Title:

Amyloid-ß plaque formation and BACE1 accumulation in the brains of a 5xFAD Alzheimer's disease mouse model is associated with altered distribution and not proteolysis of BACE1 substrates Sez6 and Sez6L

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Abstract

The formation of amyloid-ß peptides (Aß), that accumulate in Alzheimer's disease (AD) brains, involves proteolytic processing of the amyloid precursor protein (APP) firstly by ß-secretase (BACE1). Since BACE1 cleaves a plethora of other substrates, in this work we investigated whether the proteolysis and/or distribution of other BACE1 substrates, such as seizure protein 6 (Sez6) and seizure 6-like protein (Sez6L), is altered in AD. To test this we used 5xFAD mouse model brains that show an early accumulation of Aß plaques already at 2-months of age. Here we show for the first time that accumulation of BACE1 in peri-plaque regions and its enhanced levels in AD brains does not affect proteolysis of BACE1 substrates other than APP, such as Sez6 and Sez6L. We observed altered distribution of Sez6 and Sez6L in the area of Aß plaques in 5xFAD brains which is distinct to that of APP, BACE1 and/or LAMP1, suggesting different localization and/or function of these BACE1 substrates. While it is necessary to further elucidate the potential role that this may play in the course of AD, it is likely that Aß-targeted therapies may have beneficial effects against accumulation and/or altered distribution of BACE1 and its substrates, in addition to APP.

Keywords: amyloid-ß, APP, BACE1, ß-secretase, Sez6, Sez6L

1. INTRODUCTION

Accumulation and aggregation of amyloid-ß peptides (Aß) as Aß plaques in the brains is a pathological feature of Alzheimer's disease (AD), both of the most common and complex form of AD as well as the rare familial AD (FAD) [1]. The Aß peptides are generated by sequential proteolysis of the amyloid precursor protein (APP) by enzymes ß-secretase and γ-secretase [2,3] . The ß-site APP cleaving enzyme 1 (BACE1 – ß -secretase) catalyses the initial step in Aß formation [4–8], and, thus, inhibition of BACE1 has been considered as a promising strategy to reduce Aß levels and to treat AD [9–11]. However, since BACE1 cleaves number of other substrates [12–14], in addition to APP, its complete inhibition may not be an option due to potential side effects [15–17]. This is supported by the fact that BACE1 knockout (KO) mice display an array of complex neurological phenotypes, including growth retardation [18], memory deficits [11,19,20], hypomyelination [21,22] , seizures [23–25], axon guidance defects [26–28] and schizophrenia-like behaviors [29]. These BACE1 KO phenotypes likely reflect diverse functions of numerous so far identified and still unknown BACE1 substrates [30,31]. Among the BACE1 substrates, Sez6 and Sez6L were found to be preferentially cleaved by BACE1 [12,32]. The BACE1-inhibition markedly reduced the levels of Sez6 and Sez6L ectodomains (by 95 and 96%, respectively) [17]. Furthermore, BACE1-mediated inhibition of Sez6 proteolysis was shown to play a role in cognitive impairment [16,33] or hyperactivity [17]. Indeed, several clinical trials that tested BACE1 inhibitors against AD were halted due to either futility or safety reasons [15,31]. Nevertheless, it is important to better understand the biology of BACE1 and of its substrates to reveal their role in health and in disease, such as in AD [10,31].

Previous studies revealed that BACE1 levels are elevated in AD patients' and in APP transgenic mouse brains and that BACE1 accumulates in swollen presynaptic structures in neurons surrounding Aß plaques [34,35]. Studies using APP transgenic mouse models with fast (5xFAD) and slow (Tg2576) Aß plaque load demonstrated that accumulation of BACE1 occurs in parallel with Aß plaque formation and is an early event before neuronal loss [35]. Furthermore, accumulation of the lysosomal marker LAMP1 as well as the endosome marker transferrin receptor (TfR) was also observed in dystrophic neurites early upon Aß plaque formation [35,36], implying defective endolysosomal trafficking in the neuronal processes in close vicinity to Aß plaques. Moreover, enhanced levels of BACE1 were found to co-localize with TfR and/or with LAMP1 in peri-plaque regions of 5xFAD mouse brains [35–38], suggesting that in AD BACE1 accumulates in endosomal vesicles following its decreased degradation by lysosomes and its subsequent elevation. Importantly, BACE1 accumulation was found to be accompanied with enhanced immunostaining and co-localization with APP in peri-plaque regions, implying that enhanced processing of APP by BACE1 may further drive Aß production and promote AD [35].

Since BACE1 function and proteolysis of its substrate APP has been strongly linked to endolysosomal pathway both under normal and disease conditions [35–39], such as in AD, we reasoned that defects within the endolysosomes may affect proteolysis and function of other BACE1 substrates in AD, in addition to APP. Indeed, we have recently demonstrated that endolysosomal dysfunction in a rare lysosomal and lipid storage disorder Niemann-Pick type C causes enhanced cleavage of APP as well as of Sez6 and Sez6L by BACE1 in the brains of NPC1 mouse model [40]. In addition, we showed that elevated BACE1-mediated proteolysis in NPC is likely due to accumulation of its substrates in enlarged endosomal vesicles within neuronal processes [40,41]. It has been previously observed that enlarged early endosomes caused by accumulation of the BACE1-generated C-terminal APP fragment

(APP-CTFß) is one of the earliest pathological features of AD [42–45]. Here, we analyzed whether endolysosomal dysfunction in AD and BACE1 accumulation in the regions surrounding Aß plaques may lead to altered processing and/or distribution of other, more specific BACE1 substrates, Sez6 and Sez6L that are, in contrast to APP, preferentially cleaved by BACE1 [12,32].

Sez6 family members are mainly located in cell membranes of dendrites, in synaptic and postsynaptic fraction [30,46,47], while Sez6L is also found in the ER membranes [48]. In experimental mouse models, Sez6 family has been shown to participate in regulation of dendritic development, synapse maturation and excitatory postsynaptic potential in the cortex and hippocampus [46,49,50]. BACE1 inhibition or Sez6 KO leads to reduced spine density, spatial memory deficits, poor coordination and hyperactivity in mice, and intellectual disability and psychiatric disorders in humans [16,17,33,50]. The most recent study [49] reports a novel role of Sez6 family members as regulators of the system of the complement that contributes to pathological cell and synapse loss in ageing and diseases including Alzheimer's. As for the most of the BACE1 sheddome proteins, it remains unknown whether the biological function is mediated by the soluble ectodomain, or by transmembrane full length protein, but altered shedding may have direct consequences on the development of the neuropathology [49,51].

This study was performed on 5xFAD mice, the widely used transgenic mouse model of AD that recapitulates many AD-related phenotypes and has a relatively early and aggressive presentation [52–55]. 5xFAD mice develop amyloid pathology at 2-months of age, with accompanying astroglyosis, microglyosis and dystrophic neurites. Robust impairment in long-term potentiation and failure in certain behavioral tasks that begins at 4-months worsening with age suggest deficits in synaptic transmission. Also, gene expression analysis reveals that the most upregulated genes during 5xFAD life time were involved in inflammation pathways, while the most common down-regulated genes were related with pathways associated with synaptic transmission and signaling [55]. Therefore, we hypothesized that function of other BACE1 substrates, in particular those involved in synaptic function, may be impaired in the course of AD and Aß plaque formation and that this may add to the progression and the pathological features of AD. Thus, the goal of the study was to assess, using western blotting and immunochemistry, if the formation of Aß plaques and accumulation of BACE1 in 5xFAD brains over time, from 2- to 9-months of age, may affect proteolysis and/or localization of exclusive BACE1 substrates, such as Sez6 and Sez6L, in addition to APP.

Our findings suggest that formation of Aß plaques and accumulation of BACE1 in AD brains may affect localization of other BACE1 substrates, such as Sez6 and Sez6L, in addition to APP. While it is necessary to further elucidate the potential role that this may play in the course of AD, it is likely that altered brain distribution of BACE1 substrates is a consequence of the primary pathological feature of AD - Aß plaque formation. Thus, Aß-targeted therapies may have beneficial effects against accumulation and/or altered distribution of BACE1 substrates, other than APP.

2. MATERIALS AND METHODS

2.1. Animals

Transgenic 5xFAD mice and their background strain (B6SJLF1/J) used in this study were purchased from the Jackson Laboratory (Cat. No: 3484-JAX and 100012-JAX, Bar Harbor, Maine, USA) and

maintained on B6/SJL genetic background by crossing 5xFAD transgenic male mice with B6SJLF1/J female mice. The resulting F1 female offspring (heterozygous 5xFAD transgenic and non-transgenic, wild-type littermates as their controls) were used in experiments. Genotyping was performed by polymerase chain reaction (PCR) of tail DNA according to the supplier's protocol. 5xFAD mouse model bears five familial AD human mutations: three in the human amyloid precursor protein (APP) gene–Swedish (K670N, M671L), Florida (I716V), and London (V717I), and two in the human presenilin1 (PS1) gene - M146L and L286V, under transcriptional control of the neuron-specific murine Thy-1 promoter, as described by Oakley et al. [52].

The animals were housed in the plastic cages under standard conditions (23 ± 2 °C, 60–70% relative humidity, 12 h light/dark cycle), with free access to commercial rodent chow and water. Their health status was routinely controlled. All animal procedures were in compliance with the Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes and was approved by the Ethical Committee for the Use of Laboratory Animals (resolution No. 01-06/13) of the Institute for Biological Research - National Institute of Republic of Serbia, University of Belgrade. Minimal numbers of animals were used and all efforts were made to minimize animal suffering.

The 5xFAD mouse model, characterized by the earliest onset and most intense amyloid pathology, recapitulates almost all the neuropathological feature associated with human AD [52–55]. Presence of amyloid plaques is detected around 2-months of age (at asymptomatic phase), appearance of memory damages in prodromal phase occurs around 4-months of age, and cognitive deficits and neurotransmission impairments were reported around 9-months, at late AD-like stage [11,52]. Therefore, in this study we used 2-, 4- and 9-months old 5xFAD animals to examine if cleavage and distribution of Sez6 family proteins is changed at this crucial time points in 5xFAD pathology.

2.2. Tissue collection and preparation

When the 5xFAD transgenic and their non-transgenic, wild type littermates reached age of 2, 4 or 9 months (N = 6 mice per group and genotype), they were anesthetized by intraperitoneal injection of 100 mg/kg, Ketamidor (Richter Pharma, Wels, Austria) and 16 mg/kg Xylased (Bioveta, Czech Republic), transcardially perfused with ice-cold 0.1 M phosphate-buffer saline (PBS, pH = 7.4) and sacrificed. The brains were quickly removed and cerebral cortices were dissected under magnifying glass on the ice. The left hemispheres were stored at −80 °C for subsequent Western blot analyses, while the right hemispheres were further processed for immunohistochemical staining as follows. Tissues intended for immunohistochemical staining were fixed in fresh 4% paraformaldehyde (PFA) in PBS for 24 h and cryoprotected in 30% sucrose in PBS at 4 °C, until brains sink. Then the brains were frozen in isopentane cooled on dry ice and stored at −80 °C until use. Later, sagittal brain sections (18 µm thick) were cut on a cryostat (Leica, Wetzlar, Germany), according to the Allan Mouse Brain Atlas (Allen Institute for Brain Science (2008) available at: http:/alleninstitute.org/). Collection of all consecutive section started at a level ~ 0.24 mm lateral from midline, every 15th sections were mounted on the glass slides, immediately processed for immunohistochemistry staining or stored at -20°C for further use.

2.3. Western blot analysis

For analyses of different proteins cerebral cortices of five wt and five 5xFAD female mice were used at 9-weeks of age. Soluble (DEA) and membrane (Triton) fractions of brain lysates were prepared as previously described [12,40]. Brain lysates were mixed with 6 × sample buffer (60% glycerol, 12% SDS, 3% DTT, 1/8 v/v 0.5 M Tris pH 6.8, bromophenol blue or 60% glycerol, 12% SDS, 1/8 v/v 0.5 M Tris pH 6.8, bromophenol blue) and heated at 70 °C for 10 min. Equivalent amounts of protein were loaded onto Tris-Glycine gels and separated by SDS-PAGE. After SDS-PAGE, proteins were transferred onto PVDF membrane (Roche Applied Science, Basel, Switzerland) and incubated in primary antibody solution, followed by washing and HRP-conjugated secondary antibody incubation. Proteins were visualized by chemiluminescence using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, USA) on a documentation system from Uvitec, Cambridge, UK. Protein signals were quantified by using the ImageJ software (National Institutes of Health, USA). To control the same amount of protein, obtained protein signals were normalized to Amido Black (Supplementary figure 1). Comparison of protein levels between two groups of data (wt- vs.5xFAD mice) was performed by using a two-tailed, unpaired t-test. Differences were considered significant at a p value of <0.05 (* <0.05; ** <0.005).

2.4. Immunohistochemistry analysis

Cryosections were dried in the hood for 1 h, followed by 5 min incubation in 70% ethanol, 2 min in distilled H_2O and stained with Amylo-Glo dye [56] in saline solution for 5 min, then washed in distilled H_2O for 15 s and in TBS (50 mM TBS, pH 8.0), 3×5 min. Samples were then permeabilized in TBS-T (50 mM TBS, pH 8.0, 0.5% Triton X-100, Sigma-Aldrich, St. Louis, MO, USA) for 30 min, blocked in 5% goat serum (Sigma-Aldrich, St. Louis, MO, USA) in TBS-T for 1 h and incubated with primary antibodies diluted in 5% goat serum in TBS-T overnight. Antibodies used were: APP (C-terminal antibody, Epitomics), BACE-1 (Epitomics), LAMP1 (Santa Cruz), Transferrin Receptor (TfR, Life Technologies), neurofilament (NFL, Sigma-Aldrich), Sez6 and Sez6L (N-terminal antibodies, gift Dr. Lichtenthaler). Next day, sections were incubated with secondary antibody goat anti-rabbit-Alexa488, anti-mouse-Alexa594 and/or anti-rat-Alexa647 (Molecular Probes, Invitrogen, Waltham, MA, USA) for 3 h and mounted (Fluoromount, Sigma-Aldrich, St. Louis, MO, USA). Confocal images were acquired on an inverted laser scanning confocal microscope Leica TCS SP8 with the software LAS X (Leica, Wetzlar, Germany), and additional image processing and quantification were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3. RESULTS

3.1. Characterization of Aß plaque formation and distribution of BACE1 in 5xFAD mouse brains

The 5xFAD mice are characterized by an early and progressive Aß plaque pathology (already at 2-months of age), enabling monitoring of AD-related pathological processes in a timely manner [11,52]. We, thus, firstly analyzed formation of Aß plaques, neurofilament loss and lysosomal impairment in 2, 4- and 9-months old brains of 5xFAD mice vs. 9-months old wt mouse brains (Figure 1). Since our initial analysis revealed that 2-, 4- and 9-months old wt brains show similar staining (Supplementary

figure 2), in this and in our further immunohistochemistry analyses we used 9-months old wt brains only as controls. Amylo-Glo staining [56] confirmed numerous plaques at 4-months as well as 9-months old 5xFAD compared to wt brains, while in 2-months old 5xFAD brains plaques were sparse. Gradual decrease of neurofilament (Nfl) staining was observed from 2-, 4- to 9-months old 5xFAD vs. wt brains, indicating neurodegeneration. Interestingly, increased staining of the lysosomal marker LAMP1 surrounding the core of Aß plaque was detected already at 2-months of age in 5xFAD brains (zoomed figure) and was clearly observed upon further accumulation of Aß plaque pathology in 4- and 9-months old 5xFAD vs. wt brains. Enhanced LAMP1 staining in peri-plaque regions indicates accumulation of lysosomes in axonal swellings/buttons and altered lysosomal trafficking along the processes that is in accord with previous findings [36]. Since enhanced staining of LAMP1 was strongly associated with plaque pathology, we reasoned that it is likely a consequence of Aß plaque formation/accumulation that causes the plaque-induced loss of neuronal network and loss of normal endolysosomal trafficking along their processes. Defective endolysosomal trafficking due to the plaque pathology was further confirmed by staining of transferrin-receptor (TfR), a marker of recycling endosomes [35,38]. In parallel to altered LAMP1 distribution, accumulation of TfR was observed already at 2-months old 5xFAD vs. wt brains (Figure 2a). However, in contrast to LAMP1 (Figure 1), TfR accumulated in the core of Aß plaques in 2-months old 5xFAD vs. wt brains. Overall, these findings indicate plaque-induced alterations of endolysosomal transport in the surrounding neuronal processes.

Since BACE1-mediated proteolysis and function is strongly linked to endolysosomal pathway [37,39,57–61], we further analyzed distribution of BACE1 upon Aß plaque pathology in 5xFAD vs. wt mouse brains. Increased BACE1 staining was observed already at 2-months of age around the core of Aß plaques (Figure 2a) in a pattern mainly distinct to TfR immunoreactivity. Indeed, staining of BACE1 resembled the staining and accumulation of LAMP1 upon Aß plaque formation (Figures 1 and 2b), with its enhanced immunoreactivity in the area surrounding Aß plaques. BACE1 co-stained with LAMP1 marker, indicating BACE1 accumulation in LAMP1-positive vesicles in axonal swellings of neurites surrounding the Aß plaques (Figure 2b). Further western blot analysis of BACE1 and LAMP1 revealed their increased levels in 9-month old 5xFAD vs. wt brains, supporting their accumulation due to defective lysosomal function and their degradation caused by Aß plaque pathology (Figure 2c).

3.2. Distribution of BACE1 substrates APP, Sez6 and Sez6L upon Aß plaque formation in 5xFAD mouse brains

To evaluate whether altered BACE1 distribution/levels upon Aß plaque load in AD brains affects BACE1 substrates other then APP, we analyzed localization and proteolysis of Sez6 ad Sez6L, the two previously reported substrates that are preferentially cleaved by BACE1 and not like APP by α-secretase. Firstly, we showed that in 5xFAD vs. wt brains in addition to Aß plaque formation, APP accumulates in the dystrophic neurites surrounding Aß plaques (Figure 3). The pattern of APP immunostaining (using C-terminal antibody Y188) is similar to that previously observed for BACE1 and LAMP1 (Figure 2b). APP fully colocalized with LAMP1 marker, indicating its accumulation together with BACE1 in LAMP1-positive vesicles in axonal swellings that surround the Aß plaques in the 5xFAD brains [36]. These findings further support that Aß plaque formation in AD brains alters

trafficking within the endolysosomal pathway along surrounding neuronal processes leading to further accumulation of APP and BACE1 [35–38,62], the two key proteins in the pathogenesis of AD, and to further progression of the disease.

To analyze whether similar defect is observed for BACE1 substrates Sez6 and Sez6L we stained 5xFAD vs. wt brains with N-terminal antibodies against Sez6 and Sez6L, respectively. Aß plaque formation in 5xFAD brains at 2- and 4-months of age caused slightly altered, punctuate immunostaining of Sez6 in the peri-plaque regions (Figure 4a), that was different to previously described profound APP, BACE1 and/or LAMP1 accumulation in the areas surrounding the Aß plaques in 5xFAD brains (Figures 2b and 3). At 9-months Sez6 staining was observed in the of Aß plaque core, while other surrounding healthy neurons showed Sez6 signal similar to that in wt brains. In parallel, punctuate staining of Sez6L was also detected in dystrophic neurites surrounding Aß plaques in 5xFAD vs. wt brains already at 2- and 4-months of age (Figure 4b). However, similar to immunostaining of Sez6, the obtained signal of Sez6L puncta was substantially lower in peri-plaque regions compared to accumulation of APP, BACE1 and/or LAMP1. While this may be likely due to their immunohistochemistry analysis at endogenous levels, in contrast to APP that is expressed by triple transgenes in 5xFAD mouse brains, the accumulation of BACE1 and LAMP1 also at endogenous levels indicates their profoundly altered distribution upon Aß plaque formation in AD brains in contrast to that of BACE1-substrates Sez6 and Sez6L. Interestingly, Sez6 and Sez6L punctuate staining in the peri-plaque regions of 5xFAD brains at 4-months of age did not show colocalization with BACE1 (Figures 4a and 4b), in contrast to what was observed for APP (Figure 3). In addition, at 9-months of age Sez6L accumulated in the Aß core region in 5xFAD brains (Figure 4b), similar to that observed for Sez6 (Figure 4a). We, thus, conclude that formation of Aß plaques alters distribution of other BACE1-substrates in addition to APP, such as Sez6 and Se6L, but locally and most likely as a consequence of the Aß plaque load in the regions of dystrophic neurites distinct to those that accumulate BACE1.

To further evaluate this we co-stained BACE1-substrates APP and Sez6L (Figure 5). Indeed, we showed that both BACE1 substrates nicely colocalize in healthy neurons in both wt and 5xFAD brains. However, upon Aß plaque formation at 4-months of age APP and Sez6L accumulated in distinct axonal buttons surrounding the Aß plaques. Different distribution of Sez6L and APP upon the plaque load was further observed in 9-months old 5xFAD vs. wt brains. Sez6L signal was mainly found in the core of the plaque, while APP accumulated in the surrounding peri-plaque regions. This finding further supports that formation of Aß plaques alters distribution of BACE1-substrates Sez6 and Se6L in peri-plaque regions, in addition to APP, but in dystrophic neurites distinct to those that accumulate APP and BACE1, suggesting their distinct function/s.

3.3. Proteolysis of BACE1 substrates APP, Sez6 and Sez6L upon Aß plaque formation in 5xFAD mouse brains

Next, we analyzed proteolytic processing of BACE1 substrates Sez6 and Sez6L, in addition to APP, in 9-months old 5xFAD vs. wt brains, e.g. cerebral cortices. We speculated that altered endolysosomal trafficking and accumulation of BACE1, that was reported in the course of AD pathogenesis [35–38,44,45,63], may affect cleavage of BACE1-substrates, other than APP, such as Sez6 and Sez6L. However, we did not observe different levels of the soluble N-terminal BACE1-generated Sez6 and Sez6L fragments (sSez6 and sSez6L, respectively) between 9-months old 5xFAD and wt brains (Figure

6), indicating that upon AD pathogenesis and Aß plaque load BACE1-mediated proteolysis of these other BACE1 substrates Sez6 and Sez6L is likely unaffected. Indeed, we found enhanced levels of N-terminal APP fragments in 9-months old 5xFAD vs. wt brains, supporting that in AD alterations of BACE1-mediated proteolysis are likely specific for APP and most likely do not involve other BACE1-substrates, such as Sez6 and Sez6L. The enhanced proteolysis of APP in the peri-plaque regions could further contribute to the pathogenesis and progression of AD.

4. DISCUSSION

BACE1 is a central enzyme in the pathogenesis of AD as it catalyzes the first step in Aß formation by proteolytic processing of its larger precursor - protein APP [4–8]. Thus, inhibition of BACE1 has been considered as an attractive strategy to inhibit formation of Aß and to treat AD [9,10]. However, numerous BACE1 substrates [12,13], complex neurological phenotypes of BACE1-knockout mice and side effects that occurred during BACE1-inhibitor clinical trials [15,30,31], suggested an important role of the enzyme BACE1 and its substrates in normal brain function. Since previous studies have shown that BACE1 accumulates in the brain regions surrounding the Aß plaques and that its levels are enhanced in human and transgenic mouse AD brains [34,35], we reasoned to speculate that distribution and/or proteolysis of other BACE1 substrates, in addition to APP, may be altered in AD brains and that these defects may contribute to the pathogenesis and progression of AD. Furthermore, we hypothesized that BACE1 substrates involved in synaptic function and/or those that are preferentially cleaved by BACE1, like Sez6 and Sez6L [12,32], may be modified in AD brains. Indeed, in our recent work by Causevic et al. [40] we demonstrated that endolysosomal dysfunction in Niemann-Pick type C disease (NPC) mouse model brains leads to enhanced BACE1-mediated proteolysis of Sez6 and Sez6L, in addition to APP, already at the presymptomatic stage and that this defect is likely due to altered trafficking of BACE1 and of its substrates within endolysosomal compartments [40,41].

Using the well characterized transgenic AD mouse model with an early Aß plaque pathology in parallel with an early BACE1 accumulation in peri-plaque regions [11,35,52], 5xFAD mouse brains (e.g. cerebral cortices), here we show for the first time that Aß plaque formation alters distribution of BACE1 substrates Sez6 and Sez6L, in addition to APP. Although Sez6 and Sez6L immunosignals in AD brains were much lower, due to their analysis at endogenous levels, compared to that of the triple transgene expression of APP, we could observe their punctuate staining in the peri-plaque regions of 5xFAD brains at 2- and 4-months of age, indicating their altered trafficking in surrounding neuronal processes. However, Sez6L puncta did not colocalize with APP puncta in the region surrounding the Aß plaque, suggesting that these two BACE1 substrates, APP and Sez6L, likely accumulate in spatially distinct compartments. While further analysis is necessary to elucidate the identity of Sez6 and Sez6L puncta upon Aß plaque load, previous studies in Sez6 KO mice and BACE1-inhibitor treated wild-type mice revealed the role of Sez6 in maintaining normal dendritic spine dynamics [16,30,33,46,47,50], rather than presynaptic function as indicated for APP and BACE1. Our immunohistochemistry analysis of APP, BACE1 and LAMP1 confirmed previous finding of their accumulation and strong colocalization in peri-plaque regions of 5xFAD brains [35–37] . According to the findings of Kandalepas et al. [35] these may likely represent enlarged presynaptic terminals (giant buttons) as a result of defective endolysosomal transport within the axons upon AD pathogenesis [36]. Indeed, BACE1 was found to

accumulate in LAMP1-positive vesicles and to a lesser extent in TfR-positive endosomes in dystrophic neurites that surround an amyloid plaque in 5xFAD vs. wt brains. This is in line with previous study by Gowrishankar et al. [36] that reported elevated levels of BACE1 in defective lysosomes that accumulate in swollen axons found locally in the area in close vicinity to Aß plaques. Although we observed strong colocalization of APP and BACE1 in the regions surrounding Aß plaque, other BACE1-substrates Sez6 and Sez6L did not show their colocalization with BACE1 in peri-plaque regions in 5xFAD brains, respectively. Indeed, at the later stage of the disease, at 9-months of age, their immunostaining signals were clearly separated – Sez6 and Sez6L were found in the Aß core, while BACE1 was present in the area surrounding the Aß plaque regions. Thus, we conclude that observed altered distribution of BACE1 substrates Sez6 and Sez6L is likely a consequence of Aß plaque pathology and occurs at sites distinct to those of accumulation of APP, LAMP1 and BACE1, indicating their altered function. Although the function of Sez6 and Sez6L, as well as of numerous other BACE1 substrates, needs to be elucidated, the previously observed role of Sez6 in dendritic spine dynamics [16,30,46,47,50] supports our finding of distinct effect of Aß plaque accumulation on distribution of different BACE1 substrates, such as APP, Sez6 and Sez6L. Nevertheless, these results suggest that Aß-targeted therapies may indirectly revert defective Sez6/Sez6L localization in AD brains, in addition to that of APP, BACE1 and/or LAMP1.

The analysis of BACE1-mediated proteolysis of its substrates APP, Sez6 and Sez6L in 5xFAD mouse brains further supported our findings of distinct Aß-plaque effect on distribution of BACE1 and APP vs. Sez6 and Sez6L. Indeed, the ectodomain shedding of APP was substantially increased in 9-months old 5xFAD vs. wt brains, supporting that Aß-plaque load and accumulation of APP and BACE1 in the peri-plaque regions can contribute to further amyloidogenic processing of APP and the pathogenesis of AD. Indeed, recent study in human neurons derived from induced pluripotent stem cells revealed that oligomers and fibrils of Aβ enhance colocalization and physical interaction of APP and BACE1 in recycling endosomes, which leads to exacerbated amyloidogenic processing of APP and to a further accumulation of intracellular Aβ42 [62]. Although the used N-terminal APP antibody could not differentiate between α- and ß-generated increased levels of soluble APP fragments, we reasoned that due to enhanced BACE1 levels in 5xFAD brains and its accumulation in areas surrounding the Aß plaques, the increased sAPP total (α + ß) levels are likely generated by enhanced cleavage of APP by BACE1. Unfortunately, our attempts to directly analyze the levels of sAPPß fragments (using an anti-sAPPß) were unsuccessful. In contrast to observed increased ectodomain schedding of APP and increased sAPP levels in 5xFAD vs. wt brains, we did not detect altered proteolysis of Sez6 and Sez6L, the two preferential BACE1-substrates, upon Aß plaque load in 5xFAD vs. wt brains. It is very unlikely that this could be due to detection limitations of our western blot analysis of endogenous levels of Sez6/Sez6L proteins since in our previous study we could clearly demonstrate enhanced proteolysis of Sez6 and Sez6L at their endogenous levels in NPC mouse brains [40]. Thus, results of proteolytic analysis of the BACE1-substrates APP, Sez6 and Sez6L in 5xFAD vs. wt brains further confirm distinct effect of Aß plaque load on different BACE1 substrates and their (dys)function.

Overall, here we show for the first time that altered distribution of BACE1, its accumulation in peri-plaque regions and its enhanced levels in AD brains, does not affect proteolysis of BACE1 substrates other than APP, such as Sez6 and Sez6L, indicating that BACE1-related alterations in AD primarily affect function of APP. We also show that altered distribution of Sez6 and Sez6L in the area of Aß plaques in 5xFAD brains is distinct to that of APP, BACE1 and/or LAMP1, suggesting different localization and/or function of these BACE1 substrates. Moreover, our findings indicate that altered

distribution of Sez6 and Sez6L is likely a secondary effect upon Aß plaque load. While it is necessary to further elucidate the potential role that this may play in the course of AD, it is likely that Aß-targeted therapies may have beneficial effects against accumulaton and/or altered distribution of BACE1 and its substrates, in addition to APP.

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AUTHOR CONTRIBUTIONS

Conceptualization, Desanka Milanovic, Selma Kanazir and Silva Hecimovic; Formal analysis, Kristina Dominko, Ana Rastija, Lucija Lešnjaković and Kosara Smiljanic; Funding acquisition, Silva Hecimovic; Investigation, Kristina Dominko, Ana Rastija and Aleksandra Mladenovic; Resources, Selma Kanazir and Silva Hecimovic; Supervision, Desanka Milanovic, Selma Kanazir and Silva Hecimovic; Visualization, Kristina Dominko and Ana Rastija; Writing – original draft, Silva Hecimovic, Kristina Dominko and Ana Rastija; Writing – review & editing, Desanka Milanovic, Aleksandra Mladenovic and Selma Kanazir.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethical Committee for the Use of Laboratory Animals (resolution No. 01-06/13) of the Institute for Biological Research - National Institute of Republic of Serbia, University of Belgrade.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

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Figure legends

Figure 1. Characterization of pathological features in 5xFAD mouse model brains. Cerebral cortices of 5xFAD mice show Aβ plaque accumulation, neurofilament loss and lysosomal impairment already at 2-months of age. These features further progresses with age in 4- and 9-months old 5xFAD mice. Shown are the results of neurofilament (NFL, red), LAMP1 (green) and Amylo-GLO (blue) staining of wt and 5xFAD cortices at 2-, 4- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar –75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 2. BACE1 accumulates within LAMP1-positive vesicles in dystrophic neurites surrounding the Aβ plaques in the cerebral cortices of 5xFAD mice. Cryoslices were co-stained with BACE1 (green), Amylo-Glo (blue) and A) TfR (red) or B) LAMP1 (red) in wt and 5xFAD mouse cortices at 2- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures). C) Western blot analyses of LAMP1 and BACE1 levels in Triton-X fractions collected from cerebral cortices of 9-months old wt and 5xFAD (tg) female mice. Graphs represent protein levels relative to wt mice as mean ± SEM of the specific protein band density normalized to Amido black. The difference between groups was compared by two-tailed, unpaired t-test. The statistical significance of the tests was set at $p < 0.05$ (* <0.05; ** <0.005).

Figure 3. APP accumulates in LAMP1-positive axonal swellings surrounding Aβ plaques. Shown are the results of APP (green), LAMP1 (red) and Amylo-GLO (blue) staining of wt and 5xFAD mouse cortices at 2-, 4- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 4. BACE1 substrates Sez6 and Sez6L show altered distribution upon Aβ plaque accumulation in the cerebral cortices of 5xFAD mice. Sez6 and Sez6L did not colocalize with BACE1 upon Aβ plaque formation. Their immunostaining signal was found both in the core and in peri-plaque regions in the 5xFAD mouse cortices. Shown are the results of BACE1 (green) and Amylo-GLO (blue) immunostaining together with A) Sez6 (red) or B) Sez6L (red) in cortices of wt and 5xFAD mice at 2-, 4- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 5. BACE1 substrates APP and Sez6L differ in their distribution upon Aβ plaque formation in 5xFAD mouse cerebral cortices. Sez6L was mainly localized in the core of the plaques, while APP accumulated in the surrounding peri-plaque regions in 5xFAD mouse brains. Shown are the results of APP (green), Sez6L (red) and Amylo-Glo (blue) staining of wt and 5xFAD mice at 2-, 4- and 9-months

of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 6. Proteolysis of BACE1 substrates Sez6, Sez6L and APP in cerebral cortices of 9-months old female 5xFAD mice. (A-C) Western blot analyses of full length Sez6 (flSez6), Sez6L (flSez6L) and APP (flAPP) in Triton-X fractions and of N-terminal soluble Sez6 (sSez6), Sez6L (sSez6L) and soluble total APP (sAPPα/β) fragments in DEA fractions collected from cerebral cortices of 9-months old wt and 5xFAD (tg) female mice. Graphs represent protein levels shown relative to wt mice as mean ± SEM of specific protein band density normalized to Amido Black. The difference between groups was compared by two-tailed, unpaired t-test. The statistical significance of the tests was set at $p < 0.05$ (* <0.05; ** <0.005).

Figure 1

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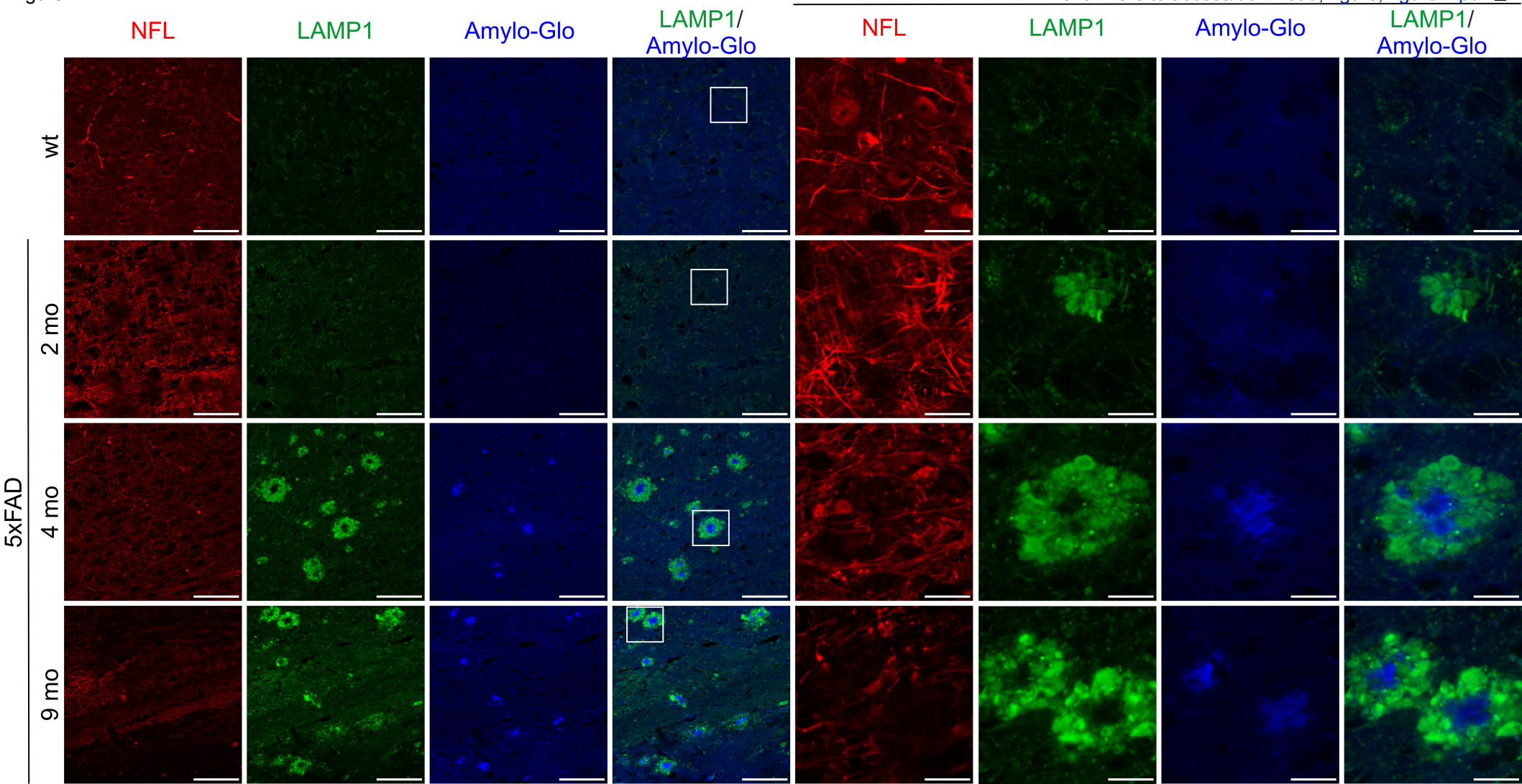

Figure 2a

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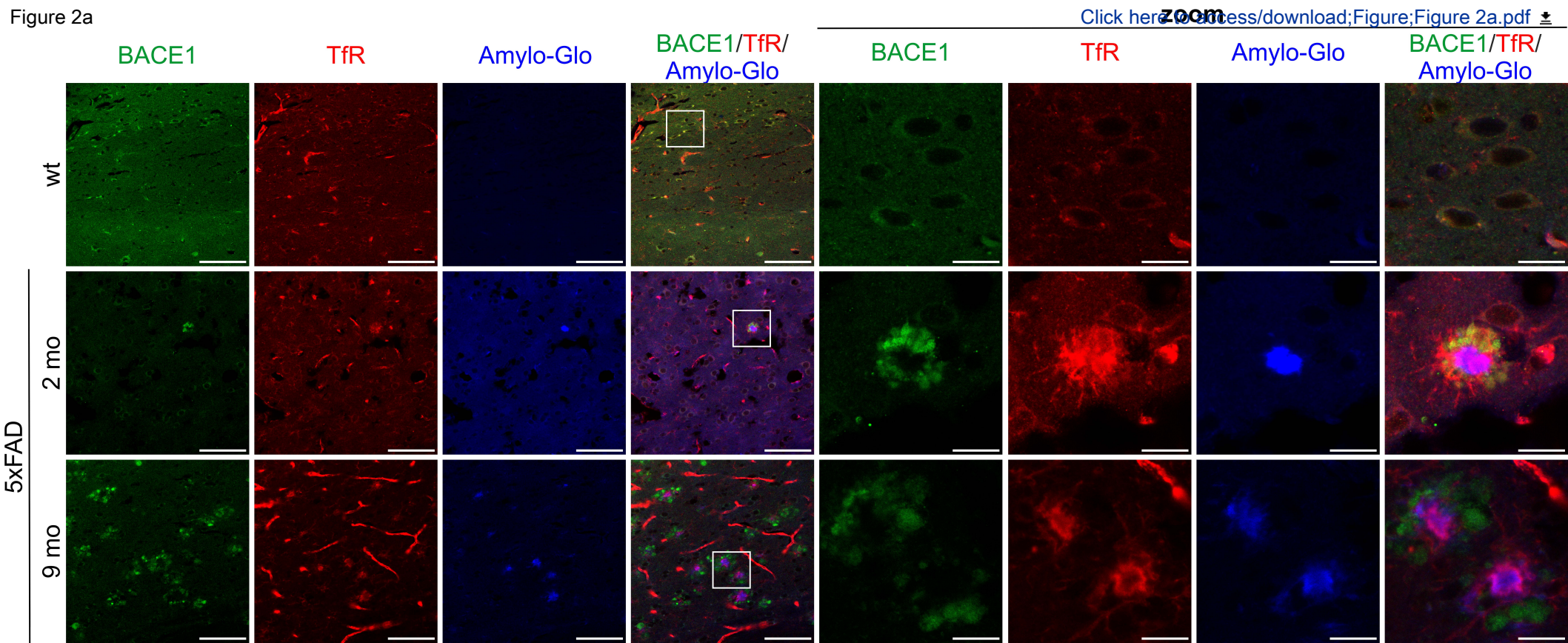

Figure 2b

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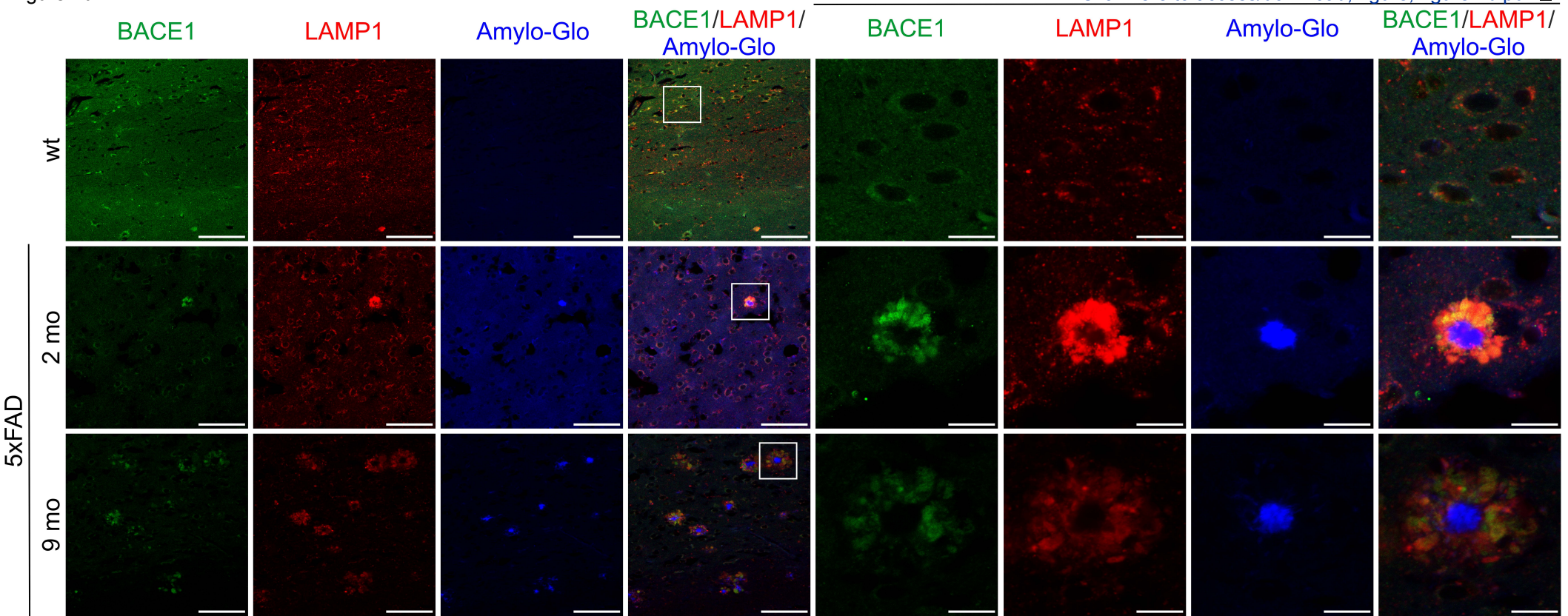
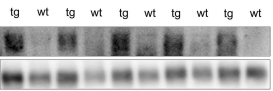
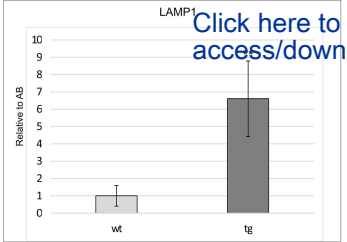


Figure 2c





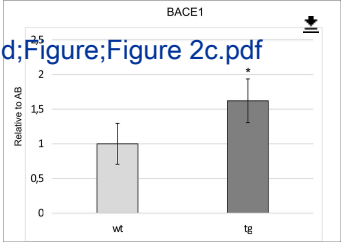
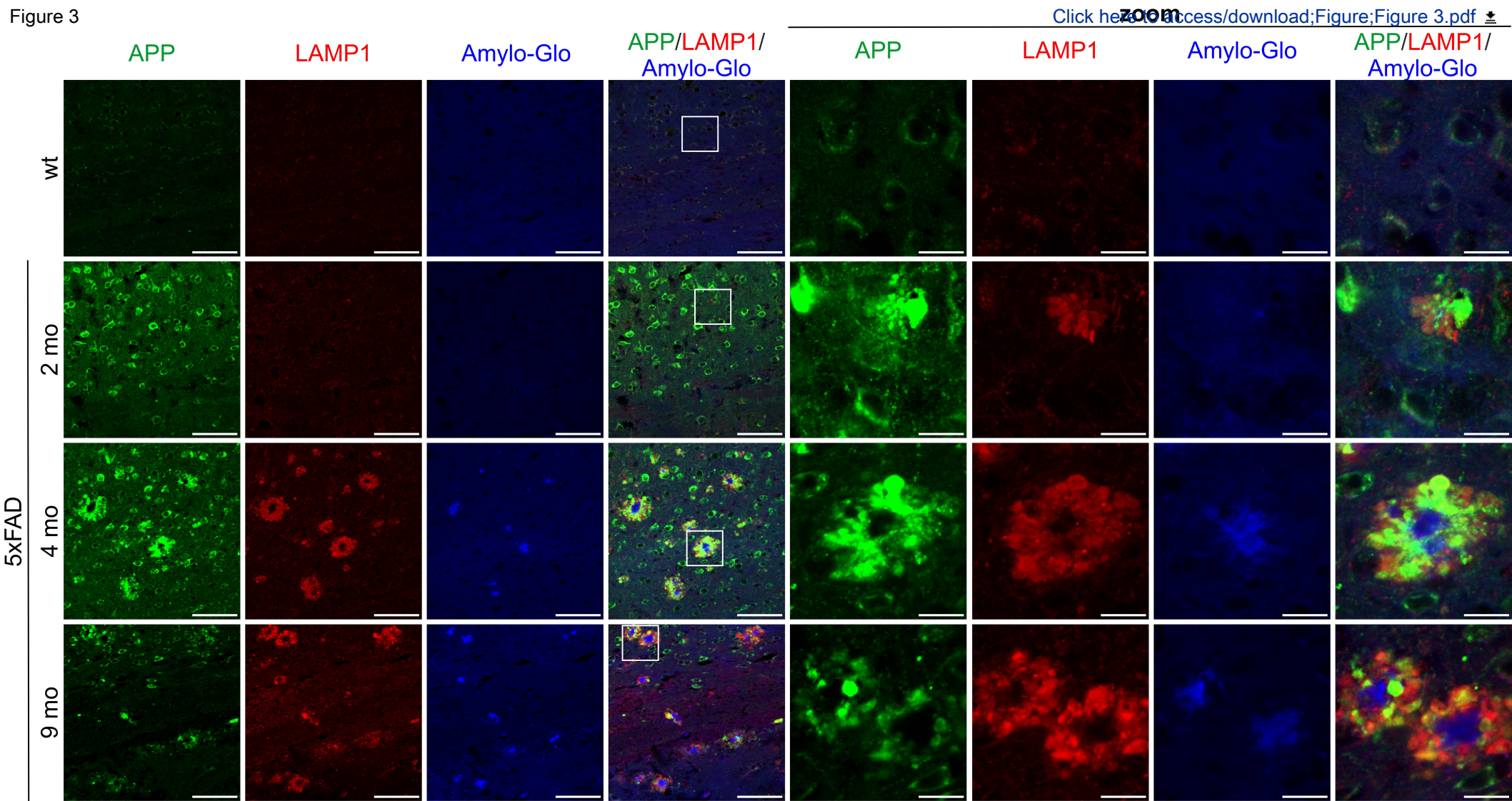


Figure 3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APP
LAMP1
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wt
5xFAD
2 mo
4 mo
9 mo

Figure 4a

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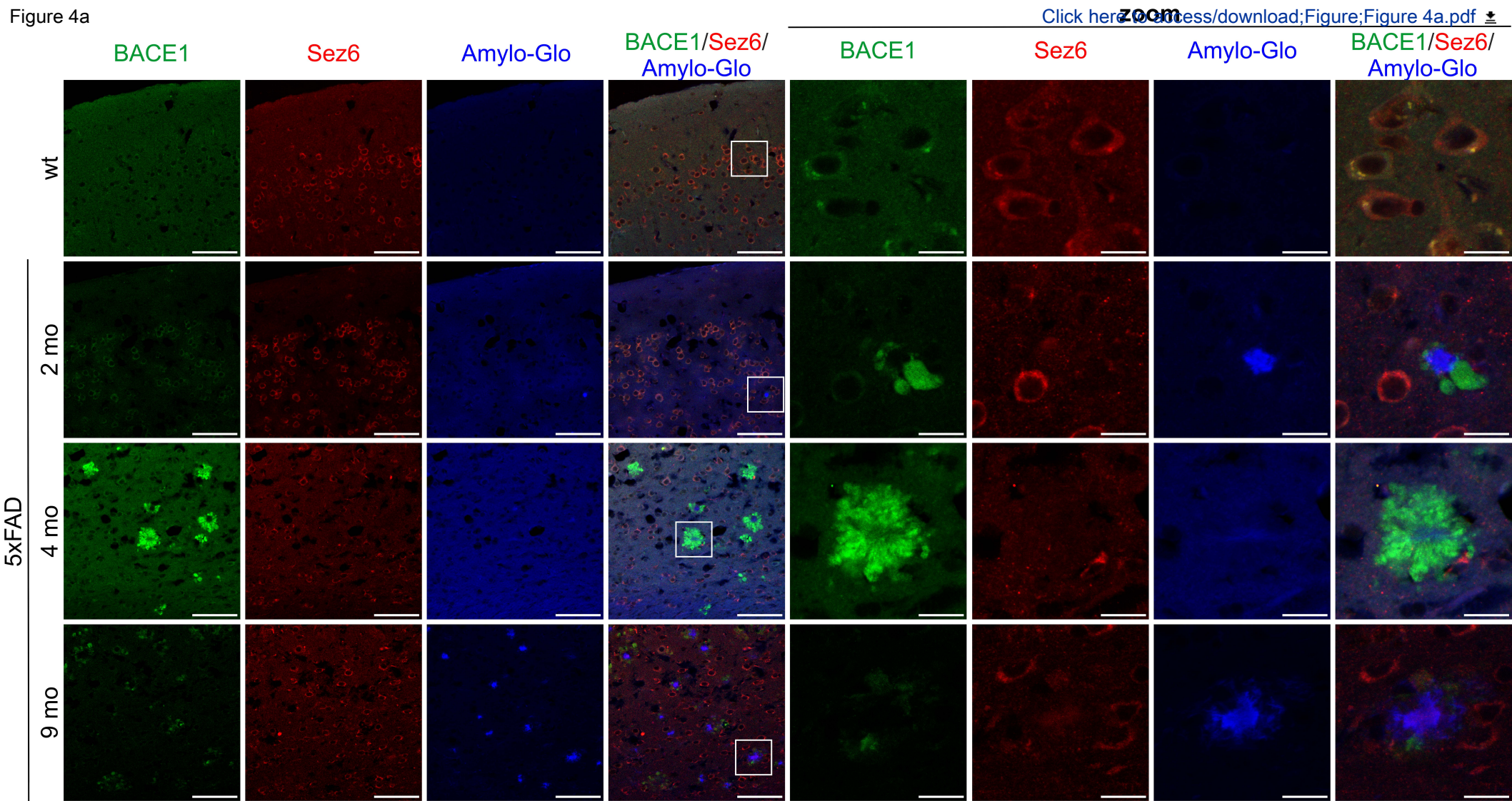

Figure 4b

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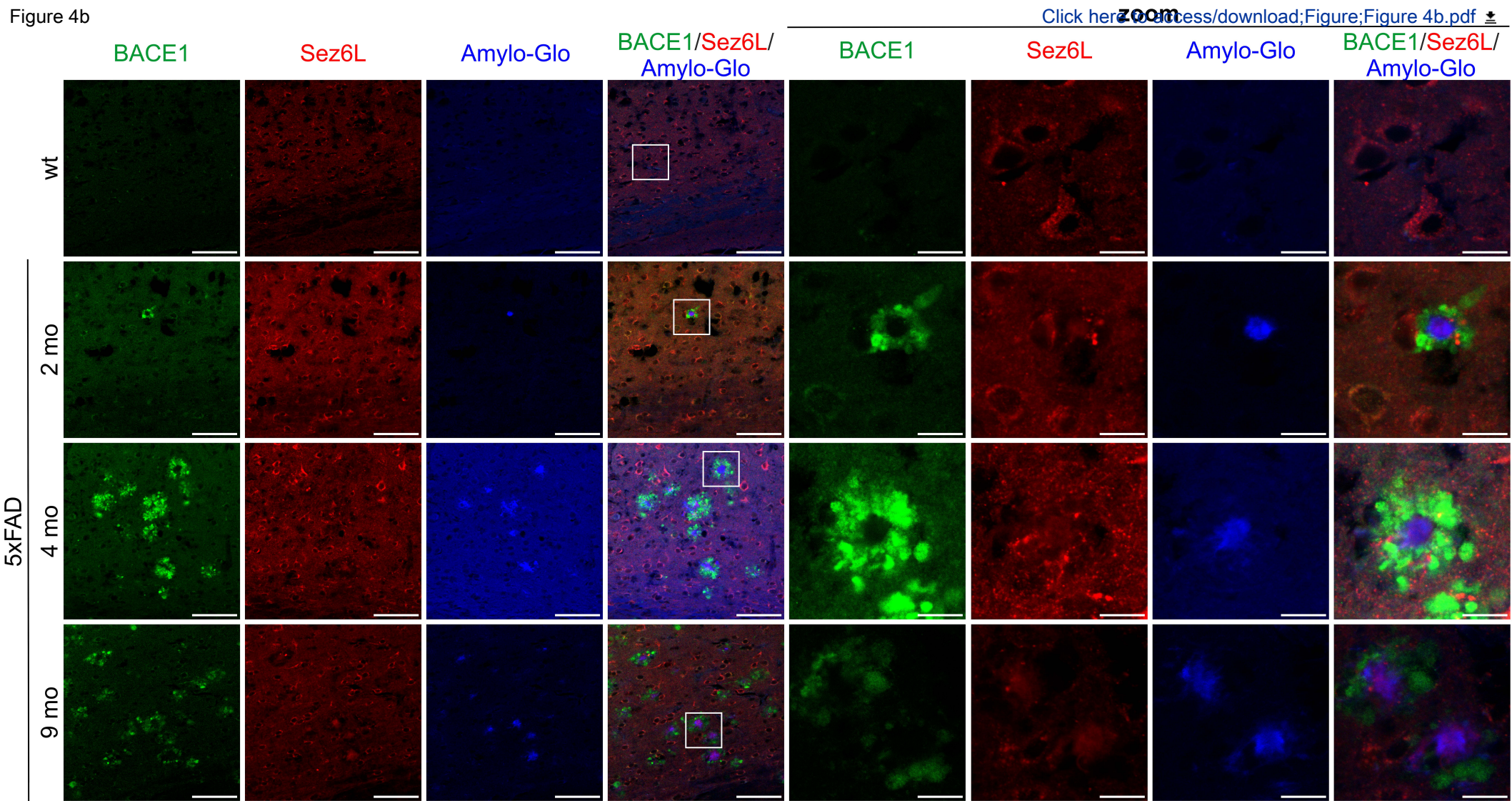
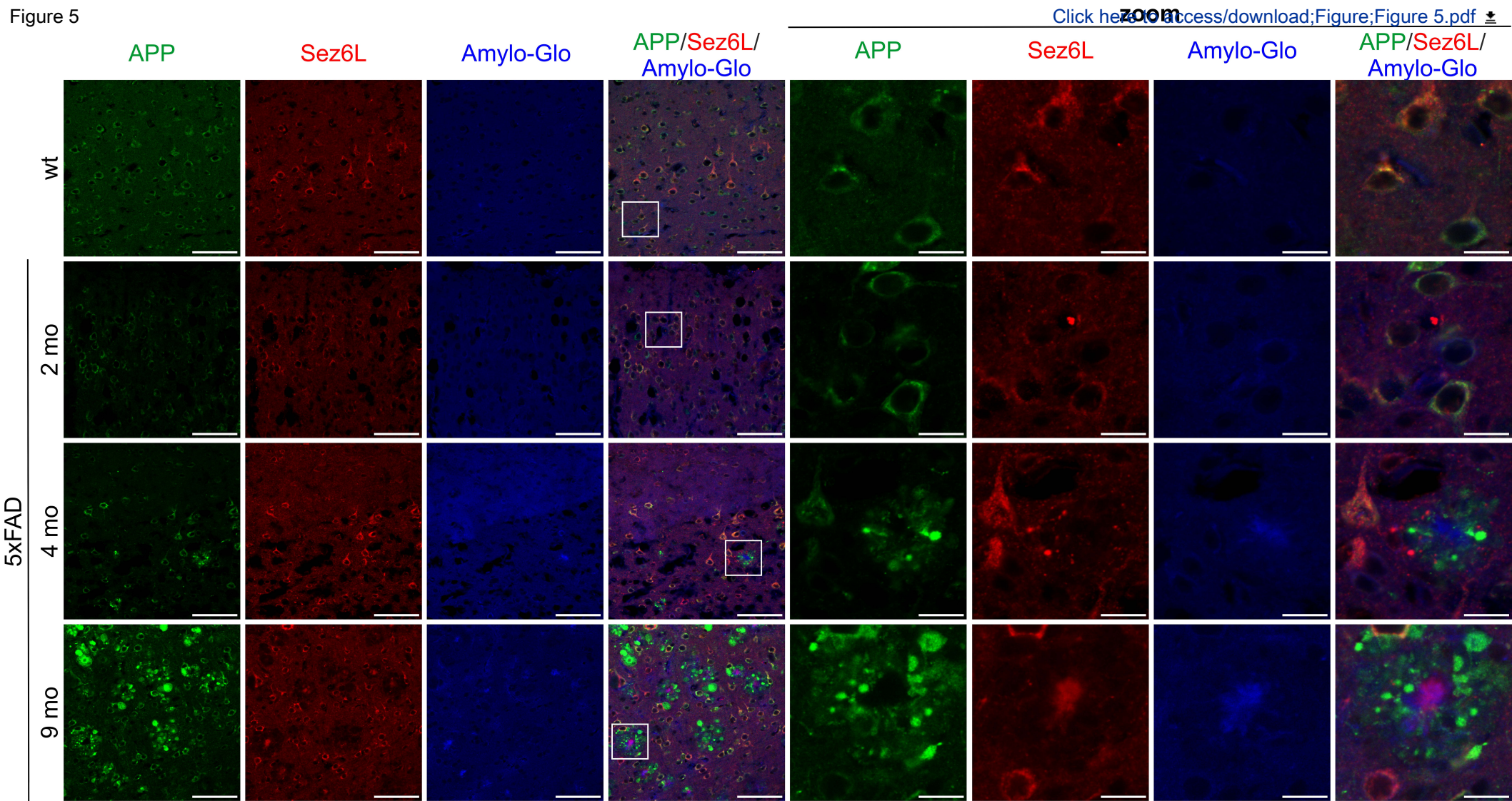


Figure 5



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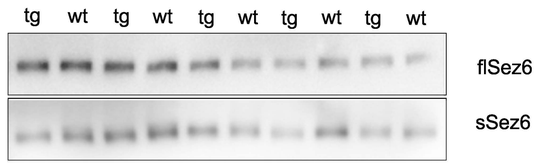

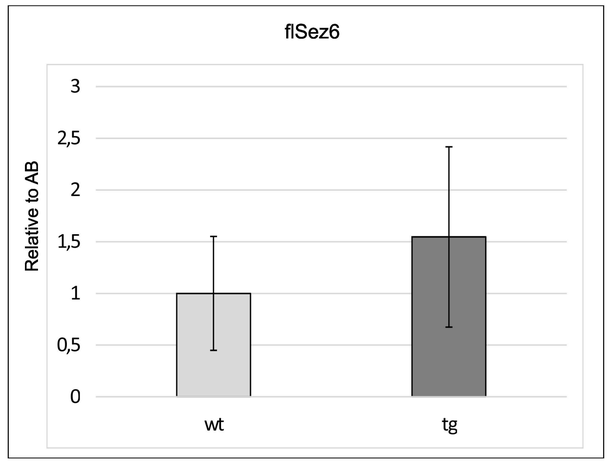

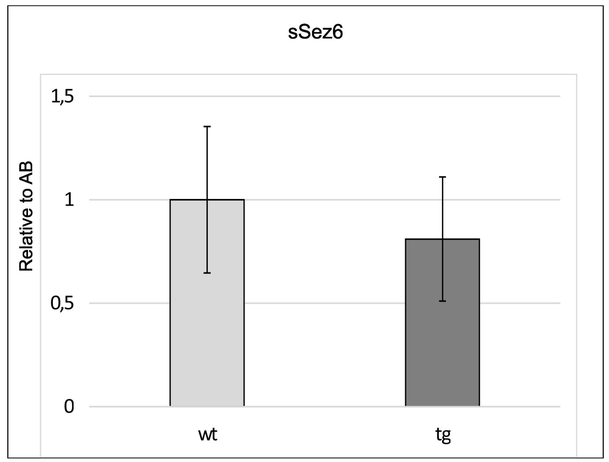

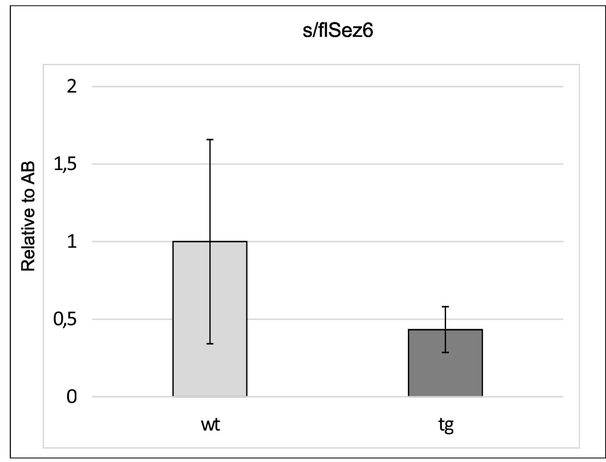

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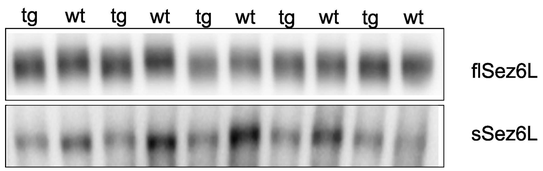

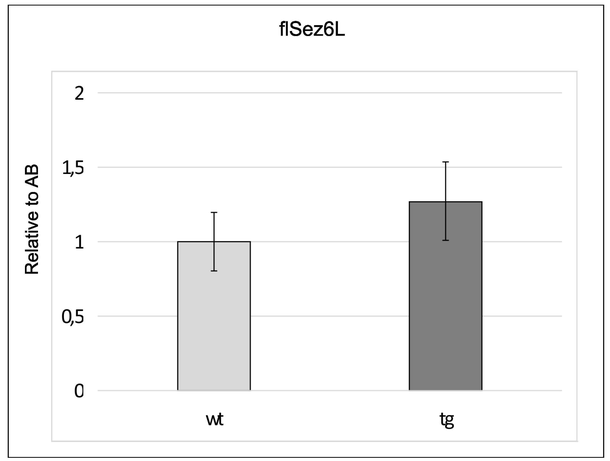

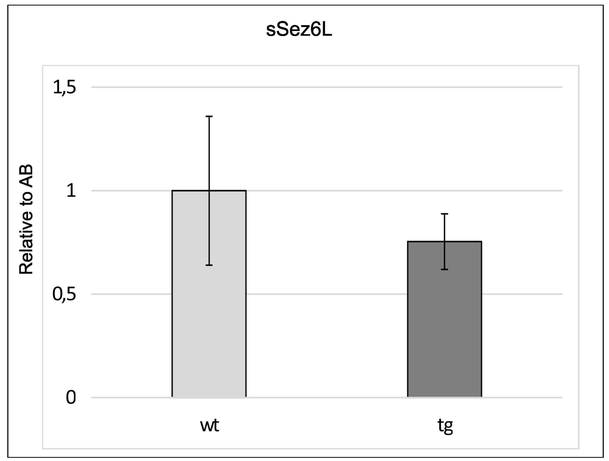

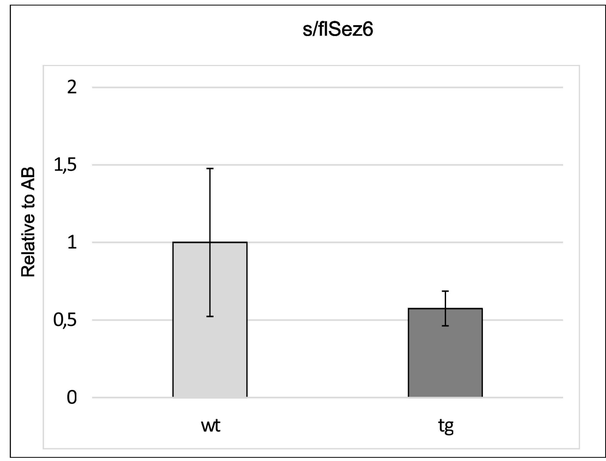

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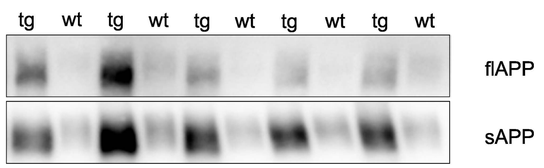

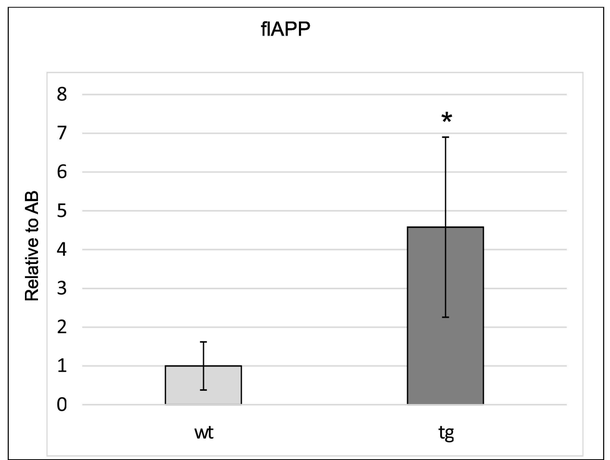

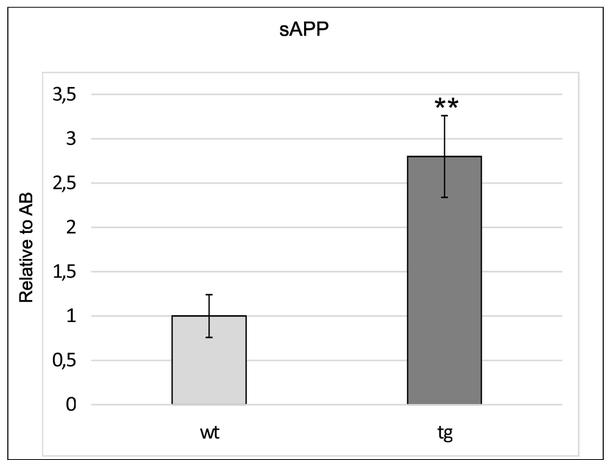

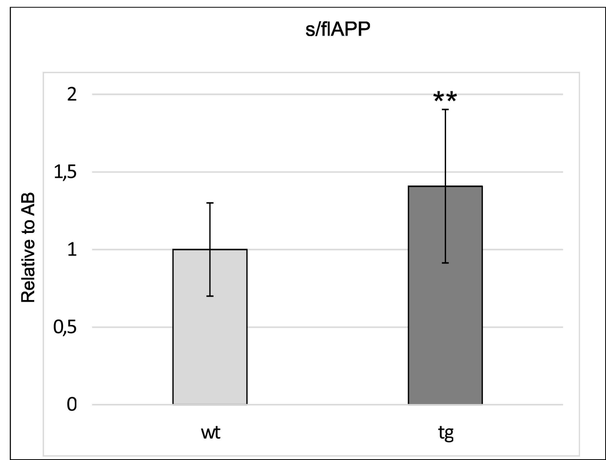

Figure legends

Figure 1. Characterization of pathological features in 5xFAD mouse model brains. Cerebral cortices of 5xFAD mice show Aβ plaque accumulation, neurofilament loss and lysosomal impairment already at 2-months of age. These features further progresses with age in 4- and 9-months old 5xFAD mice. Shown are the results of neurofilament (NFL, red), LAMP1 (green) and Amylo-GLO (blue) staining of wt and 5xFAD cortices at 2-, 4- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar –75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 2. BACE1 accumulates within LAMP1-positive vesicles in dystrophic neurites surrounding the Aβ plaques in the cerebral cortices of 5xFAD mice. Cryoslices were co-stained with BACE1 (green), Amylo-Glo (blue) and A) TfR (red) or B) LAMP1 (red) in wt and 5xFAD mouse cortices at 2- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures). C) Western blot analyses of LAMP1 and BACE1 levels in Triton-X fractions collected from cerebral cortices of 9-months old wt and 5xFAD (tg) female mice. Graphs represent protein levels relative to wt mice as mean ± SEM of the specific protein band density normalized to Amido black. The difference between groups was compared by two-tailed, unpaired t-test. The statistical significance of the tests was set at $p < 0.05$ (* <0.05; ** <0.005).

Figure 3. APP accumulates in LAMP1-positive axonal swellings surrounding Aβ plaques. Shown are the results of APP (green), LAMP1 (red) and Amylo-GLO (blue) staining of wt and 5xFAD mouse cortices at 2-, 4- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 4. BACE1 substrates Sez6 and Sez6L show altered distribution upon Aβ plaque accumulation in the cerebral cortices of 5xFAD mice. Sez6 and Sez6L did not colocalize with BACE1 upon Aβ plaque formation. Their immunostaining signal was found both in the core and in peri-plaque regions in the 5xFAD mouse cortices. Shown are the results of BACE1 (green) and Amylo-GLO (blue) immunostaining together with A) Sez6 (red) or B) Sez6L (red) in cortices of wt and 5xFAD mice at 2-, 4- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 5. BACE1 substrates APP and Sez6L differ in their distribution upon Aβ plaque formation in 5xFAD mouse cerebral cortices. Sez6L was mainly localized in the core of the plaques, while APP accumulated in the surrounding peri-plaque regions in 5xFAD mouse brains. Shown are the results of APP (green), Sez6L (red) and Amylo-Glo (blue) staining of wt and 5xFAD mice at 2-, 4- and 9-months of age representing data from three different experiments, n = 3 slices per animal, 6 animals per group. Zoomed picture is depicted by white square. Scale bar – 75 (unzoomed pictures) and 16 µm (zoomed pictures).

Figure 6. Proteolysis of BACE1 substrates Sez6, Sez6L and APP in cerebral cortices of 9-months old female 5xFAD mice. (A-C) Western blot analyses of full length Sez6 (flSez6), Sez6L (flSez6L) and APP (flAPP) in Triton-X fractions and of N-terminal soluble Sez6 (sSez6), Sez6L (sSez6L) and soluble total

APP (sAPPα/β) fragments in DEA fractions collected from cerebral cortices of 9-months old wt and 5xFAD (tg) female mice. Graphs represent protein levels shown relative to wt mice as mean ± SEM of specific protein band density normalized to Amido Black. The difference between groups was compared by two-tailed, unpaired t-test. The statistical significance of the tests was set at $p < 0.05$ (* <0.05; ** <0.005).

Supplementary Figure 1

Supplementary Figure 2

Title:

Amyloid-ß plaque formation and BACE1 accumulation in the brains of a 5xFAD Alzheimer's disease mouse model is associated with altered distribution and not proteolysis of BACE1 substrates Sez6 and Sez6L

Authors:

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Abstract

The formation of amyloid-ß peptides (Aß), that accumulate in Alzheimer's disease (AD) brains, involves proteolytic processing of the amyloid precursor protein (APP) firstly by ß-secretase (BACE1). Since BACE1 cleaves a plethora of other substrates, in this work we investigated whether the proteolysis and/or distribution of other BACE1 substrates, such as seizure protein 6 (Sez6) and seizure 6-like protein (Sez6L), is altered in AD. To test this we used 5xFAD mouse model brains that show an early accumulation of Aß plaques already at 2-months of age. Here we show for the first time that accumulation of BACE1 in peri-plaque regions and its enhanced levels in AD brains does not affect proteolysis of BACE1 substrates other than APP, such as Sez6 and Sez6L. We observed altered distribution of Sez6 and Sez6L in the area of Aß plaques in 5xFAD brains which is distinct to that of APP, BACE1 and/or LAMP1, suggesting different localization and/or function of these BACE1 substrates. While it is necessary to further elucidate the potential role that this may play in the course of AD, it is likely that Aß-targeted therapies may have beneficial effects against accumulation and/or altered distribution of BACE1 and its substrates, in addition to APP.

Keywords: amyloid-ß, APP, BACE1, ß-secretase, Sez6, Sez6L

1. INTRODUCTION

Accumulation and aggregation of amyloid-ß peptides (Aß) as Aß plaques in the brains is a pathological feature of Alzheimer's disease (AD), both of the most common and complex form of AD as well as the rare familial AD (FAD) [1]. The Aß peptides are generated by sequential proteolysis of the amyloid precursor protein (APP) by enzymes ß-secretase and γ-secretase [2,3] . The ß-site APP cleaving enzyme 1 (BACE1 – ß -secretase) catalyses the initial step in Aß formation [4–8], and, thus, inhibition of BACE1 has been considered as a promising strategy to reduce Aß levels and to treat AD [9–11]. However, since BACE1 cleaves number of other substrates [12–14], in addition to APP, its complete inhibition may not be an option due to potential side effects [15–17]. This is supported by the fact that BACE1 knockout (KO) mice display an array of complex neurological phenotypes, including growth retardation [18], memory deficits [11,19,20], hypomyelination [21,22] , seizures [23–25], axon guidance defects [26–28] and schizophrenia-like behaviors [29]. These BACE1 KO phenotypes likely reflect diverse functions of numerous so far identified and still unknown BACE1 substrates [30,31]. Among the BACE1 substrates, Sez6 and Sez6L were found to be preferentially cleaved by BACE1 [12,32]. The BACE1-inhibition markedly reduced the levels of Sez6 and Sez6L ectodomains (by 95 and 96%, respectively) [17]. Furthermore, BACE1-mediated inhibition of Sez6 proteolysis was shown to play a role in cognitive impairment [16,33] or hyperactivity [17]. Indeed, several clinical trials that tested BACE1 inhibitors against AD were halted due to either futility or safety reasons [15,31]. Nevertheless, it is important to better understand the biology of BACE1 and of its substrates to reveal their role in health and in disease, such as in AD [10,31].

Previous studies revealed that BACE1 levels are elevated in AD patients' and in APP transgenic mouse brains and that BACE1 accumulates in swollen presynaptic structures in neurons surrounding Aß plaques [34,35]. Studies using APP transgenic mouse models with fast (5xFAD) and slow (Tg2576) Aß plaque load demonstrated that accumulation of BACE1 occurs in parallel with Aß plaque formation and is an early event before neuronal loss [35]. Furthermore, accumulation of the lysosomal marker LAMP1 as well as the endosome marker transferrin receptor (TfR) was also observed in dystrophic neurites early upon Aß plaque formation [35,36], implying defective endolysosomal trafficking in the neuronal processes in close vicinity to Aß plaques. Moreover, enhanced levels of BACE1 were found to co-localize with TfR and/or with LAMP1 in peri-plaque regions of 5xFAD mouse brains [35–38], suggesting that in AD BACE1 accumulates in endosomal vesicles following its decreased degradation by lysosomes and its subsequent elevation. Importantly, BACE1 accumulation was found to be accompanied with enhanced immunostaining and co-localization with APP in peri-plaque regions, implying that enhanced processing of APP by BACE1 may further drive Aß production and promote AD [35].

Since BACE1 function and proteolysis of its substrate APP has been strongly linked to endolysosomal pathway both under normal and disease conditions [35–39], such as in AD, we reasoned that defects within the endolysosomes may affect proteolysis and function of other BACE1 substrates in AD, in addition to APP. Indeed, we have recently demonstrated that endolysosomal dysfunction in a rare lysosomal and lipid storage disorder Niemann-Pick type C causes enhanced cleavage of APP as well as of Sez6 and Sez6L by BACE1 in the brains of NPC1 mouse model [40]. In addition, we showed that elevated BACE1-mediated proteolysis in NPC is likely due to accumulation of its substrates in enlarged endosomal vesicles within neuronal processes [40,41]. It has been previously observed that enlarged early endosomes caused by accumulation of the BACE1-generated C-terminal APP fragment (APP-

CTFß) is one of the earliest pathological features of AD [42–45]. Here, we analyzed whether endolysosomal dysfunction in AD and BACE1 accumulation in the regions surrounding Aß plaques may lead to altered processing and/or distribution of other, more specific BACE1 substrates, Sez6 and Sez6L that are, in contrast to APP, preferentially cleaved by BACE1 [12,32].

Sez6 family members are mainly located in cell membranes of dendrites, in synaptic and postsynaptic fraction [30,46,47], while Sez6L is also found in the ER membranes [48]. In experimental mouse models, Sez6 family has been shown to participate in regulation of dendritic development, synapse maturation and excitatory postsynaptic potential in the cortex and hippocampus [46,49,50]. BACE1 inhibition or Sez6 KO leads to reduced spine density, spatial memory deficits, poor coordination and hyperactivity in mice, and intellectual disability and psychiatric disorders in humans [16,17,33,50]. The most recent study [49] reports a novel role of Sez6 family members as regulators of the system of the complement that contributes to pathological cell and synapse loss in ageing and diseases including Alzheimer's. As for the most of the BACE1 sheddome proteins, it remains unknown whether the biological function is mediated by the soluble ectodomain, or by transmembrane full length protein, but altered shedding may have direct consequences on the development of the neuropathology [49,51].

This study was performed on 5xFAD mice, the widely used transgenic mouse model of AD that recapitulates many AD-related phenotypes and has a relatively early and aggressive presentation [52–55]. 5xFAD mice develop amyloid pathology at 2-months of age, with accompanying astroglyosis, microglyosis and dystrophic neurites. Robust impairment in long-term potentiation and failure in certain behavioral tasks that begins at 4-months worsening with age suggest deficits in synaptic transmission. Also, gene expression analysis reveals that the most upregulated genes during 5xFAD life time were involved in inflammation pathways, while the most common down-regulated genes were related with pathways associated with synaptic transmission and signaling [55]. Therefore, we hypothesized that function of other BACE1 substrates, in particular those involved in synaptic function, may be impaired in the course of AD and Aß plaque formation and that this may add to the progression and the pathological features of AD. Thus, the goal of the study was to assess, using western blotting and immunochemistry, if the formation of Aß plaques and accumulation of BACE1 in 5xFAD brains over time, from 2- to 9-months of age, may affect proteolysis and/or localization of exclusive BACE1 substrates, such as Sez6 and Sez6L, in addition to APP.

Our findings suggest that formation of Aß plaques and accumulation of BACE1 in AD brains may affect localization of other BACE1 substrates, such as Sez6 and Sez6L, in addition to APP. While it is necessary to further elucidate the potential role that this may play in the course of AD, it is likely that altered brain distribution of BACE1 substrates is a consequence of the primary pathological feature of AD - Aß plaque formation. Thus, Aß-targeted therapies may have beneficial effects against accumulation and/or altered distribution of BACE1 substrates, other than APP.

2. MATERIALS AND METHODS

2.1. Animals

Transgenic 5xFAD mice and their background strain (B6SJLF1/J) used in this study were purchased from the Jackson Laboratory (Cat. No: 034840-JAX and 100012-JAX, Bar Harbor, Maine, USA) and maintained

on B6/SJL genetic background by crossing 5xFAD transgenic male mice with B6SJLF1/J female mice. The resulting F1 female offspring (heterozygous 5xFAD transgenic and non-transgenic, wild-type littermates as their controls) were used in experiments. Genotyping was performed by polymerase chain reaction (PCR) of tail DNA according to the supplier's protocol. 5xFAD mouse model bears five familial AD human mutations: three in the human amyloid precursor protein (APP) gene–Swedish (K670N, M671L), Florida (I716V), and London (V717I), and two in the human presenilin1 (PS1) gene - M146L and L286V, under transcriptional control of the neuron-specific murine Thy-1 promoter, as described by Oakley et al. [52].

The animals were housed in the plastic cages under standard conditions (23 ± 2 °C, 60–70% relative humidity, 12 h light/dark cycle), with free access to commercial rodent chow and water. Their health status was routinely controlled. All animal procedures were in compliance with the Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes and was approved by the Ethical Committee for the Use of Laboratory Animals (resolution No. 01-06/13) of the Institute for Biological Research - National Institute of Republic of Serbia, University of Belgrade. Minimal numbers of animals were used and all efforts were made to minimize animal suffering.

The 5xFAD mouse model, characterized by the earliest onset and most intense amyloid pathology, recapitulates almost all the neuropathological feature associated with human AD [52–55]. Presence of amyloid plaques is detected around 2-months of age (at asymptomatic phase), appearance of memory damages in prodromal phase occurs around 4-months of age, and cognitive deficits and neurotransmission impairments were reported around 9-months, at late AD-like stage [11,52]. Therefore, in this study we used 2-, 4- and 9-months old 5xFAD animals to examine if cleavage and distribution of Sez6 family proteins is changed at this crucial time points in 5xFAD pathology.

2.2. Tissue collection and preparation

When the 5xFAD transgenic and their non-transgenic, wild type littermates reached age of 2, 4 or 9 months (N = 6 mice per group and genotype), they were anesthetized by intraperitoneal injection of 100 mg/kg, Ketamidor (Richter Pharma, Wels, Austria) and 16 mg/kg Xylased (Bioveta, Czech Republic), transcardially perfused with ice-cold 0.1 M phosphate-buffer saline (PBS, pH = 7.4) and sacrificed. The brains were quickly removed and cerebral cortices were dissected under magnifying glass on the ice. The left hemispheres were stored at −80 °C for subsequent Western blot analyses, while the right hemispheres were further processed for immunohistochemical staining as follows. Tissues intended for immunohistochemical staining were fixed in fresh 4% paraformaldehyde (PFA) in PBS for 24 h and cryoprotected in 30% sucrose in PBS at 4 °C, until brains sink. Then the brains were frozen in isopentane cooled on dry ice and stored at −80 °C until use. Later, sagittal brain sections (18 µm thick) were cut on a cryostat (Leica, Wetzlar, Germany), according to the Allan Mouse Brain Atlas (Allen Institute for Brain Science (2008) available at: http:/alleninstitute.org/). Collection of all consecutive section started at a level ~ 0.24 mm lateral from midline, every 15th sections were mounted on the glass slides, immediately processed for immunohistochemistry staining or stored at -20°C for further use.

2.3. Western blot analysis

For analyses of different proteins cerebral cortices of five wt and five 5xFAD female mice were used at 9-weeks of age. Soluble (DEA) and membrane (Triton) fractions of brain lysates were prepared as previously described [12,40]. Brain lysates were mixed with 6 × sample buffer (60% glycerol, 12% SDS, 3% DTT, 1/8 v/v 0.5 M Tris pH 6.8, bromophenol blue or 60% glycerol, 12% SDS, 1/8 v/v 0.5 M Tris pH 6.8, bromophenol blue) and heated at 70 °C for 10 min. Equivalent amounts of protein were loaded onto Tris-Glycine gels and separated by SDS-PAGE. After SDS-PAGE, proteins were transferred onto PVDF membrane (Roche Applied Science, Basel, Switzerland) and incubated in primary antibody solution, followed by washing and HRP-conjugated secondary antibody incubation. Proteins were visualized by chemiluminescence using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, USA) on a documentation system from Uvitec, Cambridge, UK. Protein signals were quantified by using the ImageJ software (National Institutes of Health, USA). To control the same amount of protein, obtained protein signals were normalized to Amido Black (Supplementary figure 1). Comparison of protein levels between two groups of data (wt- vs.5xFAD mice) was performed by using a two-tailed, unpaired t-test. Differences were considered significant at a p value of <0.05 (* <0.05; ** <0.005).

2.4. Immunohistochemistry analysis

Cryosections were dried in the hood for 1 h, followed by 5 min incubation in 70% ethanol, 2 min in distilled H_2O and stained with Amylo-Glo dye [56] in saline solution for 5 min, then washed in distilled H_2O for 15 s and in TBS (50 mM TBS, pH 8.0), 3×5 min. Samples were then permeabilized in TBS-T (50 mM TBS, pH 8.0, 0.5% Triton X-100, Sigma-Aldrich, St. Louis, MO, USA) for 30 min, blocked in 5% goat serum (Sigma-Aldrich, St. Louis, MO, USA) in TBS-T for 1 h and incubated with primary antibodies diluted in 5% goat serum in TBS-T overnight. Antibodies used were: APP (C-terminal antibody, Epitomics), BACE-1 (Epitomics), LAMP1 (Santa Cruz), Transferrin Receptor (TfR, Life Technologies), neurofilament (NFL, Sigma-Aldrich), Sez6 and Sez6L (N-terminal antibodies, gift Dr. Lichtenthaler). Next day, sections were incubated with secondary antibody goat anti-rabbit-Alexa488, anti-mouse-Alexa594 and/or anti-rat-Alexa647 (Molecular Probes, Invitrogen, Waltham, MA, USA) for 3 h and mounted (Fluoromount, Sigma-Aldrich, St. Louis, MO, USA). Confocal images were acquired on an inverted laser scanning confocal microscope Leica TCS SP8 with the software LAS X (Leica, Wetzlar, Germany), and additional image processing and quantification were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3. RESULTS

3.1. Characterization of Aß plaque formation and distribution of BACE1 in 5xFAD mouse brains

The 5xFAD mice are characterized by an early and progressive Aß plaque pathology (already at 2-months of age), enabling monitoring of AD-related pathological processes in a timely manner [11,52]. We, thus, firstly analyzed formation of Aß plaques, neurofilament loss and lysosomal impairment in 2, 4- and 9-months old brains of 5xFAD mice vs. 9-months old wt mouse brains (Figure 1). Since our initial analysis revealed that 2-, 4- and 9-months old wt brains show similar staining (Supplementary figure 2), in this and in our further immunohistochemistry analyses we used 9-months old wt brains only as controls. Amylo-Glo staining [56] confirmed numerous plaques at 4-months as well as 9-months old

5xFAD compared to wt brains, while in 2-months old 5xFAD brains plaques were sparse. Gradual decrease of neurofilament (Nfl) staining was observed from 2-, 4- to 9-months old 5xFAD vs. wt brains, indicating neurodegeneration. Interestingly, increased staining of the lysosomal marker LAMP1 surrounding the core of Aß plaque was detected already at 2-months of age in 5xFAD brains (zoomed figure) and was clearly observed upon further accumulation of Aß plaque pathology in 4- and 9-months old 5xFAD vs. wt brains. Enhanced LAMP1 staining in peri-plaque regions indicates accumulation of lysosomes in axonal swellings/buttons and altered lysosomal trafficking along the processes that is in accord with previous findings [36]. Since enhanced staining of LAMP1 was strongly associated with plaque pathology, we reasoned that it is likely a consequence of Aß plaque formation/accumulation that causes the plaque-induced loss of neuronal network and loss of normal endolysosomal trafficking along their processes. Defective endolysosomal trafficking due to the plaque pathology was further confirmed by staining of transferrin-receptor (TfR), a marker of recycling endosomes [35,38]. In parallel to altered LAMP1 distribution, accumulation of TfR was observed already at 2-months old 5xFAD vs. wt brains (Figure 2a). However, in contrast to LAMP1 (Figure 1), TfR accumulated in the core of Aß plaques in 2-months old 5xFAD vs. wt brains. Overall, these findings indicate plaque-induced alterations of endolysosomal transport in the surrounding neuronal processes.

Since BACE1-mediated proteolysis and function is strongly linked to endolysosomal pathway [37,39,57–61], we further analyzed distribution of BACE1 upon Aß plaque pathology in 5xFAD vs. wt mouse brains. Increased BACE1 staining was observed already at 2-months of age around the core of Aß plaques (Figure 2a) in a pattern mainly distinct to TfR immunoreactivity. Indeed, staining of BACE1 resembled the staining and accumulation of LAMP1 upon Aß plaque formation (Figures 1 and 2b), with its enhanced immunoreactivity in the area surrounding Aß plaques. BACE1 co-stained with LAMP1 marker, indicating BACE1 accumulation in LAMP1-positive vesicles in axonal swellings of neurites surrounding the Aß plaques (Figure 2b). Further western blot analysis of BACE1 and LAMP1 revealed their increased levels in 9-month old 5xFAD vs. wt brains, supporting their accumulation due to defective lysosomal function and their degradation caused by Aß plaque pathology (Figure 2c).

3.2. Distribution of BACE1 substrates APP, Sez6 and Sez6L upon Aß plaque formation in 5xFAD mouse brains

To evaluate whether altered BACE1 distribution/levels upon Aß plaque load in AD brains affects BACE1 substrates other then APP, we analyzed localization and proteolysis of Sez6 ad Sez6L, the two previously reported substrates that are preferentially cleaved by BACE1 and not like APP by α-secretase. Firstly, we showed that in 5xFAD vs. wt brains in addition to Aß plaque formation, APP accumulates in the dystrophic neurites surrounding Aß plaques (Figure 3). The pattern of APP immunostaining (using C-terminal antibody Y188) is similar to that previously observed for BACE1 and LAMP1 (Figure 2b). APP fully colocalized with LAMP1 marker, indicating its accumulation together with BACE1 in LAMP1-positive vesicles in axonal swellings that surround the Aß plaques in the 5xFAD brains [36]. These findings further support that Aß plaque formation in AD brains alters trafficking within the endolysosomal pathway along surrounding neuronal processes leading to further accumulation of APP and BACE1 [35–38,62], the two key proteins in the pathogenesis of AD, and to further progression of the disease.

To analyze whether similar defect is observed for BACE1 substrates Sez6 and Sez6L we stained 5xFAD vs. wt brains with N-terminal antibodies against Sez6 and Sez6L, respectively. We have previously demonstrated reliable and valid analysis of Sez6 and Sez6L at their endogenous levels using these antibodies in the brains of Niemann-Pick type C disease mouse model [40]. Here, we show that Aß plaque formation in 5xFAD brains at 2- and 4-months of age caused slightly altered, punctuate immunostaining of Sez6 in the peri-plaque regions (Figure 4a), that was different to previously described profound APP, BACE1 and/or LAMP1 accumulation in the areas surrounding the Aß plaques in 5xFAD brains (Figures 2b and 3). At 9-months Sez6 staining was observed in Aß plaque core, while other surrounding healthy neurons showed Sez6 signal similar to that in wt brains. In parallel, punctuate staining of Sez6L was also detected in dystrophic neurites surrounding Aß plaques in 5xFAD vs. wt brains already at 2- and 4-months of age (Figure 4b). However, similar to immunostaining of Sez6, the obtained signal of Sez6L puncta was substantially lower in peri-plaque regions compared to accumulation of APP, BACE1 and/or LAMP1. While this may be likely due to their endogenous immunohistochemistry analysis, in contrast to APP which is exogenously expressed in a transgene in 5xFAD mouse brains, the accumulation of BACE1 and LAMP1 that are also endogenously stained indicates their profoundly altered distribution upon Aß plaque formation in AD brains . Interestingly, Sez6 and Sez6L punctuate staining in the peri-plaque regions of 5xFAD brains at 4-months of age did not show colocalization with BACE1 (Figures 4a and 4b), in contrast to what was observed for APP (Figure 3). In addition, at 9-months of age Sez6L accumulated in the Aß core region in 5xFAD brains (Figure 4b), similar to that observed for Sez6 (Figure 4a). We, thus, conclude that formation of Aß plaques alters distribution of other BACE1-substrates in addition to APP, such as Sez6 and Se6L, but locally and most likely as a consequence of the Aß plaque load in the regions of dystrophic neurites distinct to those that accumulate BACE1.

To further evaluate this we co-stained BACE1-substrates APP and Sez6L (Figure 5). Indeed, we showed that both BACE1 substrates nicely colocalize in healthy neurons in both wt and 5xFAD brains. However, upon Aß plaque formation at 4-months of age APP and Sez6L accumulated in distinct axonal buttons surrounding the Aß plaques. Different distribution of Sez6L and APP upon the plaque load was further observed in 9-months old 5xFAD vs. wt brains. Sez6L signal was mainly found in the core of the plaque, while APP accumulated in the surrounding peri-plaque regions. This finding further supports that formation of Aß plaques alters distribution of BACE1-substrates Sez6 and Se6L in peri-plaque regions, in addition to APP, but in dystrophic neurites and in distinct compartments compared to those that accumulate APP and BACE1, suggesting their distinct function/s.

3.3. Proteolysis of BACE1 substrates APP, Sez6 and Sez6L upon Aß plaque formation in 5xFAD mouse brains

Next, we analyzed proteolytic processing of BACE1 substrates Sez6 and Sez6L, in addition to APP, in 9-months old 5xFAD vs. wt brains, e.g. cerebral cortices. We speculated that altered endolysosomal trafficking and accumulation of BACE1, that was reported in the course of AD pathogenesis [35–38,44,45,63], may affect cleavage of BACE1-substrates, other than APP, such as Sez6 and Sez6L. However, we did not observe different levels of the soluble N-terminal BACE1-generated Sez6 and Sez6L fragments (sSez6 and sSez6L, respectively) between 9-months old 5xFAD and wt brains (Figure 6), indicating that upon AD pathogenesis and Aß plaque load BACE1-mediated proteolysis of these

other substrates Sez6 and Sez6L that are preferentially cleaved by BACE1 is likely unaffected. Indeed, we found enhanced levels of N-terminal APP fragments in 9-months old 5xFAD vs. wt brains, supporting that in AD alterations of BACE1-mediated proteolysis are likely specific for APP and most likely do not involve other BACE1-substrates, such as Sez6 and Sez6L. The enhanced proteolysis of APP in the peri-plaque regions could further contribute to the pathogenesis and progression of AD.

4. DISCUSSION

BACE1 is a central enzyme in the pathogenesis of AD as it catalyzes the first step in Aß formation by proteolytic processing of its larger precursor - protein APP [4–8]. Thus, inhibition of BACE1 has been considered as an attractive strategy to inhibit formation of Aß and to treat AD [9,10]. However, numerous BACE1 substrates [12,13], complex neurological phenotypes of BACE1-knockout mice and side effects that occurred during BACE1-inhibitor clinical trials [15,30,31], suggested an important role of the enzyme BACE1 and its substrates in normal brain function. Since previous studies have shown that BACE1 accumulates in the brain regions surrounding the Aß plaques and that its levels are enhanced in human and transgenic mouse AD brains [34,35], we reasoned to speculate that distribution and/or proteolysis of other BACE1 substrates, in addition to APP, may be altered in AD brains and that these defects may contribute to the pathogenesis and progression of AD. Furthermore, we hypothesized that BACE1 substrates involved in synaptic function and/or those that are preferentially cleaved by BACE1, like Sez6 and Sez6L [12,32], may be modified in AD brains. Indeed, in our recent work by Causevic et al. [40] we demonstrated that endolysosomal dysfunction in Niemann-Pick type C disease (NPC) mouse model brains leads to enhanced BACE1-mediated proteolysis of Sez6 and Sez6L, in addition to APP, already at the presymptomatic stage and that this defect is likely due to altered trafficking of BACE1 and of its substrates within endolysosomal compartments [40,41].

Using the well characterized transgenic AD mouse model with an early Aß plaque pathology in parallel with an early BACE1 accumulation in peri-plaque regions [11,35,52], 5xFAD mouse brains (e.g. cerebral cortices), here we show for the first time that Aß plaque formation alters distribution of BACE1 substrates Sez6 and Sez6L, in addition to APP. Although Sez6 and Sez6L immunosignals in AD brains were much lower, due to their analysis at endogenous levels, compared to that of a transgene expression of APP that carries triple FAD mutations, we could observe their punctuate staining in the peri-plaque regions of 5xFAD brains at 2- and 4-months of age, indicating their altered trafficking in surrounding neuronal processes. However, Sez6L puncta did not colocalize with APP puncta in the region surrounding the Aß plaque, suggesting that these two BACE1 substrates, APP and Sez6L, likely accumulate in spatially distinct compartments. While further analysis is necessary to elucidate the identity of Sez6 and Sez6L puncta upon Aß plaque load, previous studies in Sez6 KO mice and BACE1-inhibitor treated wild-type mice revealed the role of Sez6 in maintaining normal dendritic spine dynamics [16,30,33,46,47,50], rather than presynaptic function as indicated for APP and BACE1. Our immunohistochemistry analysis of APP, BACE1 and LAMP1 confirmed previous finding of their accumulation and strong colocalization in peri-plaque regions of 5xFAD brains [35–37]. According to the findings of Kandalepas et al. [35] these may likely represent enlarged presynaptic terminals (giant buttons) as a result of defective endolysosomal transport within the axons upon AD pathogenesis [36]. Indeed, BACE1 was found to accumulate in LAMP1-positive vesicles and to a lesser extent in TfR-positive endosomes in dystrophic neurites that surround an amyloid plaque in 5xFAD vs. wt brains.

This is in line with previous study by Gowrishankar et al. [36] that reported elevated levels of BACE1 in defective lysosomes that accumulate in swollen axons found locally in the area in close vicinity to Aß plaques. Although we observed strong colocalization of APP and BACE1 in the regions surrounding Aß plaque, other BACE1-substrates Sez6 and Sez6L did not show their colocalization with BACE1 in peri-plaque regions in 5xFAD brains, respectively. Indeed, at the later stage of the disease, at 9-months of age, their immunostaining signals were clearly separated – Sez6 and Sez6L were found in the Aß core, while BACE1 was present in the area surrounding the Aß plaque regions. Thus, we conclude that observed altered distribution of BACE1 substrates Sez6 and Sez6L is likely a consequence of Aß plaque pathology and occurs at sites distinct to those of accumulation of APP, LAMP1 and BACE1, indicating their altered function. Although the function of Sez6 and Sez6L, as well as of numerous other BACE1 substrates, needs to be elucidated, the previously observed role of Sez6 in dendritic spine dynamics [16,30,46,47,50] supports our finding of distinct effect of Aß plaque accumulation on distribution of different BACE1 substrates, such as APP, Sez6 and Sez6L. Nevertheless, these results suggest that Aß-targeted therapies may indirectly revert defective Sez6/Sez6L localization in AD brains, in addition to that of APP, BACE1 and/or LAMP1.

The analysis of BACE1-mediated proteolysis of its substrates APP, Sez6 and Sez6L in 5xFAD mouse brains further supported our findings of distinct Aß-plaque effect on distribution of BACE1 and APP vs. Sez6 and Sez6L. Indeed, the ectodomain shedding of APP was substantially increased in 9-months old 5xFAD vs. wt brains, supporting that Aß-plaque load and accumulation of APP and BACE1 in the peri-plaque regions can contribute to further amyloidogenic processing of APP and the pathogenesis of AD. Indeed, recent study in human neurons derived from induced pluripotent stem cells revealed that oligomers and fibrils of Aβ enhance colocalization and physical interaction of APP and BACE1 in recycling endosomes, which leads to exacerbated amyloidogenic processing of APP and to a further accumulation of intracellular Aβ42 [62]. Although the used N-terminal APP antibody could not differentiate between α- and ß-generated increased levels of soluble APP fragments, we reasoned that due to enhanced BACE1 levels in 5xFAD brains and its accumulation in areas surrounding the Aß plaques, the increased sAPP total (α + ß) levels are likely generated by enhanced cleavage of APP by BACE1. Unfortunately, our attempts to directly analyze the levels of sAPPß fragments (using an anti-sAPPß) were unsuccessful. In contrast to observed increased ectodomain schedding of APP and increased sAPP levels in 5xFAD vs. wt brains, we did not detect altered proteolysis of Sez6 and Sez6L, the two preferential BACE1-substrates, upon Aß plaque load in 5xFAD vs. wt brains. It is very unlikely that this could be due to detection limitations of our western blot analysis of endogenous levels of Sez6/Sez6L proteins since in our previous study we could clearly demonstrate enhanced proteolysis of Sez6 and Sez6L at their endogenous levels in NPC mouse brains [40]. Thus, results of proteolytic analysis of the BACE1-substrates APP, Sez6 and Sez6L in 5xFAD vs. wt brains further confirm distinct effect of Aß plaque load on different BACE1 substrates and their (dys)function.

Overall, here we show for the first time that altered distribution of BACE1, its accumulation in peri-plaque regions and its enhanced levels in AD brains, does not affect proteolysis of BACE1 substrates other than APP, such as Sez6 and Sez6L, indicating that BACE1-related alterations in AD primarily affect function of APP. We also show that altered distribution of Sez6 and Sez6L in the area of Aß plaques in 5xFAD brains is distinct to that of APP, BACE1 and/or LAMP1, suggesting different localization and/or function of these BACE1 substrates. Moreover, our findings indicate that altered distribution of Sez6 and Sez6L is likely a secondary effect upon Aß plaque load. While it is necessary to further elucidate the potential role that this may play in the course of AD, it is likely that Aß-targeted therapies may have

beneficial effects against accumulation and/or altered distribution of BACE1 and its substrates, in addition to APP.

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AUTHOR CONTRIBUTIONS
Conceptualization, Desanka Milanovic, Selma Kanazir and Silva Hecimovic; Formal analysis, Kristina Dominko, Ana Rastija, Lucija Lešnjaković and Kosara Smiljanic; Funding acquisition, Silva Hecimovic; Investigation, Kristina Dominko, Ana Rastija and Aleksandra Mladenovic; Resources, Selma Kanazir and Silva Hecimovic; Supervision, Desanka Milanovic, Selma Kanazir and Silva Hecimovic; Visualization, Kristina Dominko and Ana Rastija; Writing – original draft, Silva Hecimovic, Kristina Dominko and Ana Rastija; Writing – review & editing, Desanka Milanovic, Aleksandra Mladenovic and Selma Kanazir.

INSTITUTIONAL REVIEW BOARD STATEMENT
The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethical Committee for the Use of Laboratory Animals (resolution No. 01-06/13) of the Institute for Biological Research - National Institute of Republic of Serbia, University of Belgrade.

CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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Amyloid-ß plaque formation and BACE1 accumulation in the brains of a 5xFAD Alzheimer's disease mouse model is associated with altered distribution and not proteolysis of BACE1 substrates Sez6 and Sez6L

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