Gut Inflammation in Alzheimer's Disease Regulates Aβ and Tau Fibril Propagation from Gut into the Brain via a C/EBPβ/δ-secretase Pathway

By

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Running Title: Gut inflammation initiates AD Pathology spreading via the vagus nerve

Keywords: fibrils; inflammation; Alzheimer's disease; C/EBPβ; δ-secretase

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Legends to Supplementary figures

Appendix Figure S1. C/EBP β/δ-secretase pathway is escalated in an age-dependent manner in 3xTg mice brain.

(A) Immunoblot showing p-C/EBP β, C/EBP β, AEP, APP, and Tau expression and processing in mouse brain. (B) AEP activity assay in brain lysate from age-dependent 3xTg mice. Data represent the mean ± SEM; representative data of three samples; *P =0.0128 (*P < 0.05), **P =0.0066 (**P < 0.01), **P =0.0018 (**P < 0.01) compared with control, one-way ANOVA.

Appendix Figure S2. Chronic DSS treatment induces colonic inflammation activating C/EBP β/δ-secretase pathway in WT mice.

(A) Immunoblot showing p-C/EBP β, C/EBP β, MPO, AEP, APP, and Tau expression and proteolytic processing in WT mouse **colon**. (B) Immunoblot showing p-C/EBP β, C/EBP β, AEP, APP, and Tau expression and processing in WT mouse brain. (C) Immunoblot showing p-C/EBP β, C/EBP β, MPO, AEP, APP, and Tau expression and proteolytic processing in 3xTg mouse **colon**. (D) Immunoblot showing p-C/EBP β, C/EBP β, AEP, APP, and Tau expression and proteolytic processing in 3xTg mouse brain. (E) AEP activity assay in **colon** lysates and brain lysates of vehicle-treated or DSS-treated WT mice. Representative data of three samples; *P=0.0121, *P=0.0172（*P < 0.05）compared with control **colon** and brain, multiple unpair t test. (F) AEP activity assay with **colon** lysates and brain lysates of vehicle-treated or DSS-treated 3xTg mice. Representative data of three samples; ***P=0.0004 (***P < 0.001), **P=0.0024 (**P < 0.01) compared with control **colon** and brain, multiple unpair t test. (G) Pro-inflammatory cytokines IL1-β, IL-6, and TNFα concentrations in **colon** lysates and brain lysates of vehicle-treated 3xTg mice, DSS-treated 3xTg and DSS-treated WT mice, respectively. Representative data of three samples; ****P<0.0001, *P=0.0104 (*P<0.05) compared with control, two-way ANOVA. (H)Immunofluorescent staining of Aβ (green) in hippocampus CA1 region and cerebral cortex of Vehicle-treated WT and 3xTg mice or DSS-treated WT and 3xTg mice. (I) Quantitative analysis of Aβ positive cells. The density of Aβ positive cells was significantly increased by DSS treatment in 3xTg mice. Representative data of five samples, data are shown as mean ± SEM. **P=0.0043 (**P < 0.01), ****P < 0.0001, one-way ANOVA. (J) Immunofluorescent staining of AEP (green), Aβ (green) and AT8 (green) in colons of vehicle-treated 3xTg mice, DSS-treated 3xTg and DSS-treated WT mice, respectively. (K) Quantitative

analysis of AEP, Aβ and AT8 positive cells. The density of AEP, Aβ and AT8 positive cells was significantly increased by DSS treatment in 3xTg mice. Representative data of five samples, data are shown as mean ± SEM. ****P < 0.0001, one-way ANOVA.

Appendix Figure S3. DSS-induced Tau pathology in 3xTg mice colon is alleviated through knocking out C/EBP β/ δ-secretase or vagotomy.

(A) Immunofluorescent staining of T22 (red) and Tau 5 (green) in colons from vehicle-treated, DSS-treated and pre-vagotomy DSS-treated mice. Cytofluorograms describe the distribution of red and green pixels from respective channels of images. Pearson's correlation coefficient was applied for colocalization analysis. (B) Quantitative analysis of number of colocalization signals per visual area. Representative data of three samples, data are shown as mean ± SEM. *P=0.0106, *P=0.0205 (*P < 0.05), **P=0.0093(**P < 0.01), *P=0.0236 (*P < 0.05), one-way ANOVA. (C) Immunofluorescent staining of T22 (red) and AT8 (green) in colons from vehicle-treated, DSS-treated and pre-vagotomy DSS-treated mice. (D) Quantitative analysis of T22 positive cells. The density of T22 positive cells was significantly increased by DSS treatment and decreased through knocking out C/EBP β/AEP or vagotomy. Representative data of three samples, data are shown as mean ± SEM. ***P=0.0003, ***P=0.0004, (***P<0.001), ****P<0.0001, one-way ANOVA. (E) Quantitative analysis of AT8 positive cells. The density of AT8 positive cells was significantly increased by DSS treatment and decreased through knocking out C/EBP β/AEP or vagotomy. Representative data of three samples, data are shown as mean ± SEM. ****P < 0.0001, one-way ANOVA.

Appendix Figure S4. Vagotomy blunts C/EBPβ/δ-secretase signaling pathway in WT mice induced by chronic DSS treatment

(A) Immunoblot showing p-C/EBP β, C/EBP β, AEP, APP and Tau expression and proteolytic processing in brain lysates of different brain hemispheres from pre-vagotomy DSS treated WT mice. (B) AEP activity assay with brain lysates of different brain hemispheres from pre-vagotomy DSS treated WT mice. Data represent the mean ± SEM; representative data of three samples. **P=0.0078 (**< 0.01), unpaired t tests. (C&D) Cued and Contextual Fear-conditioning tests. Data represent the mean ± SEM of n = 8 mice per group; ***P =0.0003 (***P < 0.001), two-way ANOVA; ***P=0.0008 (***P < 0.001), unpaired t tests.

Appendix Figure S5. Characterization and verification of protein materials for colonic injection

(A) MALDI-ToF mass spectra data for the recombinant Aβ1-42 peptide purchased from the rpeptide® company. This quality control data shows no additional contamination visible in the batch that have been used within the experiments. (B) Thioflavin T binding assay. Aggregation kinetics experiments in the presence of different Aβ PFFs concentrations showed that Aβ PFFs had little effect on aggregation at concentration of 20 nM or below. (C) TEM image of Tau N368 PFFs. Scale bar: 50 nm. (D)Illustration of colonoscopy injection endoscopy. (E) Demonstration of the vagus nerve dissection. (F) SDS-PAGE following immunoblotting of Aβ fibrils, AD brain extracts and 5xFAD brain extracts (Hank's soluble fraction) with Aβ antibody; Coomassie blue staining SDS-PAGE gel (18%). (G) Aβ40 and Aβ42 concentrations in both AD brain extracts and 5xFAD brain extracts (Hank's soluble fraction) were measured using human Aβ40 and human Aβ42 ELISA kit. (H) SDS-PAGE following immunoblotting of AD brain extracts and 5xFAD brain extracts (Hank's soluble fraction) with human Tau antibody (HT7), cleaved Tau N368 antibody and phosphorylated Tau antibody (AT8).

Appendix Figure S6. Vagotomy blunts AD pathologies in 3xTg mice induced by colonic inoculated AD brain extracts

(A) Immunoblot showing p-C/EBP β, C/EBP β, APP and Tau expression and proteolytic processing in brain lysates of different brain hemispheres from pre-vagotomy colonic-injected AD brain extracts 3xTg mice. (B) AEP activity assay with brain lysates of different brain hemispheres from pre-vagotomy colonic-injected AD brain extracts 3xTg mice. Data represent the mean ± SEM; representative data of three samples, two-way ANOVA. (C) Aβ42 concentration in brain lysates prepared from cell lysis buffer (soluble) and Guanidine-HCl buffer (total) were measured using human Aβ42 ELISA kit. Triton X-100 soluble Aβ42 concentration in mice brain increased 4 months after colonic inoculation with AD brain extracts. However, pre-vagotomy diminished the escalation. Data represent the mean ± SEM; representative data of three samples; **P=0.0034 (**P < 0.01) compared with control, two-way ANOVA.

Appendix Figure S7. Colonic-injection of AD brain extracts increases oligomeric Tau and neuronal cell death in mice brain.

(A) Immunofluorescent co-staining of cleaved Tau N368 (red) and AT8 (green) in CA1 region from hippocampus of brains from colonic-injected PBS, colonic-injected AD brain extracts, and pre-Vagotomy colonic injected AD brain extracts 3xTg mice. (B) Quantitative analysis of cleaved Tau N368 positive cells and AT8 positive cells, respectively. Data are shown as mean ± SEM, representative data of three samples. The density of both cleaved Tau N368 positive cells and AT8 positive cells were significantly increased by colonic-injection of AD brain extracts and decreased through vagotomy before colonic-injection. *P=0.0289(*P<0.05), ****P<0.0001; **P=0.0045(**P<0.01), *P=0.0103 (*P<0.05), ***P=0.002 (***P<0.001), one-way ANOVA. (C) Immunofluorescent staining of cleaved Caspase 3 (red) and NeuN (green) in cerebral cortex of brains from colonic-injected PBS, colonic-injected AD brain extracts, and pre-Vagotomy colonic injected AD brain extracts 3xTg mice. (D) Quantitative analysis of cleaved-Caspase 3 and NeuN positive cells, respectively. Data are shown as mean ± SEM, representative data of three samples. The density of cleaved-Caspase 3 positive cells was significantly increased by colonic injection of AD brain extracts and decreased through vagotomy before colonic-injection, ****P < 0.0001, ***P=0.0004 (***P<0.001), one-way ANOVA; NeuN positive cells was significantly decreased by colonic-injection of AD brain extracts and increased through vagotomy before colonic-injection, ****P < 0.000, **P=0.004 (**P<0.01), one-way ANOVA.

Appendix Supplementary Figures

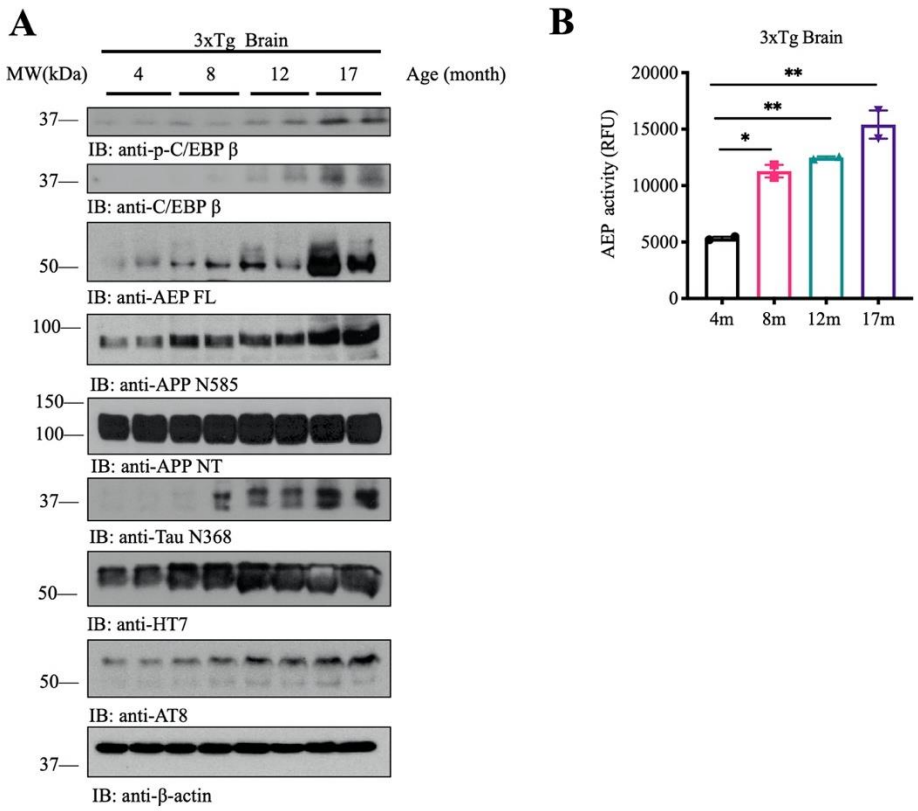


Figure S1

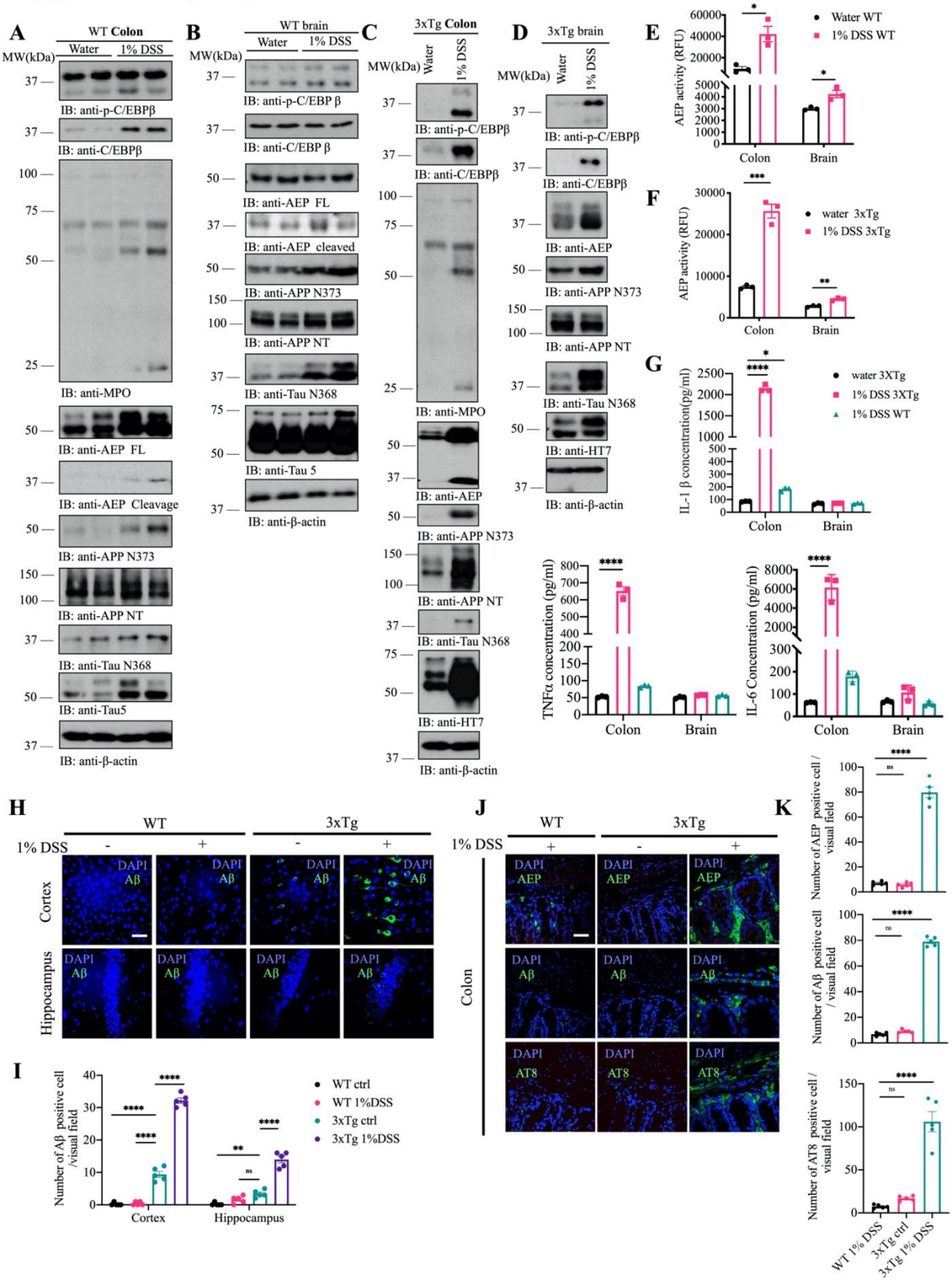

A
WT Colon
Water
1% DSS
MW(kDa)
IB: anti-p-C/EBPβ
IB: anti-C/EBPβ
IB: anti-MPO
IB: anti-AEP FL
IB: anti-AEP Cleavage
IB: anti-APP N373
IB: anti-APP NT
IB: anti-Tau N368
IB: anti-Tau5
IB: anti-β-actin
B
WT brain
IB: anti-p-C/EBP β
IB: anti-C/EBP β
IB: anti-AEP FL
IB: anti-AEP cleaved
IB: anti-APP N373
IB: anti-APP NT
IB: anti-Tau N368
IB: anti-Tau 5
IB: anti-β-actin
C
3xTg Colon
IB: anti-p-C/EBPβ
IB: anti-C/EBPβ
IB: anti-MPO
IB: anti-AEP
IB: anti-APP N373
IB: anti-APP NT
IB: anti-Tau N368
IB: anti-HT7
IB: anti-β-actin
D
3xTg brain
IB: anti-p-C/EBPβ
IB: anti-C/EBPβ
IB: anti-AEP
IB: anti-APP N373
IB: anti-APP NT
IB: anti-Tau N368
IB: anti-HT7
IB: anti-β-actin
E
AEP activity (RFU)
Water WT
1% DSS WT
Colon
Brain
F
AEP activity (RFU)
water 3xTg
1% DSS 3xTg
Colon
Brain
G
IL-1 β concentration(pg/ml)
water 3XTg
1% DSS 3XTg
1% DSS WT
Colon
Brain
TNFα concentration (pg/ml)
Colon
Brain
IL-6 Concentration (pg/ml)
Colon
Brain
H
WT
3xTg
1% DSS
Cortex
Hippocampus
DAPI
Aβ
I
Number of Aβ positive cell /visual field
WT ctrl
WT 1%DSS
3xTg ctrl
3xTg 1%DSS
Cortex
Hippocampus
J
WT
3xTg
1% DSS
Colon
DAPI
AEP
Aβ
AT8
K
Number of AEP positive cell / visual field
Number of Aβ positive cell / visual field
Number of AT8 positive cell / visual field
WT 1% DSS
3xTg ctrl
3xTg 1% DSS

Figure S2

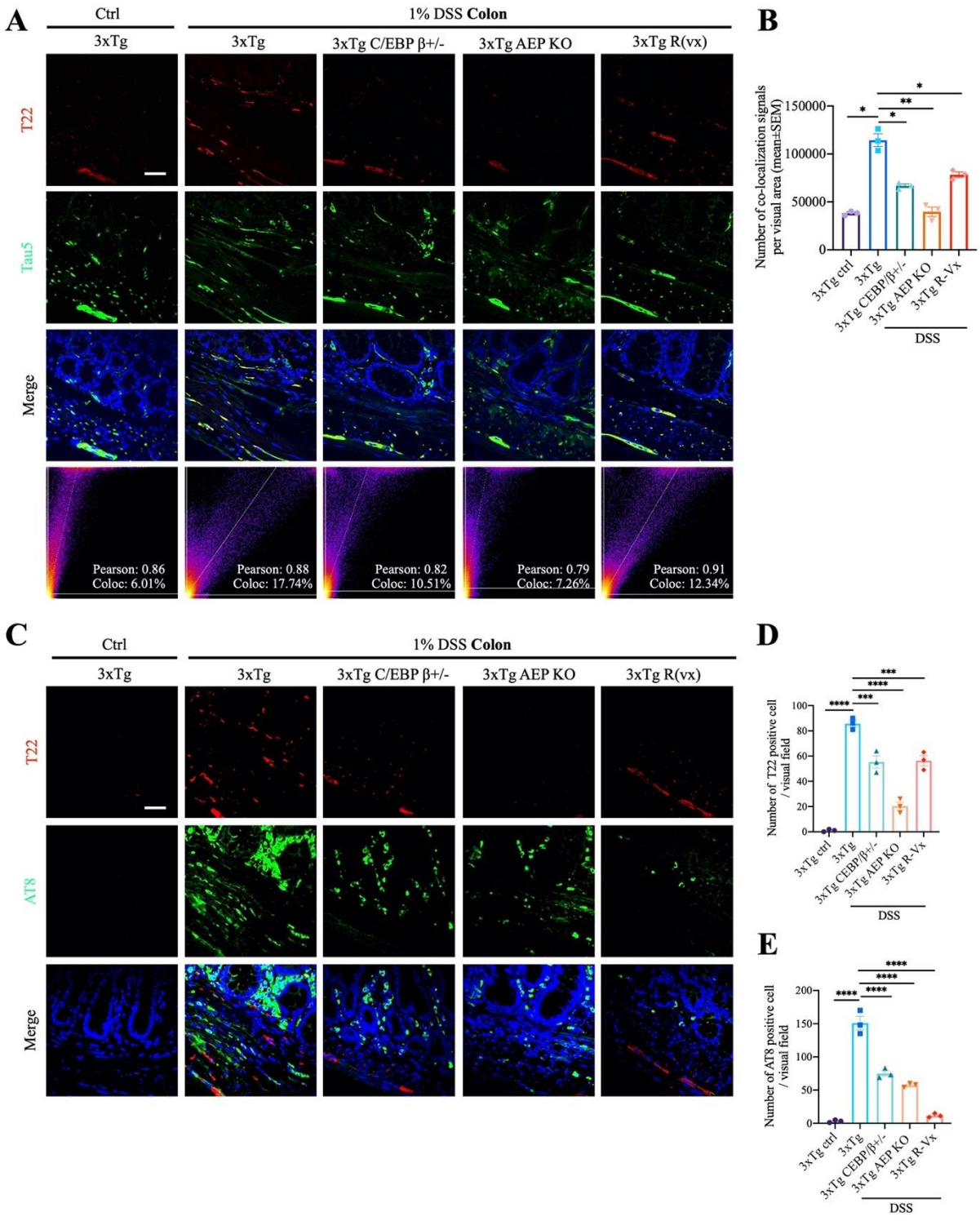

A
Ctrl
1% DSS Colon
3xTg
3xTg
3xTg C/EBP β+/-
3xTg AEP KO
3xTg R(vx)
T22
Tau5
Merge
Pearson: 0.86
Coloc: 6.01%
Pearson: 0.88
Coloc: 17.74%
Pearson: 0.82
Coloc: 10.51%
Pearson: 0.79
Coloc: 7.26%
Pearson: 0.91
Coloc: 12.34%
B
Number of co-localization signals per visual area (mean±SEM)
150000
100000
50000
0
3xTg ctrl
3xTg
3xTg CEBP/β+/-
3xTg AEP KO
3xTg R-Vx
DSS
C
Ctrl
1% DSS Colon
3xTg
3xTg
3xTg C/EBP β+/-
3xTg AEP KO
3xTg R(vx)
T22
AT8
Merge
D
Number of T22 positive cell / visual field
100
80
60
40
20
0
3xTg ctrl
3xTg
3xTg CEBP/β+/-
3xTg AEP KO
3xTg R-Vx
DSS
E
Number of AT8 positive cell / visual field
200
150
100
50
0
3xTg ctrl
3xTg
3xTg CEBP/β+/-
3xTg AEP KO
3xTg R-Vx
DSS

Figure S3

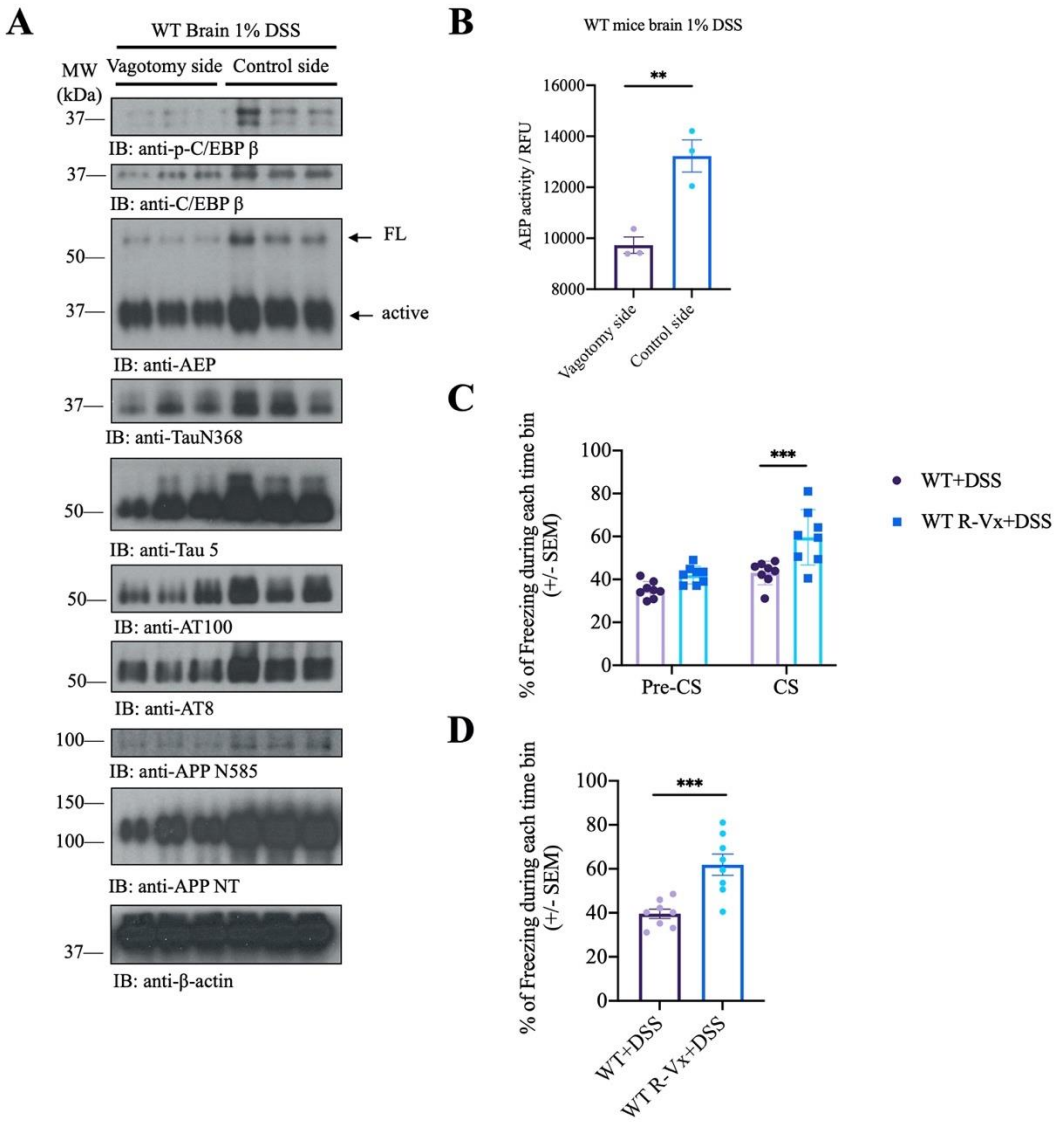
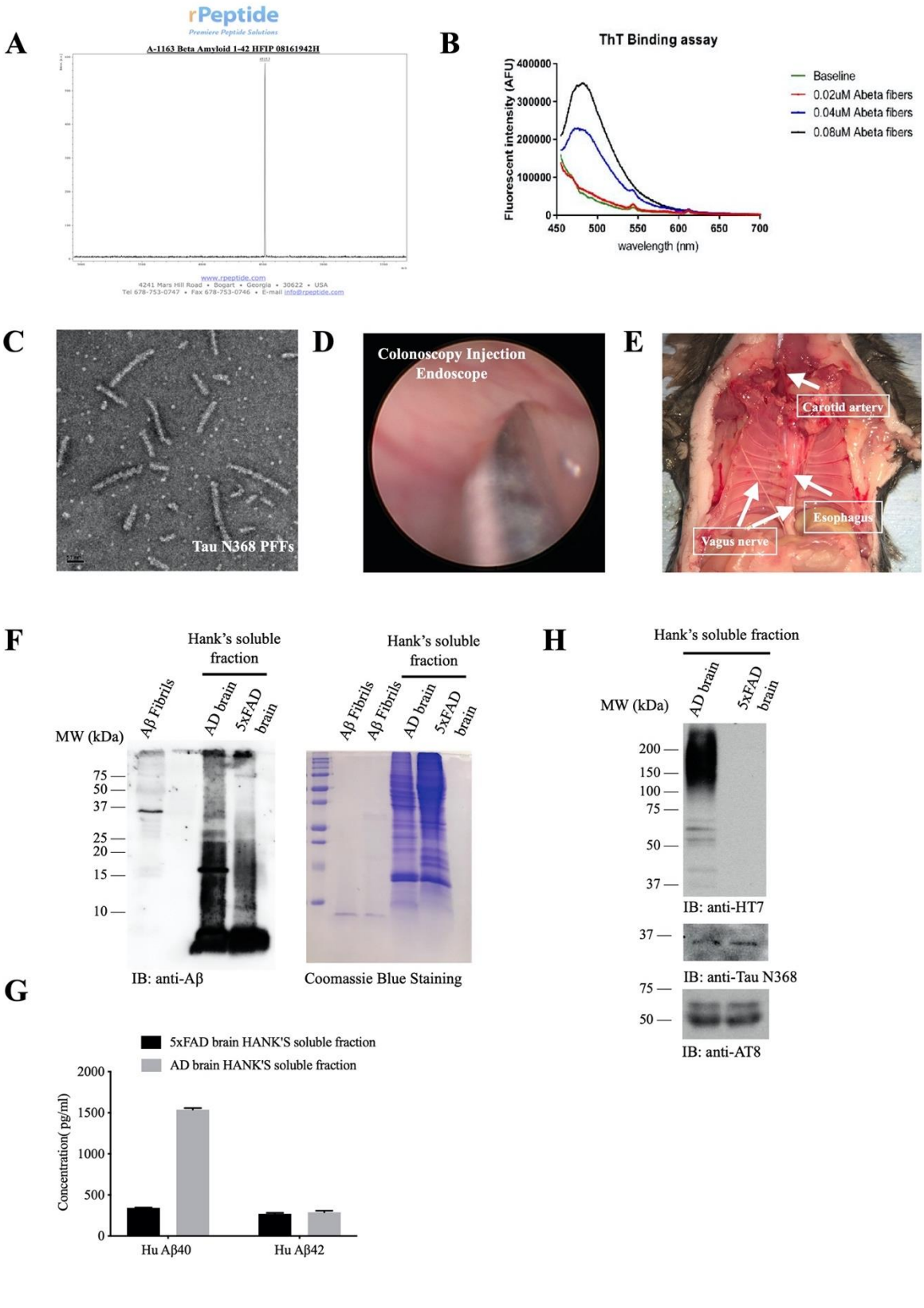

A
WT Brain 1% DSS
MW (kDa)
Vagotomy side
Control side
37—
IB: anti-p-C/EBP β
37—
IB: anti-C/EBP β
50—
37—
FL
active
IB: anti-AEP
37—
IB: anti-TauN368
50—
IB: anti-Tau 5
50—
IB: anti-AT100
50—
IB: anti-AT8
100—
IB: anti-APP N585
150—
100—
IB: anti-APP NT
37—
IB: anti-β-actin
B
WT mice brain 1% DSS
**
AEP activity / RFU
16000
14000
12000
10000
8000
Vagotomy side
Control side
C
***
% of Freezing during each time bin (+/- SEM)
100
80
60
40
20
0
Pre-CS
CS
WT+DSS
WT R-Vx+DSS
D
***
% of Freezing during each time bin (+/- SEM)
100
80
60
40
20
0
WT+DSS
WT R-Vx+DSS

Figure S4


A
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B
ThT Binding assay
Fluorescent intensity (AFU)
400000
300000
200000
100000
0
450
500
550
600
650
700
wavelength (nm)
Baseline
0.02uM Abeta fibers
0.04uM Abeta fibers
0.08uM Abeta fibers
C
Tau N368 PFFs
D
Colonoscopy Injection
Endoscope
E
Carotid arterv
Esophagus
Vagus nerve
F
Hank's soluble fraction
Aβ Fibrils
AD brain
5xFAD brain
MW (kDa)
75
50
37
25
20
15
10
IB: anti-Aβ
Hank's soluble fraction
Aβ Fibrils
Aβ Fibrils
AD brain
5xFAD brain
Coomassie Blue Staining
H
Hank's soluble fraction
AD brain
5xFAD brain
MW (kDa)
200
150
100
75
50
37
IB: anti-HT7
37
IB: anti-Tau N368
75
50
IB: anti-AT8
G
5xFAD brain HANK'S soluble fraction
AD brain HANK'S soluble fraction
Concentration(pg/ml)
2000
1500
1000
500
0
Hu Aβ40
Hu Aβ42

Figure S5

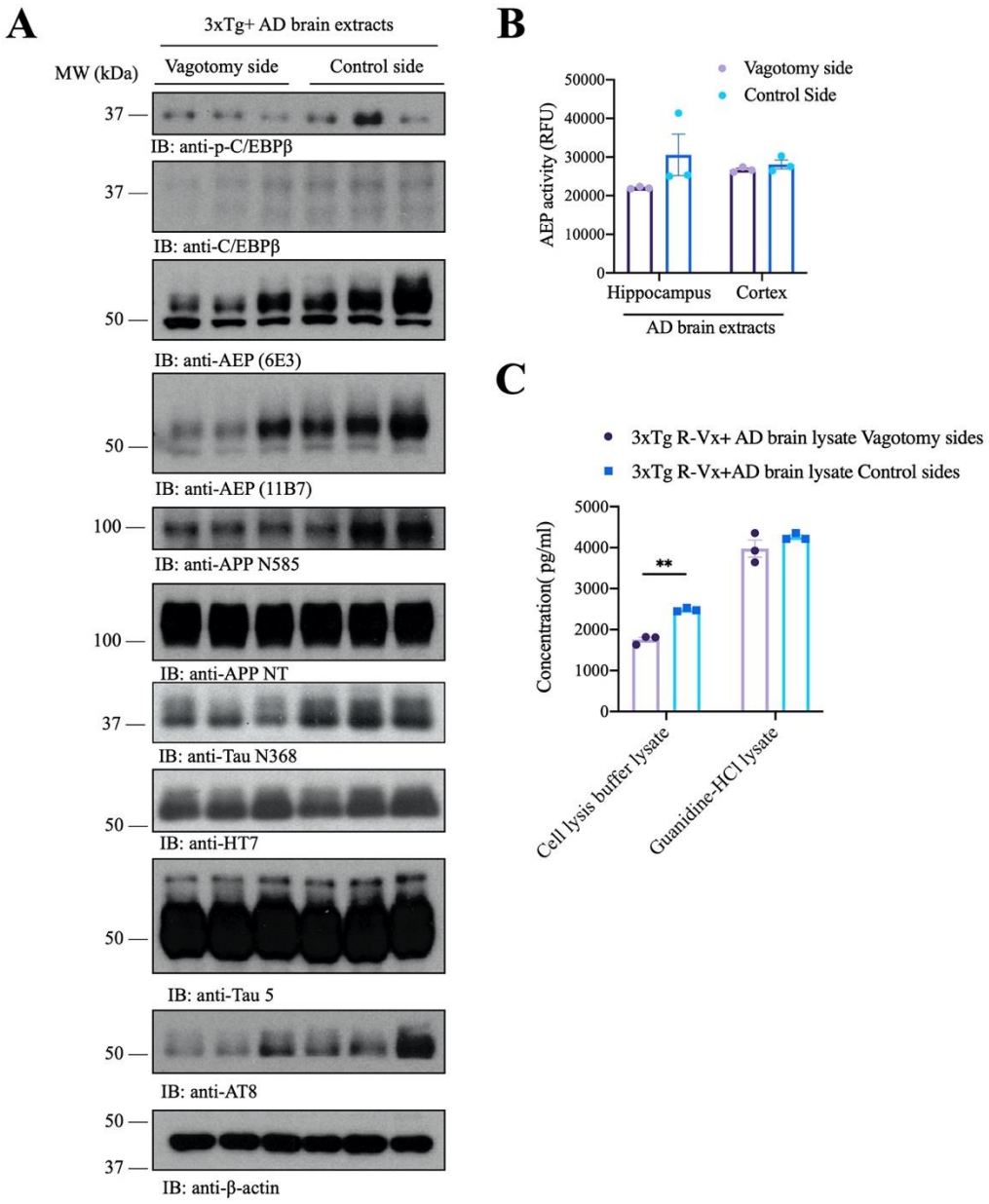
A
3xTg+ AD brain extracts
MW (kDa)
Vagotomy side
Control side
37
IB: anti-p-C/EBPβ
37
IB: anti-C/EBPβ
50
IB: anti-AEP (6E3)
50
IB: anti-AEP (11B7)
100
IB: anti-APP N585
100
IB: anti-APP NT
37
IB: anti-Tau N368
50
IB: anti-HT7
50
IB: anti-Tau 5
50
IB: anti-AT8
50
37
IB: anti-β-actin
B
Vagotomy side
Control Side
AEP activity (RFU)
50000
40000
30000
20000
10000
0
Hippocampus
Cortex
AD brain extracts
C
3xTg R-Vx+ AD brain lysate Vagotomy sides
3xTg R-Vx+AD brain lysate Control sides
Concentration(pg/ml)
5000
4000
3000
2000
1000
0
**
Cell lysis buffer lysate
Guanidine-HCl lysate

Figure S6

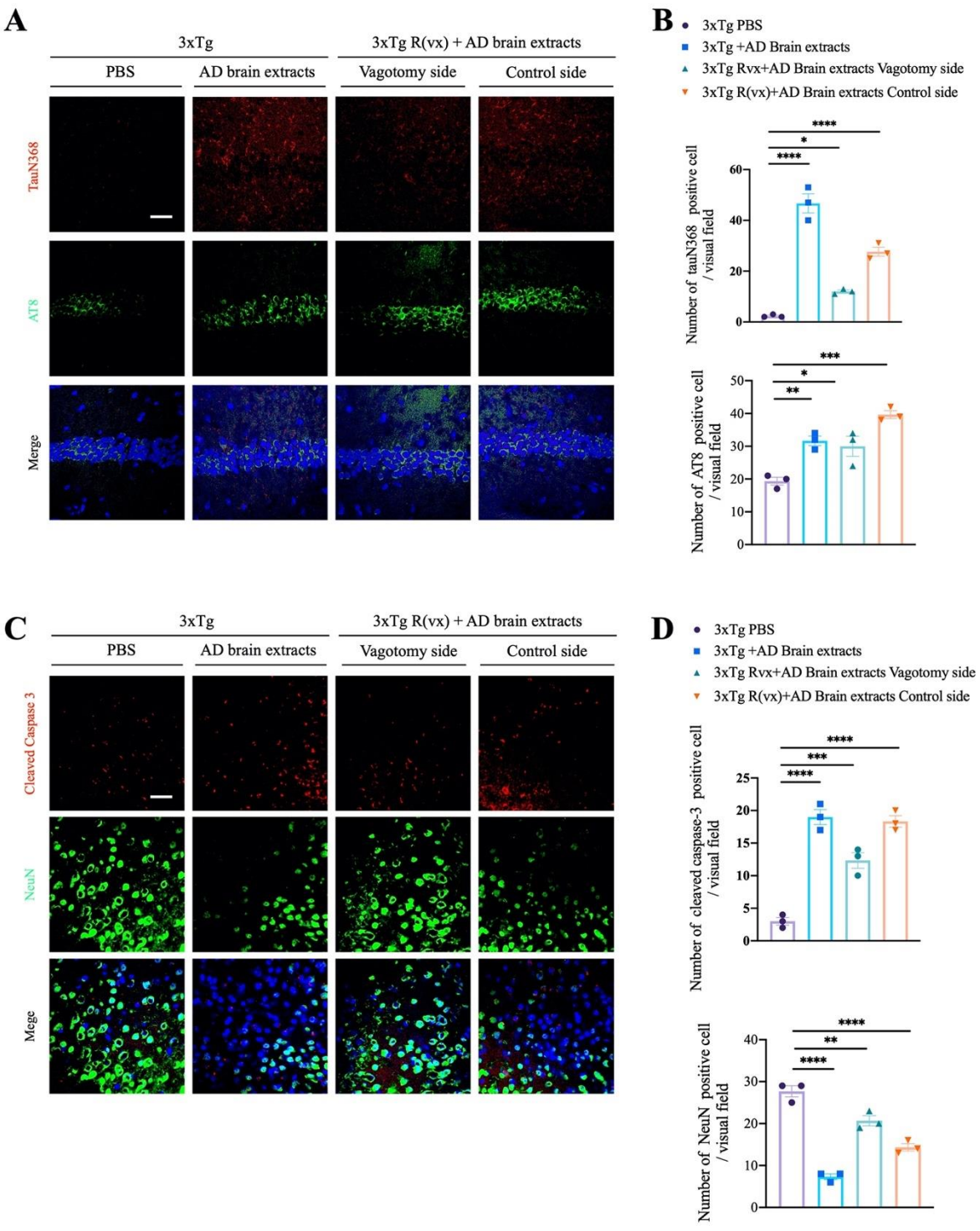
A
3xTg
3xTg R(vx) + AD brain extracts
PBS
AD brain extracts
Vagotomy side
Control side
TauN368
AT8
Merge
B
3xTg PBS
3xTg +AD Brain extracts
3xTg Rvx+AD Brain extracts Vagotomy side
3xTg R(vx)+AD Brain extracts Control side
Number of tauN368 positive cell / visual field
Number of AT8 positive cell / visual field
C
Cleaved Caspase 3
NeuN
Mege
D
Number of cleaved caspase-3 positive cell / visual field
Number of NeuN positive cell / visual field

Figure S7