

Original Article

Intestinal Organoids from Alzheimer's Disease Transgenic Mice Reveal Structural and Molecular Gut Pathology

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ABSTRACT: Alzheimer's disease (AD) is the most common form of dementia, and early alterations in the gut may contribute to disease progression. The current study focused on generating intestinal organoids from 3xTg-AD transgenic and Wild Type (WT) mice to investigate the role of the gut-brain axis in AD. Intestinal organoid cultures were produced through isolation of leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5+) crypt cells from 4–5-month-old or 1-year-old intestinal tissue. Transmission electron microscopy (TEM) analysis of aged 3xTg organoids demonstrated evidence of impaired gut epithelial viability compared to WT controls. In aged 3xTg organoids, enlarged intercellular spaces, disrupted cell adhesions, diminished tight junction complexes, cellular debris, and amyloid-like fibrils were prominent findings. Immunostaining also demonstrated decreased E-Cadherin and ZO1 expression, along with increased cellular and luminal A β in aged 3xTg organoids compared to young 3xTg and WT organoids and p-tau protein accumulation in aged 3xTg organoids compared to all other experimental groups. In the aged 3xTg cohort, increased immunoreactivity of inflammasome components, including IL-1 β , ASC, and GSDMD, implicated pyroptosis as a potential mechanism of cell death. These results support the hypothesis that familial AD pathology includes pronounced effects on gut organoid viability, junctional integrity, and abnormal protein accumulation within the intestinal epithelium. This study provides early evidence of gut organoid abnormalities occurring independently of alterations in the AD brain. These findings suggest that the gut epithelium may warrant further investigation as a potential target for future therapeutic strategies in AD.

Keywords: Alzheimer's disease, Intestinal organoids, Gut-brain axis, Stem cells, Transgenic, Amyloid- β

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia worldwide [1]. The neuropathology of AD is characterized by several hallmark features, including aggregation of amyloid- β (A β) plaques. A β is a naturally occurring protein that can become misfolded and resistant to clearance, contributing to chronic inflammation,

neuronal degeneration, and cell loss [2]. Another defining pathological feature of AD is the presence of neurofibrillary tangles composed of hyperphosphorylated tau protein (p-tau), which contribute to synapse loss and cognitive impairment [3]. Although these pathologies have long been considered primary drivers of AD, therapies targeting A β and tau have shown only limited benefit on neurological outcomes [4].

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Increasing evidence suggests that gut health may significantly influence brain health. The gut is often referred to as the “second brain” [5] because of its bidirectional communication with the central nervous system through hormonal, immunological, and neuronal pathways [6]. The gut-brain axis (GBA) plays a critical role in maintaining gut microbiome (GMB) homeostasis and preserving intestinal barrier integrity, both of which are essential for host health [7]. However, when the intestinal barrier becomes permeable, immune responses may become detrimental to the host. A “leaky gut” can develop with normal aging, but it may also be prematurely induced by chronic gut dysbiosis [8] or systemic inflammatory and endotoxic states [9]. Increased gut permeability permits enteric bacteria and pathogens to enter systemic circulation and contribute to disease progression.

Previous studies have linked gut microbiome dysfunction to worsened outcomes in several neurodegenerative diseases, including Multiple Sclerosis [10], Huntington’s Disease [11], Parkinson’s Disease [12], and Alzheimer’s Disease [13]. In particular, the inflammasome, a component of the innate immune system, has been implicated in gut-brain axis dysfunction associated with neurodegeneration [14]. The inflammasome is a multiprotein complex involved in activation of pro-inflammatory caspase-1, apoptosis-associated speck-like protein (ASC), and downstream inflammatory cytokines, resulting in Gasdermin-D (GSDMD)-mediated pyroptosis [2]. Previous work from our laboratory demonstrated that inflammasome-mediated pyroptosis contributes to bidirectional GBA dysfunction following stroke in AD mice [15]. We have also shown that traumatic brain injury (TBI) induces inflammasome activation and GSDMD-mediated pyroptotic signaling in both the brain and gastrointestinal tract [16]. Although these *in vivo* studies support a role for inflammasome signaling in gut dysfunction, the epithelial-specific mechanisms underlying gut pathology in neurodegenerative disease remain poorly understood.

The present study was designed to evaluate the ultrastructural characteristics of intestinal organoids generated from 3xTg and WT mice, assess the presence of hallmark AD-related proteins, and determine whether inflammasome-associated epithelial dysfunction occurs without systemic inflammatory input using a novel intestinal organoid model. Organoid systems represent a major advancement in *in vitro* cell culture techniques [17]. Although AD organoid research has largely focused on brain organoids [18, 19], there remains a need for comparable studies using intestinal organoids derived from experimental and clinical tissues to better define the role of the gut in AD pathogenesis [20].

The overall goal of this study was to generate intestinal organoids from 3xTg AD mice and evaluate morphological features of gut epithelial cells, including cell-cell adhesion and tight junction integrity, which are often difficult to visualize in standard histological sections [21]. We also investigated whether gut epithelial pathology and inflammasome/pyroptotic signaling could arise independently of systemic injury, immune cells, or circulating inflammatory factors. Using intestinal organoids generated from young and aged AD transgenic and WT mice, we examined the mechanistic contribution of intestinal pathology to AD progression. Immunohistochemistry (IHC) and transmission electron microscopy (TEM) analyses demonstrated disrupted cellular organization, impaired adhesion and tight junction proteins, increased accumulation of AD-related proteins, and elevated inflammatory and cell death markers in 3xTg organoids compared to WT controls. These findings support the role of a dysfunctional gut-brain axis in AD pathogenesis and may contribute to the development of novel therapeutic strategies targeting the gut in AD and related dementias.

MATERIALS AND METHODS

Animal Model

For this study, we used the 3xTg (B6;129-Tg(APP^{Swe}, tau^{P301L})1LfaPsen1^{tm1Mpm}) AD transgenic mouse model and corresponding WT (B6129SF2) controls. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami Miller School of Medicine (Animal Welfare Assurance #A3224-01, #IPROTO202300000150; effective date 09/01/23).

The 3xTg model contains three humanized gene mutations that recapitulate major hallmarks of familial AD pathology [22]. Mutations in presenilin-1 and amyloid precursor protein promote A β plaque formation similar to that observed in humans [23], while mutation of the human microtubule-associated protein tau results in neurofibrillary tangle formation. Male and female mice were harvested at either 1 year of age (aged cohort) or 4–5 months of age (young cohort). The younger cohort was selected because neuropathological symptoms in 3xTg mice generally begin at approximately 6 months of age [22], allowing examination of pre-symptomatic intestinal alterations. All diseased and healthy comparisons were age-matched.

Intestinal Organoid Culture

Isolation of leucine-rich repeat-containing G protein-coupled receptor 5-positive (Lgr5+) crypt cells from

mouse intestinal tissue was performed to generate intestinal organoids in culture [21, 24]. Crypt cells are stem cells located at the base of intestinal crypts that continuously divide and differentiate into various intestinal epithelial cell types [21]. Isolation was performed using a StemCell Technologies protocol (IntestiCult™ Organoid Growth Medium, Mouse).

Following sacrifice, approximately 20 cm of small intestine was collected and washed in cold PBS. Blood samples were collected simultaneously, centrifuged at $2000 \times g$ for 10 min at 4°C, and serum supernatants stored at -80°C for V-Plex protein analysis. Following crypt isolation, successful isolation was confirmed using inverted microscopy. Crypts appeared as small folded epithelial monolayers in suspension. Cells were then plated in Matrigel Matrix in 6-well plates and cultured for at least 5 days.

Each isolation from a single mouse yielded approximately 1500 organoids distributed across six wells. WT organoids typically yielded up to 2100 organoids per isolation, whereas 3xTg mice yielded fewer organoids, averaging approximately 900. WT and 3xTg tissues were isolated side-by-side for each experiment. A total of 16 side-by-side isolations (biological replicates) were completed, including 9 aged and 7 young cohorts. Organoids from separate isolations were used for IHC and TEM analyses.

Organoids were pooled, fixed in 4% paraformaldehyde, infiltrated with 30% sucrose, embedded in M-1 embedding matrix, and cryosectioned for immunostaining. For quantification, organoid groupings derived from a single animal and sectioned on the same slide were analyzed collectively as one biological replicate (N).

Immunohistochemistry

Organoids embedded in M-1 Matrix were stored at -20°C for at least 24 h before cryosectioning at 5 µm thickness. Antigen retrieval was performed using sodium citrate buffer (20 min in a steamer, pH 6.0), followed by permeabilization with 1% Triton X-100 for 10 min. Sections were blocked for 4 h in 5% normal goat serum in PBS and incubated overnight at 4°C with primary antibodies.

Organoids were double-labeled using antibodies against proteins of interest and cell-specific markers. Cell markers included CRP1, a cytoskeletal protein (Invitrogen, PA5-118824, 1:100), and LGR5, a marker of adult intestinal stem cells (Invitrogen, MA5-25644, 1:300). Tight junction-associated antibodies included ZO1 (Abcam, ab221547, 1:500) and E-Cadherin (Abcam, ab233766, 1:1000). AD-related protein antibodies included Tau phospho S396 (Abcam, ab32057, 1:500)

and Amyloid-β (NOVUS, NBP2-13075, 1:300). Inflammasome-associated antibodies included Gasdermin-D (GSDMD) (Invitrogen, NBP2-33422, 1:200), ASC (Santa Cruz, sc-271054, 1:300), and IL-1β (Cell Signaling Technologies, 12242S, 1:100).

Antibody validation was confirmed by manufacturers, and secondary-only negative controls were included to verify specificity. After 24 hours of primary antibody incubation, slides were incubated for 2 h at room temperature with goat anti-rabbit and goat anti-mouse secondary antibodies. Sections were washed with 0.05% Tween-20, mounted using Vectashield Vibrance Anti-fade Mounting Medium with DAPI (Vector Labs), and stored at 4°C in darkness until imaging. Confocal imaging was performed using a 63× objective, and analysis was conducted using IMARIS software. Laser intensity and saturation settings were standardized across all samples.

Transmission Electron Microscopy

Pelleted organoids were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer containing 100 mM sucrose and post-fixed for 1 h in 1% osmium tetroxide. Samples were dehydrated through graded ethanol series and embedded in EM-bed/Araldite resin (Electron Microscopy Sciences, Hatfield, PA). Semi-thin 1 µm sections were stained with Richardson's stain and examined under light microscopy. Ultrathin 100 nm sections were cut using a Leica Ultracut-R ultramicrotome (Leica Biosystems, Deerfield, IL), stained with uranyl acetate and lead citrate, and examined at 80 kV using a JEOL JEM-1400 transmission electron microscope. Images were captured using an AMT BioSprint 12 digital camera (AMT Imaging Systems, Woburn, MA).

Image Analysis

Fluorescent image analyses were performed using ImageJ software to quantify mean fluorescence intensity as an indicator of relative protein expression [25]. Images were converted to 16-bit using grayscale thresholding to isolate organoids from background fluorescence, generating regions of interest (ROIs) limited to organoid structures. Thresholding also served as an initial attempt to normalize background fluorescence and reduce noise across samples. Mean intensity was used to quantify fluorescence intensity. This metric was chosen because it accounts for both the intensity of staining and the size of the ROI, making it well-suited for comparing fluorescence between samples of varying size and morphology. Quantitative analysis of horizontal junctional distribution for E-Cadherin and ZO1 was performed using the algorithm previously developed [26].

Line-scan intensity profiles were generated from straight-line ROIs drawn perpendicular to cell-cell junctions in Fiji on preprocessed single-channel TIFF images. Approximately 10 junctional ROIs were collected per image, with 4 images analyzed per group ($n = 4$ images/group). Both TEM and IHC analyses were conducted by investigators blinded to experimental group assignments. For line-scan junctional analysis, one representative image was analyzed per biological replicate, with approximately 10 junctional ROIs collected per image and averaged to yield a single image-level (biological-replicate-level) value; four biological replicates were analyzed per group ($N = 4$ biological replicates/group).

Statistical Analysis

For tight junction analysis, raw ROI intensity profiles were reduced to one image-level mean intensity value per channel, with the image serving as the unit of analysis. Statistical analysis was performed using SciPy, a scientific computing package for Python. Profiles were aligned to a normalized distance axis and plotted as mean $\pm$ SEM for aged WT and aged 3xTg groups. For fluorescence analyses, one-way ANOVA with multiple comparisons was performed between WT and 3xTg groups across young and aged cohorts. Normality was assessed using Shapiro-Wilk test, and homogeneity of variance was assessed using Bartlett's test. Tukey post hoc testing was used to evaluate group differences ($*p \leq 0.01$, $**p \leq 0.001$, $***p \leq 0.001$). Statistical analyses and graphs were generated using Prism 10 software.

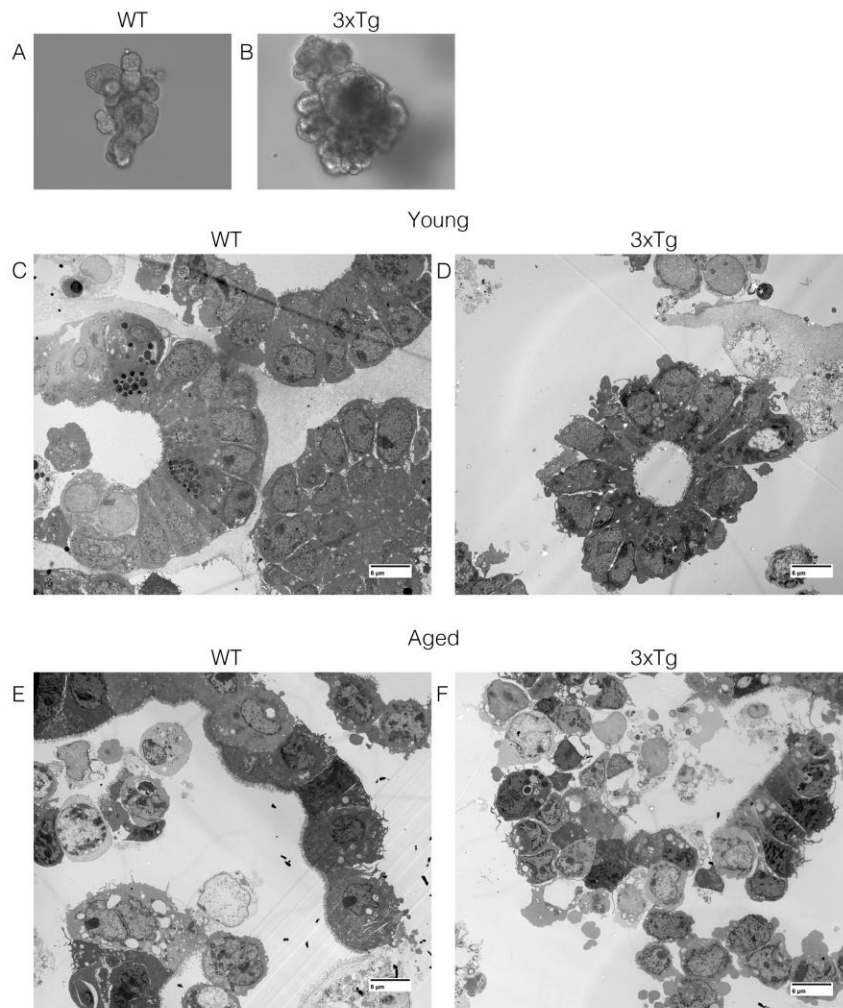


Figure 1. Morphology of young and aged WT and 3xTg intestinal organoids. Representative brightfield images of WT (A) and 3xTg (B) intestinal organoids from aged cohorts (20 $\times$ magnification). Representative transmission electron microscopy (TEM) images of young and aged WT (C, E) and 3xTg (D, F) organoids illustrating structural organization and cytoplasmic organelles. Young and aged WT organoids appear organized into a single polarized monolayer, whereas aged 3xTg organoids demonstrate reduced structural integrity and poorly defined luminal organization. Scale bars indicate image magnification.

RESULTS

Morphological Changes in Organoids Derived from 3xTg and WT Intestinal Stem Cells

As shown in Figure 1A and B, representative brightfield images show intestinal organoids across experimental cohorts. Using TEM imaging, organoids derived from Lgr5⁺ intestinal stem cells (ISCs) of transgenic 3xTg mice demonstrated evidence of impaired gut epithelial viability compared to WT controls. Distinct morphological abnormalities affecting both overall

organoid integrity and individual cellular structure were consistently observed when comparing WT organoids (Fig. 1C, E) with 3xTg organoids (Fig. 1D, F).

Young (Fig. 1C, D) and aged WT organoids (Fig. 1E) appeared more structurally organized and were more likely to develop into a single polarized epithelial monolayer with clearly defined apical and basal surfaces characteristic of intestinal organoids (Fig. 1C, E). In contrast, aged 3xTg organoids (Fig. 1F) demonstrated reduced structural stability and were less likely to maintain a well-defined lumen compared to aged WT organoids (Fig. 1E).

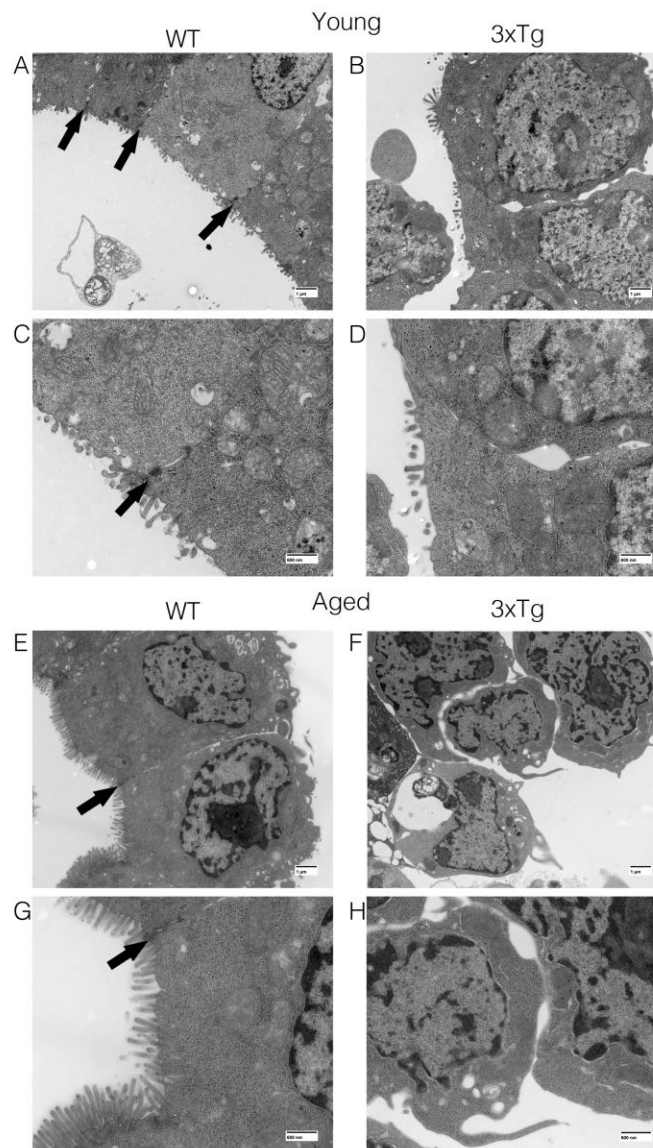


Figure 2. Ultrastructural Evidence of Epithelial Barrier Disruption in young and aged WT and 3xTg organoids. Representative TEM images illustrating microvilli morphology, tight junctions, and cell adhesions in WT and 3xTg organoids from young (A–D) and aged (E–H) cohorts. WT organoids (left column) demonstrate intact tight junction complexes in both young and aged specimens (arrows). In contrast, young and aged 3xTg organoids (right column) display disrupted cell adhesions, loss of tight junctions, and enlarged intercellular spaces between adjacent cells

Tight Junction Changes in 3xTg and WT Organoids

As shown in Figure 2, representative TEM images highlight additional structural abnormalities in young and aged 3xTg organoids, which were absent in aged WT specimens. In 3xTg organoids, cells frequently appeared to grow on top of or between adjacent cells, disrupting normal apical-basal organization. Enlarged intercellular spaces between neighboring cells (Fig. 2B, D, F, H), disrupted lateral cell-cell adhesions, and diminished tight junctions were also observed in the 3xTg groups compared to WT organoids. These morphological differences were most pronounced in the aged 3xTg group (Fig. 2F, H).

Both young and aged 3xTg organoids demonstrated fewer intact tight junctions identified by TEM compared to WT groups (Fig. 2B, D, F, H). In contrast, aged WT organoids more frequently displayed healthy microvilli populations with intact terminal web structures (Fig. 2E, G). Although the young cohorts did not show marked differences in microvilli morphology, tight junction loss was still evident in 3xTg organoids (Fig 2B). Arrows in

Fig. 2A and C identify multiple intact tight junctions along the same cell boundary in WT specimens.

To further evaluate tight junction integrity, IHC analyses for E-Cadherin and ZO1 were conducted. Immunostaining demonstrated slight reductions in E-Cadherin and ZO1 expression in young 3xTg organoids compared to young WT controls (Fig. 3A). However, quantitative fluorescence intensity analysis revealed no statistically significant differences between young WT and young 3xTg organoids for ZO1 ($p=0.618$, $F=3.501$, $df=6.929$, $\eta^2=0.038$) or E-Cadherin ($p=0.328$, $F=4.330$, $df=6.561$, $\eta^2=0.146$) (Fig. 3B). In aged specimens, reduced ZO1 and E-Cadherin staining was clearly observed in aged 3xTg organoids compared to aged WT organoids (Fig. 3C). Quantitative analysis confirmed significantly decreased ZO1 ($p=0.0006$, $F=2.975$, $df=4.812$, $\eta^2=0.928$) and E-Cadherin ($p=0.0019$, $F=2.829$, $df=4.885$, $\eta^2=0.883$) fluorescence intensity in aged 3xTg organoids relative to aged WT controls (Fig. 3D), supporting substantial loss of tight junction integrity in the aged AD cohort.

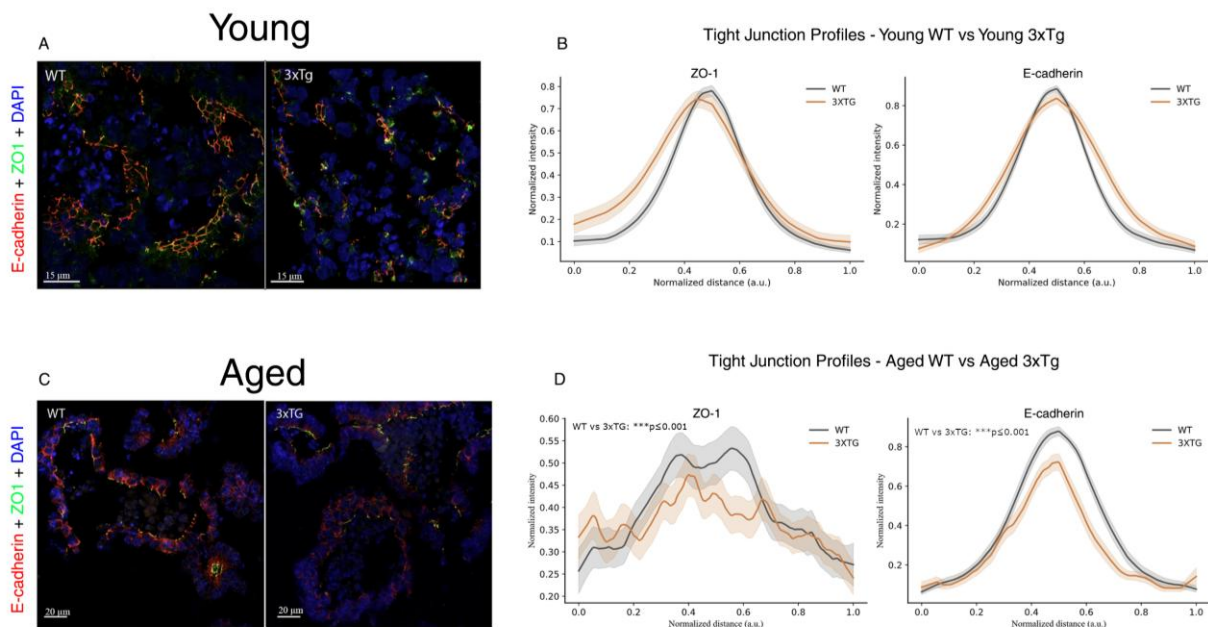


Figure 3. Reduced Tight Junction Protein Expression in Young and Aged WT and 3xTg intestinal organoids. Representative immunohistochemical (IHC) images demonstrating altered tight junction and adhesion protein expression in WT and 3xTg organoids from young (A) and aged (C) cohorts. IHC images show E-Cadherin (red) and ZO1 (green) expression in WT and 3xTg organoids. Images were acquired using confocal microscopy at 63 $\times$ magnification and analyzed using IMARIS software. (B, D) Horizontal junctional distribution analysis of ZO1 and E-Cadherin in WT and 3xTg organoids. (B) In young WT organoids (N=9) and young 3xTg organoids (N=6), Welch's t test demonstrated no significant differences in ZO1 or E-Cadherin expression between groups. (D) In aged WT organoids (N=4) and aged 3xTg organoids (N=4), significant reductions in ZO1 and E-Cadherin expression were observed in 3xTg organoids compared to WT controls. Group means were compared using Welch's t test (* $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$).

Increased presence of AD proteins in 3xTg and WT Organoids

Immunostaining revealed increased accumulation of A β (red) in 3xTg organoids, with deposits observed both intracellularly and within the organoid lumen compared to WT organoids (Fig. 4A, C). Assessment of A β staining across young and aged WT and 3xTg groups demonstrated that A β accumulation was qualitatively most prominent in aged 3xTg organoids. However, statistical analysis did not reveal significant differences between aged WT and aged 3xTg groups (Fig. 4A, B, C). A statistically significant increase in A β expression was observed in aged 3xTg organoids compared to young

3xTg and WT organoids (Fig. 4A, B, C). In addition, aged WT organoids demonstrated significantly greater A β accumulation than young WT organoids, suggesting that normal aging contributes to peripheral A β accumulation within the gut (Fig. 4A, B, C) ($p < 0.0001$, $F = 48.10$, $df = 3$, $\eta^2 = 0.935$). To further support the presence of A β pathology within organoid tissue, high-magnification TEM imaging was performed (Fig. 4D, E). Ultrastructural analysis identified fibrillar structures within aged 3xTg organoids. Figure 4E demonstrates fibrils with diameters measuring 20.2 nm, 25.4 nm, and 27.0 nm, consistent with previously established ultrastructural criteria for amyloid fibrils identified by TEM [27].

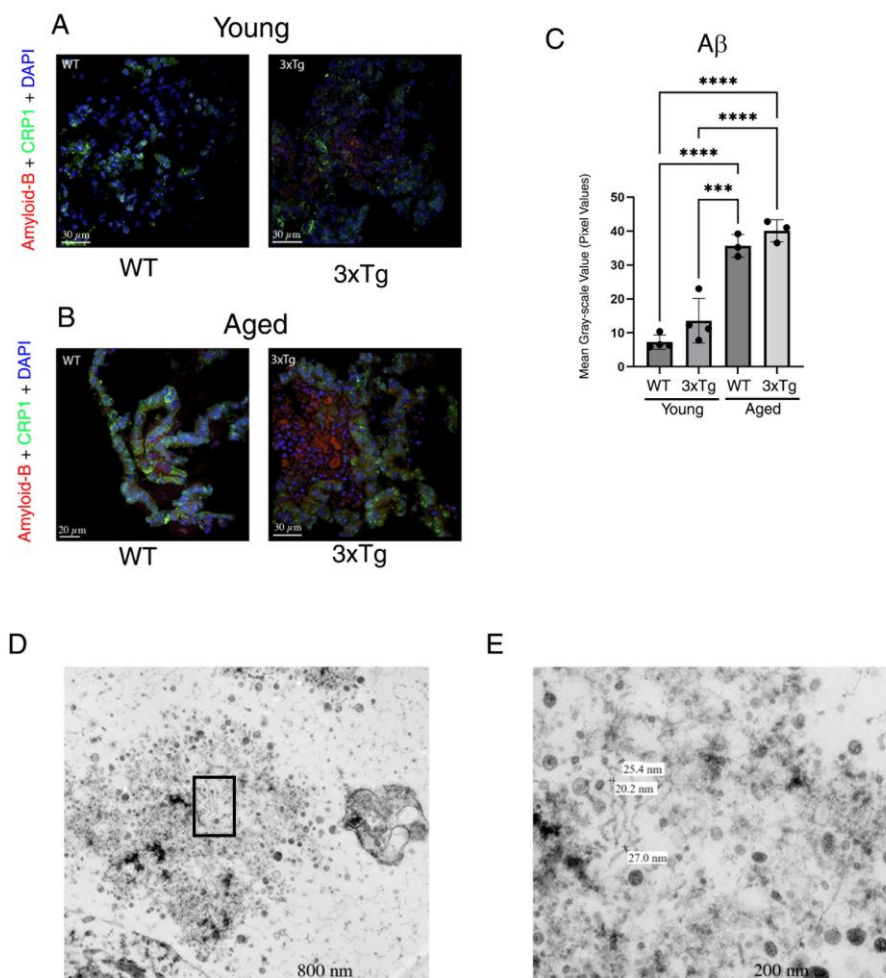


Figure 4. Changes in A β expression in intestinal organoids. (A) IHC images demonstrating A β accumulation (red) within the lumen of young WT (left) and young 3xTg (right) organoids. (B) IHC images showing A β accumulation (red) in aged WT (left) and aged 3xTg (right) organoids. All IHC images were acquired at 63 $\times$ magnification and analyzed using IMARIS software. (C) Mean A β fluorescence intensity per organoid from young WT (N=4), young 3xTg (N=4), aged WT (N=3), and aged 3xTg (N=3) groups. Bars represent mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA with multiple comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Image processing was performed using ImageJ software. (D, E) TEM images from aged 3xTg organoids demonstrate amyloid fibrils (D) with higher magnification views (E) showing labeled fibril diameters. Scale bars indicate image magnification.

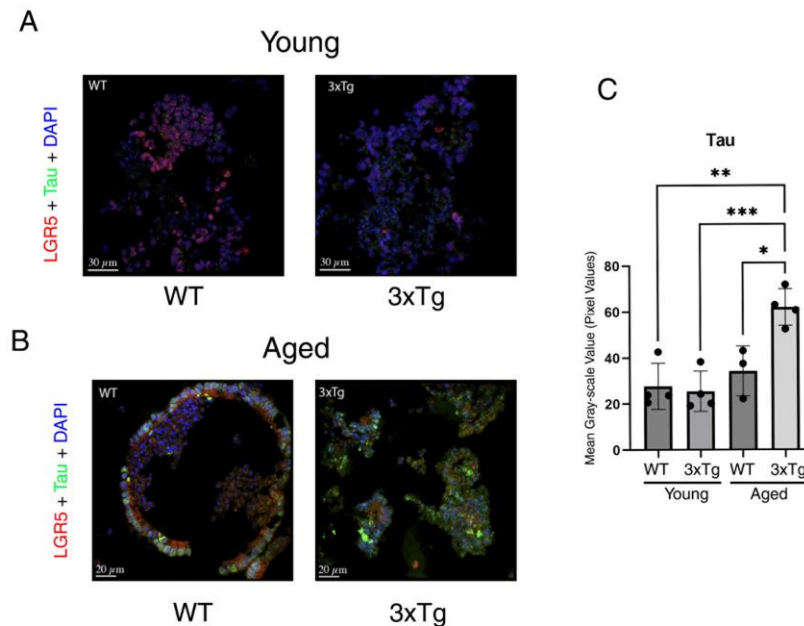


Figure 5. Changes in p-tau expression in WT and 3xTg organoids. (A) Representative IHC images showing p-tau accumulation (green) in young WT (left) and young 3xTg (right) organoids. (B) In aged specimens, increased p-tau accumulation is evident in aged 3xTg organoids compared to the other groups. All IHC images were acquired at 63 $\times$ magnification and analyzed using IMARIS software. (C) Mean p-tau fluorescence intensity per organoid from young WT (N=4), young 3xTg (N=4), aged WT (N=3), and aged 3xTg (N=4) groups. Bars represent mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with multiple comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

We also evaluated expression of phosphorylated tau (p-tau), another hallmark protein associated with AD pathology (Fig. 5). Increased p-tau accumulation was observed in aged WT and 3xTg organoids, with the most pronounced staining detected in aged 3xTg specimens (Fig. 5B). Quantitative analysis demonstrated a statistically significant increase in p-tau expression in aged 3xTg organoids compared to all other experimental groups (Fig. 5C) ($p=0.0006$, $F=12.97$, $df=3$, $\eta^2=0.780$). No statistically significant differences were detected between aged WT organoids and young WT or young 3xTg groups. (Fig. 5C). These findings suggest that intestinal p-tau accumulation may be both genotype- and age-dependent.

Increased inflammation and cell death markers in 3xTg and WT Organoids

TEM analysis also demonstrated normal intracellular organelle morphology in young WT organoids, whereas cellular abnormalities, stress, and evidence of cell death were increasingly observed in the remaining groups (Fig. 6). These pathological changes were especially pronounced in young and aged 3xTg organoids (Fig. 6B, D). Aged 3xTg organoids demonstrated greater organelle disruption and degradation compared to WT controls (Fig. 6C, D).

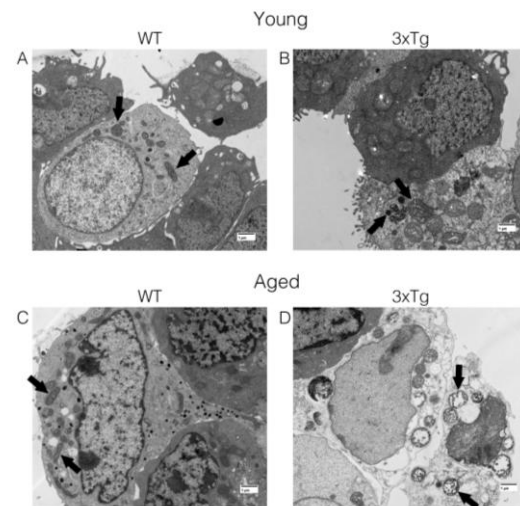


Figure 6. Ultrastructural Changes in young and aged WT and 3xTg Organoids. Representative TEM images of WT (A, C) and 3xTg (B, D) organoids demonstrating healthy cellular morphology as well as evidence of cytoplasmic abnormalities and mitochondrial degradation (arrows) in specific groups. In aged 3xTg organoids (D), late-stage mitochondrial swelling and degradation are evident as balloon-like structures with disruption of inner mitochondrial membranes. Increased cytoplasmic and nuclear electron density is also observed in both aged groups. Scale bars are shown below each micrograph.

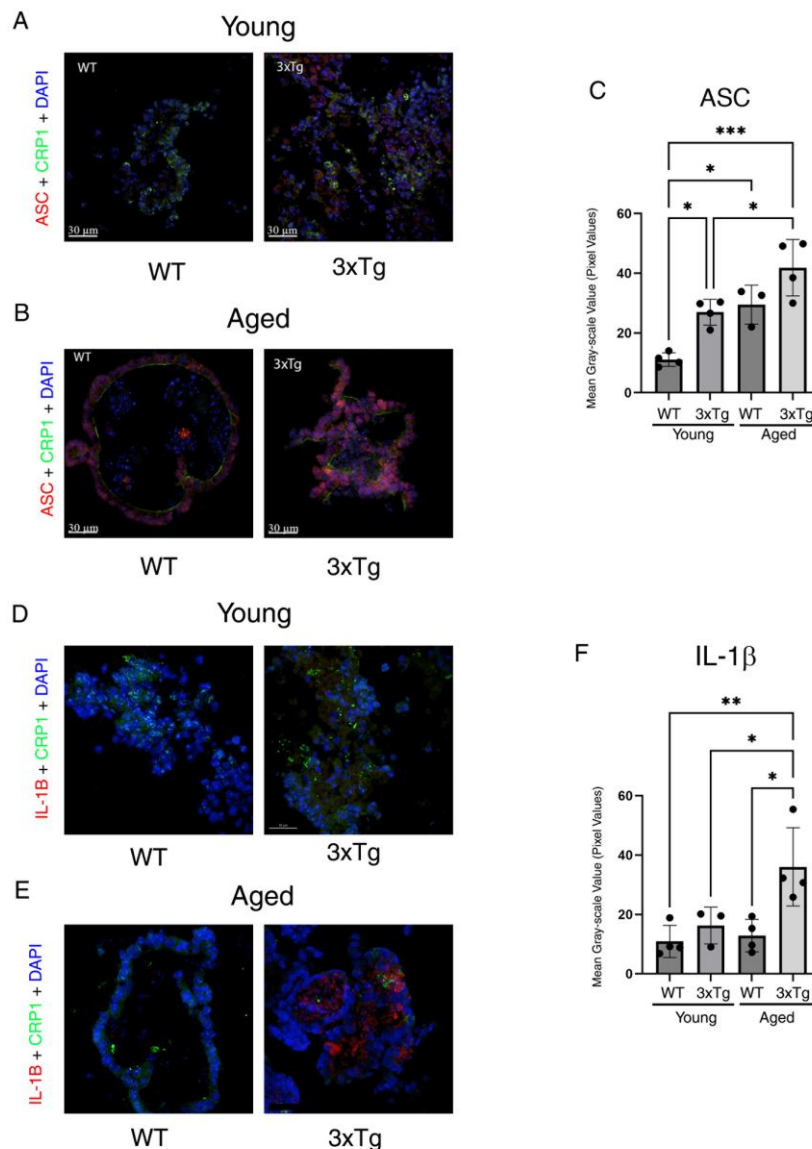


Figure 7. Inflammation Protein Expression in Young and Aged WT and 3xTg Intestinal Organoids. Inflammation markers identified in young (left) and aged (right) WT and 3xTg organoid specimens. **(A, B)** Representative IHC images of ASC (red) and CRP1 (green) demonstrate increased ASC accumulation in aged 3xTg organoids. **(C)** Mean ASC fluorescence intensity per organoid from young WT (N=4), young 3xTg (N=4), aged WT (N=3), and aged 3xTg (N=4) groups. Bars represent mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA with multiple comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). **(D, E)** Representative IHC images of IL-1 β (red) and CRP1 (green) showing increased IL-1 β expression in aged 3xTg organoids. All IHC images were acquired using confocal microscopy at 63 $\times$ magnification and analyzed with IMARIS software. **(F)** Mean IL-1 β fluorescence intensity per organoid from young WT (N=4), young 3xTg (N=3), aged WT (N=4), and aged 3xTg (N=4) groups. Bars represent mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA with multiple comparisons (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Mitochondrial abnormalities, considered strong indicators of impaired cellular viability [28], were particularly evident in aged 3xTg organoids. TEM demonstrated mitochondrial swelling and inner membrane alterations suggestive of programmed cell

death and mitochondrial dysfunction [29]. Increased numbers of mitochondria exhibiting vesicular or swollen morphologies were identified in aged 3xTg samples, indicating advanced stages of degeneration (Fig. 6D).

Additional ultrastructural indicators of cell death included darkened cytoplasm, condensed nuclear chromatin, ruptured cellular membranes, and cellular debris within the organoid lumen.

To further investigate programmed cell death pathways, IHC analyses of inflammasome-associated proteins were performed. ASC, the adaptor protein of the inflammasome complex, was predominantly observed in 3xTg organoids, although staining was also present in aged WT specimens (Fig. 7A, B). Quantification of ASC fluorescence intensity demonstrated significant increases across multiple experimental groups relative to young WT controls (Fig. 7C) ($p=0.0002$, $F=16.50$, $df=3$, $\eta^2=0.818$). The greatest increase was observed in aged 3xTg organoids compared to young WT organoids, indicating combined effects of aging and genotype on ASC

expression. No significant differences were observed between aged WT and aged 3xTg groups (Fig. 7C). Robust cleaved IL-1 β immunostaining was also observed in aged 3xTg organoids compared to all other experimental groups (Fig. 7D, E). Quantitative analysis demonstrated significantly elevated IL-1 β expression in aged 3xTg organoids compared to all other experimental groups (Fig. 7F) ($p=0.0052$, $F=7.510$, $df=3$, $\eta^2=0.672$). Finally, because GSDMD cleavage mediates IL-1 β release through pyroptosis, cleaved GSDMD expression was evaluated. Increased cleaved GSDMD immunostaining was primarily observed in aged WT and aged 3xTg organoids (Fig. 8A, B). Quantitative analysis demonstrated significantly increased GSDMD expression in aged 3xTg organoids compared to young 3xTg organoids (Fig. 8C) ($p=0.0250$, $F=5.076$, $df=3$, $\eta^2=0.629$).

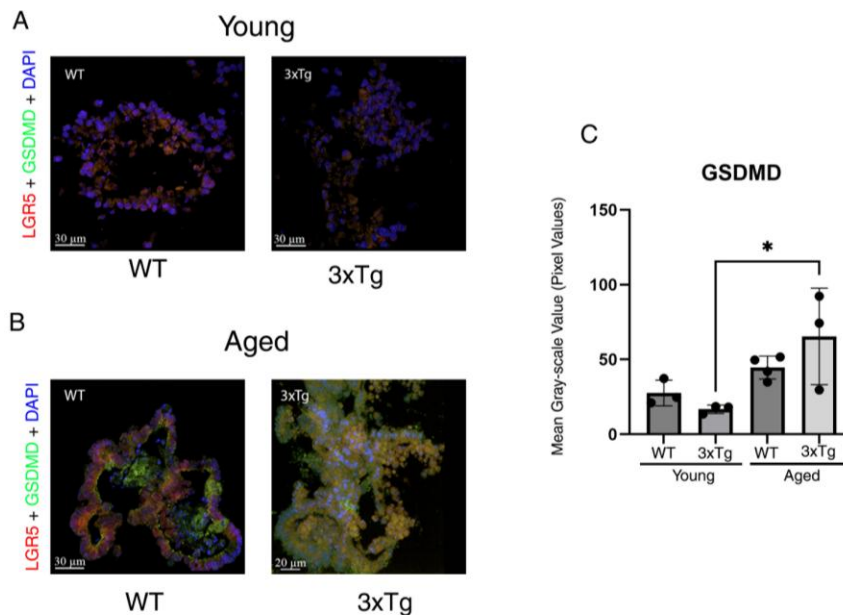


Figure 8. Gasdermin-D Expression in Young and Aged 3xTg and WT Organoids. (A, B) Representative IHC images of GSDMD (red) and CRP1 (green) in young and aged organoids demonstrating increase evidence for pyroptotic mechanisms in aged 3xTg organoids. All IHC images were acquired using confocal microscopy at 63 $\times$ magnification and analyzed with IMARIS software. (C) Mean GSDMD fluorescence intensity per organoid from young WT (N=4), young 3xTg (N=3), aged WT (N=4), and aged 3xTg (N=4) groups. Bars represent mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

DISCUSSION

The present study characterized pathological alterations in intestinal organoids generated from crypt cells isolated from 3xTg and WT mice. Ultrastructural analyses evaluated cellular organization, tight junction integrity, organelle morphology, and epithelial polarity, while immunohistochemical analyses assessed AD-related proteins and inflammatory signaling pathways. Using these complementary approaches, we demonstrated, (a) abnormal morphological changes in intestinal organoids

derived from aged 3xTg mice compared to WT controls, (b) reduced tight junction integrity in aged 3xTg organoids compared to aged WT organoids, (c) increased expression of AD-related proteins, including elevated p-tau and increased A β accumulation with aging, (d) increased inflammasome-associated signaling, including elevated ASC and cleaved IL-1 β expression, and (e) evidence of pyroptosis demonstrated by increased cleaved GSDMD expression in aged 3xTg organoids. Together, these findings support previous reports suggesting that

AD pathology extends beyond the brain and significantly affects gut epithelial integrity and viability.

We identified several morphological abnormalities associated with aging and AD pathology. Structural defects, including disrupted tight junctions, as shown by reduced E-Cadherin and ZO1 expression, were evident in both young and aged 3xTg organoids. In contrast, A β accumulation, p-tau expression, inflammasome activation, and mitochondrial damage were most pronounced in aged 3xTg organoids compared to other experimental groups. Importantly, A β and p-tau expression occurred intrinsically within organoids derived from 3xTg intestinal crypt cells, independent of systemic inflammatory signaling or brain-derived influences. Although A β accumulation was increased in aged 3xTg organoids than aged WT controls, this difference did not reach statistical significance, possibly reflecting normal age-related peripheral A β accumulation in the gut. Supporting this interpretation, aged WT organoids demonstrated significantly increased A β accumulation than young WT organoids. A recent review by Kumari and colleagues (2025) also reported elevated intestinal A β levels in AD mouse models and human AD patients [30]. In contrast to A β , p-tau expression demonstrated a stronger genotype-dependent effect, with significant increases observed specifically in aged 3xTg organoids. These findings suggest that intestinal tau phosphorylation may contribute directly to the structural abnormalities observed in aged 3xTg organoids and play a role in GBA signaling in AD. Our data further suggest that aging independently alters intestinal biology in both WT and 3xTg organoids, while the AD genotype accelerates these pathological processes. The combined effects of aging and AD genotype appear to exacerbate epithelial dysfunction more rapidly than aging alone.

A growing body of evidence implicates inflammasome signaling in the pathogenesis of AD and disorders associated with gut microbiome dysfunction [31]. It has also been shown that abnormal inflammasome activation plays a mechanistic role in gut-brain axis dysfunction in neurodegenerative disease [32]. In the current study, inflammasome-associated proteins were most prominent in aged 3xTg organoids compared to all other experimental groups. ASC expression demonstrated both age- and genotype-dependent increases, with significant elevation already evident in young 3xTg organoids, suggesting that inflammasome activation may occur early in intestinal AD pathology. Previous studies from our laboratory demonstrated that pyroptosis contributes to gut-brain axis dysfunction following CNS injury in aged 3xTg mice [15]. To our knowledge, this study is among the first to evaluate pyroptotic signaling in young and aged 3xTg intestinal organoids. Increased cleaved GSDMD expression supports pyroptosis as a

mechanism of epithelial cell death associated with aging and AD genotype. Elevated cleaved IL-1 β and cleaved GSDMD expression in aged 3xTg organoids suggest enhanced activation of the inflammasome-pyroptosis pathway within the intestinal epithelium during AD progression. Increased ASC, cleaved IL-1 β , and cleaved GSDMD staining highlights the inflammasome pathway as a potential therapeutic target for gut-brain axis dysfunction associated with neurodegenerative disease. Mitochondrial abnormalities observed in aged WT and aged 3xTg organoids support the role of cellular stress and cell death in intestinal aging and AD pathology. Further studies examining mitochondrial dynamics are needed to examine organelle specific contribution of pyroptosis to AD pathology in intestinal organoids.

This study supports intestinal organoids as a novel platform for investigating gut-brain axis dysfunction in AD. Lgr5+ intestinal stem cells isolated from young and aged 3xTg mice developed structural abnormalities in vitro that recapitulate pathological changes associated with impaired gut epithelial function in vivo [15]. These findings support the concept of genotype-dependent intestinal stem cell dysfunction in AD-prone mice. Our findings also indicate that intestinal cell differentiation declines with aging, a process accelerated by the 3xTg genotype. We focused particularly on Paneth cells because of their critical role in stem cell regulation, microbiome homeostasis, immune defense, and cytokine-mediated tissue repair [33, 34]. Previous studies have shown increased IL-1 β , NLRP3, and caspase-1 expression during Paneth cell pyroptosis following intestinal infections [31]. Consistent with these reports, we observed increased IL-1 β expression in Paneth cells within aged 3xTg organoids. TEM analyses further demonstrated disruption of Paneth cell integrity, including loss of secretory granules and mitochondrial abnormalities. Although these alterations were present in aged WT organoids, they were substantially more severe in aged 3xTg specimens. Paneth cells have also been implicated in amyloidogenesis and disease progression, highlighting their potential as therapeutic targets for reducing gut inflammation and AD-related intestinal pathology [35, 36].

The fragile nature of these organoids often resulted in fragmented spheroid structures, particularly in aged specimens. Nevertheless, aged WT organoids retained structural organization more effectively than aged 3xTg organoids. One of the most notable TEM findings was the severe impairment of intercellular adhesions and tight junctions in both young and aged 3xTg cohorts. Such abnormalities may facilitate translocation of microorganisms through enlarged intercellular spaces while disrupting cell-cell communication and epithelial polarity [37]. Interestingly, tight junction abnormalities

were already apparent in young 3xTg mice prior to the typical onset of cognitive symptoms [38]. These findings suggest that intestinal barrier dysfunction may represent an early event in AD pathogenesis and may contribute to disease progression before overt neurological symptoms emerge.

In the present study, intestinal stem cells from both young and aged mice were used to generate organoids. Aging itself is known to alter stem cell function and intestinal epithelial biology [39-41]. However, the consistent differences observed between WT and 3xTg organoids support a genotype-dependent effect beyond normal aging alone. The pronounced abnormalities observed in aged 3xTg organoids suggest an interaction between aging and AD pathology that exacerbates intestinal dysfunction.

These findings may have important therapeutic implications. Organoid systems provide a controlled in vitro environment and thus may also serve as useful platforms for evaluating therapies targeting inflammasome signaling pathways. Strategies targeting age-associated gut epithelial dysfunction or intestinal stem cell decline could potentially reduce AD-related intestinal pathology and improve gut integrity [41, 42]. Importantly, intestinal organoids lack immune cells, microbiome components, and neural inputs. Future studies incorporating co-culture systems with immune cells, microbiota, or brain organoids will therefore be necessary to more fully model the physiological complexity of the gut-brain axis. In turn, therapies targeting the gut-brain axis may help reduce systemic inflammation and slow progression of AD-related neuropathology.

Although this study provides insights for a novel intestinal organoid model for investigating gut pathology in AD, certain limitations should be acknowledged. Our studies of intestinal organoids encountered limitations involving preserving the intact organoids for imaging. A limitation of this study is the relatively small number of biological replicates per group. While we were able to generate organoids from mice in large amounts for each experimental group, liberating them from the Matrigel matrix in which they were embedded for subsequent fixation, immunostaining and electron microscopy resulted in disruption and or damage to the structural integrity of a significant number of the organoids, thus leading to a smaller sample size. To address these limitations we choose intact or minimally disrupted organoids for the images included and representative intact or minimally disrupted specimen from replicate experiments were selected for analysis. Furthermore, it is important to note that results should therefore also interpreted as preliminary evidence of genotype- and age-associated trends rather than definitive confirmation of

underlying mechanisms. In this study both male and female mice were included, future studies are needed to assess sex-specific effects on intestinal AD pathology. TEM identified amyloid-like fibrils in aged 3xTg organoids, however additional studies using immunogold labeling will be necessary to confirm fibril identity and distribution. Future investigations should also incorporate functional barrier permeability assays, cytokine release analyses, and studies evaluating inflammasome inhibitors or probiotic-based therapies. Co-culture systems involving brain organoids and microbiome components will also help establish more physiologically relevant models of AD-associated gut pathology.

In conclusion, the present study provides new evidence of structural and molecular abnormalities in intestinal organoids derived from 3xTg AD transgenic mice compared to WT controls. These abnormalities included altered epithelial viability and polarity, disrupted junctional complexes, mitochondrial pathology, and accumulation of AD-related proteins. A β and p-tau expression were most prominent in organoids generated from aged 3xTg mice. In addition, elevated inflammasome-associated proteins and increased GSDMD expression supported a role for pyroptotic cell death in intestinal pathology associated with AD. Collectively, these findings demonstrate that organoids generated from genetic disease models provide valuable opportunities to investigate mechanisms contributing to pathological progression. Intestinal organoids may therefore represent promising translational tools for precision medicine, drug screening, and therapeutic development targeting the gut-brain axis in neurodegenerative disease. Human iPSC-derived intestinal organoids may further validate these findings within a clinically relevant human-specific model system.

Availability of Data and Materials

Available upon reasonable request

Competing Interests

H.M.B. and W.D.D. are co-founders and managing members of InflamaCORE, LLC and have licensed patents on inflammasome proteins as biomarkers of injury and disease as well as on targeting inflammasome proteins for therapeutic purposes. H.M.B. and W.D.D. are Scientific Advisory Board Members of ZyVersa Therapeutics.

Microsoft Copilot was solely used as an AI-assisted editing tool to improve grammar, clarity and readability; all scientific content and references were generated and verified by the human authors.

Author Contributions

K. Matisse Gilson Conceptualization, Methodology, Investigation, Data collection and analysis, Visualization, Writing – original draft, review, & editing. Alfredo Fernandez Higuera Data collection and analysis. Winston M. Walters Methodology, Data collection and analysis, Writing – editing. Juliana Sanchez-Molano Methodology, Data collection. Vania A. Wolf Data collection and analysis. Zane Zeier Data analysis, Writing- review, & editing. W. Dalton Dietrich Conceptualization, Methodology, Writing review, & editing, Resources. Helen M. Bramlett Conceptualization, Methodology, Writing review, & editing, Resources. Nadine A. Kerr. Conceptualization, Methodology, Writing review, & editing, Resources.

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References

- [1] Zysk M, Clausen F, Aguilar X, Sehlin D, Syvanen S, Erlandsson A (2019). Long-Term Effects of Traumatic Brain Injury in a Mouse Model of Alzheimer's Disease. *J Alzheimers Dis*, 72:161-180.
- [2] Johnson NH, Kerr NA, de Rivero Vaccari JP, Bramlett HM, Keane RW, Dietrich WD (2023). Genetic predisposition to Alzheimer's disease alters inflammasome activity after traumatic brain injury. *Transl Res*, 257:66-77.
- [3] Hanslik KL, Ulland TK (2020). The Role of Microglia and the Nlrp3 Inflammasome in Alzheimer's Disease. *Front Neurol*, 11:570711.
- [4] Frolich L (2020). Alzheimer's disease - the 'microbial hypothesis' from a clinical and neuroimaging perspective. *Psychiatry Res Neuroimaging*, 306:111181.
- [5] He J, Liu Y, Li J, Zhao Y, Jiang H, Luo S, et al. (2023). Intestinal changes in permeability, tight junction and mucin synthesis in a mouse model of Alzheimer's disease. *Int J Mol Med*, 52.
- [6] Sochocka M, Donskow-Lysoniewska K, Diniz BS, Kurpas D, Brzozowska E, Leszek J (2019). The Gut Microbiome Alterations and Inflammation-Driven Pathogenesis of Alzheimer's Disease-a Critical Review. *Mol Neurobiol*, 56:1841-1851.
- [7] Gomaa EZ (2020). Human gut microbiota/microbiome in health and diseases: a review. *Antonie Van Leeuwenhoek*, 113:2019-2040.
- [8] Di Tommaso N, Gasbarrini A, Ponziani FR (2021). Intestinal Barrier in Human Health and Disease. *Int J Environ Res Public Health*, 18.
- [9] Hietbrink F, Besselink MG, Renooij W, de Smet MB, Draisma A, van der Hoeven H, et al. (2009). Systemic inflammation increases intestinal permeability during experimental human endotoxemia. *Shock*, 32:374-378.
- [10] Belvoncikova P, Maronek M, Gardlik R (2022). Gut Dysbiosis and Fecal Microbiota Transplantation in Autoimmune Diseases. *Int J Mol Sci*, 23.
- [11] Gubert C, Choo JM, Love CJ, Kodikara S, Masson BA, Liew JJM, et al. (2022). Faecal microbiota transplant ameliorates gut dysbiosis and cognitive deficits in Huntington's disease mice. *Brain Commun*, 4:fcac205.
- [12] Klann EM, Dissanayake U, Gurralla A, Farrer M, Wagle Shukla A, Ramirez-Zamora A, et al. (2021). The Gut-Brain Axis and Its Relation to Parkinson's Disease: A Review. *Front Aging Neurosci*, 13:782082.
- [13] Kapoor B, Biswas P, Gulati M, Rani P, Gupta R (2024). Gut microbiome and Alzheimer's disease: What we know and what remains to be explored. *Ageing Res Rev*, 102:102570.
- [14] Chen C, Wang GQ, Li DD, Zhang F (2025). Microbiota-gut-brain axis in neurodegenerative diseases: molecular mechanisms and therapeutic targets. *Mol Biomed*, 6:64.
- [15] Kerr NA, Bramlett HM, Sanchez-Molano J, Higuera AF, Walters W, de Rivero Vaccari JP, et al. (2025). Stool-derived extracellular vesicles increase inflammasome signaling and regulate the gut-brain axis after stroke in Alzheimer's disease transgenic mice. *Exp Neurol*, 390:115269.
- [16] Cabrera Ranaldi E, Bramlett HM, Umland O, Levine LI, Keane RW, de Rivero Vaccari JP, et al. (2025). Gasdermin-D Genetic Knockout Reduces Inflammasome-Induced Disruption of the Gut-Brain Axis After Traumatic Brain Injury. *Int J Mol Sci*, 26.
- [17] Clevers H (2016). Modeling Development and Disease with Organoids. *Cell*, 165:1586-1597.
- [18] Papaspyropoulos A, Tsolaki M, Foroglou N, Pantazaki AA (2020). Modeling and Targeting Alzheimer's Disease With Organoids. *Front Pharmacol*, 11:396.
- [19] Gerakis Y, Hetz C (2019). Brain organoids: a next step for humanized Alzheimer's disease models? *Mol Psychiatry*, 24:474-478.
- [20] Cerneckis J, Bu G, Shi Y (2023). Pushing the boundaries of brain organoids to study Alzheimer's disease. *Trends Mol Med*, 29:659-672.
- [21] Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, et al. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*, 459:262-265.
- [22] Belfiore R, Rodin A, Ferreira E, Velazquez R, Branca C, Caccamo A, et al. (2019). Temporal and regional progression of Alzheimer's disease-like pathology in 3xTg-AD mice. *Aging Cell*, 18:e12873.
- [23] Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, et al. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron*, 39:409-421.
- [24] Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, et al. (2010). Lgr5(+ve) stem

- cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell*, 6:25-36.
- [25] Crowe AR, Yue W (2019). Semi-quantitative Determination of Protein Expression using Immunohistochemistry Staining and Analysis: An Integrated Protocol. *Bio Protoc*, 9.
- [26] Lovejoy M, Mucci A, Rabino A, Garcia-Mata R (2025). Quantification of horizontal and vertical distribution of junctional proteins in fixed epithelial cells. *bioRxiv*.
- [27] Tosoni A. BdBGaNM. 2011. Electron Microscopy in the Diagnosis of Amyloidosis. In *Amyloidosis - Mechanisms and Prospects for Therapy*: InTech. S. Sarantseva, editor: InTechOpen. 121-144.
- [28] Vringer E, Tait SWG (2023). Mitochondria and cell death-associated inflammation. *Cell Death Differ*, 30:304-312.
- [29] Eustaquio T, Wang C, Dugard CK, George NI, Liu F, Slikker W, Jr., et al. (2018). Electron microscopy techniques employed to explore mitochondrial defects in the developing rat brain following ketamine treatment. *Exp Cell Res*, 373:164-170.
- [30] Sonam Kumari VD, Sivakumar S, Sikri K, Kumar M (2025). Association between gut microbiial dysbiosis and Alzheimer's disease: an umbrella review. *npj Dementia*, 1:41.
- [31] Shukla PK, Delotterie DF, Xiao J, Pierre JF, Rao R, McDonald MP, et al. (2021). Alterations in the Gut-Microbial-Inflammasome-Brain Axis in a Mouse Model of Alzheimer's Disease. *Cells*, 10.
- [32] Rutsch A, Kantsjo JB, Ronchi F (2020). The Gut-Brain Axis: How Microbiota and Host Inflammasome Influence Brain Physiology and Pathology. *Front Immunol*, 11:604179.
- [33] Wallaey C, Garcia-Gonzalez N, Libert C (2023). Paneth cells as the cornerstones of intestinal and organismal health: a primer. *EMBO Mol Med*, 15:e16427.
- [34] Nakamura K, Yokoi Y, Fukaya R, Ohira S, Shinozaki R, Nishida T, et al. (2020). Expression and Localization of Paneth Cells and Their alpha-Defensins in the Small Intestine of Adult Mouse. *Front Immunol*, 11:570296.
- [35] Cui C, Wang F, Zheng Y, Wei H, Peng J (2023). From birth to death: The hardworking life of Paneth cell in the small intestine. *Front Immunol*, 14:1122258.
- [36] Bergman P, Roan NR, Romling U, Bevins CL, Munch J (2016). Amyloid formation: functional friend or fearful foe? *J Intern Med*, 280:139-152.
- [37] Schneeberger K, Roth S, Nieuwenhuis EES, Middendorp S (2018). Intestinal epithelial cell polarity defects in disease: lessons from microvillus inclusion disease. *Dis Model Mech*, 11.
- [38] Wei Z, Li D, Shi J (2023). Alterations of Spatial Memory and Gut Microbiota Composition in Alzheimer's Disease Triple-Transgenic Mice at 3, 6, and 9 Months of Age. *Am J Alzheimers Dis Other Dement*, 38:15333175231174193.
- [39] Penttimikko N, Iqbal S, Mana M, Andersson S, Cognetta AB, 3rd, Suci RM, et al. (2019). Notum produced by Paneth cells attenuates regeneration of aged intestinal epithelium. *Nature*, 571:398-402.
- [40] Martin K, Kirkwood TB, Potten CS (1998). Age changes in stem cells of murine small intestinal crypts. *Exp Cell Res*, 241:316-323.
- [41] Nalapareddy K, Zheng Y, Geiger H (2022). Aging of intestinal stem cells. *Stem Cell Reports*, 17:734-740.
- [42] Geiger H, de Haan G, Florian MC (2013). The ageing haematopoietic stem cell compartment. *Nat Rev Immunol*, 13:376-389.