

## ORIGINAL ARTICLE

# Lysosomal dysfunction in the brain of a mouse model with intraneuronal accumulation of carboxyl terminal fragments of the amyloid precursor protein

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Recent data suggest that intraneuronal accumulation of metabolites of the amyloid- $\beta$ -precursor protein (APP) is neurotoxic. We observed that transgenic mice overexpressing in neurons a human APP gene harboring the APP<sup>E693Q</sup> (Dutch) mutation have intraneuronal lysosomal accumulation of APP carboxylterminal fragments (APP-CTFs) and oligomeric amyloid  $\beta$  (oA $\beta$ ) but no histological evidence of amyloid deposition. Morphometric quantification using the lysosomal marker protein 2 (LAMP-2) immunolabeling showed higher neuronal lysosomal counts in brain of 12-months-old APP<sup>E693Q</sup> as compared with age-matched non-transgenic littermates, and western blots showed increased lysosomal proteins including LAMP-2, cathepsin D and LC3. At 24 months of age, these mice also exhibited an accumulation of  $\alpha$ -synuclein in the brain, along with increased conversion of LC3-I to LC3-II, an autophagosomal/autolysosomal marker. In addition to lysosomal changes at 12 months of age, these mice developed cholinergic neuronal loss in the basal forebrain, GABAergic neuronal loss in the cortex, hippocampus and basal forebrain and gliosis and microgliosis in the hippocampus. These findings suggest a role for the intraneuronal accumulation of oA $\beta$  and APP-CTFs and resultant lysosomal pathology at early stages of Alzheimer's disease-related pathology.

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## INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that typically begins with mild short-term memory deficits and culminates in total loss of cognition and executive functions. The neuropathological features of AD include brain parenchymal senile plaques, cerebral amyloid angiopathy and neurofibrillary tangles.<sup>1</sup> Hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D), a familial form of AD,<sup>2,3</sup> is caused by an E693Q substitution in the amyloid- $\beta$ -precursor protein (APP).<sup>4</sup> The disease is characterized by cerebral amyloid angiopathy leading to hemorrhagic strokes,<sup>5,6</sup> as well as cerebral lesions caused by the hemorrhages,<sup>7</sup> non-fibrillar parenchymal deposits of amyloid- $\beta$  (A $\beta$ )<sup>6,8–10</sup> and dementia.<sup>11,12</sup> A $\beta$  harboring the Dutch substitution at position 22 has been associated with enhanced aggregation and oligomerization<sup>13,14</sup> and reduced clearance as compared with wild-type A $\beta$ .<sup>15,16</sup>

Two mouse lines overexpressing the E693Q-substituted human APP under the neuron-specific Thy1.1 expression cassette were generated.<sup>16,17</sup> Similar to the brains of HCHWA-D patients,<sup>18</sup> higher A $\beta$ 40 to A $\beta$ 42 ratio was observed in the brains of the APP<sup>E693Q</sup> transgenic mice as compared with non-transgenic littermates.<sup>16,17</sup> It was shown that neurons in the brain of 12-month-old APP<sup>E693Q</sup> mice contain vesicles immunopositive for antibodies that interact with full-length APP (fAPP), amyloid precursor protein (APP-CTFs) and A $\beta$ .<sup>17</sup> A $\beta$  has been shown to exist in different forms, including soluble A $\beta$  monomers and oligomers, as well as insoluble amyloid fibrils and multiple studies

have documented the correlation between A $\beta$  pathology and cognitive decline in AD.<sup>19–21</sup> Synaptic dysfunction and neuronal degeneration have been demonstrated in the brains lacking amyloid plaques and cerebral amyloid angiopathy during the early stages of AD, and several lines of evidence have suggested that soluble oligomers of A $\beta$ , but not monomers, cause the synaptic dysfunction and neurodegeneration in the brains of AD patients and animal models.<sup>19–21</sup> In addition to A $\beta$ , APP-CTFs have been shown to exhibit cytotoxicity in the absence of amyloid deposition in the brain.<sup>22–38</sup> APP<sup>E693Q</sup> mice have behavioral deficits at ages when APP metabolites accumulate within neurons but no fibrillar amyloid deposits are detectable in either brain vasculature or parenchyma.<sup>17,39–41</sup>

Although the cellular events triggered by neurotoxic oA $\beta$  accumulation that lead to synaptic deficits may vary in different forms of AD, data suggest that neuronal endocytic-lysosomal alterations are either an underlying pathological mechanism in the disease or a maladaptive response of neurons to other disease stresses in AD.<sup>42–46</sup> Given that endosomal abnormalities were reported in cerebral endothelia in the brain of HCHWA-D patients<sup>43</sup> and that we have documented intraneuronal punctate accumulation of APP metabolites in cortical and hippocampal brain regions of APP<sup>E93Q</sup> transgenic mice,<sup>17</sup> we investigated the endosomal-lysosomal pathway in the brain of these APP<sup>E93Q</sup> transgenic mice. Here we demonstrate the intraneuronal lysosomal accumulation of APP-CTFs associated with intraneuronal

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lysosomal abnormalities and neuronal loss in the absence of fibrillar A $\beta$ .

## MATERIALS AND METHODS

### Animals

A breeding colony of APP<sup>E693Q</sup> mice<sup>17</sup> on C57BL/6J background was maintained at Nathan S. Kline Institute for Psychiatric Research. Both male and female APP<sup>E693Q</sup> transgenic mice and littermate controls were studied at 12 and 24 months of age. No gender differences between the genotypes were found. All animal procedures were performed following the National Institutes of Health guidelines with approval from the Institutional Animal Care and Use Committee at the Nathan S. Kline Institute for Psychiatric Research. All the experiments were performed with a sample size of 4–5 mice per genotype per age. The sample size was determined on the basis of findings from our previous APP<sup>E693Q</sup> transgenic mouse studies.<sup>17</sup>

### Thioflavin S staining and immunocytochemistry

For all histological procedures, identification cards were coded to render the experimenter blind to the sex and genotype of each subject. Mice were anesthetized with ketamine (50 g kg<sup>-1</sup>)/xylazine (5 mg kg<sup>-1</sup>) and transcardially perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer.<sup>47</sup> To detect fibrillar amyloid deposits, brain sections from 12- and 24-month-old mice were slide-mounted and immersed in a solution of filtered 1% thioflavin S in ddH<sub>2</sub>O for 10 min (Sigma Aldrich, St. Louis, MO, USA), rinsed ( $\times 3$ ) for 1 min in ddH<sub>2</sub>O and subsequently coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Immunolabeling was performed using 4G8 antibody (which recognizes  $\beta$ APP/APP-CTF/A $\beta$ ; 1:200; Covance ImmunoTechnologies, Denver, PA, USA; Cat # SIG-39220), C1/6.1 to the carboxyl-terminal cytoplasmic domain of APP (1:100; provided by Dr Paul M Mathews), 22C11 antibody (which recognizes full-length and soluble amino terminal fragment of APP, sAPP) (1:200; EMD Millipore, Billerica, MA, USA; Cat # MAB 348), anti-glial fibrillary acidic protein (GFAP) (1:500; Dako, Carpinteria, CA, USA; Cat # Z0334), anti-Iba1 (1:500, Wako Chemicals, Richmond, VA, USA; Cat # 019-1974), anti-rab5a (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat # SC-309), anti-cathepsin D (Cat D) (1:500; Scripps, San Diego, CA, USA; Cat # 90236), anti-LC3 (1:500; Novus Biologicals, Littleton, CO, USA; Cat # NB600-1384), anti-lysosomal marker protein 2 (LAMP-2) (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, USA; Cat # 93 S), and anti-ChAT antibody (1:500; Millipore; Cat # AB144P) antibodies. Double immunofluorescent labeling was performed to identify coincidence of localization of  $\beta$ APP/APP-CTFs/A $\beta$  using 4G8 (1:200) or  $\beta$ APP/APP-CTFs using antibody to the carboxyl-terminal cytoplasmic domain of APP using C1/6.1<sup>48</sup> (1:100) with antibody to the lysosomal marker LAMP-2 (1:100). Anti-parvalbumin (PV) (1:5000; Swant Technologies, Marly, Switzerland; Cat # PV25) staining was performed to detect GABAergic interneurons. Secondary antibodies used were: fluorescence-conjugated anti-rabbit, anti-sheep, and anti-mouse antibodies (Invitrogen, Carlsbad, CA, USA).

PV-positive GABAergic interneurons were quantified in the hippocampus of four individual brain sections per mouse, five mice per genotype, at the age of 12 months using ImageJ (NIH, Bethesda, MD, USA). For lysosomal quantification, approximately 30 neurons per mouse, four mice per genotype, were analyzed and LAMP-2 signal was measured using ImageJ randomly by a genotype-blinded observer.<sup>47</sup> LAMP-2 labeled brain sections were further stained with Nissl (Neurotrace) (1:100; Molecular Probes, Eugene, OR, USA) to quantify lysosomes in the large neurons of the medial septal nucleus (MSN), counting  $\sim 30$  neurons per mouse, four mice per genotype. LAMP-2 signal was measured using ImageJ. Qualitative analysis of GFAP- or Iba1-positive staining was performed on four individual brain sections per mouse, five mice per genotype, at the ages of 12 and 24 months.

### Optical fractionator procedure

The number of immunofluorescence-labeled ChAT cells in the MSN was estimated using the optical fractionator method,<sup>49</sup> utilizing ImageJ software as previously described.<sup>50</sup> Briefly, the MSN was sampled in every 3rd consecutive 40- $\mu$ m-thick vibratome section rostral to the anterior commissure. A grid of optical disector sampling sites with 0.25 mm spacing was placed on each section. At each sampling site, a  $\times 40$  oil-immersion objective with 1.3 numerical aperture was used to collect a z-stack of 15

3- $\mu$ m-spaced images starting at a focal plane just beneath the tissue surface. Cell counting was done on 198  $\times$  143  $\mu$ m counting boxes drawn onto each z-stack, using the top two slices as the upper guard zone, the next 10 slices as a 30  $\mu$ m deep counting box and the subsequent slices as the lower guard zone. Section thickness (40.5  $\pm$  1.7  $\mu$ m mean  $\pm$  s.d.,  $n = 8$  animals), measured in triplicate on each section, showed no evidence of shrinkage from the original 40  $\mu$ m section thickness. On average, 191  $\pm$  69 cells were counted per MSN, and the coefficient of error<sup>51</sup> was 0.08  $\pm$  0.01 (mean  $\pm$  s.d.).

### Western blot analysis

Mice were anesthetized with ketamine (50 g kg<sup>-1</sup>)/xylazine (5 mg kg<sup>-1</sup>) and brains were rapidly removed over ice. Brains of 12- ( $n = 4$ ) and 24- ( $n = 5$ ) month-old mice were snap frozen and stored at  $-80^\circ\text{C}$  before biochemical analyses. Entorhinal cortices were dissected out from fresh brains of 12- ( $n = 4$ ) and 24- ( $n = 4$ ) month-old mice and stored at  $-80^\circ\text{C}$ . Western blot immunolabeling was performed as previously described.<sup>52</sup> Primary antibodies used were: anti-LAMP-2 (1:1000), anti-rab4a (1:1000; Santa Cruz Biotechnology; Cat # SC-312), anti-rab5a (1:1000), anti-rab7 (1:1000; Sigma; Cat # R8779), anti-rabaptin5 (1:1000; Santa Cruz Biotechnology; Cat # SC-6162), anti-LC3 (1:1000), anti-Cat D (1:1000), C1/6.1 (1:1000),<sup>48</sup> anti-mTOR and anti-AKT (phosphorylated and total protein) (1:1000, Cell Signaling Technology, Danvers, MA, USA; Cat # 2983 S and Cat # 9272 S respectively), anti- $\alpha$ -synuclein (1:1000; Sigma; Cat # S3062) and anti- $\beta$ -tubulin (1:10 000; Sigma; Cat # T8535). Secondary antibodies used were: horseradish peroxidase-conjugated anti-rabbit and mouse antibodies (1:5000; GE Healthcare, Pittsburgh, PA, USA). The protein bands were scanned, optical density was calculated using the Image J, and the ratio of protein intensity to  $\beta$ -tubulin in the same lane was calculated. Western blot analyses were repeated three times.

### Statistical analyses

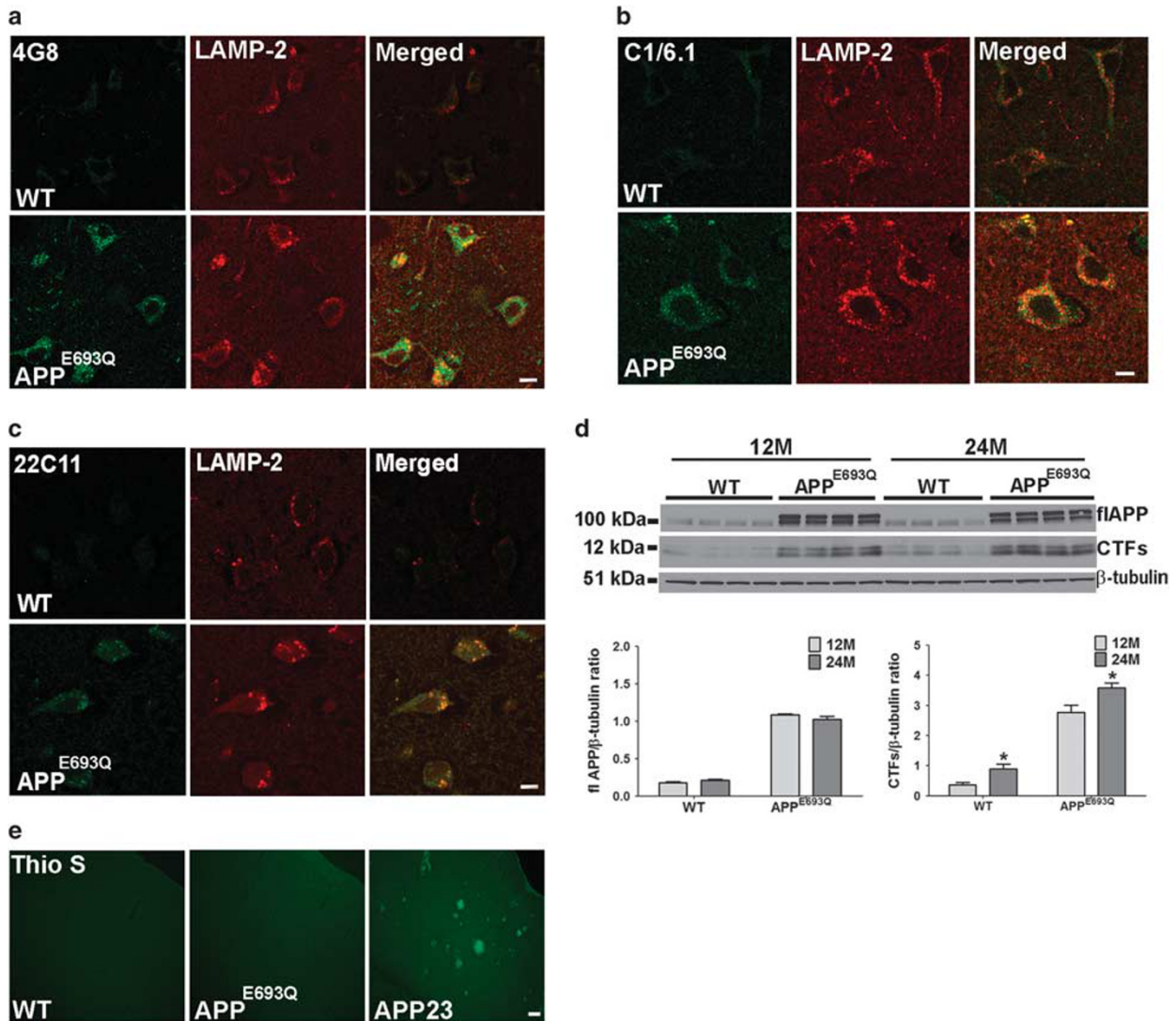
Results were evaluated using one-way analysis of variance followed by *post hoc* multiple-comparison Bonferroni's tests to determine the disparity among different age groups between the genotypes. Unpaired, two-tailed Student's *t*-test was used to analyze results among genotypes. A *P*-value of  $P < 0.05$  was considered statistically significant. None of the animals were excluded from analysis. No randomization was used in this study. The data from each group showed similar variance, presented in the study as mean  $\pm$  s.e.m.

## RESULTS

Lysosomal localization of APP metabolites in the brain of APP<sup>E693Q</sup> mice

Immunocytochemistry revealed brain region-specific high levels of staining by antibodies to APP and its metabolites in APP<sup>E693Q</sup> mice as compared with their wild-type (WT) littermate controls. Strong intraneuronal, punctate, 4G8-immuno-positive staining that identifies  $\beta$ APP, APP-CTFs and A $\beta$  was detected in the entorhinal and frontoparietal cortices of 12-month-old APP<sup>E693Q</sup> mice as compared with WT mice (Figure 1a). Similarly, robust intraneuronal, punctate staining was observed with C1/6.1, an antibody that binds  $\beta$ APP and APP-CTFs, in 12-month-old APP<sup>E693Q</sup> mice as compared with WT mice (Figure 1b). Staining with an antibody against the amino-terminus of APP (22C11) that binds both  $\beta$ APP and amino terminal soluble fragments of APP (sAPP) showed a lower difference between APP<sup>E693Q</sup> and WT mice as compared with the staining with 4G8 and C1/6.1 in the brain of the APP<sup>E693Q</sup> mice (Figure 1c). Dual immunostaining with an antibody to the lysosomal protein LAMP-2 showed that staining with both 4G8 and C1/6.1 was largely localized to lysosomes in the brains of APP<sup>E693Q</sup> transgenic mice (Figures 1a and b). Compared with the staining with these two antibodies, fewer LAMP-2 lysosomes were stained with 22C11 (Figure 1c). These data indicate the accumulation of APP-CTFs, but not of  $\beta$ APP or sAPP, in lysosomes in the brain of APP<sup>E693Q</sup> transgenic mice.

Western blot analysis with the antibody C1/6.1 demonstrated that while there was no change in the expression level of  $\beta$ APP, APP-CTFs levels were significantly elevated in the brains of



**Figure 1.** Age-dependent intraneuronal lysosomal accumulation of APP-CTFs and lysosomal abnormality in the brain of APP<sup>E693Q</sup> mice. Double immunostaining of the entorhinal cortices of 12-month-old APP<sup>E693Q</sup> and WT mice with antibodies to the lysosomal marker LAMP-2 (red) and 4G8 (green), which reacts with flAPP, APP-CTFs and A $\beta$  (a), with C1/6.1, which reacts with flAPP and APP-CTFs (green) (b) or 22C11 which reacts with flAPP and sAPP (green) (c), ( $n = 4$ ) (scale bars, 10  $\mu$ m), revealing intra-lysosomal accumulation of APP-CTFs. (d) Western blot analysis of cerebral homogenates of brains of 12- and 24-month-old APP<sup>E693Q</sup> mice and WT littermate controls using the C1/6.1 antibody revealed an increase in APP-CTFs levels in 24-month-old as compared with 12-month-old mice. Different exposure times of the same membrane are shown.  $\beta$ -tubulin reactivity is shown as a loading control. Densitometric analysis of the ratio of APP-CTFs signals to  $\beta$ -tubulin. Measurements are presented as the mean  $\pm$  s.e.m. ( $n = 4/5$ ). The difference among different age groups in mice were significant at  $*P < 0.05$ . (e) Thioflavin-S (Thio-S) staining showing no fibrillar A $\beta$  deposits in the frontoparietal cortex of APP<sup>E693Q</sup> mice at 24 months of age as compared with fibrillar A $\beta$ -positive 24-month-old APP23 mouse brain (scale bar, 100  $\mu$ m). A $\beta$ , amyloid- $\beta$ ; APP, amyloid- $\beta$ -precursor protein; CTF, APP carboxyl-terminal fragment; flAPP, full-length APP; WT, wild type.

24-month-old compared with 12-month-old APP<sup>E693Q</sup> mice (Figure 1d). This reveals accumulation of APP-CTFs in the brains of APP<sup>E693Q</sup> mice with age, in addition to the previous demonstration of accumulation of oA $\beta$ .<sup>17,41</sup> No fibrillar amyloid deposits were observed in the brains of APP<sup>E693Q</sup> mice up to 24 months of age (Figure 1e).

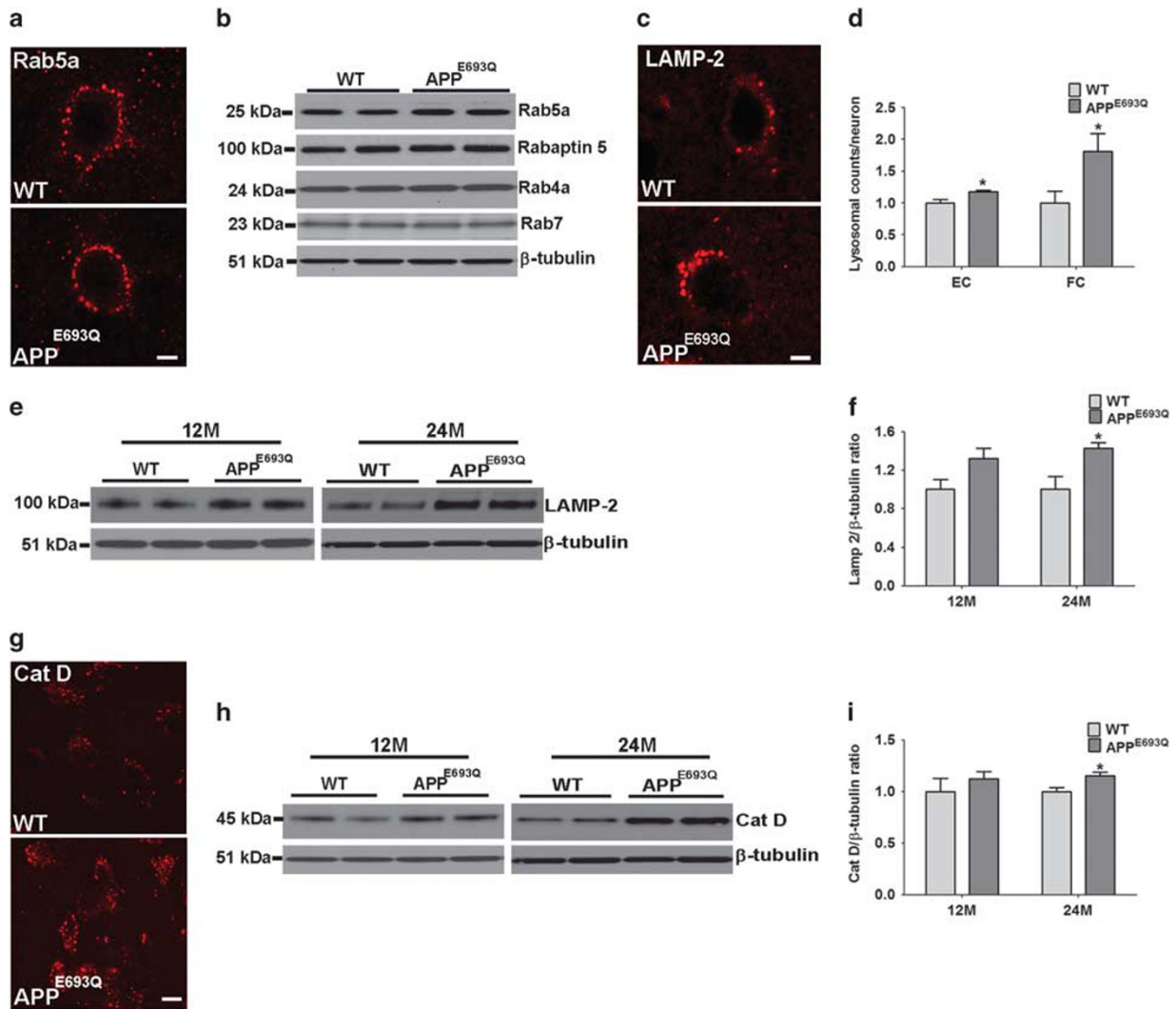
#### Lysosomal pathology in the brain of APP<sup>E693Q</sup> mice

Immunocytochemical and western blot analyses were conducted in order to investigate whether accumulation of APP metabolites in neuronal lysosomes affects the endocytic pathway in APP<sup>E693Q</sup> mouse brains. Immunostaining with an antibody to the early endosomal protein rab5 showed no difference in endosomal size

between APP<sup>E693Q</sup> mice and WT littermate control mice (Figure 2a). Furthermore, no differences were observed in 12- and 24-month-old APP<sup>E693Q</sup> mice by western blot analysis of hemibrain homogenates with antibodies to the early endosomal marker, rab5a, the rab5 effector protein, rabaptin 5, the late endosomal marker, rab7 and the recycling endosomal marker, rab4a (Figure 2b), suggesting that the endosomal pathway is unaffected.

Immunocytochemistry of lysosomal proteins have shown higher intensity staining and higher number of LAMP-2-positive vesicles per neuron in the entorhinal and frontoparietal cortices in the brains of 12-month-old APP<sup>E693Q</sup> compared with WT mice (Figures 1a–c and 2c). Quantification of LAMP-2 positive lysosomes in the frontoparietal and entorhinal cortices revealed higher

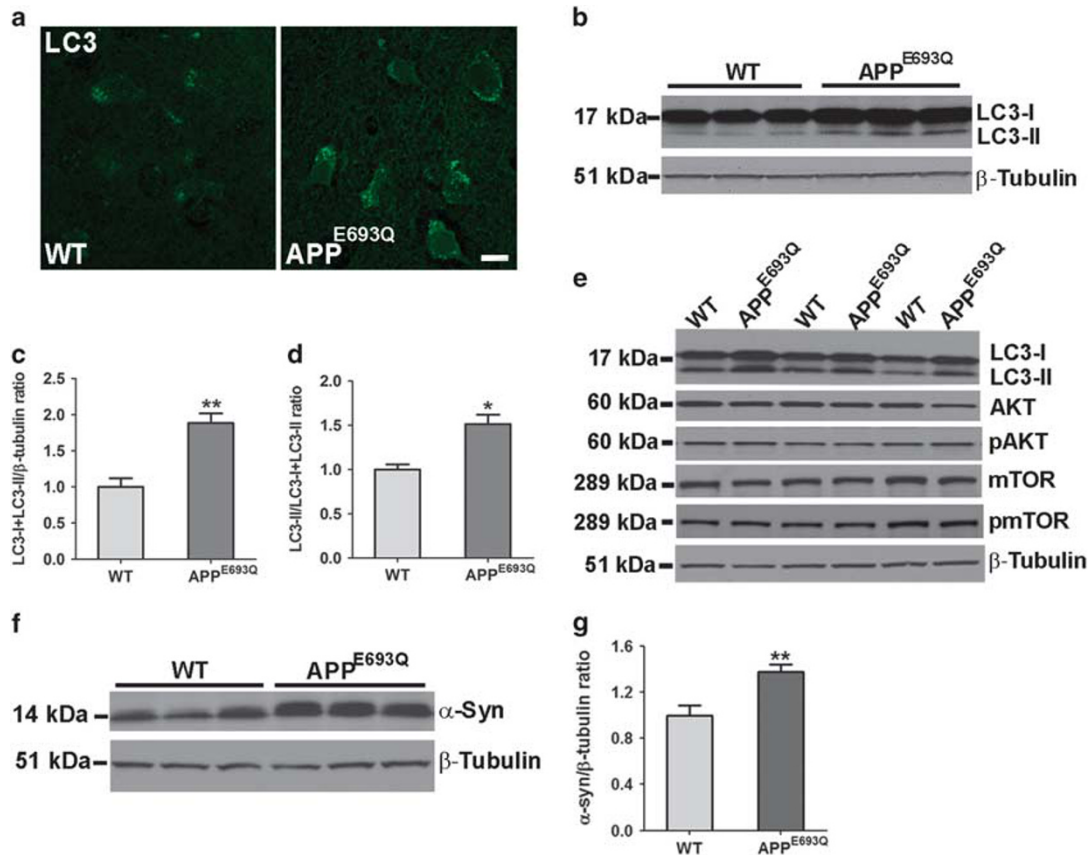




**Figure 2.** Higher LAMP-2 and Cat D levels in the brain of APP<sup>E693Q</sup> mice compared with WT control mice. (a) No difference was observed between APP<sup>E693Q</sup> mice and littermate control neurons immunostained for the early endosomal marker, rab5, shown here in the frontoparietal cortex (scale bar, 5 μm). (b) Western blot analysis using anti-rab4a, rab5a, rab7 and rabaplin 5 antibodies did not show differences in the levels of these proteins in hemibrain homogenates of APP<sup>E693Q</sup> as compared with WT mice (n = 4). (c) Higher signal for LAMP-2 immunoreactivity was observed in the frontoparietal cortex of 12-month-old APP<sup>E693Q</sup> mice compared with age-matched WT controls as found in the entorhinal cortex (Figures 1a–c). (d) Morphometric measurements of LAMP-2-labeled lysosomes revealed higher numbers of lysosomes per neuron in APP<sup>E693Q</sup> as compared with WT mice. The ratio of LAMP-2-positive lysosomes per neuron in APP<sup>E693Q</sup> mice was normalized to control mice. (e) Western blot of proteins in the entorhinal cortex of 12- and 24-month-old WT and APP<sup>E693Q</sup> mice, using anti-LAMP-2 antibody. β-tubulin reactivity is shown as a loading control. (f) The ratio of LAMP-2 to β-tubulin reactivity in APP<sup>E693Q</sup> mice was normalized to control mice. (g) Immunocytochemical staining of the entorhinal cortex of mice at 12 months of age using anti-Cat D antibody (scale bar, 50 μm). (h) Western blot analysis of homogenates of the entorhinal cortex confirmed higher active Cat D level in 12- and 24-month-old APP<sup>E693Q</sup> mice compared with WT mice. β-tubulin reactivity is shown as a loading control. (i) The ratio of Cat D to β-tubulin reactivity in APP<sup>E693Q</sup> mice was normalized to control mice. Measurements are presented as the mean ± s.e.m. (n = 4/5). The difference from WT mice was significant at \*P < 0.05. APP, amyloid-β-precursor protein; EC, entorhinal cortex; FC, frontoparietal cortex; LAMP-2, lysosomal marker protein 2; WT, wild type.

lysosomal counts (Figure 2d) in brain neurons of APP<sup>E693Q</sup> mice as compared with WT controls mice. Similar to LAMP-2, higher intensity staining and higher number of stained vesicles were observed with the antibody to the lysosomal hydrolase Cat D in the entorhinal cortices of 12-month-old APP<sup>E693Q</sup> as compared with WT mice (Figure 2g). While western blot analysis of entorhinal cortical homogenates of 12-month-old mice with antibodies to LAMP-2 and Cat D showed a non-significant trend toward greater levels in APP<sup>E693Q</sup> compared with WT mice, western blot analysis of entorhinal cortical homogenates of 24-months-old mice showed significantly higher levels of LAMP-2, and active Cat D in APP<sup>E693Q</sup> as compared with WT mice (Figures 2e, f, h and i).

These data show that immunocytochemical staining revealed lysosomal changes in specific neuronal populations, apparent already in the brain of 12-month-old transgenic mice, whereas western blot analysis of brain tissue that contains multiple cellular populations, including astrocytes and microglia, did not show lysosomal changes at 12 months of old. Significantly higher levels of lysosomal proteins were observed by western blot analysis of entorhinal cortices of APP<sup>E693Q</sup> compared with WT mice at 24 months of age. These data reveal an age-dependent increase in lysosomal pathology in entorhinal cortical neurons of APP<sup>E693Q</sup> mice. Although similar changes in the lysosomal markers LAMP-2 and Cat D were observed in multiple brain regions, the entorhinal



**Figure 3.** Higher LC3 levels and conversion to LC3-II in the brain of 24-month-old APP<sup>E693Q</sup> mice compared with WT controls. **(a)** Brain sections immunostained with anti-LC3 antibody showed higher intensity in the entorhinal cortices of APP<sup>E693Q</sup> as compared with WT mice (scale bar, 10 μm). **(b)** Western blot analysis of entorhinal cortical homogenates of APP<sup>E693Q</sup> as compared with WT mice using an anti-LC3 antibody showed higher intensity of both the LC3-I and LC3-II bands. **(c)** Quantification of the ratio of total LC3 (LC3-I+LC3-II) to β-tubulin bands in APP<sup>E693Q</sup> normalized to WT mice, (*n* = 4). **(d)** Quantification of the ratio of the LC3-II to total LC3 bands in APP<sup>E693Q</sup> normalized to WT mice, (*n* = 4). **(e)** Western blot analysis of hemibrain homogenates using anti-LC3, mTOR and AKT antibodies (total and phosphorylated protein) showed higher intensity of both the LC3-I and LC3-II bands in APP<sup>E693Q</sup> as compared with WT mice whereas no differences were observed in mTOR and AKT expression and phosphorylation levels (*n* = 4). **(f)** Western blot analysis of brain homogenates showed higher α-synuclein protein levels in hemibrain homogenates of APP<sup>E693Q</sup> compared with WT mice. **(g)** Quantification of the ratio of α-synuclein to β-tubulin bands (*n* = 5). All data are presented as the mean ± s.e.m. The differences from WT mice were significant at \**P* < 0.05 and \*\**P* < 0.01. α-Syn, α-synuclein; APP, amyloid-β-precursor protein; mTOR, mammalian target of rapamycin; WT, wild type.

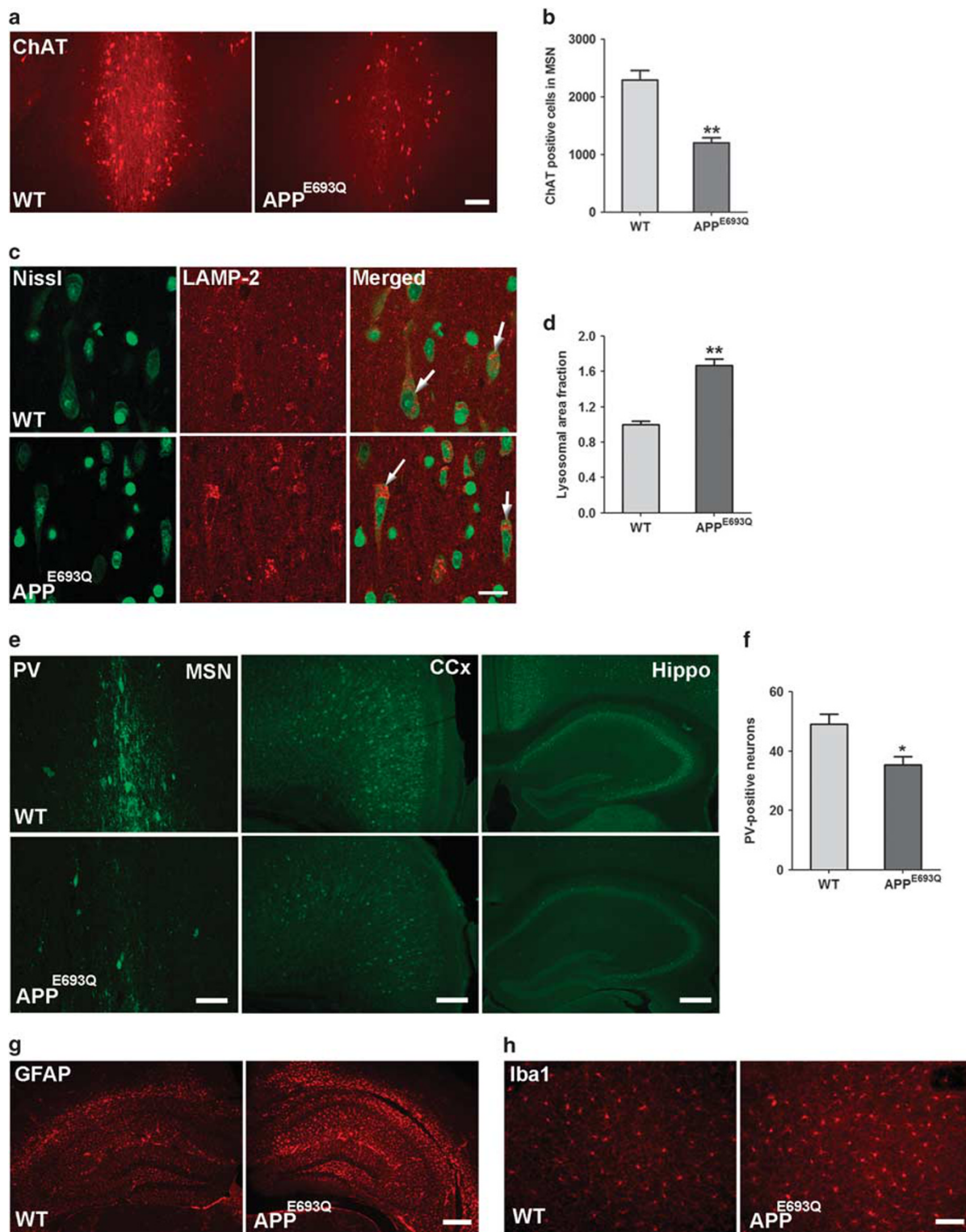
cortex was the region most affected by lysosomal pathology in the brain of APP<sup>E693Q</sup> mice. Furthermore, immunostaining and western blots showed higher LC3 expression levels in the entorhinal cortex of APP<sup>E693Q</sup> as compared with WT mice at 24 months of age (Figures 3a-c). In addition, higher levels of conversion from LC3-I to LC3-II were observed in APP<sup>E693Q</sup> mice as compared with WT littermate controls (Figure 3d). Although higher levels of LC3 and conversion from LC3-I to LC3-II were observed in brain homogenate of APP<sup>E693Q</sup> mice as compared with WT littermate controls, study of the mammalian target of rapamycin (mTOR) signaling pathway to test its involvement in autophagic abnormalities in APP<sup>E693Q</sup> mice did not show differences in the phosphorylation of proteins such as P70S6K, AKT, and mTOR between APP<sup>E693Q</sup> mice and littermate controls (Figure 3e), suggesting lysosomal dysfunction, but not autophagic pathology, that leads to defective clearance of neuronal autophagic substrates in APP<sup>E693Q</sup> mice.

On the basis of recent indications that impaired lysosomal clearance may also be associated with accumulation of neurodegeneration-related proteins, including α-synuclein, in an APP transgenic mouse model,<sup>53</sup> we sought to determine the levels of α-synuclein in the brains of APP<sup>E693Q</sup> mice. Western blot

analysis of 24-month-old APP<sup>E693Q</sup> mouse brains exhibited higher levels of α-synuclein as compared with WT mice (Figures 3f and g), supporting impaired lysosomal function.

#### Cholinergic neuronal loss in the MSN of APP<sup>E693Q</sup> mice

Basal forebrain cholinergic neurons are particularly vulnerable to degeneration in AD.<sup>54</sup> We undertook to investigate whether lysosomal abnormality in neurons affects basal forebrain cholinergic neuron in the MSN of APP<sup>E693Q</sup> mice. Comparison of cholinergic neurons in the MSN of APP<sup>E693Q</sup> and WT mice by immunostaining with antibody to ChAT revealed lower number of immunopositive cells in APP<sup>E693Q</sup> mice at 12 months of age as compared with littermate controls (Figure 4a). Using quantitative unbiased stereology, we found that APP<sup>E693Q</sup> mice had significantly fewer ChAT-positive cells in the MSN as compared with WT mice (Figures 4b). We further investigated the lysosomal abnormalities in large neurons of the MSN immunostained with LAMP-2, counter-stained with Nissl (Figure 4c). Quantification of LAMP-2-positive lysosomes in these large septal neurons showed bigger lysosomal size (Figure 4d) in brains of APP<sup>E693Q</sup> mice as compared with WT controls mice.



Loss of GABAergic interneurons in the brain of APP<sup>E693Q</sup> mice  
Immunostaining of GABAergic interneurons with an antibody to PV has shown that the GABAergic cell population was decreased in the cortex, hippocampus, and MSN areas in 12 months-old APP<sup>E693Q</sup> as compared with WT mice (Figure 4e). PV-positive cells in the hippocampi of APP<sup>E693Q</sup> and WT mice were quantified revealing a lower number of these inhibitory interneurons in the

hippocampus of APP<sup>E693Q</sup> mice as compared with WT controls (Figure 4f).

Activation of astrocytes and microglia in the brain of APP<sup>E693Q</sup> mice  
In 29-month-old mice of the model described by Herzig *et al.*,<sup>16</sup> microglial activation demonstrated by Iba-1 immunostaining was



**Figure 4.** Loss of cholinergic neurons, GABAergic interneurons and gliosis in the brain of APP<sup>E693Q</sup> mice at 12 months of age. **(a)** Loss of ChAT-immunopositive neurons in the MSN of APP<sup>E693Q</sup> mice compared with age-matched WT controls (scale bar, 50  $\mu$ m). **(b)** Unbiased stereological quantification of ChAT-immunopositive cells in the MSN of WT and APP<sup>E693Q</sup> mice is depicted as cell number  $\pm$  s.e.m. ( $n = 5$ ). **(c)** Nissl counterstained neurons in the MSN of APP<sup>E693Q</sup> mice immunostained for LAMP-2 showed higher signal for LAMP-2 compared with age-matched WT controls (scale bars, 20  $\mu$ m). **(d)** Morphometric measurements of LAMP-2 labeled lysosomes revealed higher lysosomal area per neuronal area in the Nissl stained large neurons of the MSN (arrowheads) in APP<sup>E693Q</sup> normalized to WT mice ( $n = 4$ ). **(e)** Representative pictures of PV-positive GABAergic cells in the MSN (scale bar, 50  $\mu$ m), cingulate cortex (scale bar, 100  $\mu$ m) and hippocampus (scale bar, 200  $\mu$ m) in WT and APP<sup>E693Q</sup> mice. **(f)** Quantification of PV-positive cells in the hippocampus ( $n = 5$ ). **(g)** Immunocytochemical staining with anti-GFAP antibody of sections of APP<sup>E693Q</sup> mouse brains revealed astrogliosis in the hippocampal region as compared with WT mice (scale bar, 200  $\mu$ m). **(h)** APP<sup>E693Q</sup> mice also exhibited microgliosis in the hippocampal region when immunostained with anti-Iba1 antibody (Scale bar, 50  $\mu$ m). Differences from WT were significant at  $*P < 0.05$  and  $**P < 0.01$ . APP, amyloid- $\beta$ -precursor protein; CCx, cingulate cortex; GFAP, glial fibrillary acidic protein; Hippo, hippocampus; LAMP-2, parvalbumin; MSN, medial septal nucleus; PV, parvalbumin; WT, wild type.

observed in the immediate vicinity of amyloid-laden vessels and adjacent areas, and GFAP-positive-activated astrocytes were observed in neocortical areas affected by cerebral amyloid angiopathy. In the APP<sup>E693Q</sup> mice studied here we observed higher intensity of GFAP and Iba1 immunostaining, suggesting activated glial and microglial cells in the hippocampus, particularly in the hilus of dentate gyrus and extended to the granular cell layer and corpus callosum (Figures 4g and h) in the absence of amyloid deposition.

## DISCUSSION

This study revealed age-dependent intraneuronal accumulation of APP-CTFs in the brains of APP<sup>E693Q</sup> mice in the absence of cerebral amyloid deposition, in addition to the previously demonstrated intraneuronal accumulation of  $\alpha$ A $\beta$ .<sup>17,39–41</sup> Characterization of cellular APP/CTFs accumulation in the absence of parenchymal A $\beta$  deposits and resultant neuropathology are valuable for understanding the pathological determinants of cognitive dysfunction in AD. Our extensive study of the intraneuronal vesicular localization of APP metabolites revealed their accumulation within lysosomes in the brain of 12-month-old APP<sup>E693Q</sup> mice. Dysfunction of lysosomes containing APP-CTFs and/or  $\alpha$ A $\beta$  aggregates was demonstrated by the observation of higher staining intensity of LAMP-2 and increased numbers of enlarged LAMP-2-positive lysosomes in neurons dually stained with antibodies to flAPP/APP-CTFs/A $\beta$  of 12-month-old APP<sup>E693Q</sup> as compared with WT controls. Neurons in the brains of APP<sup>E693Q</sup> mice also had higher levels of the lysosomal hydrolase Cat D. A previous study showed no accumulation of soluble LC3, p62 or  $\alpha$ -synuclein in the brains of APP<sup>E693Q</sup> mice at 6 months of age,<sup>41</sup> suggesting an age-dependent lysosomal dysfunction due to the ineffective clearance of APP metabolites. The lysosomal abnormality became more severe at an older age, as revealed by the intraneuronal accumulation of the autophagosomal/autolysosomal marker LC3 as well as by the increased conversion of LC3-I into LC3-II in entorhinal cortical neurons at 24 months of age. These data suggest early mobilization of the lysosomal system, followed by delayed turnover of intra-lysosomal proteins such as LC3-II that is normally rapidly degraded after fusion of autophagosomes with lysosomes. In addition, we found increased  $\alpha$ -synuclein in lysosomes of 24-month-old APP<sup>E693Q</sup> mice.  $\alpha$ -Synuclein forms insoluble fibrils under pathological conditions in Parkinson's disease, dementia with Lewy bodies,<sup>55,56</sup> and accumulates in the cortex of 30–50% AD patients.<sup>57</sup> These data show that APP-CTFs accumulation is involved in the age-related changes in the brains of aged mice, and could be a cause of AD-relevant lysosomal dysfunction.

Evidence is mounting from transgenic mice and human patients, indicating that A $\beta$  as well as APP-CTFs accumulate intraneuronally and contribute to AD progression.<sup>22–38</sup> An association between elevated APP-CTFs levels, abnormal endosomal morphology, and basal forebrain cholinergic neuron

degenerative changes have also been observed in mouse models of familial AD due to other APP mutations.<sup>47</sup> Earlier studies using isotropic fractionator showed no evidence for total neuronal loss in the hippocampus, neocortex, and cerebellum of APP<sup>E693Q</sup> mice,<sup>58</sup> but reduced dendritic arborization of apical dendrites and a decrease in postsynaptic density length in individual hippocampal neurons of 12 months-old APP<sup>E693Q</sup> mice was observed.<sup>59</sup> However, our data show loss of cholinergic neurons in the MSN of our APP<sup>E693Q</sup> mice. Dysregulated cholinergic signaling is an early hallmark of AD.<sup>60</sup> The severity and magnitude of cholinergic dysfunction appears to depend, at least in part, on age and brain region, in many APP-overexpressing mouse models, including APP23,<sup>47,61,62</sup> TgCRND8,<sup>63</sup> Tg2576<sup>64</sup> and APP/PS1K1.<sup>65</sup> The cholinergic fiber loss was attributed to higher A $\beta$  load.<sup>66</sup> However, the observed shrinkage of cholinergic neurons<sup>61</sup> and reduced enzymatic activity in the basal forebrain in 6–8-month-old APP23 transgenic mice<sup>62</sup> and the cholinergic degenerative pathology in PDAPP mice,<sup>67</sup> indicate the presence of an early cholinergic dysfunction prior to A $\beta$  amyloid deposition. Oligomeric forms of A $\beta$  have been implicated in the reduction in ChAT activity<sup>60</sup> and loss of cholinergic innervation in the neocortex,<sup>68</sup> preceding the loss of cholinergic neurons commonly observed in AD brains. However, the data presented here suggest that the neuronal lysosomal dysfunction in MSN cholinergic neurons and cortical regions of APP<sup>E693Q</sup> mice is due at least in part to the accumulation of APP-CTFs.

Besides showing significant loss of cholinergic cells in the MSN, 12-month-old APP<sup>E693Q</sup> mice also showed GABAergic neurotransmission deficits, similar to other APP transgenic mice, including TgCRND8,<sup>69</sup> AbPPdE9<sup>70</sup> and J20 transgenic mice.<sup>71</sup> Both cholinergic and GABAergic cell loss in the brain of these transgenic mice, in the absence of parenchymal and vascular  $\beta$  amyloid deposition, implies a role for intracellular accumulation of APP-CTFs/ $\alpha$ A $\beta$  in neuronal degeneration and subsequent impaired spatial learning and memory, novel object recognition and increased anxiety in APP<sup>E693Q</sup> transgenic mice.<sup>17,39–41</sup>

In conclusion, our results suggest that an age-dependent intraneuronal accumulation of APP-CTFs and  $\alpha$ A $\beta$  initiates a cascade of pathogenic events, including lysosomal dysfunction in vulnerable brain regions and leads to inflammatory activation, cholinergic and GABAergic neuronal loss in transgenic mice overexpressing human APP gene harboring the E693Q mutation, leading to memory deficits.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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