). These results showed that insoluble mutant αSYN aggregation selectively decreased the DA level at 10 and 17 months of age.

3. Discussion

Several groups have already reported animal models of PD, such as wild-type αSYN Tg mice (Masliah et al., 2000), mutant αSYN Tg mice (van der Putten et al., 2000, Kahle et al., 2000, Giasson et al., 2002, Lee et al., 2002, Richfield et al., 2002, Neumann et al., 2002, Thiruchelvam et al., 2004, Tofaris et al. 2006, Wakamatsu et al., 2008), *Drosophila melanogaster* (Pendleton et al., 2002) and *C. elegans* (Kuwahara et al., 2006). The first αSYN Tg mice expressed wild-type human αSYN driven by the PDGF-β promoter (Masliah et al., 2000). This mouse displayed intraneuronal inclusions immunoreactive for αSYN and ubiquitin in several regions typically affected in α-synucleinopathies, while lacking the characteristic fibrillar components of Lewy bodies. The Tg mice overexpressing αSYN A53T developed under the murine Thy-1 regulatory sequence showed an early and dramatic decline in motor function (van der Putten et al., 2000). Transgenic wild-type and A30P αSYN abnormally accumulated in neuronal cell bodies and neurites throughout the brain (Kahle et al., 2000). Mice expressing wild-type or A53T αSYN under the mouse prion promoter developed motor deficits by eight months of age (Giasson et al., 2002). Neuropathological assessment of these Tg mice revealed a wide distribution of αSYN, with a pathological sparing of the motor cortex and a total sparing of the substantia nigra. Another group developed Tg mice harboring αSYN A53T using a mouse prion promoter showing motor dysfunction and αSYN accumulation (Lee et al., 2002). Truncated human αSYN (C-120) Tg mice under the TH promoter led to pathological changes in dopaminergic nerve cells of the substantia nigra and olfactory bulb (Tofaris et al., 2006). Recently, truncated human αSYN (C-130) Tg mice also led to selective loss of dopaminergic neurons and accumulation of phosphorylated αSYN (Wakamatsu et al., 2007, 2008).

One of the differences between these models and our TgαSYN was a novel combination of a promoter and mutation of αSYN. TgαSYN expressed double mutant αSYN with A30P +A53T under the human Thy-1 promoter. As expected, our TgαSYN demonstrated widespread αSYN accumulation in the brainstem, caudate putamen, cerebellum, hippocampus and cerebral cortex. Eosinophilic inclusion bodies in the substantia nigra and dentate nucleus of the cerebellum corresponded to accumulation of αSYN. Accumulated αSYN was ubiquitinated, nitrated and phosphorylated at the histological levels as shown in PD and DLB brain. Unfortunately, these inclusion bodies were not compatible with typical LBs because of the absence of a halo structure. At the EM level, fibrillar structure was not observed in inclusion bodies, but they were composed of massive aberrant fine granular structures. Aberrant inclusion bodies with modified αSYN were also observed widely. Since Gallyas–Braak staining labeled these LNs, accumulated αSYN in these neurites may have characteristics of those in β-sheet pleated structures.

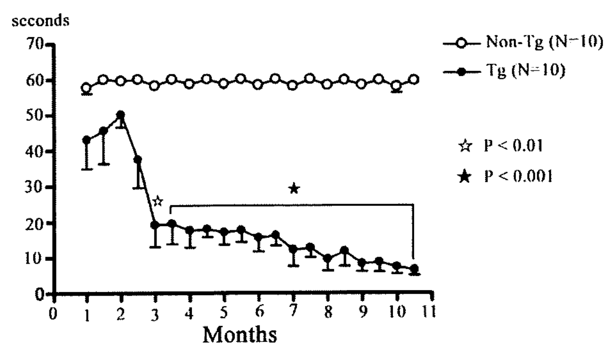

Fig. 4 – Rotarod test. The retention time of Tg mice on the rotarod significantly decreased in TgαSYN. The significant difference began to decrease at three months old (☆: $p<0.01$) and progressively deteriorated in an age-dependent manner from six months (★: $p<0.001$). Statistics were analyzed by two-way repeated measures ANOVA.

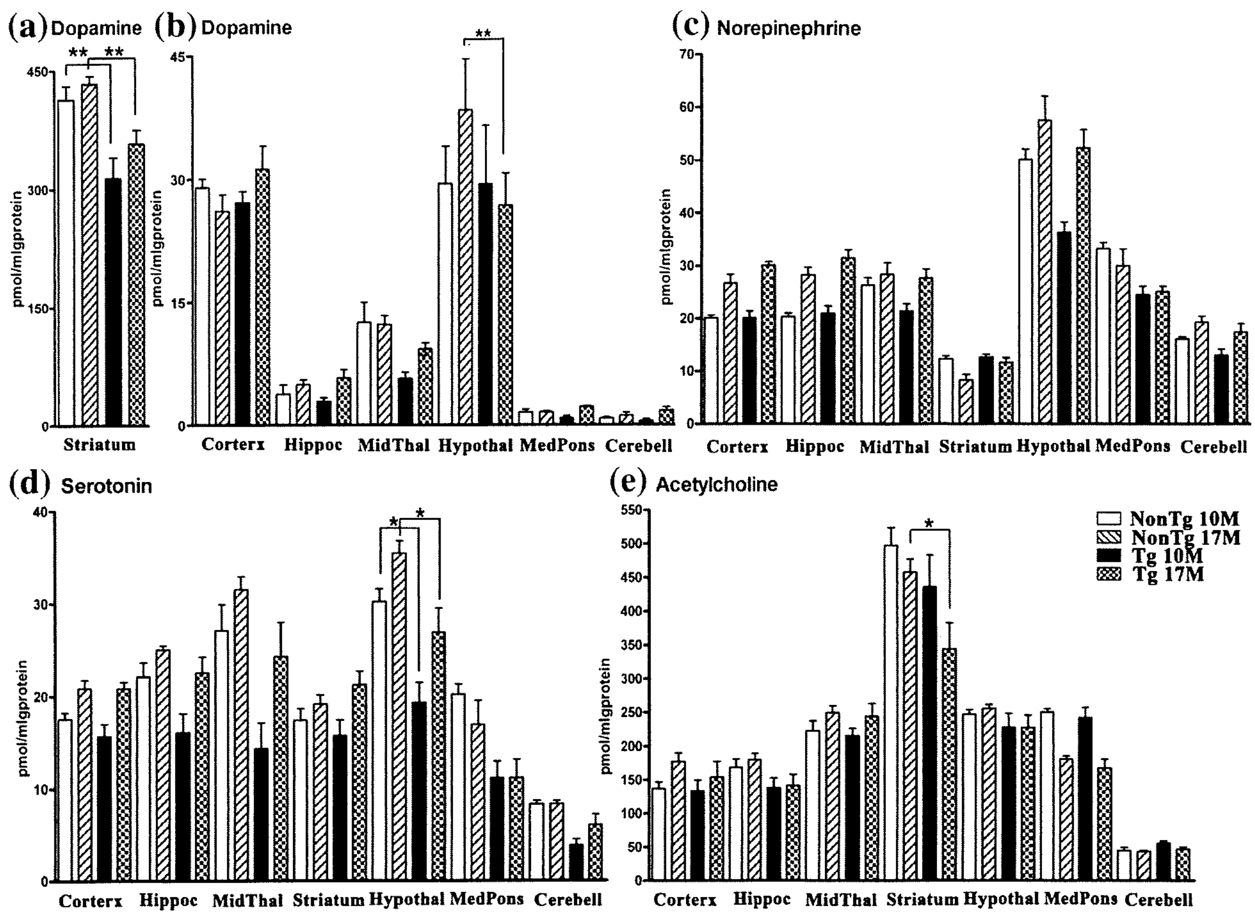

Fig. 5 – Measurement of neurochemicals. Opened column: 10-month-old non-Tg mice, Oblique column: 17-month-old non-Tg mice; Closed black colored column: 10-month-old TgαSYN mice, Crossed column: 17-month-old non-Tg mice. Cortex: cerebral cortex, Hippoc: hippocampus, MidThal: midbrain–thalamus, Hypothal: hypothalamus, MedPons: medulla–pons, Cerebell: cerebellum. *$p<0.01$, **$p<0.05$. Statistical analysis of neurochemicals between the TgαSYN and non-Tg control groups at the same age was conducted by a two-way repeated measure ANOVA (GraphPad Prism 4). (a) DA was significantly decreased in the striatum in 10- and 17-month-old TgαSYN compared with non-Tg control mice. (b) A decrease in dopamine was detected significantly in the hypothalamuses of 17-month-old TgαSYN brains ($p=0.0079$). (c) Norepinephrine (NE) was not decreased in 10- and 17-month-old TgαSYN brains. (d) Serotonin (5-HT) was decreased in the hypothalamus of 10- and 17-month-old TgαSYN brains ($p=0.0079$, $p=0.0286$, respectively). (e) ACh was decreased in the striatum of 17-month-old TgαSYN ($p=0.0286$).

The α-synuclein pathologies in TgαSYN were accompanied by decreased tyrosine hydroxylase-positive neurons, Substance P synapses and severe astrocytosis. These histological α-synuclein pathologies were also detected at the biochemical level. Accumulated αSYN was phosphorylated, ubiquitinated and sarcosyl-insoluble, suggesting that it may be conformationally changed as reported in PD/DLB brains (Hasegawa et al., 2002). The presence of higher molecule phosphorylated and ubiquitinated bands (22/29 kD) on Western blots also indicated that accumulated αSYN was modified and aggregated.

The severe decrease in DA and loss of dopaminergic neurons in SNc and the striata of PD brains is widely believed to be the pathological and biochemical cause of PD. Notably, our TgαSYN demonstrated decreased DA production in a disturbed DA system which was measured in the liquid chromatographic systems. Although other neurochemicals were altered slightly, a prominently decreased level of DA was revealed in the striatum of TgαSYN. These findings suggest selective neurotoxicity with αSYN accumulation.

In our mouse model, approximately a 20% reduction in DA in the striatum was observed when motor impairment existed. Since the rotarod test revealed significant decreased spontaneous movement, the phenotype of TgαSYN is quite similar to the cardinal clinical symptom of PD, akinesia. A decreased level of TH-positive neurons and substance P synapses also suggested that the motor impairment in TgαSYN may be caused by aberrant αSYN processes.

Our TgαSYN is a mammalian model animal showing decreased DA and motor deficits, which were certainly detected by the liquid chromatographic systems and rotarod test. For this reason, TgαSYN is a useful model for analyzing the aberrant cascade induced by pathological metabolism and aggregation of mutant αSYN, and may be useful for developing essential treatments for α-synucleinopathies such as PD and DLB.

4. Experimental procedures

4.1. *Transgene construction, generation of transgenic mice and analyses of RT-PCR*

Human αSYN A30P+A53T cDNA (450 bp) was ligated into Hind III sites in the human Thy-1 genome gene. The transgene hThy1-αSYN A30P+A53T consisted of an 8.1 kb XhoI–NcoI fragment of pBluescript II KS kidney enhancer (Fig. 1a). The CX-EGFP transgene consisted of a 3 kb Xba I/BamH I fragment of pCAGGS containing the CMV enhancer, β-actin promoter, a part of the rabbit β-globin gene, a part of the second intron, the third exon and 3′-untranslated region and cDNA of EGFP (Enhanced green fluorescent protein) with the Kozak sequence (Imai et al., 1999). Approximately 2,000 copies of the transgene with a 1:1 mole rate mixture of the hThy1-αSYN A30P+A53T and CX-EGFP as a transgene marker were micro-injected into the pronuclei of fertilized BDF1 eggs. To analyze gene expression of human αSYN, RT-PCR was performed using 2 μl of mRNA, isolated using the QuickPrep Micro mRNA purification kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), from the brains of TgαSYN (#8713) and non-Tg mice brains at 3, 8, 17 months of age ($n=3$, respectively) in the reaction tube of Ready-To-Go RT-PCR Beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) with PCR primer sets as follows: (αSYN forward primer: TG GAT GTA TTC ATG AAA GGA, αSYN reverse primer: CC AGT GGC TGC TGC AAT GCT C; EGFP forward primer: TGG TGA GCA AGG GCG AGG AG; EGFP reverse primer: TCG TGC TGC TTC ATG TGG TC). For semi-quantification, RT-PCR of β-actin was performed as an internal control (Elliott, 2001). Ten microliters of PCR products were analyzed by 2.5% agarose gel electrophoresis. The intensity of ethidium-stained bands was analyzed by Scion Image (Scion Corporation, Frederick, MD).

4.2. *Histological examinations*

After mice were sacrificed under anesthesia, the brains were removed and cut sagittally along the midline. One hemisphere was fixed in 0.1 mol/L phosphate buffer (PB, pH 7.6) containing 4% paraformaldehyde, and embedded in paraffin. For immunostaining, 5-μm sections were treated with 99% formic acid for 3 min, or treated in a microwave at 500 W for 5 min three times in 10 mmol/L citrate buffer (pH 6.0). After blocking with 5% normal goat or horse serum in 50 mmol/L phosphate buffered saline (PBS) containing 0.05% Tween-20 and 4% Block-Ace (Snow brand, Sapporo, Japan), sections were incubated with primary antibodies for 6 h. Specific labeling was visualized using a Vectastain Elite ABC kit (Vector, Burlingame, CA). Immunostained tissue sections were counterstained with hematoxylin. Nissl, Hematoxylin–eosin (H–E), and Gallyas–Braak stains were also done.

The following antibodies were used: mouse monoclonal antibody to human αSYN, LB509 (×4, residues 115–121/122) (Baba et al., 1998); mouse monoclonal antibody to αSYN, 42/α-Synuclein (×50, BD Transduction Laboratories, San Jose, CA); rabbit polyclonal antibody to phosphorylated Serine at residue 129 of human αSYN, PSer129 (×200) (Fujiwara et al., 2002; Hasegawa et al., 2002); rabbit polyclonal antibody to tyrosine hydroxylase (TH), AB151 (×2,000, CHEMICON, Temecula, CA); rabbit polyclonal antibody to substance P, AB1566 (×1,000, CHEMICON, Temecula, CA); rabbit polyclonal antibody to ubiquitin, UbiQ (×500) (Ikeda et al., 2005; Murakami et al., 2006); mouse monoclonal antibody to nitrated α/β SYN, Syn12 (×400, Invitrogen, Corps., Carlsbad, CA); rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP, ×20,000, DAKO, Carpinteria, CA).

For electron microscopic (EM) studies, the brain tissues were immersed in a fixative solution (2.5% glutaraldehyde, 0.1 mol/L phosphate buffer (PB), pH 7.4) for 4 h and washed several times in 0.1 mol/L PB containing 7% sucrose. Blocks were then postfixed in 2% osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead acetate prior to observation.

4.3. *Western blot analysis*

Half of each brain was homogenized in 3 ml/g of low-salt buffer (LS: 10 mmol/L Tris, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), 10% sucrose, and a cocktail of protease inhibitors (Complete®, Roche Diagnostics, Indianapolis, IN), pH 7.5) and centrifuged at 25,000 g for 30 min at 4 °C (LS-soluble fraction). Pellets were treated with 3 ml/g of LS with 1% Triton X-100 and 0.5 mol/L NaCl, and centrifuged at 180,000 g for 30 min at 4 °C (Triton-soluble fraction). Pellets were then homogenized again in 2 ml/g LS with 1% N-lauroylsarcosine (SIGMA CHEMICAL CO. St Louis, MO) and 0.5 mol/L NaCl, incubated at 22 °C on a shaker for 1 h, and centrifuged at 180,000 g for 30 min at 22 °C. Supernatants were analyzed as sarcosyl-soluble fraction (Iwatsubo et al., 1996; Hasegawa et al., 2002; Sampathu et al., 2003; Ikeda et al., 2005; Murakami et al., 2006). The remaining pellets obtained from each sarcosyl-insoluble fraction were boiled at 70 °C in 20 μl of NuPAGE® LDS Sample Buffer for 10 min. Each fraction was separated on 4 to 12% NuPAGE Bis–Tris Gels (Invitrogen, Corps., Carlsbad, CA) and the blots were labeled by a mouse monoclonal antibody to human αSYN (LB509, ×4), and a mouse monoclonal antibody to phosphorylated Serine at residue 129 of human αSYN (pSyn#64, ×200, Wako, Japan). Signals were visualized with an enhanced chemiluminescence detection system (SuperSignal West® Dura Extended Duration Substrate, PIERCE, Rockford, IL) and quantified by a luminoimage analyzer (LAS 1000-mini, Fuji film, Tokyo, Japan).

4.4. *Measurement of neurochemicals*

Neurochemicals, including dopaminergic (dopamine: DA, 3,4-dihydroxyphenylacetic acid: DOPAC, homovanillic acid: HVA), noradrenergic (norepinephrine: NE, 3-methoxy-4hydroxyphenylglycol: MHPG), serotonergic (5-hydroxytryptamine: 5-HT, 5-hydroxyindoleacetic acid: 5-HIAA) and cholinergic (acetylcholine: ACh, choline: Ch) systems in the brain were measured in Tg mice ($n=10$) and non-Tg control mice ($n=10$) at 10 and 17 months old, respectively. In brief, each animal was anesthetized with Nembutal® (Dainippon Pharmaceutical Co. Ltd., pentobarbital sodium), sacrificed, and irradiated with microwaves (NJE 2603 Microwave device, New Japan Radio, Kamifukuoka, Japan) at 9.0 kW for 0.42 s to prevent post-mortem

changes in the neurochemicals during brain sampling (Ikarashi et al., 1985, 2004). The brain was removed from the skull and dissected into seven regions (cerebral cortex, hippocampus, midbrain–thalamus, striatum, hypothalamus, medulla-pons and cerebellum) according to the established method (Glowinski and Iversen, 1966), and then each region was weighed. Each dissected brain region was homogenized with 0.5 ml of 0.1 mol/L perchloric acid containing 0.8 nmol isoproterenol hydrochloride as an internal standard for the determinations of catecholamines, indoleamines and related metabolites, and 5 nmol ethylhomocholine iodide as an internal standard for the determinations of ACh and Ch, using an ultrasonic cell disrupter (US-300T, Nissei, Tokyo, Japan). The homogenate was centrifuged at 17,300 g at 4 °C for 15 min. The supernatant was filtered through a 0.45 μm millipore filter. Aliquots, typically 10 μl of the filtrates, were injected into the liquid chromatographic systems (Eicom HPLC-ECD system, Eicom Co. Ltd., Kyoto, Japan) to determine catecholamines-, indoleamines-, and acetylcholine-related substances (Ikarashi et al., 1992). The sediment was re-homogenized with 2 ml of 1 mol/L NaOH for a protein assay, which was performed using the method of Lowry et al. (1951). The concentrations of neurochemicals in the brain were expressed as the values per milligram of protein.

4.5. *Behavioral experiments*

4.5.1. *Rotarod test*

TgαSYN (n=10) and age-matched control mice (n=10) at one month to 10.5 months old were examined by the rotarod performance test according to a previous method (Kuribara et al., 1977; Ikarashi et al., 2004). Mice were placed on the rotating rod of the apparatus (Ugo basile, biological research apparatus, Milan, Italy) at a speed of 16 rpm for 300 s, and the time they stayed on the rotating rod was measured. Each set of three trials was performed at 10 minute intervals each day for every mouse.

4.6. *Statistical analyses*

Two-way repeated measures ANOVA followed by the Mann–Whitney U test for the rotarod test and open field test, and two-way ANOVA followed by Student's t test to analyze neurochemicals were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) and SPSS 10.0 (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL). The means of all data are presented with their standard errors (mean±S.E.).

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第2回 アルツハイマー病の治療薬－現状と将来展望

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1. アルツハイマー病

アルツハイマー病は、細胞内外への線維性構造物の蓄積を認める、いわゆる“原因蛋白の蓄積病”をその基本病態とする。このアルツハイマー病の病理過程でみられる最も早期の変化は細胞外に認められ、βアミロイド(Aβ)を主要構成成分とする斑状の嗜銀性構造物（老人斑）として知られている。アルツハイマー病患者脳では何らかの原因でこの本来可溶型の生理的Aβの産生・分解・クリアランスの代謝機構が破綻し、不溶性の高まった病的Aβが脳実質に蓄積し、老人斑としての脳アミロイド蓄積・沈着をきたすと考えられている。こうした形成機序から、従来、老人斑自体は免疫原性に乏しく、蛋白分解にも抵抗性で生理的機能のない負の産物ととらえられ、長らくその除去を目的とした治療標的からは除外されていた。しかし、典型的老人斑においては、脳在住のミクログリアが活性化されており、アストロサイトとともに炎症性サイトカインや補体、その他の生体防御因子を産生・分泌していることが知られ、老人斑は低レベルの炎症が持続する慢性炎症病変と捕らえる考え方や、老人斑自体が生体防御反応の結果としてこうした炎症を惹起しているとの考え方が提唱され、アルツハイマー病治療標的の幅が広がってきた。しかしながら、アルツハイマー病の治療戦略の標的はその病態生理に基づいており、その理解は本稿を考える上で不可欠である。

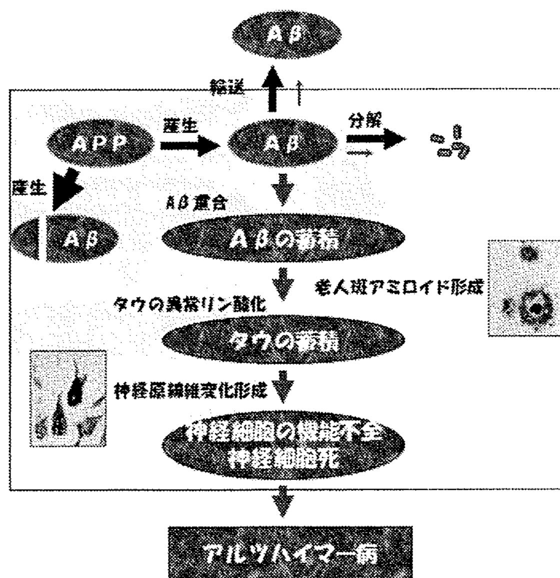

図1 アミロイドカスケード仮説

2. アルツハイマー病の病態生理

アルツハイマー病の病態生理においては、Aβの重合ならびに神経細胞毒性発現が、神経原線維変化、神経細胞脱落へと続く一連の病的カスケードの引き金となり、認知症を引き起こすとの考え方が広く受け入れられている。このアミロイドカスケード仮説[1]（図1）を支持する理論的根拠は、

(1)Aβの脳内蓄積は老人斑として形態学的に捉えうる最も早期の変化であり、神経原線維変化、神経細胞脱落に先行する
(2)Aβの前駆体をコードするアミロイド前駆体蛋白amyloid precursor protein (APP)遺伝子の変異や重複をもつ家族性アルツハイマー病が存在する
(3)これまでに家族性アルツハイマー病の原因として発見されたAPP遺伝子変異・重複及びプレセニリン遺伝子変異の全てによって、Aβの産生異常が誘導される
(4)培養神経細胞や動物実験において、重合したAβは細胞毒性やタウ蛋白のリン酸化亢進を引き起こす
(5)重合したAβは記憶に関連するシナプス電位である海馬における長期増強(Long Term Potential, LTP)を阻害し、シナプス毒性を発揮する
(6) Aβの脳内異常沈着を示すAPP遺伝子導入トランスジェニックマウスに抗原としてAβを投与することで（Aβワクチン療法)、Aβの脳内沈着が抑制されるとともに、このモデルマウスが示す様々な機能障害(学習・記憶障害など)も軽減する
(7)このモデルマウスに直接Aβに対する抗体を投与すると（受動免疫療法)、Aβの脳内沈着には影響することなく、記憶障害が可逆的に回復する

との知見であるが、厳格な意味でこのアミロイドカスケード仮説に修正が加えられる結果となった。現在、老人斑を構成するアミロイド線維よりもむしろ重合Aβ（Aβオリゴマー）がアルツハイマー病患者脳における神経細胞障害誘導の物質的基盤と考えられ、新たにAβオリゴマーカスケード仮説として市民権を得ている。

3. アルツハイマー病治療薬の現状

前述したアルツハイマー病の病態生理をもとに、アルツハイマー病治療薬・発症抑止薬の研究が行われている。大多数がアミロイドカスケード仮説の上流に位置するAβを標的とした薬剤開発であるが、現状（図2）は対症療法の域をでず、臨床現場ではその力不足が否めない。

4. 開発途上のアルツハイマー病治療薬

開発途上の治療薬を図3に示す。このうち本稿ではアルツハイマー病の免疫療法に焦点を絞り概説したい。

アルツハイマー病の免疫療法

生体の免疫系を駆使し一旦沈着した脳アミロイド除去を標的とした治療が免疫療法（Aβワクチン療法)

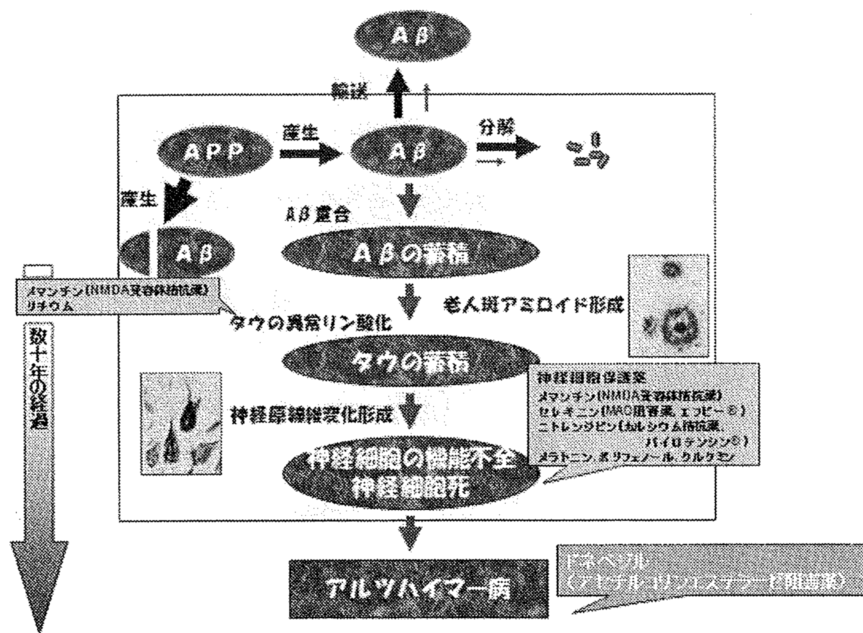

図2 アミロイドカスケード仮説に基づくアルツハイマー病治療薬の現況

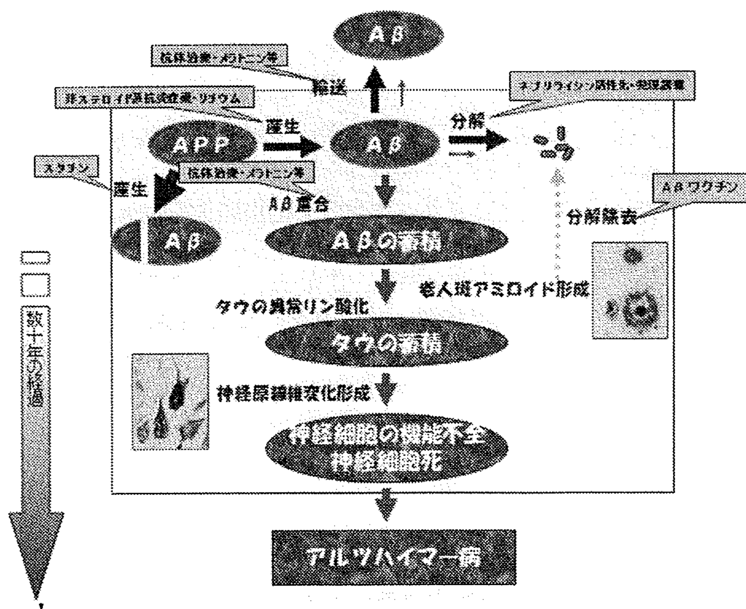

図3 アミロイドカスケード仮説に基づく開発途上のアルツハイマー病治療薬

である。いわゆる老人斑除去を抗体で行う治療法であり、従来の老人斑自体は免疫原性に乏しく、蛋白分解にも抵抗性で生理的機能のない負の産物でありその除去自体できるはずがないとの誤った常識を打破した、いわゆるパラダイム変換に基づきSchenk等[2]により提唱された治療法である。厳格な意味でのワクチン療法とは異なり、アルツハイマー病の免疫療法(Aβワクチン療法)は、Aβペプチドをadjuvantとともに投与し抗体産生を誘導する能動免疫と直接抗体を投与する受動免疫、全身性免疫でなく粘膜免疫を用いた免疫療法の3種を包括した概念として使用されている。

① 能動免疫療法（厳格な定義のAβワクチン療法）

ヒトの変異型APPを強発現させたトランスジェニックマウスを、凝集させた合成Aβで免疫し、脳内に異常沈着したAβが果たして抗体によって排除可能か否かの検討がなされた。その結果、このAβワクチン療法はマウス脳内でのAβ沈着抑制[3]ならびにAβ沈着によると考えられる学習・記憶障害の緩和に有効であることが確認された[4]。Aβワクチンの作用機序としては、①これまで、抗体は血液脳関門を容易に通過しないと考えられてきたが、わずかな抗体がこのバリアーを突破し、脳実質内に異常沈着したAβ凝集塊と結合することがシグナルとなり、ミクログリアによる貪食が促進される。②脳内移行した抗体が直接Aβと結合し、その重合抑制・線維溶解・毒性中和をする。③抗体が血液中からのAβクリアランス誘導を介し、Aβの脳から血液への移行を増加させる、④抗体と結合したAβがその免疫複合体として、Fc受容体を介して血液中に移行する、以上４つの作用機序が想定された。こうした前臨床試験の結果を踏まえ施行されたヒトにおけるAβワクチン(AN-1792)第II相臨床治験は、6%の症例で髄膜脳炎が発症し中止となった。髄膜脳炎が直接の原因となった死亡例は報告されていないが、不幸にして亡くなられた髄膜脳炎合併例[5]・非合併例の２剖検脳による検討から老人斑アミロイドの除去効果が確認された。しかしながら神経原線維変化の除去には至らないことも明らかとなった。残念なことに、その後１年の追跡結果[6]から認知症評価スケール個々の値は、約20%のワクチン反応群（抗体上昇群）とプラセボ投与群との間で有意差を認めなかったが、神経心理学テスト全体の標準化スコア（zスコア）はワクチン反応群で有意によく、また少数ながら同群では脳脊髄液中タウの減少も認められたと報告されている。最近になりAβワクチン(AN-1792)長期経過観察例での剖検所見の報告がなされ、老人斑が消失しても認知症は進行することが明らかとされた[7]。この結果を好意的に解釈すれば、Aβワクチン療法も中等症以上にまで進行してしまうとその認知機能改善治療効果が望めず、やはり予防的な効果を期待して施行すべき治療法である可能性も考えられる。またAβワクチン接種者

に認められた抗体は老人斑認識抗体であった点からは、老人斑を標的としたAβワクチン療法には効果が認められないことへ警鐘をならしたとも考えられる。免疫療法の大事な点はどのような抗体が結果的に産生されるかという点であり、この点は能動免疫療法の克服されるべき重大な弱点である。

② 受動免疫療法（抗体投与療法）

この受動免疫療法が最も注目を集めた所以は[8]、①この抗体投与でアルツハイマー病モデルマウスで発症した記憶障害の回復ができることと、②この発見に端を発し、その病態解析の過程で、実は老人斑Aβアミロイド線維自体のアルツハイマー病発症病態への関与は低く、アミロイド線維形成して沈着・蓄積する前に形成されるAβオリゴマーこそがその本態で、アルツハイマー病の治療標的であることが認識された点にある[9]。これまでさまざまな抗体による受動免疫療法の前臨床試験がアルツハイマー病モデル動物で施行され、その有用性が確認されてきた。老人斑アミロイド除去を標的とする抗体や脳アミロイド除去とは無関係に記憶障害改善効果をも発揮する抗体、さらに老人斑除去のみでなく、Aβのアミロイド線維形成や毒性などの抑制活性を念頭に考案されたAβオリゴマーやシードに対する抗体がこれに含まれるが、これまで沈着した脳アミロイドに結合性を示す抗体を使用した受動免疫療法で微少出血と頻度は低いものの髄膜脳炎の副作用発生がアルツハイマー病モデルマウスで報告されており、使用抗体選択の重要性と副作用を未然に防ぐための教訓として、如何にアミロイドが相当量沈着する前に治療を開始することが出来るか、またそうした対象を如何にして知り得る術をもつかが大事なポイントであることが明らかとなった。

5. アルツハイマー病治療の将来展望

先に開催されたICAD2008ではヒト化モノクローナル抗Aβ抗体２種、nativeな抗Aβ抗体を含有する免疫グロブリン製剤でのヒト第II相臨床治験の途中経過報告がなされ、老人斑を主に認識する抗体では認知機能改善効果が乏しく、抗Aβオリゴマー抗体を含有する免疫グロブリン製剤でのみ認知機能改善効果が得られたことが確認された。本結果からも、いわゆる病態発症を特異的に制御する目的で、生理的な分子には反応しない、病態惹起分子特異的な抗体による治療法の開発が肝要であると考えられ、アルツハイマー病を“治療可能な認知症”とするため、今後より一層研きをかけた抗体治療が世に送り出されることを期待したい。

文　献

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PET化学ワークショップ2009

理化学研究所分子イメージング
科学研究センター　高橋和弘

2月10日～12日にPET化学ワークショップ2009（通称冬の学校）が行なわれました。今年は、昨年と同じ神戸市六甲山での開催となりました。今回は穏やかな天候に恵まれ人工スキー場を除けば雪はほんのわずかしかありませんでした。とはいえ昨年同様、山の上閉ざされた静かな環境の中で、突っ込んだ議論ができ大いに盛り上がった会でした。

このワークショップは「PET薬剤のルーチンな合成に従事する者が集まり、学術集会では取上げにくい“現場が抱える諸問題”について自由に討議し、PETの現状や日進月歩の“PET化学の新しい展開”に触れる啓蒙的な場を提供する」ことを目的に、平成4年に開始されました。今回は第18回にあたり、108人が参加しました。プログラムは次の通りです。

10日◇　PET化学への招待
◇サイクロメンテ中の被曝事故
◇　各施設のチョッと良いもの
◇　新しい薬剤導入（FLT）
◇　施設運営状況アンケートから
◇　情報交流会
11日◇　フリーディスカッション
◇　耳寄り情報
^{64}Cu、添加物、超純水
◇　「てびき」をナナメに読む脳編
◇　初心者のためのわかる合成装置入門
◇　情報交流会
12日◇　PET施設の放射線管理問題と対応について
◇　核医学認定薬剤師について
◇　製品情報・業界情報

「チョッと良いもの」ではUSBカメラの利用法から始まり分注装置や手軽な遮蔽箱、入手が危ぶまれるアセトニトリルの話題、可溶化剤として多用されるポリソルベート80さらにサイクロトロンの壊し方まで広範な内容に楽しませてもらいました。「薬剤導入（FLT）」では本ワークショップらしい最も現場的な内容に突っ込んだ議論ができました。フリーディスカッションはケーブルカーがメンテナンスのため運休となり、有馬温泉への移動が困難だったため、メイン会場は灘の酒蔵巡りと昨年とはまた違った議論に花が咲いたようです。「耳寄り情報」は気にはなりつつも普段調べることの少ない話題について専門家から教えてもらいました。「てびきをナナメに読む（脳編）」では日頃何となく眺めることの多いPET化学の応用編（臨床利用）を若い発表者に解説していただきました。「わかる合成装置入門」では最近要望が増えつつある多目的合成装置の現状について各社から報告してもらいました。また今年も深夜遅くまで及んだ情報交流会では皆さん有益な議論と親睦を多いに深められた模様です。

FDGブームによるPETセンター数の激増や、分子イメージングの興隆によりPET化学を取り巻く環境も変わりつつあります。その中でこの会への期待が今まで以上に高まっているように思います。このことは参加者の3分の1が初参加だったことからも覗えます。

次回開催案内をご希望の方は籏野（hatanok@nils.go.jp）または高橋（kazu.takahashi@riken.jp）までご連絡下さい。

Standard Textbook of Internal Medicine

7

内科学書

Vol. 1

内科学総論
臨床症状

◉総編集
小川　聡
◉部門編集
伴　信太郎
山本　和利

中山書店