Alterations in endocytic protein expression with increasing age in the transgenic APP_{695} V717I London mouse model of amyloid pathology – implications for Alzheimer's disease.

Running head: Altered endocytic protein expression with age

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Conflicts of interest

None of the authors have any conflicts of interest.

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Authors' contributions

EJK, RST, MHE and MG conceived the study and designed the experiments. MHE bred the mice. MA and JSB performed all the experiments. MA analysed the data and EJK assisted with data interpretation. EJK and MA wrote the manuscript. All authors read and commented on the text and approved the final version of the manuscript.

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Abstract

A major risk factor for the development of Alzheimer's disease is increasing age but the reason behind this association has not been identified. It is thought that the changes in endocytosis seen in Alzheimer's disease patients are causal for this condition. Thus we hypothesised that the increased risk of developing Alzheimer's disease associated with ageing may be due to changes in endocytosis. We investigated using Western blotting whether the expression of endocytic proteins involved in clathrin-mediated and clathrin-independent endocytosis are altered by increasing age in a mouse model of amyloid pathology. We used mice transgenic for human amyloid precursor protein containing the V717I London mutation. We compared London mutation mice with age-matched wild-type controls at three ages, 3, 9 and 18 months, representing different stages in the development of pathology in this model. Having verified that the London mutation mice over-expressed amyloid precursor protein and β-amyloid, we found that the expression of the smallest isoform of PICALM, a key protein involved in the regulation of clathrin-coated pit formation, was significantly increased in wild-type mice but decreased in London mutation mice with age. PICALM levels in wild-type 18-month mice and clathrin levels in wild-type 9-month mice were significantly higher than those in London mutation mice of the same ages. The expression of caveolin-1, involved in clathrin-independent endocytosis, was significantly increased with age in all mice. Our results suggest that endocytic processes could be altered by the ageing process and such changes could partly explain the association between ageing and Alzheimer's disease.

Keywords

Alzheimer's disease; β-amyloid; amyloid precursor protein; caveolin; clathrin-independent endocytosis; clathrin-mediated endocytosis; endocytosis; London mutation; mouse model; PICALM; V717I

Introduction

Dementia currently affects about 47.5 million people worldwide [1] with Alzheimer's disease (AD) being the most common cause [1,2]. The major risk factor for developing AD is increasing age, with about 11% of people aged 65 and over having the disease, rising to 32% over the age of 85 [2]. It is estimated that by 2050 there will be over 115 million people in the world with AD [3]. However, few studies have considered how ageing may contribute to the aetiology of the disease.

Alterations in endocytosis represent a potential mechanism which could underlie the association of AD with age. Changes in endocytosis were first identified in cases of AD twenty years ago [4]. Since 2009, a number of Genome Wide Association Studies have described polymorphisms in genes associated with a small increased risk of developing AD and at least three of the proteins encoded by these genes, *PICALM, BIN1* and *SORL1*, are involved in endocytosis [5], emphasising the importance of this pathway in AD. Amyloid precursor protein (APP), the source of β–amyloid (Aβ), a key protein involved in the pathology of AD, is transported via the secretory pathway to the cell surface and is then internalised by endocytosis. Most amyloidogenic processing occurs only after this event, within the endocytic/lysosomal system [6,7]. Endocytosis is thus central to the production of Aβ.

Clathrin-mediated endocytosis (CME) involves many different proteins including the scission protein dynamin and regulatory accessory or adaptor proteins such

as AP180, PICALM, amphiphysin I and BIN1 [8]. Changes in CME have been seen in AD and associated models of amyloid pathology. Early endocytic changes, as evidenced by an increase in the number and size of Rab5-positive endosomes, are present in Down Syndrome and AD brains [9]. Importantly, inhibition of both CME in vivo in APP transgenic mice and dynamin-dependent endocytosis in vitro lowered Aβ levels [10,11], while up-regulation of endocytosis increased APP metabolism to sAPPβ and βCTF and increased Aβ secretion [7].

Lipid rafts in the plasma membrane are also important for modulating Aβ production [12,13]. Caveolae are a type of lipid raft enriched with caveolins-1-3 and are associated with processes including clathrin-independent endocytosis (CIE) [14]. Flotillin-1 and -2, also found in lipid rafts, non-caveolar lipid raft microdomains in neurones that may also be implicated in endocytosis [15,16]. Alterations in caveolins have been associated with AD as the expression of caveolin-1 is elevated in the hippocampus in AD compared to non-AD brains [17].

We have previously considered the importance of changes in endocytosis with ageing for AD pathophysiology by examining the expression of several CME and CIE-related endocytic proteins in the cortex of aged transgenic (Tg) Tg2576 mice expressing the Swedish mutation of human APP at the β-secretase cleavage site [18]. Using 22 month-old male mice, we found significantly higher levels of clathrin heavy chain (CHC), dynamin II and PICALM compared to wild-

type (WT) mice but no changes in proteins involved in CIE [18]. However, we did not compare different ages. Therefore we have now investigated how ageing and the presence of amyloid pathology affect the expression of a range of proteins involved in CME and CIE. We have used the London V717I mouse, a well-characterised model of amyloid pathology that overexpresses human APP_{695} with a mutation at V717I [19]. In contrast to our earlier data from the Tg2576 mouse [18], we did not see any changes in clathrin expression with age or between genotypes but we did identify an increase in the expression of caveolin-1 with age in WT and Tg mice. Furthermore, interestingly we saw an increase in the expression of an isoform of PICALM in WT mice but a decrease in Tg mice with age.

Methods

Materials

All chemicals and reagents were purchased from Sigma-Aldrich, Poole, U.K. or Fisher Scientific, Leicester, U.K unless specified. Antibodies used in Western blotting were: anti N-APP, 22C11 (Millipore, Watford, U.K.); anti-clathrin heavy chain (CHC, Clone 23), anti-caveolin-1, anti-caveolin-2 (Clone 65), anti-flotillin-1 (Clone 18), (BD Biosciences, Oxford, UK); anti-GAPDH (Sigma-Aldrich); anti-BIN1 (Santa Cruz, Wembley, U.K.); anti-PICALM, anti-flotillin-2 (Novus Biologicals, Littleton, CO, USA); anti-dynamin-1 (Abcam, Cambridge, MA).

Mice

Tg mice carrying the London V717I mutation in human APP [19] were maintained on the in-bred C57Bl/6 background. All work described here complied with the guidelines for the care and use of laboratory animals according to the Animals (Scientific Procedures) Act 1986 and in accordance with Home Office (U.K.) regulations and European Union directive 2010/63/EU.

Protein Extraction

Soluble and insoluble proteins were extracted from the total cortices of male 3, 9 and 18-month old London Tg mice and WT aged-matched littermates following the method of Rees et al. [18,20]. Total protein concentration was determined with the BCA Protein Assay Kit (Thermo Scientific, Waltham, USA).

Western Blotting

Western Blotting was performed using standard methods. Briefly, after protein analysis, 10µg of all samples were resolved on 10% polyacrylamide gels, and detected with the relevant antibody as previously described [18].

ELISA

Soluble and insoluble human Aβ40 and 42 were detected by ELISA as previously described [18].

Statistical analyses

The Western blot shown for each protein was quantified using Image J (www.imagej.nih.gov). All protein bands were expressed as the relative density of WT cortex sample 1 and then normalised for relative GAPDH levels. ELISA data were expressed as ng Aβ/mg total protein. The blotting data were analysed by one-way ANOVA followed by Tukey's post-hoc tests or by unpaired Student's t-tests to determine if protein levels differed significantly between ages or between Tg and age-matched WT mice, respectively. ELISA data were analysed using Kruskal-Wallis followed by Dunn's multiple comparisons test (soluble Aβ) or one-way ANOVA followed by Fisher's LSD test (insoluble Aβ40). Where necessary, data were transformed to fit the assumptions of normality.

Results

Expression of APP, Aβ40 and Aβ42 in the Tg and WT mice

As expected the expression of APP was significantly increased by about 3-fold in the cortex of 18-month Tg mice compared to WT mice ($p<0.05$) (Fig. 1A). The levels of APP were not altered by ageing in either WT or Tg mice (see Figure Supplemental Digital Content 1). Soluble and insoluble Aβ40 and soluble Aβ42 levels from the overexpressed human APP were all significantly increased in 18-month Tg mice compared to younger mice ($p<0.05$) (Fig. 2A, B).

Levels of proteins involved in CME are altered by ageing and APP genotype

No significant differences in the levels of clathrin heavy chain (CHC) with increasing age were detected in either WT or Tg mice (Fig. 1B,C). In contrast,

when the level of clathrin was compared between WT and Tg mice brains, a significant decrease was seen in 9-month Tg mice, 0.3 ± 0.02 compared to 0.8 ± 0.1 in WT mice ($p < 0.001$, OD ratio relative to GAPDH). There were no significant changes in the levels of clathrin between WT and Tg mice aged 3 and 18 months (see Table Supplemental Digital Content 2).

The levels of dynamin I were not significantly altered by ageing in either WT or Tg mice (see Figure Supplemental Digital Content 1). Furthermore, no changes in dynamin were observed between WT and Tg mice of the same age (see Table Supplemental Digital Content 2).

At least 6 isoforms of *Mus musculus* PICALM have been found with predicted molecular masses ranging from approximately 64 to 72 kDa (NCBI RefSeq). We identified PICALM as 3 distinct bands at 72, 68, and 62 kDa (Fig. 1D-F). The largest bands (bands 1 and 2) were analysed together as they were not fully resolved. There was no detectable change in the levels of bands 1 and 2 with ageing in either WT or Tg mice (Fig. 1E,F). There were also no changes in the expression of bands 1 and 2 between WT and Tg mice aged 3, 9 and 18 months (Fig. 1D, see Table Supplemental Digital Content 2). However, expression of the smallest band of PICALM (band 3) was significantly increased in 18-month WT mice compared to 9-month mice (Fig. 1E) but was significantly decreased in 18-month Tg mice compared to 3-month mice (Fig. 1F). Furthermore, the levels of band 3 were significantly reduced by approximately 6 times in 18-month Tg mice when compared to WT mice (Fig. 1D). In contrast,

there were no changes in the expression of band 3 between WT and Tg mice aged 3 and 9 months (see Table Supplemental Digital Content 2).

At least 15 different isoforms of bridging integrator 1 (Bin-1) have been identified. Here, two bands were observed for Bin-1 (see Figure Supplemental Digital Content 1). The levels of Bin-1 were not significantly altered in Tg mice compared to WT mice at any age point (see Table Supplemental Digital Content 2). Similarly, no significant changes were detected in the levels of Bin-1 with ageing in either WT or Tg mice (see Figure Supplemental Digital Content 1).

Levels of proteins involved in CIE are altered by ageing and genotype

The levels of caveolin-1 were significantly higher in both 9- and -18 month WT and Tg mice compared to 3-month mice (Fig. 2A,B). However, the levels of caveolin-1 were not altered between 3- and 9-month old WT and Tg mice (see Table Supplemental Digital Content 2) but were significantly decreased by approximately 1.4 times in 18-month Tg compared to WT mice (Fig. 2C). Caveolin-2 expression in WT and Tg mice was not altered by age or genotype (see Figure and Table Supplemental Digital Content 1 and 2). Neither flotillin-1 nor flotillin-2 in WT and Tg mice were affected by age or genotype (see Figure and Table Supplemental Digital Content 1 and 2).

Discussion

The results presented here show that both ageing and genotype affected the expression of endocytic proteins in the cortex of WT and Tg V717I London

mutation mice. The data obtained for APP expression confirmed firstly that the Tg London mutation mice over-expressed APP compared to the WT mice and secondly showed no change in APP expression with age, as expected from other studies [21]. This overexpression of APP led to the expected increase in human Aβ40 and 42 in the 18-month Tg mice.

Interestingly, although there is much evidence, reviewed above, to show that CME is affected in AD and implicated in the pathogenesis of the disease, we saw limited changes in clathrin itself with a decrease only in 9-month Tg mice compared to WT mice. We know that cognitive deficits start to appear around 6-9 months of age in these mice which could be linked to the change in clathrin seen here (unpublished data). Interestingly, the decrease in clathrin precedes the increase in Aβ in these mice so a small change in endocytosis in earlier life might be linked to the subsequent rise in Aβ levels in these mice. However, in the cortex of the London mutation mice neither age nor the presence of amyloid pathology appeared to have a large effect on CME as determined by the expression levels of clathrin. Support is provided for this conclusion by the data for dynamin-1, PICALM bands 1 and 2 and Bin-1 where no changes in expression were seen. This is particularly significant for dynamin-1, crucial for CME to occur but also for many forms of CIE. The data for band 3 for PICALM, however, do not fit with this conclusion as its expression was differentially affected by both age and genotype. Currently, the function of the different PICALM isoforms is not understood and we have previously shown that at least human isoforms 1 and 2 are required for PICALM to affect functional

endocytosis in the H4 cell line [22]. The most likely explanation for these findings lies in another role of PICALM, in addition to its involvement in CME. More specifically, PICALM controls the endocytosis of R-SNAREs (Soluble NSF Attachment Protein Receptors) necessary for the fusion of endocytic vesicles with endosomes or the plasma membrane [23].

There does appear to be some involvement of ageing and genotype in CIE as the expression of caveolin-1 was increased in both genotypes with age but to a larger extent in the WT mice. Furthermore, this increase in caveolin-1 preceded the increase in Aβ40 and 42 in the Tg mice supporting the data with clathrin and possibly suggesting that changes in endocytosis could be linked to subsequent increases in Aβ accumulation. Another study also found an increase in caveolin-1 with age in WT mice [17]. This is an interesting result as loss of caveolin-1 is associated with accelerated aging and neurodegeneration in mice [24]. These data support those for caveolin-1 expression in the human brain where higher expression was seen in the hippocampus from AD brains compared to non-AD brains [17]. We also detected a significant rise in caveolin-1 expression in human AD frontal cortex compared to age-matched controls (unpublished data). Another study found no changes in caveolin-1 in human cortex comparing AD and control individuals [25] but the effect of age was not considered. The other CIE proteins examined here, caveolin-2, flotillin-1 and -2 were not affected by ageing or genotype suggesting that any effect of these factors on CIE in mice is not wide-spread.

The results we have obtained here for various endocytic proteins in the V717I London mutation mice contrast with those we obtained for the same proteins in old Tg2576 mice [18]. One possible explanation for these differences lies in the different mutations affecting the β-secretase (Tg2575) and γ-secretase (V717I) cleavage sites in APP leading to higher levels of APP and 10 to over 1400-fold increases in Aβ in the Tg2576 mice [18]. In addition, the different genetic backgrounds of the two mice strains probably also affect other proteins and biochemical pathways such as those involved in endocytosis.

In conclusion, we have shown that proteins involved in both CME and CIE are affected by ageing and also by the presence of amyloid pathology in mice. Our data provide support for the idea that changes in endocytosis are involved in the pathogenesis of AD.

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Figure 1

1

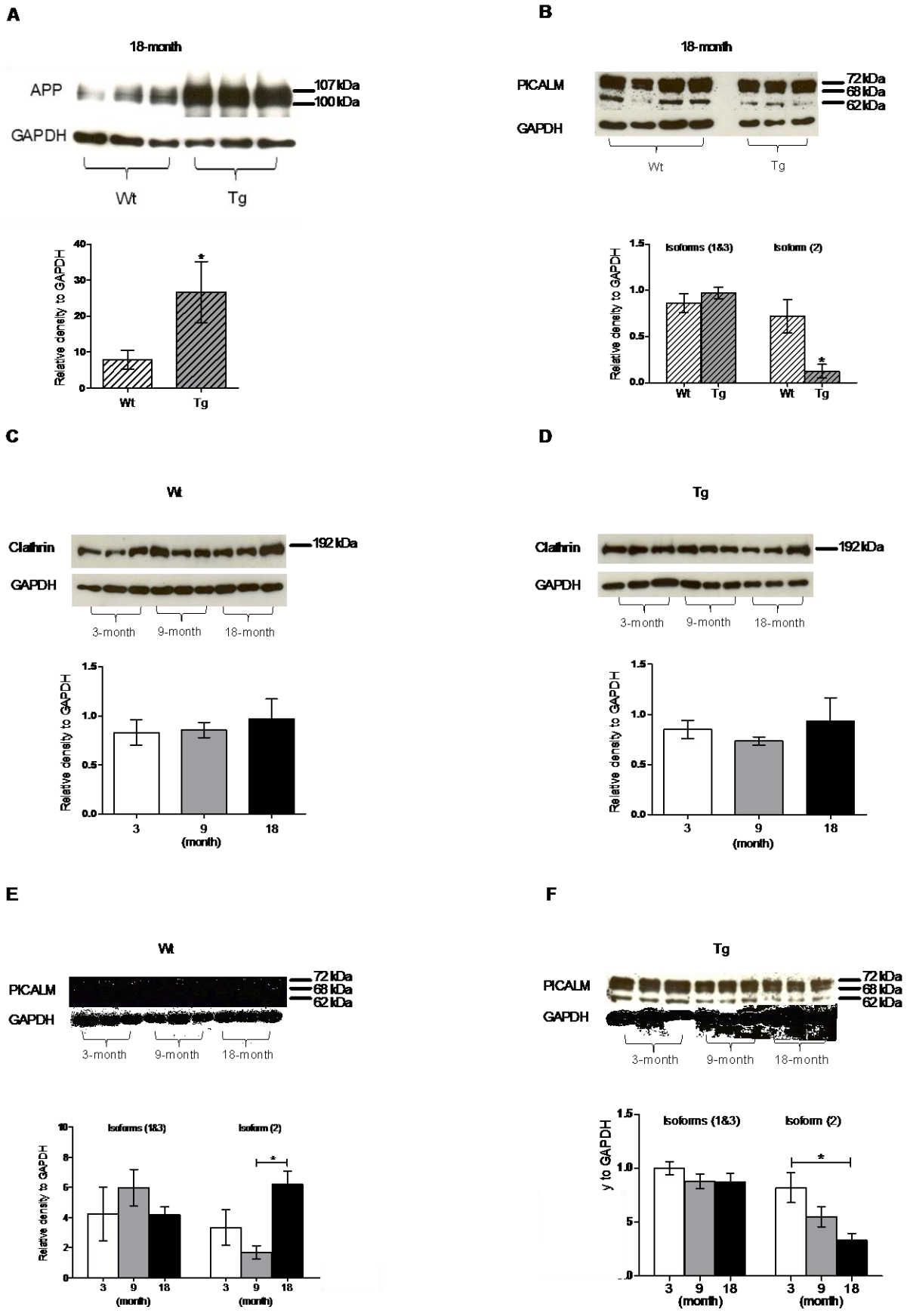
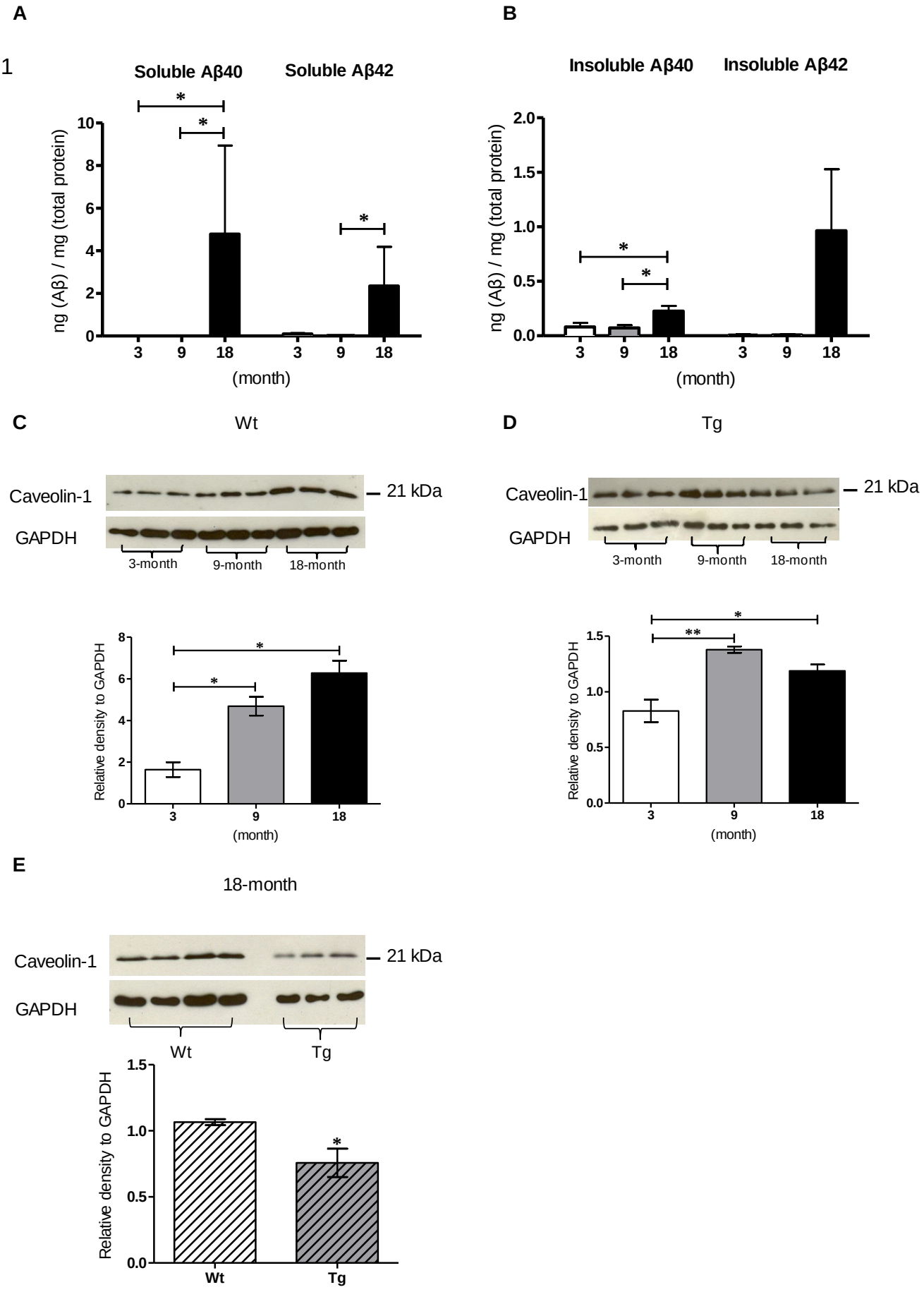

A
18-month
APP
GAPDH
107 kDa
100 kDa
Wt
Tg
Relative density to GAPDH
B
18-month
PICALM
GAPDH
72 kDa
68 kDa
62 kDa
Wt
Tg
Isoforms (1&3)
Isoform (2)
C
Wt
Clathrin
GAPDH
192 kDa
3-month
9-month
18-month
(month)
D
Tg
E
F

Figure 1. Comparison of APP, clathrin heavy chain, and 3 bands for PICALM in the cortex of male WT and Tg mice aged 3, 9 and 18 months. Each section shows an immunoblot and densitometric analysis of the immunoblot. (A) Comparison of APP expression in 18 month WT and Tg mice; comparison of clathrin expression in (B) WT mice and (C) Tg mice aged 3, 9 and 18 months; comparison of PICALM bands 1&2 and 3 between (D) 18 month WT and Tg mice and in (E) WT mice and (F) Tg mice aged 3, 9 and 18 months. Levels of APP were significantly increased in 18 month Tg mice compared to WT mice. PICALM band 3 expression was significantly decreased in 18 month Tg mice compared to WT mice and in 18 month Tg mice compared to 3 and 9 month Tg mice but was increased in 18 month WT mice compared to 3 and 9 month WT mice. Data are represented as mean ± S.E.M. *$p < 0.05$, one-way ANOVA followed by Tukey's post-hoc tests or unpaired Student's t-tests. n=3-4 mice for each age group.

Figure 2


A
1
Soluble Aβ40
Soluble Aβ42
ng (Aβ) / mg (total protein)
(month)
B
Insoluble Aβ40
Insoluble Aβ42
ng (Aβ) / mg (total protein)
(month)
C
Wt
Caveolin-1
21 kDa
GAPDH
3-month
9-month
18-month
Relative density to GAPDH
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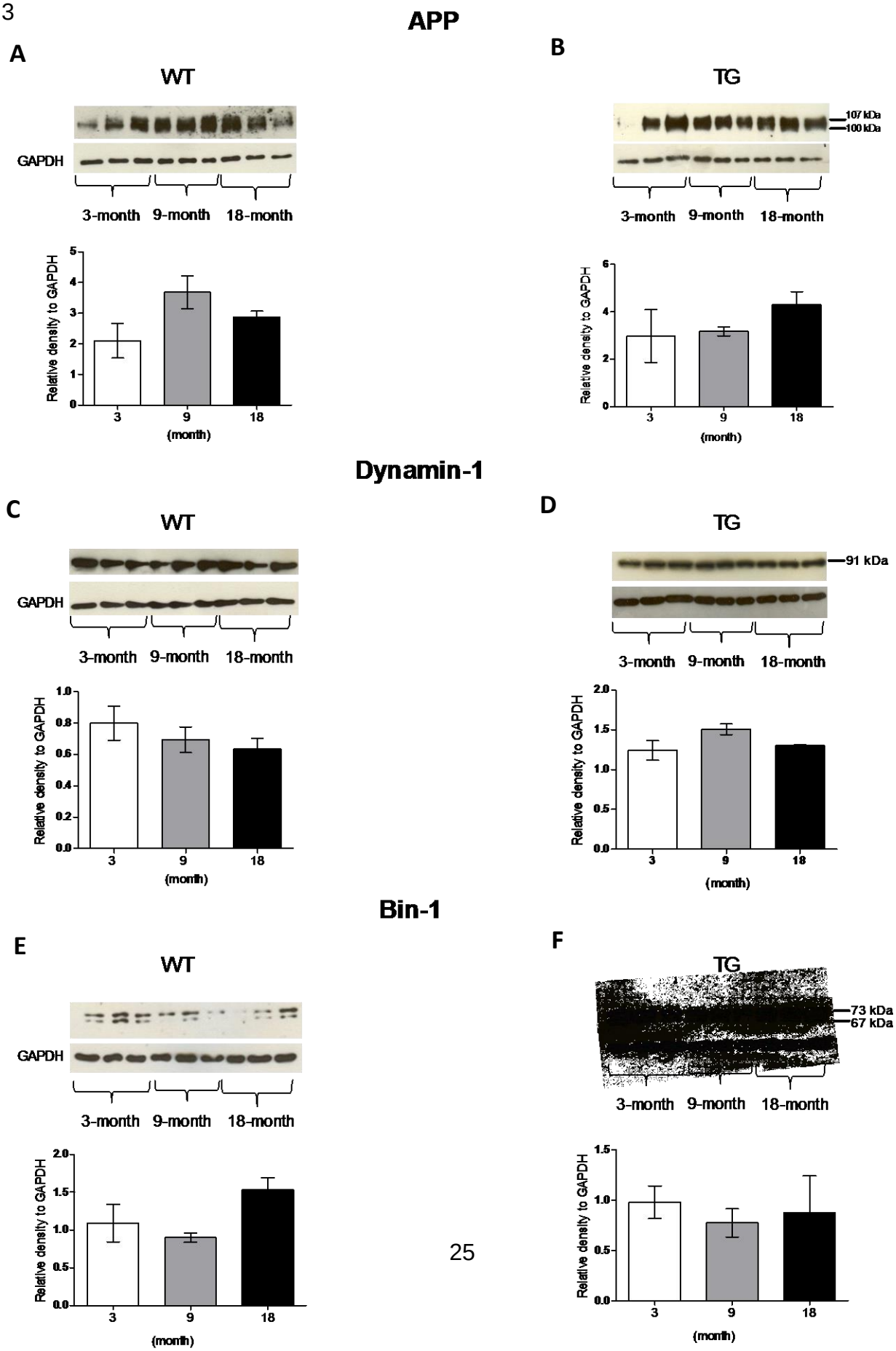
Wt
Tg
Relative density to GAPDH
Wt
Tg

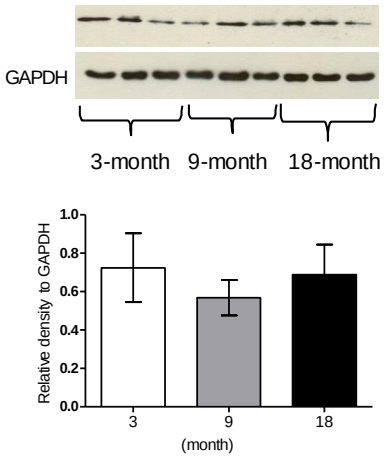
Figure 2. Comparison of Aβ40, Aβ42 and caveolin-1 in the cortex of male WT and Tg mice aged 3, 9 and 18 months. The caveolin-1 sections each show an immunoblot and densitometric analysis of the immunoblot. Comparison of soluble (A) and insoluble (B) Aβ40 and 42 in Tg mice aged 3, 9 and 18 months; comparison of caveolin-1 expression in (C) WT mice and (D) Tg mice aged 3, 9 and 18 months; (E) comparison of caveolin-1 expression in 18 month WT and Tg mice; Soluble and insoluble Aβ40 was significantly increased in 18 month Tg mice compared to 3 and 9 month mice while soluble Aβ42 was significantly increased in 18 month Tg mice compared to 9 month mice. Levels of caveolin-1 were significantly increased in 9 and 18 month WT and Tg mice compared to the corresponding 3 month mice. Caveolin-1 expression was significantly decreased in 18 month Tg mice compared to WT mice. Data are represented as mean ± S.E.M. *$p < 0.05$, **$p<0.01$, ELISAs Kruskal-Wallis followed by Dunn's multiple comparisons test (soluble Aβ) or one-way ANOVA followed by Fisher's LSD test (insoluble Aβ40); Western blots one-way ANOVA followed by Tukey's post-hoc tests or unpaired Student's t-tests. n=3-4 mice for each age group.

Supplemental Digital Content 1



Caveolin-2

G WT



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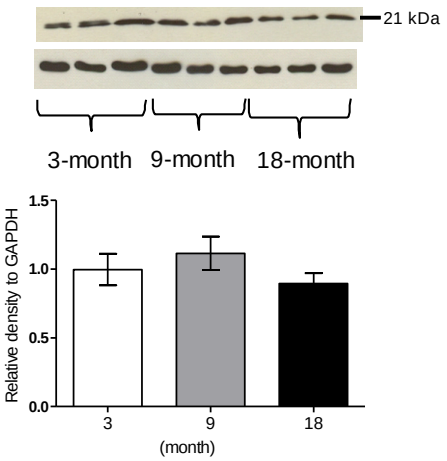

Flotillin-1

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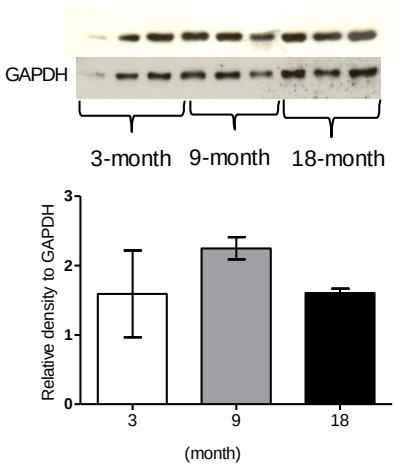

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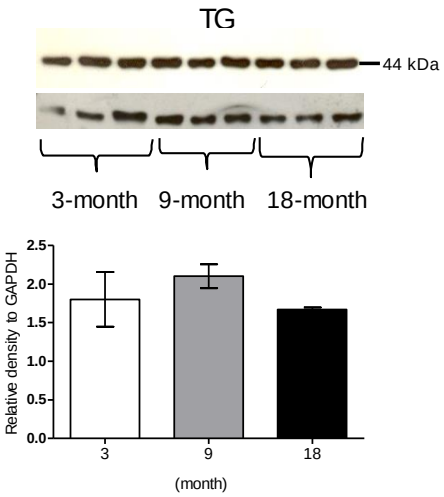
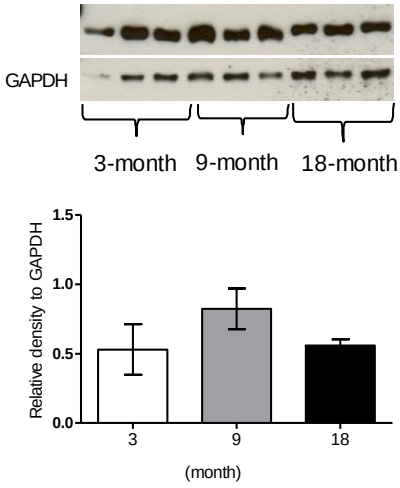
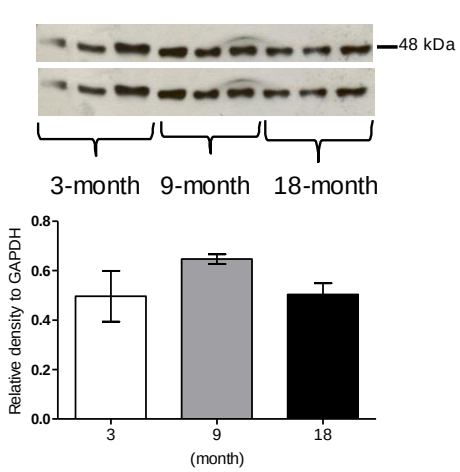


Flotillin-2

K WT



L TG



Supplementary Figure 1 Comparison of the expression of several proteins in the cortex of male WT and Tg mice aged 3, 9 and 18 months. Each section shows an immunoblot and densitometric analysis of the immunoblot. Comparison of APP expression in (A) WT mice and (B) Tg mice; comparison of Dynamin-1 expression in (C) WT mice and (D) Tg mice; comparison of Bin-1 expression in (E) WT mice and (F) Tg mice; comparison of Caveolin-2 expression in (G) WT mice and (H) Tg mice; comparison of Flotillin-1 expression in (I) WT mice and (J) Tg mice; comparison of Flotillin-2 expression in (K) WT mice and (L) Tg mice. There were no significant differences between any groups for all proteins. n=3 mice for each age group.

Supplemental Digital content 2

Table showing the comparison of expression levels of endocytic proteins between WT and Tg London V717I mice

Protein/Age	WT mice[1]	Tg mice[1]
	OD ratio relative to GAPDH	
Clathrin, 3 month	0.7 ± 0.1	0.7 ± 0.1
Clathrin, 9 month	0.8 ± 0.1*	0.3 ± 0.1
Clathrin, 18 month	1.3 ± 0.2	1.9 ± 0.5
Dynamin 1, 3 month	1.1 ± 0.03	0.9 ± 0.1
Dynamin 1, 9 month	1.1 ± 0.02	0.9 ± 0.1
Dynamin 1, 18 month	1.1 ± 0.1	0.9 ± 0.1
PICALM bands 1&2, 3 month	0.8 ± 0.1	0.8 ± 0.1
PICALM band 3, 3 month	0.8 ± 0.1	0.7 ± 0.04
PICALM bands 1&2, 9 month	0.8 ± 0.1	0.9 ± 0.1
PICALM band 3, 9 month	0.8 ± 0.2	0.7 ± 0.1
Bin-1, 3 month	0.8 ± 0.1	0.7 ± 0.2
Bin-1, 9 month	2.3 ± 0.5	2.5 ± 0.5
Bin-1, 18 month	1.1 ± 0.2	0.9 ± 0.2
Caveolin-1, 3 month	1.1 ± 0.1	0.9 ± 0.3
Caveolin-1, 9 month	1.0 ± 0.2	0.9 ± 0.1
Caveolin-2, 3 month	0.9 ± 0.03	0.8 ± 0.1
Caveolin-2, 9 month	0.9 ± 0.04	0.9 ± 0.1
Caveolin-2, 18 month	1.0 ± 0.1	1.1 ± 0.1
Flotillin-1, 3 month	1.2 ± 0.1	1.2 ± 0.1
Flotillin-1, 9 month	1.6 ± 0.3	1.5 ± 0.1
Flotillin-1, 18 month	0.9 ± 0.1	0.8 ± 0.1
Flotillin-2, 3 month	1.0 ± 0.04	1.1 ± 0.2
Flotillin-2, 9 month	1.1 ± 0.1	0.9 ± 0.1
Flotillin-2, 18 month	0.9 ± 0.2	0.9 ± 0.1

[1] * p,0.001, clathrin expression was significantly higher in 9 month WT mice compared to Tg mice analysed with an unpaired Student's t-test. There were no other significant differences between WT and Tg mice for any of the proteins at the different ages.

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