

Potent amyloidogenicity and pathogenicity of A β 43

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ABSTRACT

A β 42 is known to be a primary amyloidogenic and pathogenic agent in Alzheimer's disease. However, the role of A β 43, found just as frequently in patient brains, remains unresolved. We generated knockin mice containing a pathogenic presenilin-1 R278I mutation that causes overproduction of A β 43. Homozygous mice exhibited embryonic lethality, indicating that the mutation involves loss of function. Crossing amyloid precursor protein transgenic mice with heterozygous mutant mice resulted in elevation of A β 43 levels, impairment of short-term memory, and acceleration of A β pathology, accompanying pronounced accumulation of A β 43 in plaque cores similar to the biochemical composition observed in patient brains. Consistently, A β 43 showed a higher propensity to aggregate and was more neurotoxic than A β 42. Other pathogenic presenilin mutations also caused overproduction of A β 43 in a manner correlating with A β 42 and with age of disease onset. These findings indicate that A β 43, an overlooked species, is potently amyloidogenic, neurotoxic, and abundant in vivo.

Alzheimer's disease, the most common form of dementia, is characterized by two pathological features in the brain, extracellular senile plaques and intracellular neurofibrillary tangles. Senile plaques consist of amyloid- β peptide ($A\beta$) generated from amyloid precursor protein (APP) through sequential proteolytic processing by β -secretase and γ -secretase. Two major forms of $A\beta$ exist, $A\beta_{40}$ and $A\beta_{42}$, with $A\beta_{42}$ being more neurotoxic due to its higher hydrophobicity, which results in faster oligomerization and aggregation¹. A number of mutations associated with early-onset familial Alzheimer's disease (FAD) have been identified in the *APP*, *PSEN1* and *PSEN2* genes, and these mutations lead to accelerated production of $A\beta_{42}$ or an increase in the $A\beta_{42}/A\beta_{40}$ ratio. Together these findings indicate that $A\beta_{42}$ plays an essential role in the initiation of pathogenesis. However, the possible involvement of longer $A\beta$ species that also exist in Alzheimer's disease brains has not yet been fully investigated.

Thus far, various longer $A\beta$ species, such as $A\beta_{43}$, $A\beta_{45}$, $A\beta_{48}$, $A\beta_{49}$ and $A\beta_{50}$, have been qualitatively described in Alzheimer's disease brains². Similar $A\beta$ species have also been found in transgenic mice that overexpress *APP* carrying FAD-linked mutations³. Further quantitative studies have revealed that $A\beta_{43}$ is deposited more frequently than $A\beta_{40}$ in both sporadic Alzheimer's disease (SAD) and FAD⁴⁻⁷.

How these $A\beta$ species with different C-terminal ends are generated from the precursor has mainly been investigated by cell biological and biochemical methods. A number of studies^{8,9} demonstrated that γ/ϵ -cleavage by γ -secretase activity controls the fate of the C-terminal end. $A\beta_{43}$, generated from $A\beta_{49}$ via $A\beta_{46}$, is subsequently converted to $A\beta_{40}$ by γ -secretase whereas $A\beta_{42}$ is independently generated from $A\beta_{48}$ via $A\beta_{45}$. It has also been reported that the FAD-associated I213T mutation in the *PSEN1* gene increases the generation of longer $A\beta$ species, such as $A\beta_{43}$, $A\beta_{45}$ and

those even longer than A β 46, in addition to A β 42¹⁰. It is also noteworthy that A β 43 appears to be equally as prone to aggregate *in vitro* as A β 42¹¹, leading to faster formation of oligomers than occurs in the case of A β 40¹². These observations imply that A β 43 could be produced as a physiological or pathological metabolite of γ -secretase activity and may affect A β amyloidosis in the brain.

In order to address whether A β 43 contributes to Alzheimer's disease pathology, we decided to take advantage of the molecular phenotype of the presenilin-1 (PS1)-R278I mutation, as this mutation results in selective overt production of A β 43 *in vitro*¹³, an effect which occurs to a much greater extent than in the case of other mutations such as R278K, R278S and R278T. The R278I mutation had independently been reported in a pedigree suffering from atypical Alzheimer's disease with language impairment¹⁴. A follow-up survey revealed that one of the patients subsequently progressed to more severe cognitive impairment, and another patient from a different branch of the family with the mutation showed Alzheimer's disease-associated symptoms with an early loss of episodic memory and with a clinical onset of the disease at the age of 59 (Rossor M.N., personal communication).

In this study, we generated PS1-R278I knockin mice to assess the biological significance of the mutation and the pathological effect of A β 43 on A β amyloidosis. Surprisingly, homozygous knockin mice showed embryonic lethality, presumably due to a partial loss of γ -secretase activity resulting in a failure in Notch1 processing. Consistent with this, mouse embryonic fibroblasts (MEFs) derived from the homozygous knockin mice exhibited a failure in PS1 endoproteolysis and Notch1 processing, implying that the particular selectivity of the PS1-R278I mutation for A β 43 production is closely associated with the partial loss of γ -secretase activity, *i.e.*

suppression of the A β 43-to-A β 40 conversion, which could also be caused by some of the other PS1 mutations. We then demonstrated that heterozygous knockin mice crossbred with APP transgenic (Tg) mice exhibit short-term memory loss prior to plaque formation and develop considerably accelerated amyloid pathology, establishing that A β 43 is potently amyloidogenic and pathogenic *in vivo*.

RESULTS

Generation of PS1-R278I knockin mice

To generate PS1 knockin mice, we constructed a targeting vector carrying a point mutation that results in amino-acid replacement of R278I in exon 8 of the *PSEN1* allele (**Supplementary Fig. 1a**). Homologous recombination, germline transmission and genotype were confirmed by Southern blotting and PCR (**Supplementary Fig. 1b,c**). The expression levels of the mutant and wild-type PS1 in the embryonic mouse brains were identical (**Supplementary Fig. 1d**). Unexpectedly, homozygous knockin (R278I/R278I) mice exhibited embryonic lethality at E15–18 (**Fig. 1a**). The mutant embryos showed an overall size reduction, a stubby tail, limb ateliosis and hemorrhage in the central nervous system (CNS) as compared to wild-type (+/+) littermate controls (**Fig. 1a**).

This phenotype is similar to that of PS1-deficient mice and Notch1-related mutant mice^{15,16}, although the adverse phenotype of the PS1-R278I knockin animals appeared a few days later than that of PS1-deficient mice. In contrast, we observed no developmental deficits in heterozygous knockin (+/R278I) mice (**Fig. 1a and Supplementary Fig. 2**). The lethal phenotype of the R278I mutation appears to be caused by a loss of developmental function that manifests only under the recessive condition. In addition, we generated two lines of double mutant mice: R278I knockin/PS1 knockout and M146V knockin/PS1 knockout. The former was embryonic lethal, the latter normal (**Supplementary Fig. 3a–d**). This observation suggests the characteristic loss-of-function nature of the R278 mutation. To our knowledge, this is the first case of developmental abnormality caused by a FAD-linked PS1 point mutation.

Abnormal PS1 endoproteolysis and Notch1 processing

In order to assess the functional significance of the R278I mutation in the PS1 knockin line, we analyzed the biochemical properties of PS1-R278I γ -secretase in the embryonic brains prior to degeneration (**Fig. 1a**). Western blotting revealed a dramatic decrease in the levels of the N-terminal fragment (NTF) and C-terminal fragment (CTF) of PS1, indicating a failure of PS1 endoproteolysis, in the homozygous knockin brains, whereas the γ -secretase components, including Nicastrin (Nct), presenilin enhancer-2 (Pen-2) and anterior pharynx defective-1 protein (Aph-1), were expressed at normal levels (**Fig. 1b**). The NTF and CTF in the homozygous knockin mice were, however, clearly detectable, indicating that a fraction of the endoproteolytic activity of PS1 still remained (**Fig. 1b and Supplementary Fig. 3e**). It is also noteworthy that the endoproteolysis was partially blocked in the heterozygous PS1-R278I brain, suggesting that the process is at least in part autolytic.

We next investigated whether the PS1-R278I mutation affects the assembly of the γ -secretase complex by Blue Native PAGE (BN-PAGE)¹⁷. A major signal corresponding to a molecular weight of 360 kDa, the normal molecular weight of the native PS1 γ -secretase complex, was detected in both the wild-type and knockin brains in a manner similar to that of the PS2 γ -secretase complex (**Fig. 1c**). Immunoprecipitation experiments further demonstrated that the mutant PS1 formed a complex with Nct, Pen-2 and Aph-1 (**Fig. 1d**). These data indicate that the PS1-R278I mutation does not affect the formation of the γ -secretase complex. Interestingly, BN-PAGE detected a minor signal corresponding to a higher molecular weight of 750 kDa in the homozygous knockin brains (**Fig. 1c: arrowhead**). A similar higher

molecular weight signal has previously been described in preparations from a patient with a deletion of exon 9 in the *PSEN1* gene (PS1- Δ E9)¹⁷ and from SH-SY5Y cells treated with a γ -secretase inhibitor, L-685,458¹⁸. PS1- Δ E9 and PS1- Δ T440 also cause PS1 endoproteolytic abnormality in a similar manner to the PS1-R278I mutation^{19–21}. The presence of this high molecular weight γ -secretase may reflect a conformational change in the multi-component complex or binding of additional factor(s) to the complex, although its mechanistic involvement remains unclear.

We then examined the effect of the mutation on the metabolism of the γ -secretase substrates. Both the CTF- α and CTF- β of APP and the CTF of N-cadherin accumulated at significant levels in the homozygous PS1-R278I knockin mouse brain but not in the wild-type or heterozygous brains (**Fig. 1e**). Conversely, APP intracellular domain (AICD) and Notch1 intracellular domain (NICD) could not be detected in the homozygous knockin. An additional signal smaller than that of CTF- α appeared in the knockin mice (**Fig. 1e: asterisk**), presumably representing an aberrant proteolytic product of CTF- α and β . These data indicate that the PS1-R278I mutation leads to a significant loss of γ -secretase activity in a recessive manner.

In order to further analyze the mutant γ -secretase, we established MEFs from knockin mice and littermate controls. Western blotting (**Supplementary Fig. 4a**), BN-PAGE (**Supplementary Fig. 4b**) and Immunoprecipitation experiments (**Supplementary Fig. 4c**) demonstrated that the biochemical properties of mutant PS in MEFs were identical to those in the embryonic brains. We then expressed Myc-tagged Δ Notch1 in the mutant and wild-type MEFs. Western blot analysis revealed that conversion of Myc- Δ Notch1 to Myc-NICD by limited proteolysis takes place in wild-type and heterozygous knockin MEFs but not in the homozygous knockin or PS1

knockout MEFs (**Fig. 1f**). These results indicate that the R287I mutation induces developmental deficits by abolishing of γ -secretase-dependent Notch1 processing.

A β 40, A β 42 and A β 43 in PS1-R278I knockin brains and MEFs

Because homozygous R278I knockin mice are embryonic lethal, we went on to analyze adult heterozygous knockin mice. The adult heterozygous mice at 3 and 24 months of age were normal in development and anatomy (**Supplementary Fig. 2**), whereas various biochemical properties of PS1, such as partial abnormality of endoproteolysis, the molecular weight of γ -secretase and the identity of the complex components, were identical to those of the heterozygous embryonic brain (**Supplementary Fig. 5**). We then established a specific and highly sensitive ELISA systems that could distinguish between A β 40, A β 42 and A β 43 over a broad concentration range (**Fig. 2a,b and Supplementary Figs. 6a,b and 7**). The antibodies used were highly specific to each A β species (**Fig. 2c–e and Supplementary Fig. 6c–e**). Brain tissue fractions soluble in Tris-HCl buffered saline (TS) and those that could be dissolved in 6M guanidine-HCl (GuHCl) were subjected to quantification.

There was a significant decrease in the steady state levels of GuHCl-A β 40 in the brains of aged (24-months-old) heterozygous PS1-R278I knockin mice as compared to wild-type animals, although no differences were recorded in the levels of TS-A β s, GuHCl-A β 42 and GuHCl-A β 43 (**Fig. 2f,g**). This reduction of GuHCl-A β 40 resulted in a significant elevation of the A β 42/A β 40 and A β 43/A β 40 ratios in the GuHCl-soluble fraction (**Fig. 2h,i**). Notably, the A β 43/A β 42 ratio was also significantly increased in the GuHCl-soluble fraction of the heterozygous PS1-R278I knockin mouse brain (**Fig. 2j**). In younger PS1-R278I knockin mice (3 months old), A β 43 levels were too low to

detect, although the GuHCl-A β 40 levels were again significantly reduced in the knockin mice (**Supplementary Fig. 8**). These results indicate that A β 43 levels in the mouse brain increase upon aging, and that the increase in the A β 42/A β 40 and A β 43/A β 40 ratios observed in the older heterozygous mice appears to be primarily caused by a decrease in A β 40. Furthermore, the R278I mutation leads to an elevation in the A β 43/A β 42 ratio in aged mice. Taken together, these findings indicate that the PS1-R278I mutation gave rise to a modest *in vivo* effect in terms of the levels of endogenous A β species under heterozygous conditions.

We next quantified the A β variants in conditioned medium from knockin MEFs (**Fig. 2k–n**). The steady state levels of A β 40 were significantly reduced in a gene-dose-dependent manner in the PS1-R278I MEFs as compared to wild-type MEFs. In contrast, A β 43 markedly increased in the homozygous knockin MEFs, whereas A β 42 levels remained unchanged in all genotypes (**Fig. 2k**). Thus, the ratios of longer A β species significantly increased in homozygous PS1-R278I knockin MEFs (**Fig. 2l–n**). Intriguingly, there was no increase in A β 43 levels in conditioned media from heterozygous knockin MEFs (**Fig. 2k**). To unravel the underlying mechanism, we generated heterozygous R278I knockin x PS1 knockout mice (R278I/–) and measured the levels of A β s in conditioned media from cultured MEFs. Indeed A β 43 levels were increased, implying that, in heterozygous PS1-R278I knockin MEFs, wild type PS1 process A β 43 to A β 40 (**Fig. 2k**). Furthermore, in heterozygous PS1 knockout, no A β 43 could be detected (data not shown). Together, the data suggests that the γ -secretase substrate can be transferred between separate PS1 molecules or between dimers of PS1 as previously suggested²² or even between PS1 molecules within the PS1 complexes, for further processing. The fact that total A β (A β 40 + A β 42 + A β 43) was

decreased in heterozygous knockin MEFs, compared to homozygous knockin MEFs, is of particular interest. It might be due to a dysfunctional PS1 heterodimer where wild type PS1 is either directly affected by PS1-R278I or overloaded with A β 43 generated by PS1-R278I. Further experiments are required to resolve the reason behind the decreased total A β level (**Fig. 2k and Supplementary Fig. 9b**). Taken together, the R278I mutation inhibits A β 43 to A β 40 conversion leading to increased A β 43 levels and concomitant decrease of A β 40, without altering A β 42 levels. A similar A β -processing pathway has been previously described by Takami et al⁹ (**Fig. 2k and Supplementary Fig. 10**).

A β pathology and memory impairment of APP Tg mice

Overexpression of wild-type human APP (hAPPwt) in the above-stated MEFs using a semliki-forest virus vector²³ resulted in a significant increase in A β 43 in the heterozygous R278I knockin cells (**Supplementary Fig. 9**). The presence of the excessive γ -secretase substrates, *i.e.* APP CTF- α and β , appears to have forced the mutant PS1 to participate in APP processing. These observations prompted us to crossbreed heterozygous PS1-R278I knockin mice with APP Tg mice (APP23) to assess the effect of A β 43 *in vivo*. APP Tg x PS1-R278I knockin mice started to accumulate pathological A β deposits at around 6 months of age, whereas it took about 12 months for single APP-Tg mice to begin to show signs of such deposition (**Fig. 3a–d,g,h**). Massive astrocytosis was also detected around the amyloid plaques already at 9 months age in the APP Tg x PS1-R278I knockin mice but not in single Tg mice (**Fig. 3e,f**). Behaviorally, 3–4 month old APP Tg x PS1-R278I knockin mice exhibited short-term memory impairment as compared to single Tg mice when their performance was tested

in a Y-maze test (**Fig. 3i,j**). A similar tendency was also observed in the Morris water maze test, although the difference in this case did not reach statistic significance (**data not shown**). Taken together, the above findings indicate that the PS1-R278I mutation leads to accelerated A β pathology with an accompanying inflammatory response, and that the significant cognitive impairment occurs even prior to plaque formation.

We next quantified the steady state levels of A β 40, A β 42 and A β 43 in the brains of APP Tg and APP Tg x PS1-R278I knockin mice at 3 and 9 months. Notably, only the double mutant mice exhibited selective elevation of A β 43 in both TS-soluble and GuHCl-extractable brain fractions at 3 months, prior to the pathological deposition of A β (**Fig. 4a–f**), but at a time-point when the double mutant mice already showed short-term memory impairment (**Fig. 3i,j**). In contrast, A β 40 and A β 42 levels started to increase at around 9 months. Consequently, both the A β 43/A β 40 and A β 43/A β 42 ratios were higher in the double-mutant mice than in the single APP Tg mice at both 3 and 9 months, whereas the A β 42/A β 40 ratio remained unaltered (**Fig. 4g–i**). It is worth noting that the elevation of biochemically detectable A β 43 levels preceded plaque formation, implying that A β 43 may be the initial seeding species and the trigger of memory impairment in this mouse model. The steady state level of A β 43 also increased in an age-dependent manner in the single APP Tg mouse brains, beginning prior to the plaque formation (**Fig. 3g,h and Supplementary Fig. 11**).

In addition, we observed that a variety of FAD-associated PS1 mutations resulted in overproduction of A β 43 in a manner correlating with the quantity of A β 42 as well as with the age of onset of the patients²⁴ (**Fig. 5 and Supplementary Fig. 12**). These observations indicate the presence of an intrinsic mechanism by which A β 43 is physiologically generated and that not only A β 42 but also A β 43 may be involved in

Alzheimer's disease pathogenesis. The reason for the correlation between A β 42 and A β 43 remains elusive.

APP Tg x PS1-R278I versus APP Tg x PS1-M146V knockin mice

We also made another line of double-mutant mice by crossbreeding the APP Tg mice with PS1-M146V knockin mice as a positive control with which to compare the APP Tg x PS1-R278I knockin mice, given that the former mutation results in overproduction of A β 42 rather than A β 43²⁵. As expected, the PS1-M146V mutation, unlike the PS1-R278I mutation, resulted in selective accumulation of A β 42 (**Supplementary Fig. 13**). Although the steady state levels of A β 42 in the APP Tg x PS1-M146V mice was about 10-fold greater than that of A β 43 in APP-Tg x PS1-R278I mice at 9 months, the total plaque areas, as determined by immunohistochemistry, were similar (**Fig. 6**). Both double-mutant lines accumulated A β 40 and A β 42, whereas A β 43 occurred much more abundantly in the APP Tg x PS1-R278I mice (**Fig. 6a,b**). Quantitative image analyses gave consistent results (**Fig. 6c–e**). A β 43 immunoreactivity colocalized with the plaque cores in a manner similar to that of A β 42 but not A β 40 (**Fig. 6f–h**). Interestingly, A β species with the 3rd N-terminal residue converted to pyroglutamate (N3pE-A β), a potentially pathogenic A β subspecies^{26–29}, also colocalized with plaque cores and deposited more abundantly in APP Tg x PS1-R278I mice than in APP Tg x PS1-M146V animals (**Supplementary Fig. 14**). Although the underlying mechanism that accounts for the elevated N3pE-A β generation in the APP Tg x PS1-R278I mice remains unclear, the observation is consistent with a previous report that some presenilin mutations increase the quantity of N-terminally truncated A β in the brains of FAD patients³⁰.

Although APP Tg x PS1-M146V mice accumulated greater numbers of A β

plaques in the cortical and hippocampal areas than APP Tg x PS1-R278I mice, the density of thioflavin S-positive plaques per total plaques was significantly greater in the APP Tg x PS1-R278I knockin mice (**Fig. 7a–f**). This observation indicates that A β 43 is even more prone to seed cores in plaque formation than A β 42. To test this hypothesis *in vitro*, we performed thioflavin T binding experiments using an equal amount of A β 40, A β 42 and A β 43 (20 μ M each). A β 43 induced the highest incorporation of thioflavin T into A β aggregates (**Fig. 7g**). In addition, stoichiometric experiments, in which we added a relatively small quantity of A β 40, A β 42 or A β 43 (0.2 μ M) to a mixture of A β 40 (20 μ M) and A β 42 (2 μ M), also showed that A β 43 most potently accelerated incorporation of thioflavin T (**Fig. 7h**). These indicate that A β 43 contributes to the formation of the thioflavin T-positive β -sheeted structure to a greater extent than either A β 40 or A β 42, a finding that may account for the observation that a relatively small amount of A β 43 is sufficient to accelerate A β amyloidosis and induce plaque core formation *in vivo*.

Neural toxicity and amyloid pathology of A β 43

In agreement with A β 42 having higher hydrophobicity and higher toxicity than A β 40 *in vitro* and *in vivo*, a large number of reports also show that A β 42 contributes to synaptic dysfunctions^{31–34}. Herein, we compared the toxicity of A β 40, A β 42 and A β 43. A β 43 and showed a higher potent neural toxicity in a dose-dependent manner as compared to A β 40 and A β 42 (**Fig. 7i,j**). These results anticipate that A β 43 directly affects neural toxicity and induces synaptic dysfunction, which would contribute to short-term memory impairments prior to the amyloidogenesis (**Fig. 3i,j**).

Finally, we performed immunohistochemical experiments on brain sections from SAD patients to explore the possible involvement and significance of A β 43 in human neuropathology. A β 43 accumulated in the brains more frequently than A β 40 (**Fig. 8a–e and Supplementary Fig. 15**), and was present in both diffuse (**Fig. 8f,g**) and dense-cored (**Fig. 8h,i**) plaques, similar to A β 42 and N3pE-A β (**Supplementary Fig. 16a–d**). Furthermore, thioflavin S fluorescence signals colocalized well with A β 43 immunoreactivity (**Fig. 8j–m**), as well as with N3pE-A β (**Supplementary Fig. 16e–g**). These observations are consistent with those of previous studies showing that a substantial amount of A β 43 accumulates in SAD and FAD brains^{4–7}.

DISCUSSION

Previous studies have elegantly demonstrated that A β 42 is essential for amyloid deposition *in vivo*³¹ and that A β 40 inhibits this deposition³², based on the use of Bri-A β fusion proteins. The difference between A β 40 and A β 42 lies in the carboxyl-terminal amino-acid sequence, *i.e.* the additional presence of isoleucine and alanine residues in A β 42. Because both isoleucine and alanine are hydrophobic amino acids, it is reasonable to assume that A β 42 is more prone to form a β -sheet structure than A β 40. In contrast, the carboxyl-terminal amino acid of A β 43 is threonine, which carries a hydrophilic alcohol group (together with a hydrophobic methyl group) and thus could reverse the hydrophobicity of A β 42. Therefore, the amyloidogenicity of A β 43, a natural product of γ -secretase activity^{8,9}, has remained elusive.

Herein, we focused on A β 43, an overlooked species in AD research, to investigate a crucial role in amyloidogenesis and pathogenesis of AD. So far, the major focus has been placed on the amyloidogenicity of A β 42 and in numerous studies; BC05, an anti-A β 42 antibody has been used to demonstrate that A β 42 is the major pathogenic species in AD. Since partial crossreactivity of BC05 against to A β 43 had already been reported⁴⁷, A β 42(43), like this orthography, was noted in some of the carefully designed and interpreted studies using BC05, as a tacit understanding. However, many of other studies apparently overestimated the A β 42 levels and ignored the possible changes in A β 43 levels. Almost all FAD-associated PS1 mutations result in an increase of the A β 42/A β 40 ratio caused by an increase of A β 42 levels. However, some of the PS1 mutations lead to a decrease of A β 40 with or without alteration of A β 42 levels which also leads to an increase of the A β 42/A β 40 ratio. One explanation of the association between decreased A β 40 and FAD could possibly be that A β 40 plays a role in

protection from plaque formation³². We here report that in PS1-R278I knockin mice, A β 40 levels are decreased accompanying increased levels of A β 43. Furthermore, the increased A β 43 levels accelerated A β pathology, thus contributing to the early onset of the disease. Therefore, we propose that A β 43 be separately analyzed apart from A β 42.

In an effort to explore the role of A β 43 in A β amyloidosis, we generated PS1-R278I knockin mice based on the knowledge that this mutation causes overproduction of A β 43 *in vitro*¹³. We chose to use this PS mutation knockin paradigm rather than the overexpression strategy for the following reasons. First, the R278I mutation is known to be clinically pathogenic. The knockin paradigm is also less artificial than Tg overexpression approaches in general, and we considered that the knockin mice could potentially be utilized to generate a relevant AD model by crossbreeding with other mice such as mutant APP Tg or knockin mice. Unexpectedly, the phenotype of the homozygous knockin mice proved to be embryonic lethal in association with abnormal PS1 endoproteolysis. Limited proteolysis of APP CTF- α , CTF- β , N-cadherin and Notch1 was also hampered in the homozygous knockin embryos, although the γ -secretase components appeared to have been properly assembled to a 360 kDa complex. Based on previous studies, it appears that the disturbance in Notch1 processing represents the primary cause of the premature death that we observed^{16,35}. Compared to PS1 knockout, the embryonic lethality of PS1-R278I knockin mice occurs at a slightly later stage. Taking into account that a 50 % reduction of γ -secretase activity in heterozygous PS1-R278I or in heterozygous PS1 knockout does not lead to embryonic lethality while the 90% reduction in homozygous PS1-R278I does so, it seems that the γ -secretase activity threshold for survival is

somewhere between 10–50 % of wild type. The remaining 10% γ -secretase activity in homozygous PS1-R278I knockin could account for the delayed lethality compared to PS1 knockout (**Supplementary Fig. 10c**). Taken together, these results strongly suggested that the primary phenotype of the R278I mutation was a partial loss of function in terms of γ -secretase activity.

Despite this, MEFs prepared from homozygous embryos produced an extremely large steady state levels of A β 43 (approximately 20-fold greater than that of wild-type MEFs); this accompanied a significant decrease in A β 40 production and no changes in A β 42 levels. Previous *in vitro* studies have also demonstrated that A β 43 is processed to A β 40 whereas A β 42 is independently produced from A β 45 in the presence of γ -secretase^{8,9}. In agreement with these studies, our results from crossbreeding heterozygous PS1-R278I mice with PS1 knockout mice, which showed significant levels of both A β 40 and A β 43, indicated that A β 43 was indeed converted to A β 40 independently of A β 42 production (**Fig. 2k**). Furthermore, we found that the ratio of production of A β 46 in homozygous PS1-R278I MEFs was increased with a concomitant increase of A β 43 and decrease of A β 40 in *in vitro* γ -secretase assays (**Supplementary Fig. 10**), suggesting that production of A β 40 and A β 43 also depends on A β 46 production as previously postulated⁸⁻¹⁰. Thus, inhibition of this A β 43-to-A β 40 conversion could account for the increase in A β 43 and the concomitant decrease in A β 40 in the knockin MEFs. Interestingly, treatment of PS1- Δ E9-expressing cells with L-685,458 resulted in elevated A β 43 production³⁶, consistent with the notion that multiple processes are involved in the generation of various A β species and that a partial loss of γ -secretase activity might give rise to a particular A β species. However, *in vitro* γ -secretase activity of heterozygous and homozygous PS1-R278I was drastically

reduced in a gene-dose-dependent manner, while there were no significant differences of the steady state levels of total MEF-produced A β compared to wild type MEFs. To elucidate the reason behind this contradiction, it will be necessary to investigate other mechanisms *e.g.* intracellular trafficking and secretion of A β in depth.

The molecular mechanism that allows A β 43 production but not other proteolytic processes remains to be clarified, but it is likely to involve specific conformational changes of the γ -secretase complex³⁷. Because A β 42 is produced independently of A β 43 in the presence of γ -secretase, some of the FAD-associated PS1 mutations that cause a decrease in A β 40 without an increase in A β 42, such as A79V, A231V, C263F, L282V, L166P and G384A^{24,38}, might actually result in the elevation of A β 43 in a manner similar to the R278I mutation. In addition, PS1- Δ E10, an artificial PS1 mutation located to the loop domain of PS1 where R278I is present, showed a significant reduction of the steady state levels of A β 40 without any alteration of the A β 42 levels similar to our results, however A β 43 levels were not measured³⁹. It will therefore be important to investigate if these FAD-associated mutations give rise to increased A β 43 levels and importantly, scrutinize their amyloidogenicity. In fact, I143T, L262F, L282V and G384A mutations did lead to significant production of A β 43 in our transfection assays. Notably, A β 43 levels and the ratio of A β 43/A β 40 significantly correlated well with the age of disease onset in a manner similar to A β 42 levels and the ratio of A β 42/A β 40. Additionally, a PS1-I143T carrier in a Swedish family with FAD gave rise to high levels of A β 43⁷. These observations highlighted the possibility that compounds that facilitate the A β 43-to-A β 40 and A β 42-to-A β 38 conversion might be beneficial for prevention and treatment of AD by decreasing not only A β 42 but also A β 43. In support of this notion, an oral vaccination with adeno-associated virus vector

carrying A β 1–43 cDNA was reported to result in a marked reduction of A β burdens and improvement of behavioral performances in APP transgenic mice^{40,41}.

Although our original plan had been to establish APP Tg x homozygous PS1-R278I knockin mice, we also explored the possible utility of heterozygous PS1-R278I knockin mice, given that overexpression of APP in heterozygous PS1-R278I knockin MEFs resulted in selective elevation of A β 43. Consistent with this, APP Tg x heterozygous PS1-R278I knockin mice exhibited short-term memory impairment, selective biochemical accumulation of A β 43 at an early stage prior to plaque formation and significant acceleration of A β pathology thereafter as compared to single APP Tg mice. It is also important to note that the APP Tg x PS1-R278I knockin mice exhibited a greater density of the thioflavin S-positive signal per plaque than APP Tg x PS1-M146V knockin mice, which overproduce A β 42 instead of A β 43. Consistent with previous reports^{6,7}, we have observed A β 43-positive plaques more often than A β 40-positive ones in AD brains. A β 43 has previously been found in amyloid plaques not only in AD patients^{4,6,7} but also in aged gorillas⁴² and in some AD model mice harboring PS1 or APP FAD-mutations^{3,10}. In addition, it has been suggested that the amount of A β 43 in plaques correlates with cognitive decline⁵. We also showed in this report that A β 43 exhibited potent neural toxicity, comparable to or even greater than that of A β 42. These observations establish that A β 43 is indeed amyloidogenic *in vivo* and likely to be pathogenic. Thus, the carboxyl-terminal amino acid residue of A β 43, *i.e.* threonine, appears to strengthen the hydrophobicity of the peptide rather than reversing it.

Notably, biochemical accumulation of A β 43 preceded pathological deposition in the APP Tg x PS1-R278I knockin mice and in the single APP Tg mice. In addition, the basal A β 43 levels substantially increased with aging in wild-type mice up to at least

18 months of age (data not shown). These observations point to the possible value of A β 43 as a biomarker for presymptomatic diagnosis of AD. We therefore believe that it would be worth trying to quantify A β 43 levels in cerebrospinal fluid from AD patients and controls although this is beyond the scope of the present study. We also detected the presence of N3pE-A β in APP Tg x PS1-R278I knockin mouse brains, a finding that is supported by a report quantitatively describing N3pE-A β 42 and N3pE-A β 43 in the brains of FAD and SAD patients². It is of particular interest that Pittsburgh Compound B, a probe for amyloid imaging by positron emission tomography, selectively binds to N3pE-A β ²⁶, implying that N3pE-A β 42/43 could be particularly prone to seed deposition of other A β species, consistent with previous reports²⁸. It is also possible that the mutation might affect the interaction of PS1 with other substrates or alter its property of non- γ -secretase activity²⁹.

In summary, our observations establish that A β 43, which has to large extent been overlooked, is potently amyloidogenic and toxic and highlight the potential value of A β 43, i.e. CSF A β 43 levels, as an early marker for some of the detrimental effects of aging in the adult brain. We propose that inhibition of A β 43 generation, for instance, by facilitating the conversion of A β 43 to A β 40 in the γ -secretase complex should be beneficial for prevention of A β amyloidosis.

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Author Contributions

This study was jointly designed by T. Saito, T. Suemoto and T.C.S.; experiments were performed by T. Saito, T. Suemoto, N.M., Y.M., K.Y. and S.F.; and T. Saito, T. Suemoto, S.F., K.Y., P.N., J.T., M.N., N.I., C.V.B., Y.I. and T.C.S. jointly analyzed and interpreted data. N.B., K.S. and C.V.B. identified pathogenic PS1 mutations in patients/families and generated *PSENI* vector constructs for expression studies.

Figure Legends

Figure 1. Phenotypic and biochemical characterization of PS1-R278I knockin mice.

(a) Embryonic lethality of homozygous PS1-R278I knockin mice. An overall size reduction, stubby tail (arrowheads), limb ateliosis (yellow arrows) and hemorrhage in the CNS (arrows) were observed. Scale bars: 2 mm. (b–e) Embryonic brains and (f) MEFs were subjected to Western blot analyses (see **Supplementary Fig. 4** for more information). Antibodies used are listed on the left side of each panel. (b) Expression of γ -secretase components. FL: full-length PS1. (c) BN-PAGE analysis of native γ -secretase complexes. The symbol (–/–) is used for the homozygous PS1 knockout mice used as a negative control. Arrows indicate the position of the native wild-type 360 kDa PS1 and PS2 γ -secretase complexes, whereas arrowheads point to the atypical high molecular weight (750 kDa) γ -secretase complex. (d) Immunoprecipitation by antibodies to PS1-NTF. IgG(H) and IgG(L) indicate immunoglobulin heavy and light chains, respectively. (e) γ -secretase activity in PS1-R278I knockin brains. Brain extracts were analyzed by Western blotting to detect endogenous APP-CTF- β , APP-CTF- α , AICD, full-length N-cadherin, N-cadherin-CTF and NICD products. (f) Notch1-processing in PS1-R278I knockin MEFs. Myc-tagged Δ Notch was transiently expressed in the MEFs, and cell lysates were subjected to Western blot analysis using anti-Myc antibody. β -actin levels are shown as internal controls.

Figure 2. A β levels in adult PS1-R278I knockin mouse brains and MEFs.

(a,b) Establishment of ELISA system to specifically quantify A β 42 and A β 43 (see more **Supplementary Figs 6a,b and 7**). (c–e) Specificity of the antibodies to A β 40, A β 42

and A β 43 used in this ELISA system. Synthetic A β 1–40, A β 1–42 and A β 1–43 were separated by Tris/Tricine PAGE (15% polyacrylamide gel) and subjected to Western blotting. Anti-A β antibodies, C40, C42 and C43, specifically recognized the A β s, respectively (see more **Supplementary Fig. 6c–e**). **(f–n)** Quantification of A β 40, A β 42 and A β 43 by ELISA in adult mouse brains **(f–j)** and MEFs **(k–n)**. **(f–j)** Cortical hemispheres from 24 month old wild-type and heterozygous knockin mice were homogenized and fractionated into TS-soluble and GuHCl-soluble fractions. Data represent mean \pm s.e.m. ($n = 9$). $*P < 0.05$ and $**P < 0.01$ between wild type and heterozygous knockin mice; Student-t test. **(k–n)** A β concentrations in conditioned medium from MEFs. 8×10^5 cells were inoculated in a 1 ml culture. The conditioned medium was collected after 24 hrs and subjected to ELISA. The symbol (R278I/–) is used for the double heterozygote of PS1-R278I knockin crossbred with PS1 knockout mice. Data represent mean \pm s.d. from two independent experiments ($n = 16$). $**P < 0.01$ compared with wild type; One-way ANOVA with Scheffe's F test.

Figure 3. Acceleration of A β pathology and short-term memory impairment by the R278I knockin mutation in APP Tg mice.

(a–f) Brain sections from APP Tg x PS1-R278I knockin mice (**a**: 3- , **b**: 6- , and **c**, **e**: 9-month old) and 9-month old single APP Tg mice (**d**,**f**) were immunostained with 4G8 anti-A β antibody (**a–d**) and anti-GFAP antibody (green) with 4G8 counterstaining (red) (**e**,**f**). A β immunostained brain sections from cortex (**g**) and hippocampus (**h**) of 3-, 6-, 9- and 12-month old wild-type, APP Tg, and heterozygous PS1-R278I knockin mice, as well as APP Tg x PS1-R278I knockin mice were analyzed ($n = 5–6$ each genotype); $*P < 0.05$ and $**P < 0.01$ compared with APP Tg mice; Two-way ANOVA with Scheffe's F

test. Scale bars: 500 μm (**a–d**); 50 μm (**e,f**). (**i,j**) Y-maze test was performed prior to plaque formation using 3–4 month old male wild-type, PS1-R278I knockin, APP Tg and APP Tg x PS1-R278I knockin mice. Data represent mean \pm s.e.m. ($n = 10$ each genotype), $*P < 0.05$ compared with wild type or PS1-R278I knockin mice; One-way ANOVA with Scheffe's F test.

Figure 4 A β 40, A β 42 and A β 43 in APP Tg x PS1-R278I knockin mice.

(**a,d**) The levels of A β 40, (**b,e**) A β 42 and (**c,f**) A β 43 were quantified by ELISA and (**g-i**) the ratios of the A β species were subsequently determined. Cortical hemispheres from single APP Tg and APP Tg x PS1-R278I knockin mouse brain (3 and 9 month old) were homogenized and fractionated into TS-soluble fractions (**a–c**) and GuHCl-extractable fractions (**d–f**). Data represent mean \pm s.e.m. ($n = 7$: 3 months old; $n = 5$: 9 months old); $*P < 0.05$ and $**P < 0.01$ between APP Tg mice and APP Tg x PS1-R278I knockin mice; Student-t test.

Figure 5. Effect of various pathogenic PS1 mutations on A β 43 production.

The constructs containing *PSEN1* with FAD-associated mutations were transfected into HEK293 cells stably expressing APP with the Swedish mutation: (**a**) Expression levels of PS1-FAD mutants. (**b–g**) Quantification of the steady state levels of A β 40, A β 42 and A β 43 and the correlation between A β levels and the age of disease onset. Age of onset is shown as follows: wild type (75 years old), A79V (59.3), V94M (53), I143T (32.5), A231V (58), L262F (50.3), L282V (44) and G384A (34.9)^{24,50}. Data represent mean \pm s.d. from five independent series each consisting of duplicate measurements; $*P < 0.05$ and $**P < 0.01$ compared with wild type; One-way ANOVA with Dunnett test.

Figure 6. Localization of A β species in amyloid plaques of APP Tg x PS1-R278I knockin mice.

(a) A set of serial brain sections from 9 month old APP Tg x PS1-M146V knockin mice and (b) APP Tg x PS1-R278I knockin mice were immunostained with the following anti-A β antibodies as indicated: 4G8 (total A β), C40 (A β _{x-40}), C42 (A β _{x-42}) and C43 (A β _{x-43}). (c–e) The immunoreactive areas in single APP Tg (left column), APP Tg x PS1-M146V knockin (middle column) and APP Tg x PS1-R278I knockin (right column) mice were quantified as indicated ($n = 6$); $**P < 0.01$ between APP Tg x PS1-M146V knockin mice and APP Tg x PS1-R278I knockin mice; One-way ANOVA with Scheffe's F test. N.D.: not detected. (f–h) Double-staining with 4G8 (green) and the following A β species-specific antibodies (red): A β ₄₀ (f), A β ₄₂ (g) and A β ₄₃ (h). The images in the left (green) and middle (red) are merged (yellow) in panels on the right. Scale bars: 500 μm (a,b); 50 μm (f–h).

Figure 7. Mature amyloid plaques in APP Tg x PS1-R278I knockin mice and *in vitro* aggregation property and neural cell toxicity of A β ₄₃.

(a–f) A set of serial brain sections from 9 month old APP Tg x PS1-M146V knockin mice (a,c) and APP Tg x PS1-R278I knockin mice (b,d) were stained with thioflavin S (a,b) and immunostained with 4G8 (c,d). Thioflavin S positive plaque are marked with arrows (a,b) and the corresponding plaques in the serial brain sections are also marked (c,d), respectively. Scale bars: 500 μm (a–d). Intensity of cortical and hippocampal A β immunoreactivity and thioflavin S signals were quantified (e), and the ratio of thioflavin S/total A β signal of amyloid plaques was determined (f) ($n = 12$); $**P < 0.01$ between

APP Tg x PS1-M146V knockin mice and APP Tg x PS1-R278I knockin mice; Student-t test. **(g,h)** *In vitro* A β aggregation experiments. Incorporation of thioflavin T into A β aggregates was measured by fluorescence spectroscopy. **(g)** The aggregation property of 20 μ M A β 40, A β 42 and A β 43 at 20 μ M was measured individually. **(h)** The effect of A β 40, A β 42 and A β 43 at a concentration of 0.2 μ M on the mixture of 20 μ M A β 40 and 2 μ M A β 42 was then assessed. Data represent mean \pm s.d. from three independent series each consisting of 6–8 individual measurements; $**P < 0.01$ **(g)** between A β 40 and A β 42 or between A β 42 and A β 43, $^{\#}P < 0.05$ **(h)** between A β 40 and A β 43; One-way ANOVA with Scheffe's F test. **(i,j)** Neural cell toxicity of A β 43. Cell viability **(i)** and LDH release as a measure of cell toxicity **(j)** were assayed. A β s were administrated at 1, 3 and 10 μ M, respectively. The results obtained after treatment with A β 40 (open column), A β 42 (gray column) and A β 43 (filled column) are indicated, and vehicle (veh) treatment was also indicated by open column in **(j)**. Data represent mean \pm s.d. from three independent series each consisting of 6 individual measurements; $*P < 0.05$ and $**P < 0.01$ between A β 40 and A β 42 or between A β 42 and A β 43, and $^{\#}P < 0.05$ between A β 40 and A β 43; Two-way ANOVA with Scheffe's F test or Dunnett test.

Figure 8. A β 43 in amyloid plaques in AD brains.

(a–d,f–i) Serial sections of the hippocampal region **(a–d, h,i and j–m)** and the frontal cortical region of AD brains **(f,g)** were stained with the anti-A β antibodies 4G8 (total A β), C40 (A β _{x–40}), C42 (A β _{x–42}), and C43 (A β _{x–43}), as well as thioflavin S, as indicated. The single staining **(a–d,f–i)** was developed using 3,3'-diaminobenzidine, whereas the double-staining **(j–m)** utilized the fluorescent dyes fluorescein (green, A β) and rhodamine (red, A β 43). The images in panels **(j)** and **(k)** are merged (yellow) in

panel **(I)**. Scale bars: 250 μm (**a–d**); 25 μm (**f–m**). **(e)** Ratio of A β 40, A β 42 and A β 43 of the plaque areas in the hippocampal region of AD brain sections from 4 individuals were quantified; $**P < 0.01$ between A β 40 and A β 43; One-way ANOVA with Scheffe's F test (see **Supplementary Fig. 15** for more details).

Methods

Generation of PS1-R278I knockin mice

As shown in **Supplementary Fig. 1**, the genomic DNA of mouse *PSEN1* was isolated from the bacterial artificial chromosome (BAC) library from the 129/Sv mouse genome, and one BAC clone which included intron 5 to intron 11 of the *PSEN1* gene was obtained. The fragment from the *ApaI* site of intron 5 to the *HindIII* site of intron 11 provided the basis for construction of the targeting vector. To introduce the PS1-R278I mutation, the *SmaI/BamHI* fragment containing introns 7 and 8 of the *PSEN1* gene was subcloned into pBluescript vector. To introduce the R278I mutation the following primer was used (underlined: original G to T), 5'-GGTTGAAACAGCTCAGGAAATTAAATGAGACTCTCTTTCCAGC-3', using GeneEditor Mutagenesis System (Promega) according to the manufacturer's protocol. This fragment was used to replace the original sequence of the *PSEN1* gene. Finally, a *pgk-neo* gene cassette was inserted for positive selection at the *EcoRI/SmaI* sites located in intron 7, and a Diphtheria toxin-A fragment cassette was inserted for negative selection at the *HindIII* site in intron 11. We used the fragment of *ApaI/EcoRI* spanning from intron 5 to intron 7 (4.3kbp) as the long arm and the fragment of *BamHI/HindIII* spanning from intron 8 to intron 11 (3.8kbp) as the short arm of the targeting vector.

Embryonic stem (ES) cell cultures and gene-targeting experiments were carried out as previously described. Targeted ES cells were microinjected into 129/Sv blastocysts. DNA was extracted from the biopsied tail of mouse pups, and the F1 generation of the mutant animals was identified by Southern blot analysis with a 3' external probe which was produced by PCR using the following primer sets, 5'-AATGGATAATCAGAGCCTGCC-3' and

5'-TCCTCACA ACTAACTACCCAAGG-3'.

The heterozygous mice were crossbred with EIIa-Cre transgenic mice to remove the *pgk-neo* gene, after which the generated PS1-R278I knockin mice were backcrossed to the C57BL6/J strain. When the *pgk-neo* gene was removed by the *cre-loxP* system, a short sequence ranging from the EcoRI to the SmaI sites of intron 7 was also removed. Deletion of such a short sequence in intron 7 leads to feasible detection of the genotype of mutant mice. To genotype the PS1-R278I knockin mouse, tail DNA was isolated and subjected to PCR analysis using the following primer sets: 5'-AGTTTCAGACCAGCCTAGGCCAC-3' and 5'-AGGAAGGGAGACTTGACAGC-3'.

Other mutant mice

PS1 knockout mice and PS1-M146V knockin mice were purchased from the Jackson Laboratory. APP23 mice carrying the human APP695 transgene harboring the Swedish mutation (KM670/671NL)⁴³ have been described previously⁴⁴. All animal experiments were carried out according to the RIKEN Brain Science Institute's guidelines for animal experimentation.

Mouse embryonic fibroblasts (MEFs)

MEFs were prepared from E13–14 embryos of wild-type, PS1-R278I knockin and PS1 knockout mice, and inoculated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The conditioned medium and cell lysates from MEFs (passage <8) were subjected to biochemical analyses, including ELISA, native-PAGE and Western blotting. Transfection of the MEFs with the Myc-tagged Δ Notch

construct⁴⁵ was performed using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's instructions.

Blue native-polyacrylamide gel electrophoresis (BN-PAGE)

Non-denaturing native PAGE was performed to confirm the integrity of the γ -secretase complexes¹⁷ using the Novex Bis-Tris gel system (Invitrogen) according to the manufacturer's instructions. Samples were extracted from embryonic brains and MEFs using the sample buffer from the Novex Bis-Tris gel system that contains 1% digitonin. Equal amounts of proteins as determined using the BCA Protein Assay Kit (Pierce) were loaded on a 3–12% gradient Bis-Tris acrylamide gel. Immunoblotting was performed using H70 (anti-PS1 N-terminal antibody, Santa Cruz) and Ab-2 (anti-PS2 antibody, Calbiochem) antibodies.

Immunoprecipitation assay and Western blot analysis

Brain homogenates from embryonic brains (E14–16) and cell lysates of MEFs were immunoprecipitated with H70, and then captured by Dynabead conjugating protein G (Invitrogen). Immunoprecipitants were subjected to Western blot analysis using H70, MAB5232 (anti-PS1-Loop antibody, Chemicon), Ab-2, PA1-758 (anti-Nicastrin antibody, Affinity Bioreagents), anti-Pen-2 (Zymed), and ACS-01 (anti-Aph1 antibody)¹⁰ antibodies. The following antibodies were also used in Western blot analysis: 6E10 (anti-A β 1–12 antibody, Covance), 22C11 (anti-APP-N-terminal antibody, Chemicon), anti-APP-CTF antibody (Sigma), 9B11 (anti-Myc antibody, Cell Signaling), mN1A (anti-Notch1 antibody, BD Bioscience), and AC-15 (anti- β -actin antibody, Sigma).

Enzyme-linked immunosorbent assay (ELISA)

Soluble materials from mouse cortical hemispheres were dissolved in Tris-HCl buffered saline (TS fraction) and the insoluble materials were dissolved in guanidine-HCl solution (GuHCl fraction) as previously described⁴⁶. Samples from the brains and from the conditioned medium of MEFs were analyzed using an A β -ELISA kit (Wako) to quantify A β 40. In order to specifically quantify the levels of A β 42 and A β 43, we established an A β 42- and A β 43-specific sandwich ELISA system utilizing the A β -ELISA kit (Wako). Since BC05, a detection antibody of this kit, cross-reacts with A β 42 and A β 43 as previously described⁴⁷, we used the A β 42- and A β 43-specific antibodies, C42 (anti-A β 42 specific antibody, IBL) and C43 (anti-A β 43 specific antibody, IBL). The specificities of these antibodies are shown in **Fig.2c–e and Supplementary Fig. 6c–e**. Samples were incubated overnight at 4 °C in a 96-well plate coated with the capture antibody, BNT77 (anti-A β 11–28 antibody⁴⁸). A β from samples captured in the ELISA were incubated with C42 or C43 (1:100, for 3 hr at room temperature), respectively, after which HRP-conjugated anti-rabbit IgG (1:500, for 2 hr at room temperature) was added as a detection antibody. Synthesized A β 42 or A β 43 peptide (Peptide Institute) was used for the preparation of a standard curve, and diluted with the diluents solutions provided in the kit. For consistency, when we quantified the amount of A β 40, a synthesized A β 40 peptide (Peptide Institute) was also used for the preparation of a standard curve. This system also worked in broader concentration range of A β 42 and A β 43 (**Supplementary Fig. 6a,b**). Furthermore, a high sensitive A β 43 system, based on modified protocols, was established for the measurement of samples containing low amount of A β 43, *e.g.* samples derived from non APP Tg mice and non

APP-overexpressing cells (**Supplementary Fig. 7.**)

Immunohistochemical and histochemical studies

Paraffin-embedded mouse brain sections were immunostained with the following antibodies: 4G8 (anti-A β 17–24 antibody, Covance), C40 (anti-A β 40 specific antibody, IBL). C42, C43 and MAB3402 (anti-GFAP antibody, Chemicon), with or without tyramide signal amplification (PerkinElmer Life Sciences) as previously described⁴⁶.

Quantification of immunoreactivity from brain sections were carried out using MetaMorph imaging software (Universal Imaging Corp.) as previously described⁴⁶.

Y-maze test

Mice were housed individually before transferring to the behavioral laboratory. They were kept during the behavioral analysis. The light condition was 12h:12h (lights on 8:00). The laboratory was air-conditioned and maintained temperature and humidity within approximately 22–23 °C and 50–55%. Food and water were freely available except during experimentation. Large tweezers were used to handle mice in order to avoid individual differences in the handling procedure. All of the experiments were conducted in the light phase (9:00–18:00), and the starting time of the experiments was kept constant.

The Y-maze apparatus (O'Hara, Tokyo, Japan) was made of gray plastic, and consisted of three compartments (3 cm (W) bottom and 10 cm (W) top, 40 cm (L) and 12 cm (H)) radiating out from the center platform (3 x 3 x 3 cm triangle). The maze was positioned 80 cm above the floor, surrounded by a number of desks and test apparatuses around the maze to act as spatial cues. In this test, each mouse was placed in the center

of the maze facing towards one of the arms, and was then allowed to explore freely for 5 min. Experiments were performed at a light intensity of 150 lux at the platform. An arm entry was defined as four legs entering one of the arms, and the experimenter counted the sequence of entries by watching a TV monitor behind a partition. An alternation was defined as entry into all three arms on consecutive choices (the maximum number of alternations was the total number of entries minus 2). The percent alternation was calculated as (actual alternations/maximum alternations) x 100. The percent alternation was designated as the spontaneous alternation behavior of the mouse, was taken as a measure of memory performance.

Thioflavin T binding assay

The thioflavin T binding assay was performed by mixing aliquots of A β . Amyloid β -protein (human, 1–40), amyloid β -protein (human, 1–42), and amyloid β -protein (human, 1–43) were purchased from the Peptide Institute. We first examined aggregation property of individual A β 40, A β 42 and A β 43 by incubating the peptide separately at 20 μ M in 50 mM potassium phosphate buffer (PPB) (pH 7.4) at 37 °C for 24 hrs with agitation. The stoichiometric effect of different A β species on aggregation was investigated in the mixture of A β 40 and A β 42 by adding and mixing A β s in 50 mM PPB (pH 7.4) at molar concentrations of 20 μ M : 2 μ M : 0.2 μ M (A β 40 : A β 42 : A β 40/A β 42/A β 43 = 100 : 10 : 1) and incubating them at 37 °C for 24 hrs with agitation. After incubation, thioflavin T was added to a final concentration of 5 μ M, and thioflavin T fluorescence was measured at excitation and emission wavelengths of 442 nm and 485 nm were used, respectively.

Neural cell toxicity assay

Primary cortical neurons were isolated as previously described²³ and plated at a density of 5×10^4 cells/well in 96 well plate ($n = 6$ wells in each experimental conditions). Day in vitro (DIV) 10 to DIV14 cultures were treated with synthesized A β 40, A β 42 and A β 43 peptide (Peptide Institute) at 0.1 to 10 μ M of A β s for 72 hr. These A β peptides were dissolved in 10% (v/v) of 60 mM NaOH and 90% (v/v) of 10 mM phosphate buffer, pH7.4, which was used as the vehicle³³. SH-SY5Y cells were plated at a density of 2×10^4 cells/well with 10% fetal bovine serum (FBS) supplemented medium in 96 well plate ($n = 6$ wells in each experimental conditions), and incubated for 24 hr. Then, the media was replaced with media containing 1% of FBS, and treated with each A β peptides for 48 hr. Cell viability was determined using MTS assay (CellTiter96 Aqueous One Solution Cell Proliferation Assay Kit: Promega)⁴⁹, and LDH release as cell toxicity was performed using CytoTox-ONE Homogeneous Membrane Integrity Assay Kit (Promega)³³, according to the manufacturer's instructions and compared to vehicle treated cells, respectively.

AD brain sections

Postmortem AD brain tissues were kindly provided by J.Q. Trojanowski and V.M.-Y. Lee (University of Pennsylvania, Philadelphia, PA). The tissues had been fixed with ethanol or formalin and embedded in paraffin. This study was approved by the Institutional Review Board of the RIKEN Brain Science Institute.

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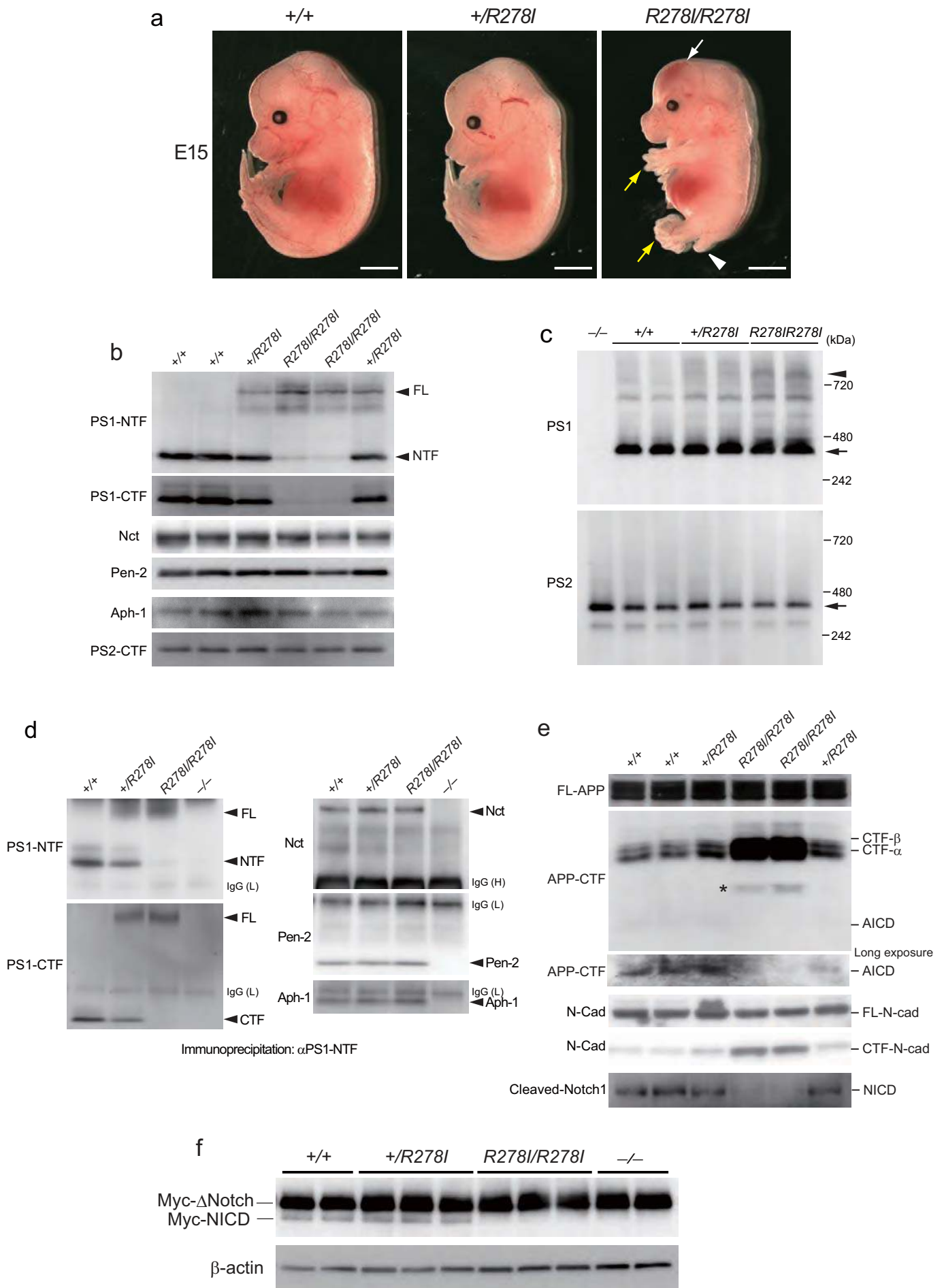
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Saido T.C.: Fig. 1



Saido T.C.: Fig. 2

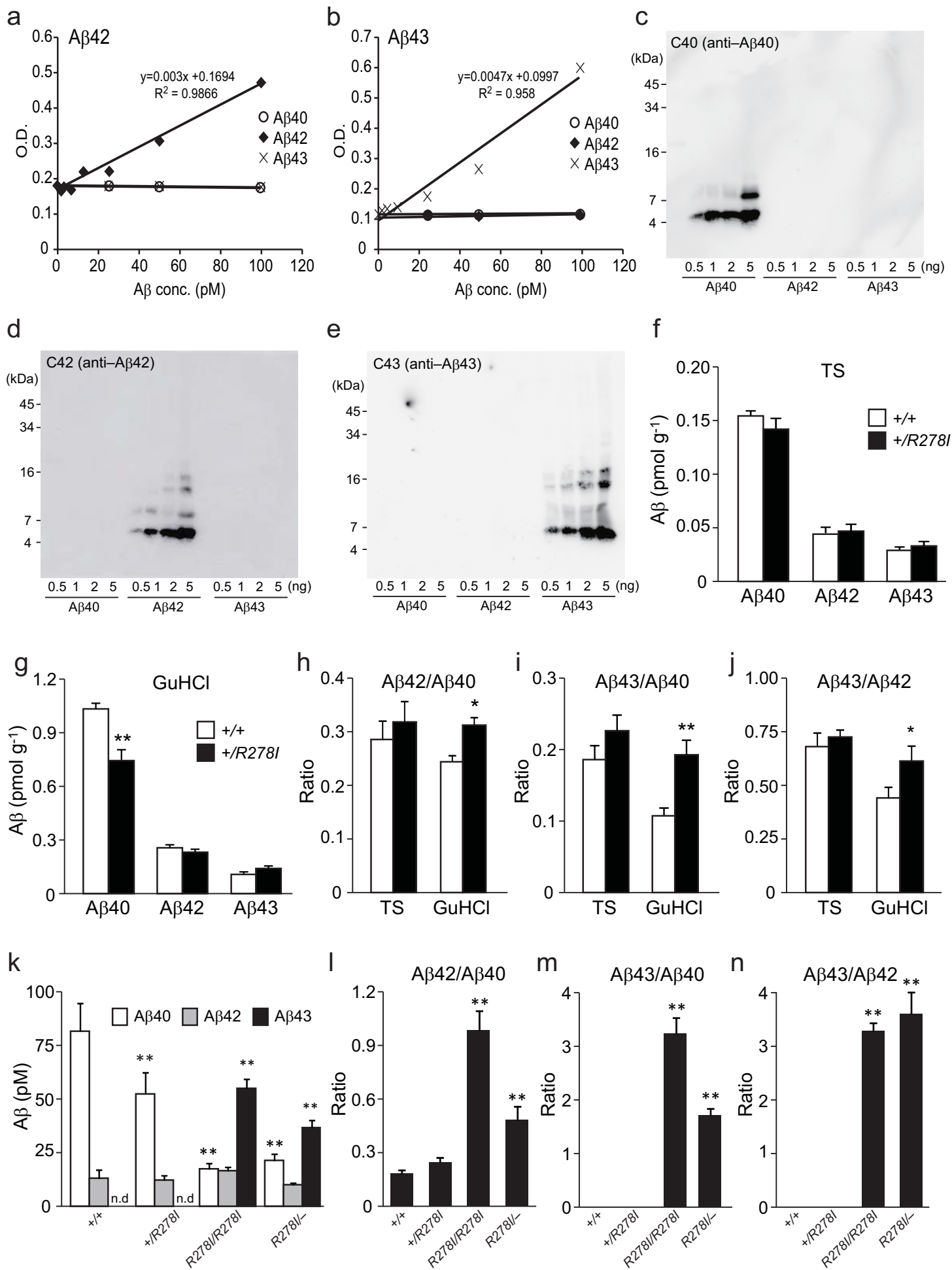
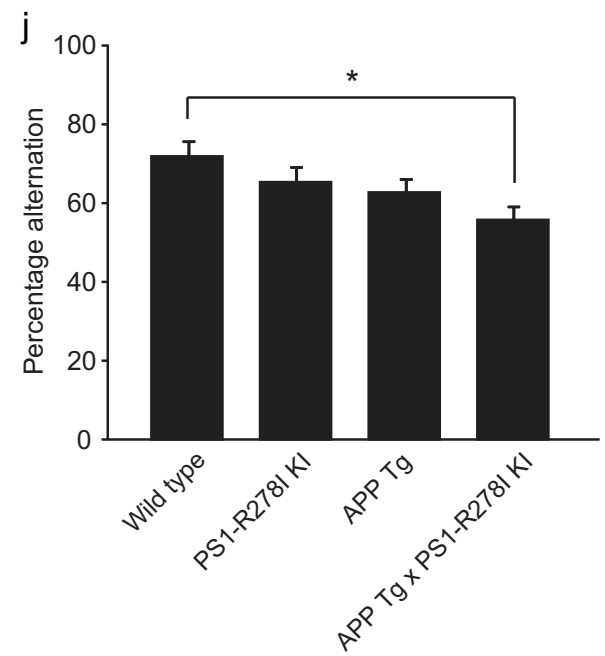
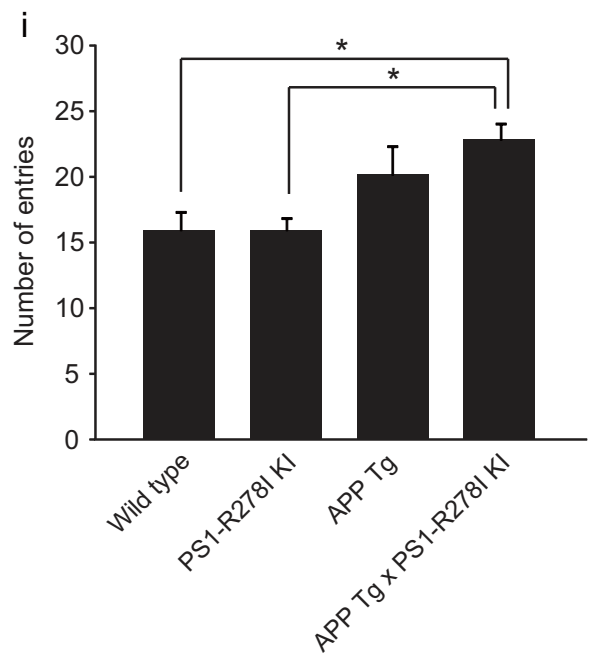
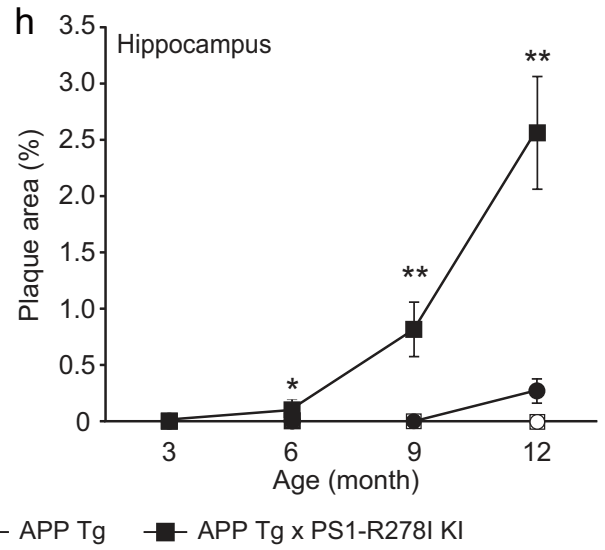
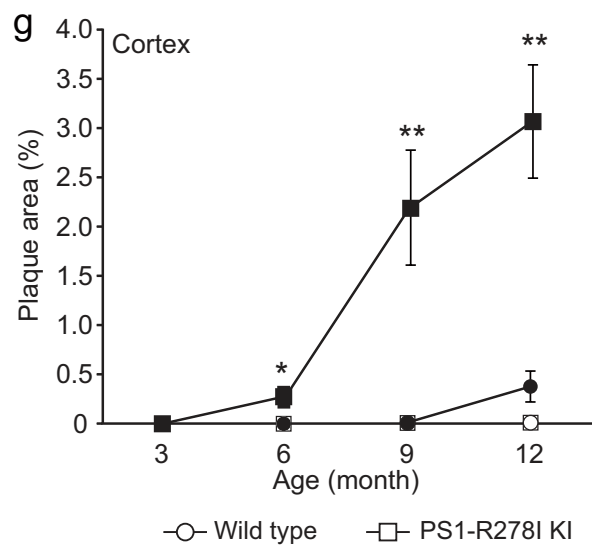
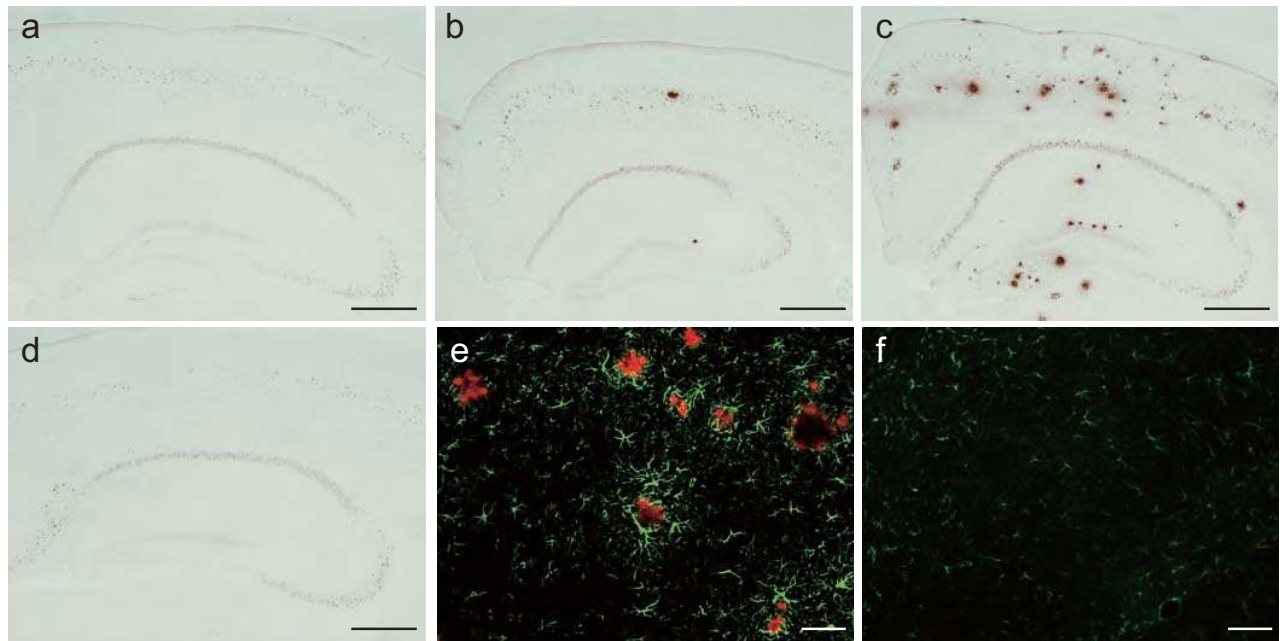
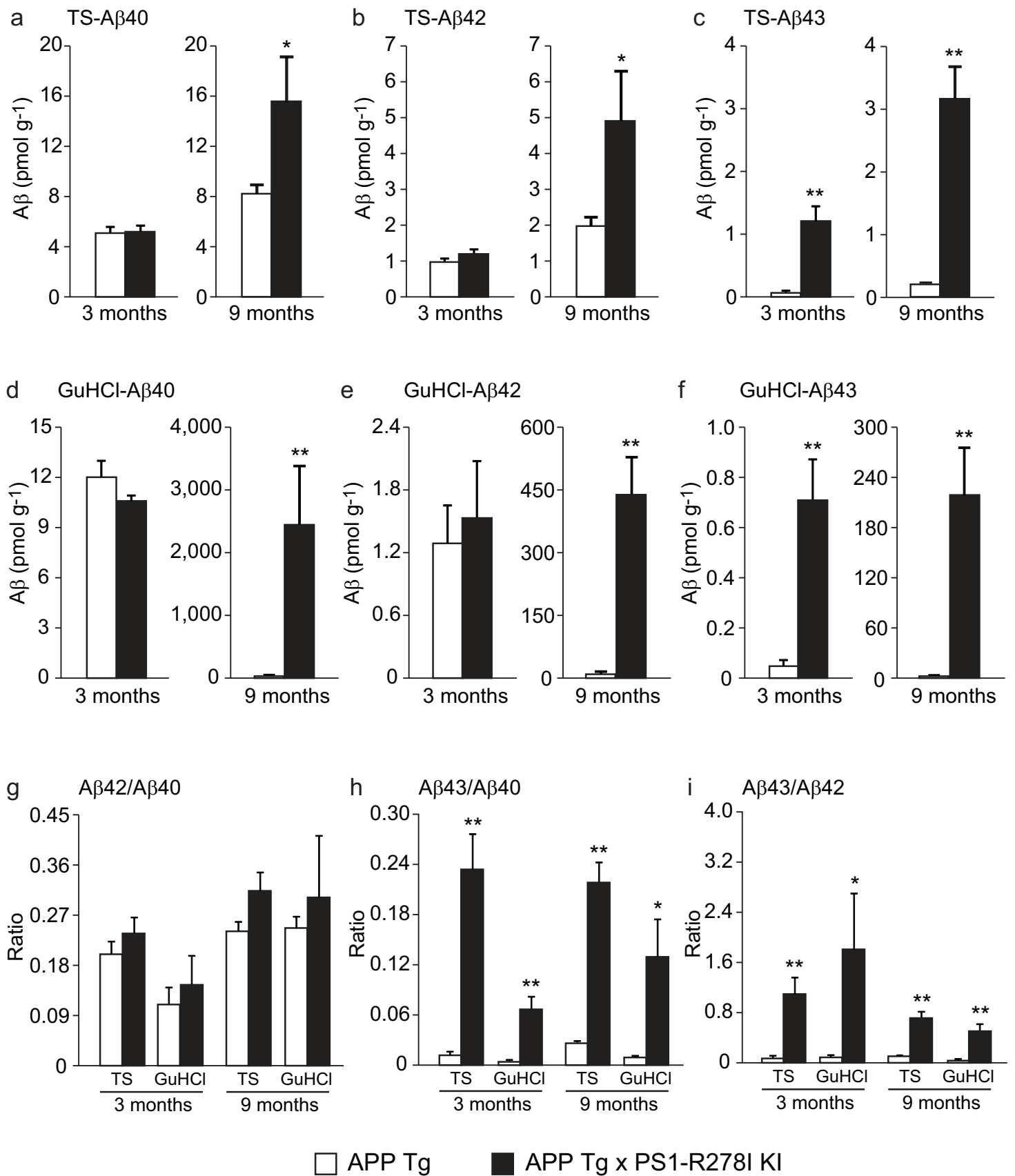


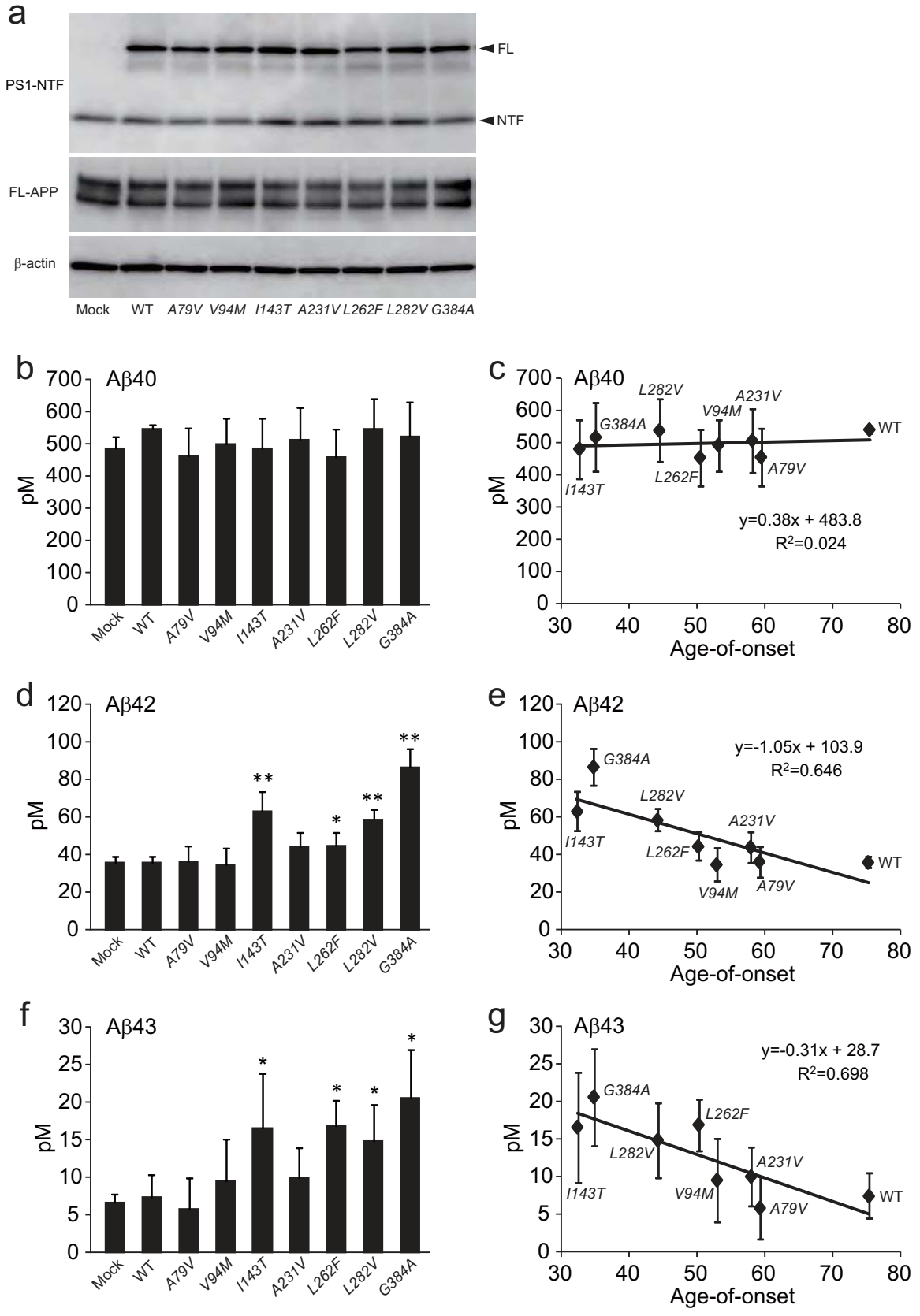
Fig. 3



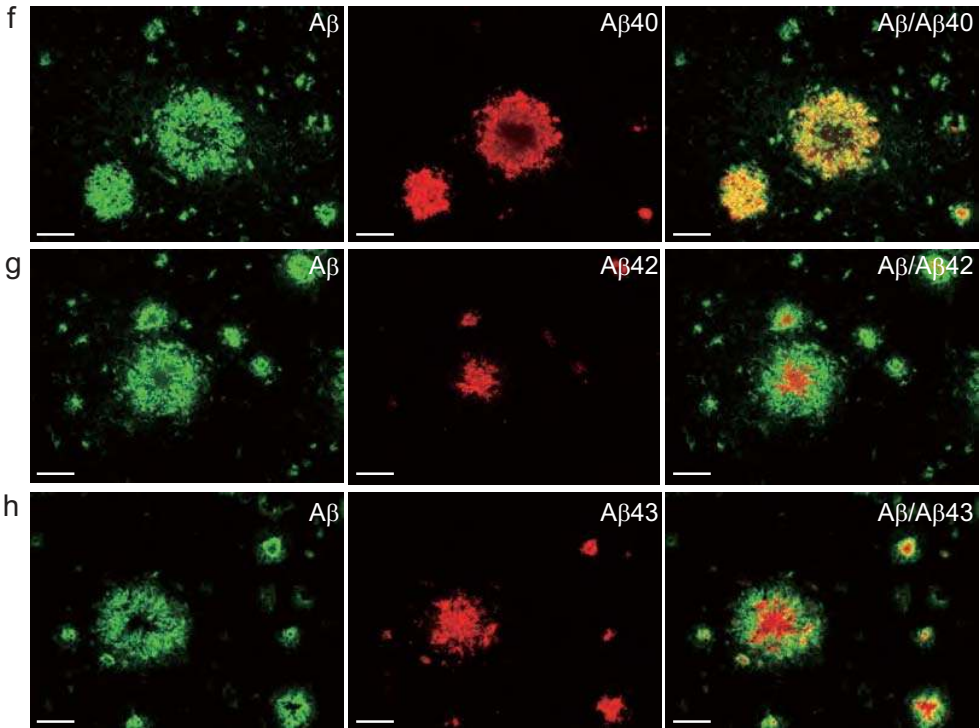
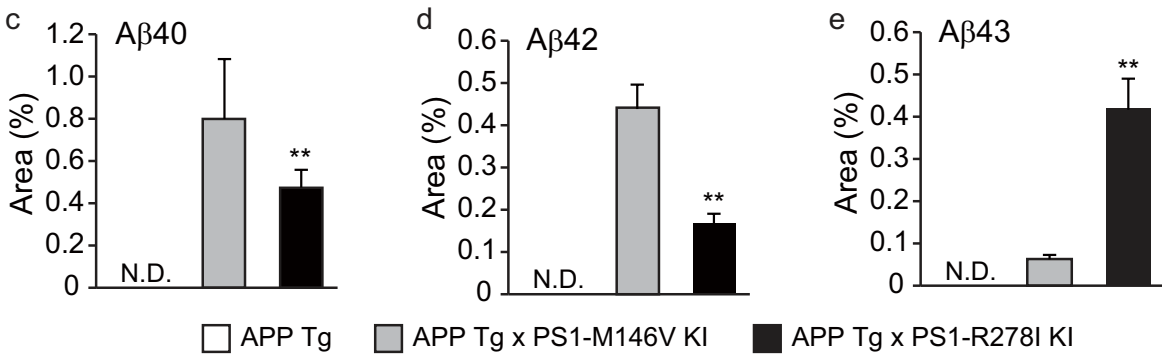
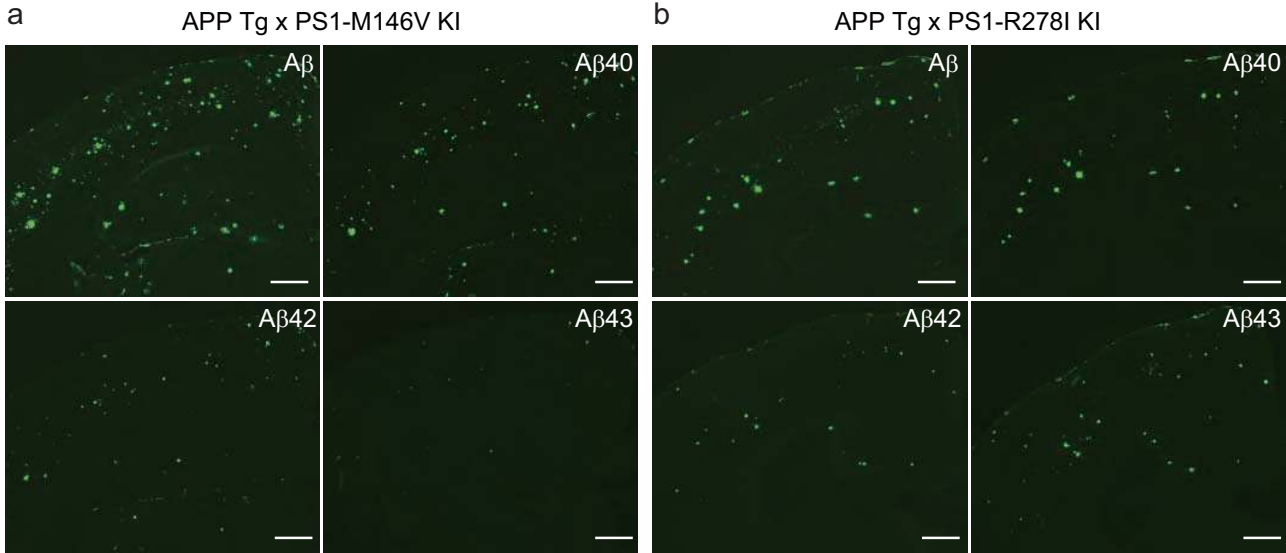
Saido T.C.: Fig. 4

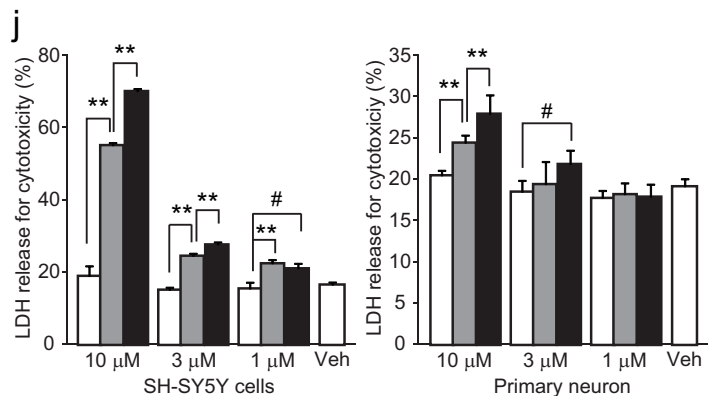
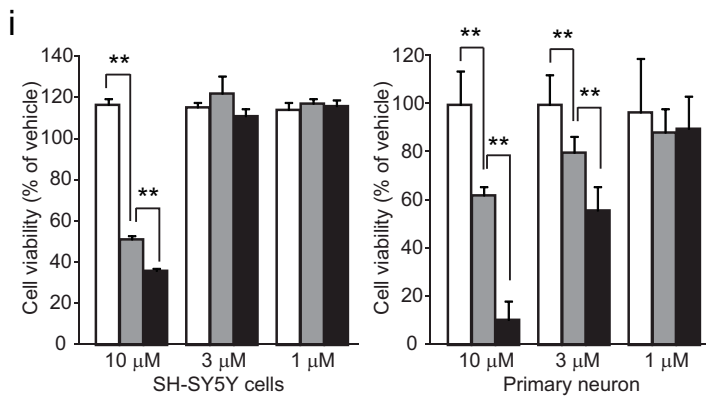
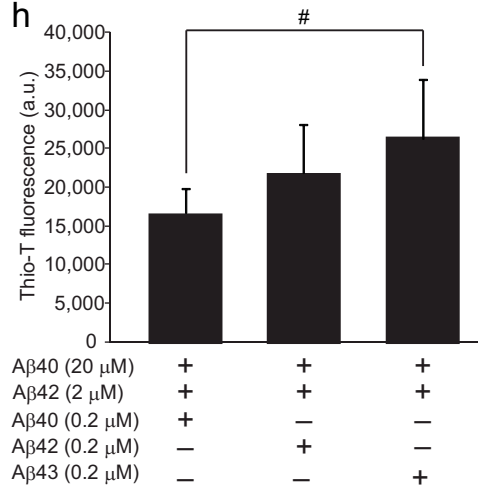
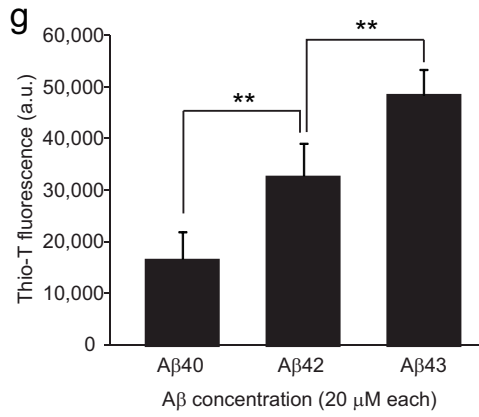
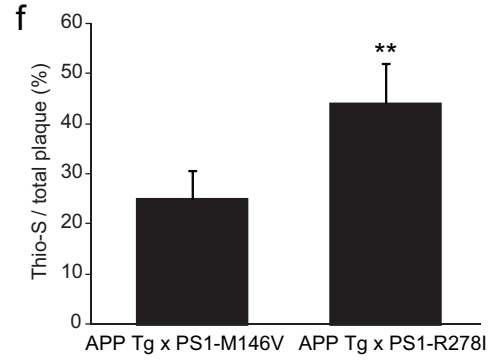
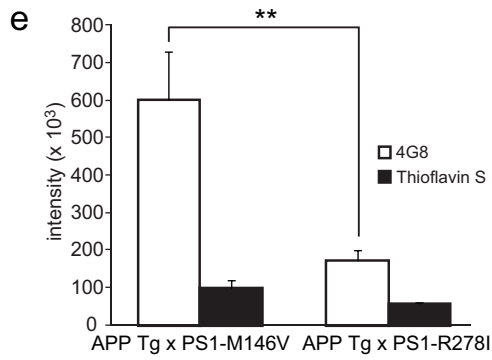
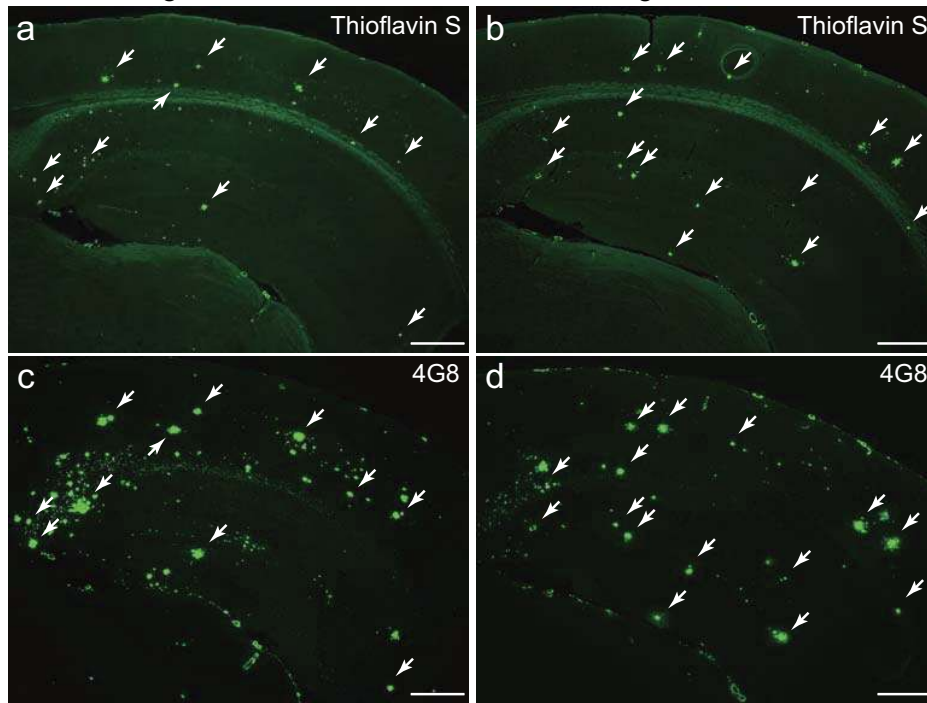


Saido T.C.: Fig. 5



Saido T.C.: Fig. 6





Saido T.C.: Fig. 8

