Supplementary Figures

Connexin43 hemichannel blockade turns microglia neuroprotective and mitigates Alzheimer's disease-related cognitive deficits

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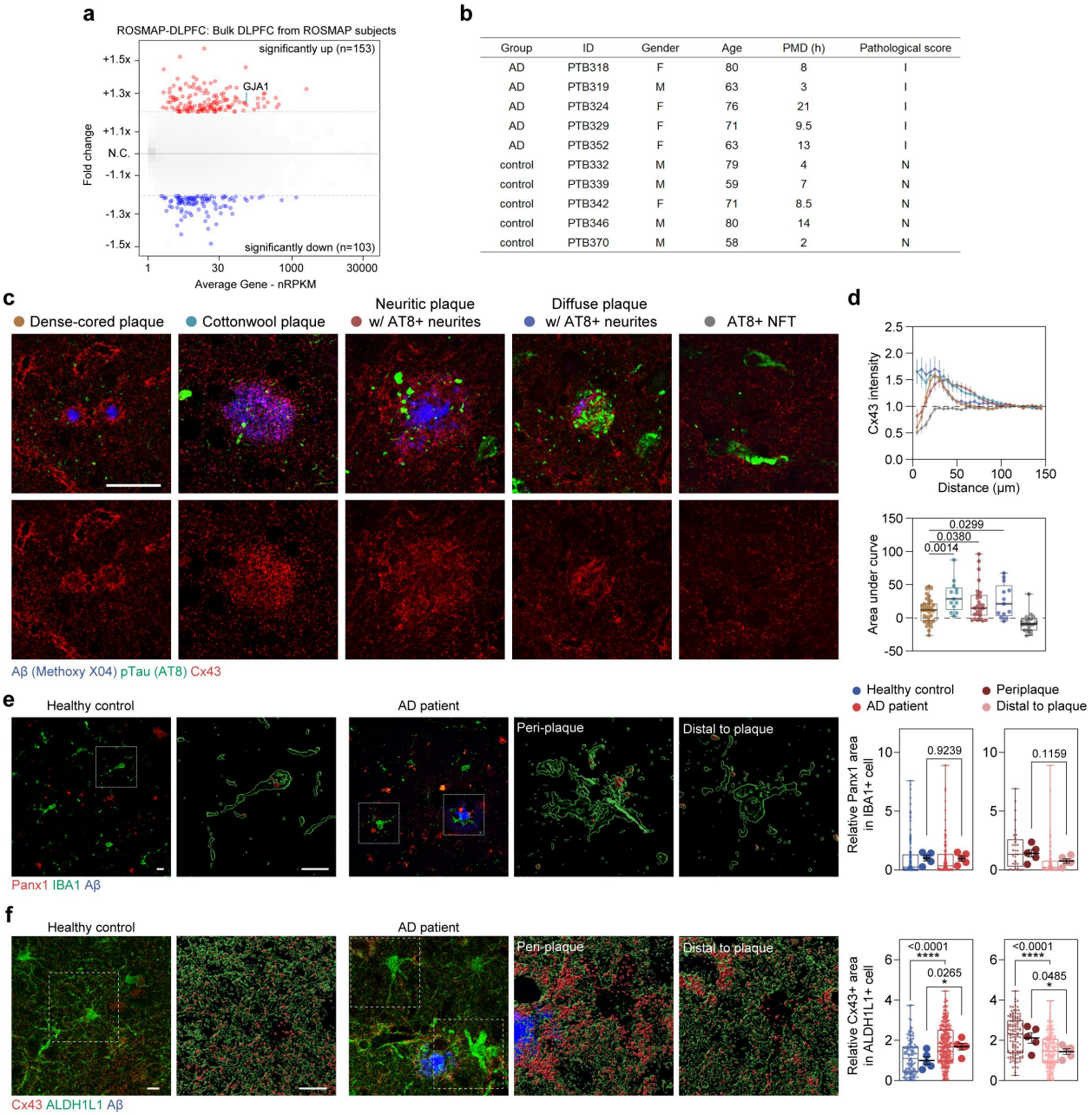
Supplementary Fig. 1: Cx43 expression in AD patients.

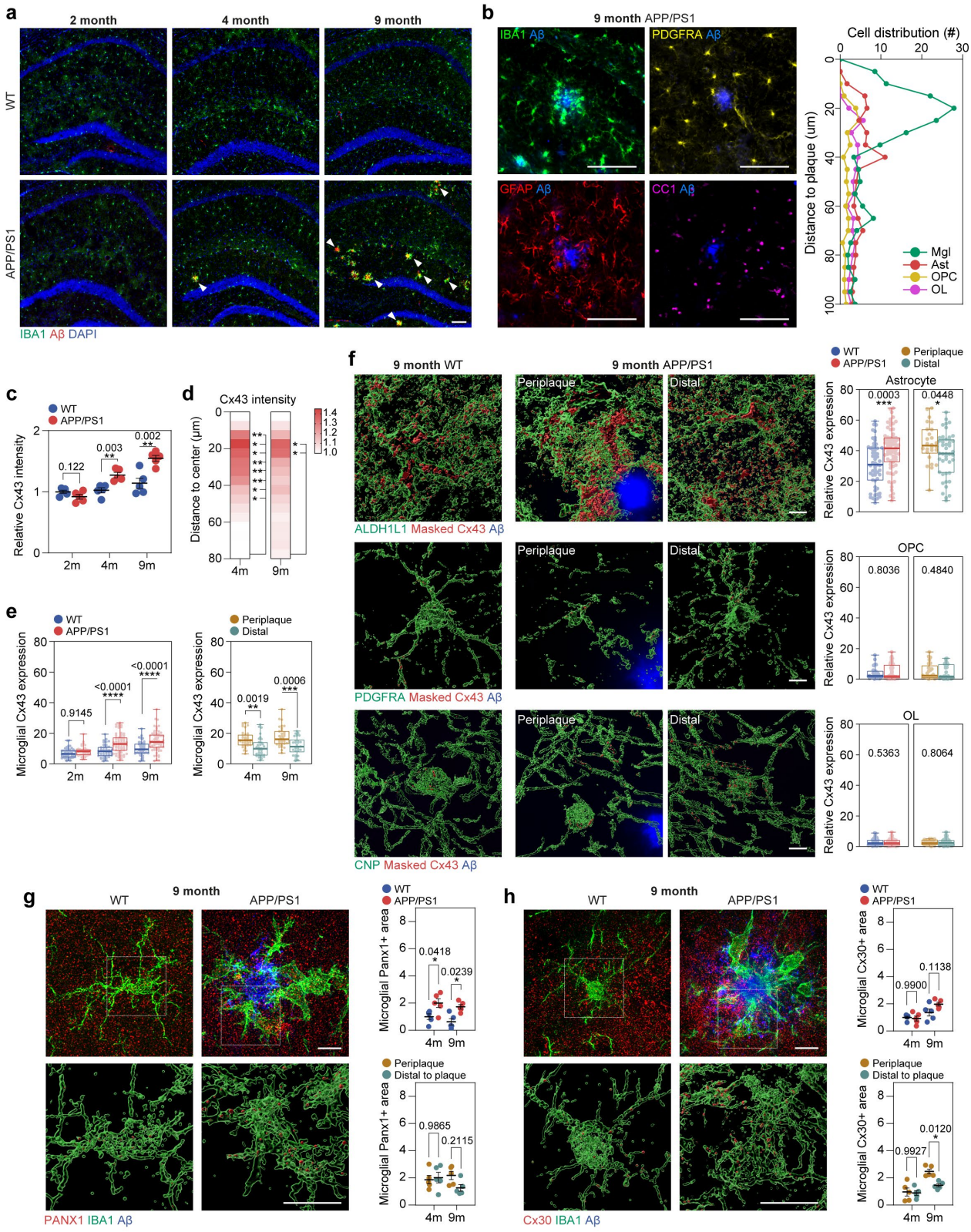


Group	ID	Gender	Age	PMD (h)	Pathological score
AD	PTB318	F	80	8	I
AD	PTB319	M	63	3	I
AD	PTB324	F	76	21	I
AD	PTB329	F	71	9.5	I
AD	PTB352	F	63	13	I
control	PTB332	M	79	4	N
control	PTB339	M	59	7	N
control	PTB342	F	71	8.5	N
control	PTB346	M	80	14	N
control	PTB370	M	58	2	N

a Volcano plot highlighting differential expressed genes in the ROSMAP-DLPFC-bulk RNA-seq dataset. Cx43/*GJA1* is among the upregulated genes in AD patients. **b** Information on human samples used in this project. **c** Immunostaining of pTau (AT8 antibody), Cx43, and Aβ of human samples. Representative image of Dense-cored plaque, cottonwool plaque, neuritic plaque with AT8+ neurites, diffuse plaque with AT8+ neurites, and AT8+ neurofilament tangle (NFT). Scale bar: 50 μm. **d** Quantification of Cx43 intensity at different distance to plaque (or NFT). Lower panel: area under curve of the upper panel. n = 37, 13, 27, 13, 23 objects from 5 patients. Statistical test: two-sided t-test. **e** Immunostaining of Panx1, IBA1, and Aβ of human samples, and 3D reconstruction of Panx1 signals within IBA1+ domain. Scale bar: 10 μm. Below: quantification of Panx1+ area in microglia

normalized to the controls. N = 5 subjects, n = 133 (control), 119 (AD), 33 (periplaque), 86 (distal) cells. Statistical test: two-sided t-test. **f** Immunostaining of Cx43, ALDH1L1, and Aβ of human samples, and 3D reconstruction of Cx43 signals within ALDH1L1+ astrocyte domain. Scale bar: 10 μm. Below: quantification of Cx43+ area in astrocyte normalized to the controls. N = 5 subjects, n = 129 (control), 321 (AD), 124 (periplaque), 197 (distal) cells. Statistical test: two-sided t-test. Dot plots show mean ± SEM, each data point, and *p*-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided as a Source Data file.

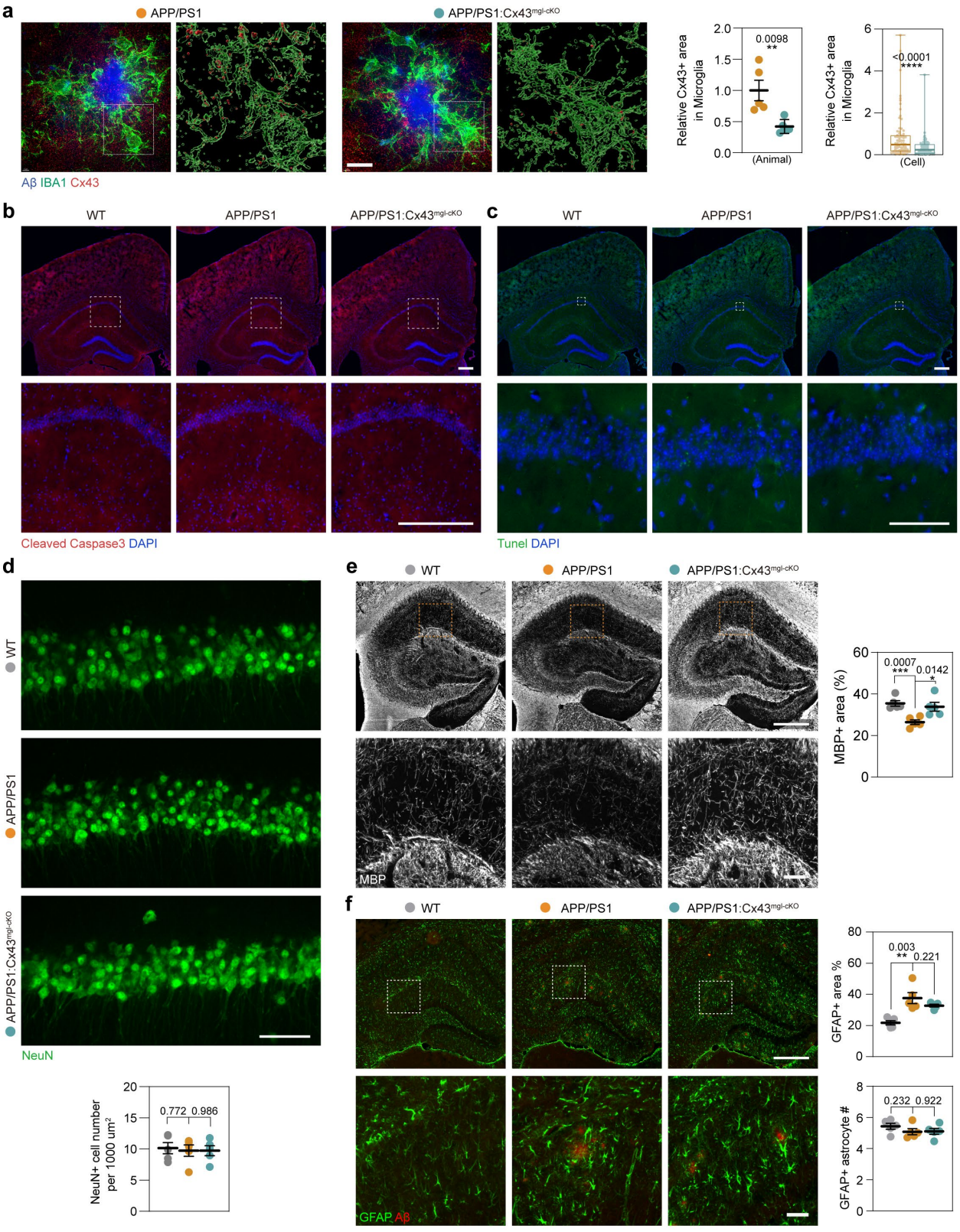
Supplementary Fig. 2: Microglial Cx43 expression and function in APP/PS1 mice.



a Representative IBA1, Aβ, and DAPI staining in 2-, 4-, and 9-month-old WT and APP/PS1 mouse hippocampus. Arrowheads highlight Aβ plaque. Scale bar: 100 μm. **b** Distribution of glial cells in relation to plaque. Images show IBA1 (microglia), GFAP (astrocyte), PDGFRA (oligodendrocyte

precursor cell, OPC), CC1 (oligodendrocyte, OL) and Aβ staining in 9-month-old APP/PS1 mouse hippocampus. Scale bar: 100 μm. Right, quantification of cell distribution at different distances from Aβ plaques (N > 15 plaques). **c** Quantification of Cx43 intensity in the cortex of WT and APP/PS1 mice at 2-, 4-, and 9-month-old. N = 5 mice. Statistical test: two-sided t-test. **d** Quantification of periplaque Cx43 expression in the cortex of APP/PS1 mice. Cx43 staining intensity at different distances from Aβ plaques (n = 13, 14) was quantified and compared to the area distal to plaque. Statistical test: two-sided t-test. **e** Quantification of microglial Cx43 expression in the cortex. Left: quantification of Cx43+ area within IBA1+ domain in WT and APP/PS1 mice. N = 50, 41, 41, 66, 50, 65 cells respectively from 5 mice. Right: comparison of Cx43+ area within IBA1+ domain between periplaque and distal microglia. N = 33, 33, 35, 30 cells respectively from 5 mice. Statistical test: two-sided t-test. **f** Representative images and quantification of Cx43 expression in ALDH1L1+ astrocyte, PDGFRA+ OPC, or CNP+ OL domain, in WT and APP/PS1 mouse hippocampus at 9-month-old. Cx43+ volume was normalized to the cell domain volume, display in percentage. Scale bar: 5 μm. For astrocyte, N = 57 (WT), 71 (APP/PS1, 26 periplaque, 45 distal) cells. For OPC, N = 27 (WT), 56 (APP/PS1, 25 periplaque, 31 distal) cells. For OL, N = 33 (WT), 52 (APP/PS1, 20 periplaque, 32 distal) cells. Statistical test: two-sided t-test. **g** Representative super-resolution images of PANX1, IBA1, Aβ staining in 4-, 9-month-old WT and APP/PS1 mouse hippocampus, and 3D reconstruction of PANX1 signals within IBA1+ domain. Scale bar: 10 μm. N = 5 mice. Statistical test: two-sided t-test. **h** Representative super-resolution images of Cx30, IBA1, Aβ staining in 4-, 9-month-old WT and APP/PS1 mouse hippocampus, and 3D reconstruction of Cx30 signals within IBA1+ domain. Scale bar: 10 μm. N = 5 mice. Statistical test: two-sided t-test. Dot plots show mean ± SEM, each data point, and *p*-value. Box plots show minima, maxima, 25~75% percentile, medium, each data point, and *p*-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided as a Source Data file.

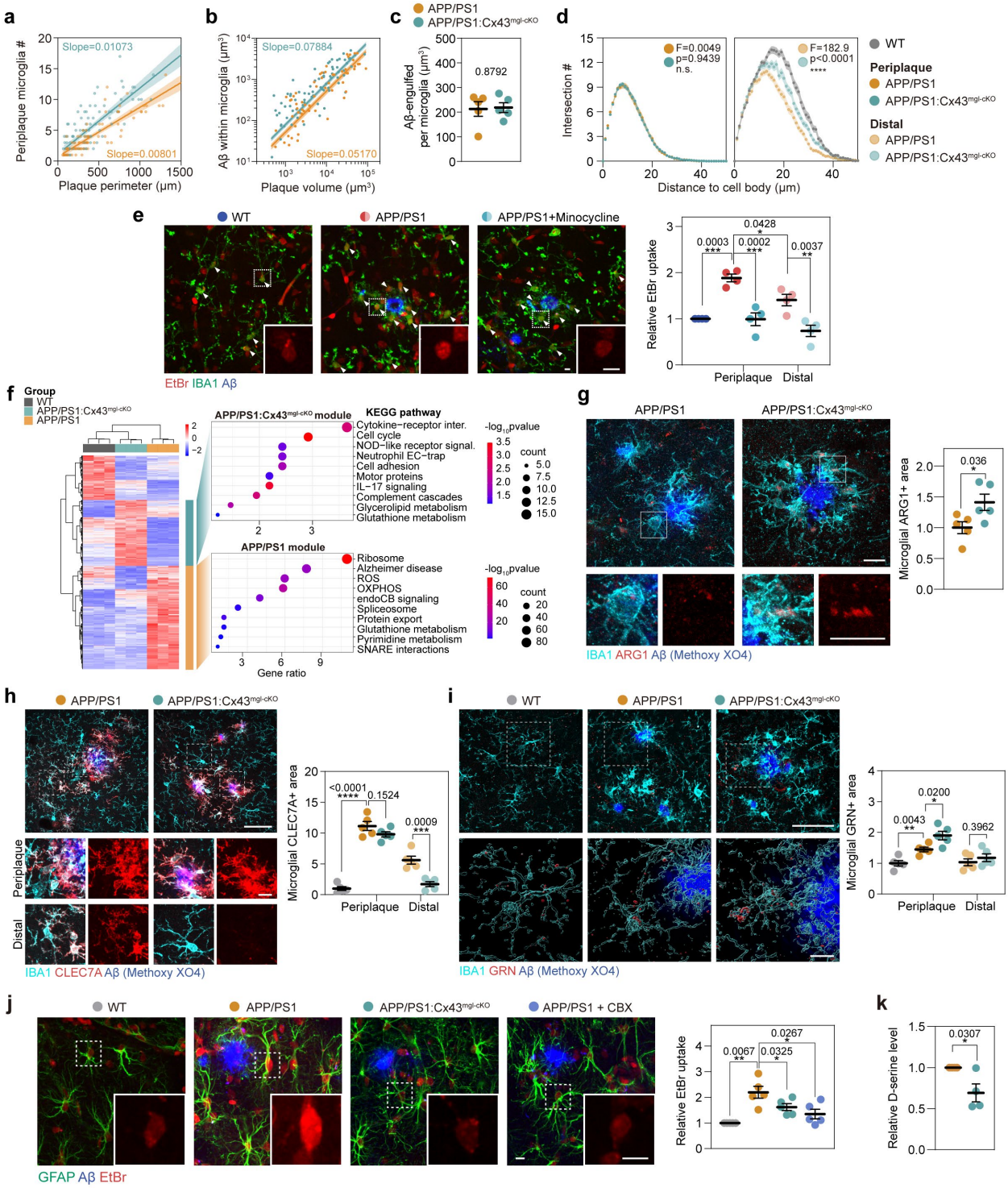
Supplementary Fig. 3: Histological analysis of Cx43$^{mgl\text{-}cKO}$ in APP/PS1 mice.



a Super-resolution images of IBA1, Cx43, and Aβ immunostaining of 9-month-old APP/PS1 and APP/PS1:Cx43$^{mgl\text{-}cKO}$ mouse hippocampus, accompanied with 3D reconstruction of Cx43 signal within IBA1+ domain. Scale bar: 10 µm. Cx43+ signal area within microglia was quantified to verify the success of Cx43 knockout. N = 5 mice, or 78 and 86 cells. Statistical test: two-sided t-test. **b-c**

Representative images of cleaved Caspase3 staining and Tunel labelling of 9-month-old WT, APP/PS1 and APP/PS1:Cx43$^{mgl\text{-}cKO}$ mouse brain. Scale bar: 250 μm, except magnification of c: 50 μm. Cleaved Caspase3-positive or Tunel-positive cell was absent in all three groups. **d** Representative images of NeuN staining of 9-month-old WT, APP/PS1 and APP/PS1:Cx43$^{mgl\text{-}cKO}$ mouse hippocampus. Scale bar: 50 μm. NeuN+ neuron number in hippocampal CA1 region was quantified. N = 5 mice. Statistical test: two-sided t-test. **e** Representative image of myelin marker MBP staining. Scale bar: 250 μm (upper) and 50 μm (magnification). The MBP+ area was quantified. N = 5 mice. Statistical test: two-sided t-test. **f** Representative image of astrocytic marker GFAP staining. Scale bar: 250 μm (upper) and 50 μm (magnification). The GFAP+ area and GFAP+ cell number were quantified. N = 5 mice. Statistical test: two-sided t-test. Dot plots show mean ± SEM, each data point, and *p*-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Source data are provided as a Source Data file.

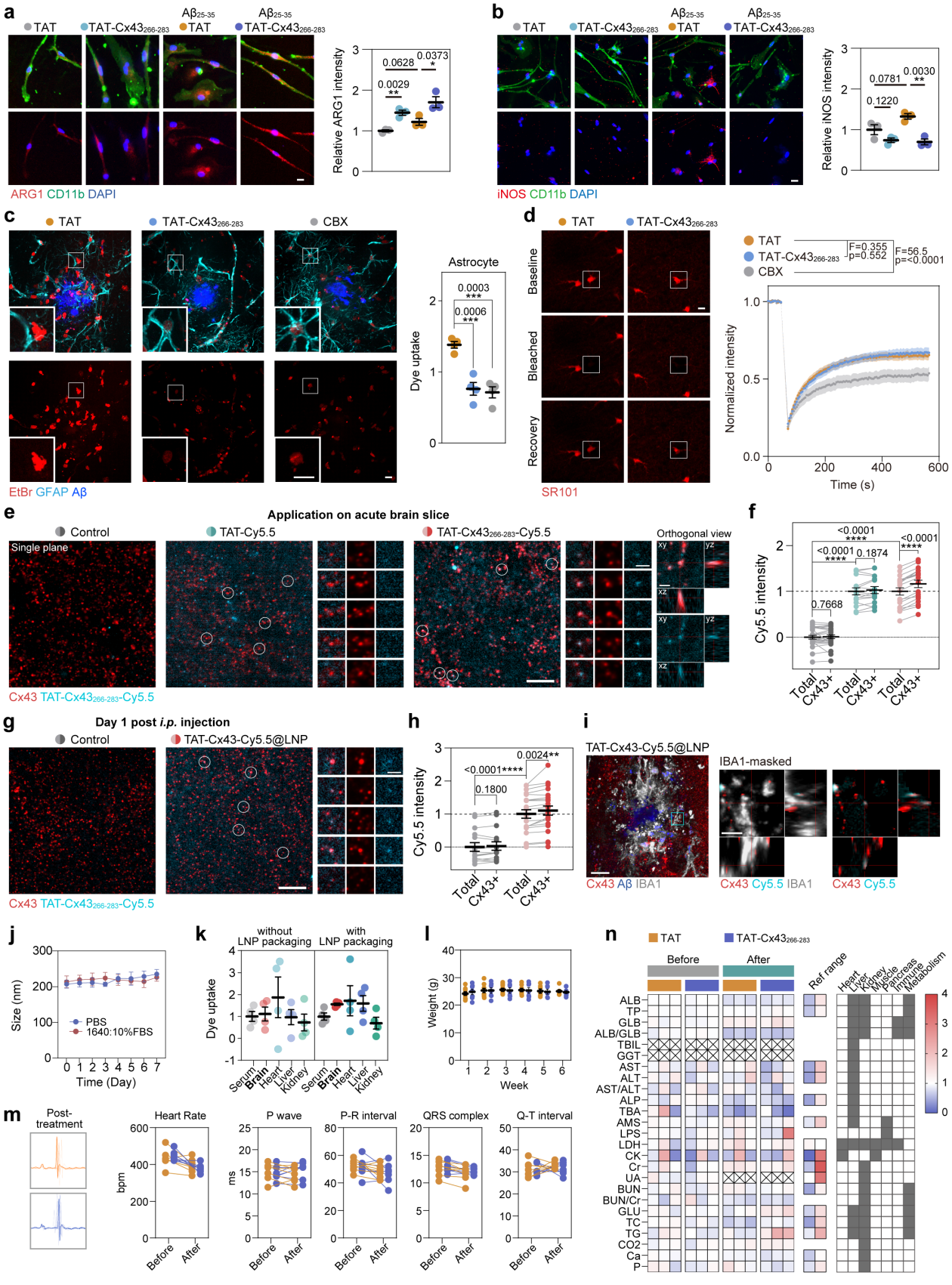
Supplementary Fig. 4: Cx43 ablation altered microglia reactive state in APP/PS1 mice.



a Periplaque microglia number was plotted against the plaque perimeter, and the linear regression with 95% confidence interval was shown. N = 76 (APP/PS1), 106 (APP/PS1:Cx43$^{mgl\text{-}cKO}$) cells. Related to Fig. 4c. **b** The fraction of Aβ plaque within the microglia domain was plotted against plaque volume with the linear regression and 95% confidence interval shown. N = 62 (APP/PS1), 77 (APP/PS1:Cx43$^{mgl\text{-}cKO}$) cells. **c** Quantification of Aβ within microglia domain normalized to periplaque microglia number. N = 5 mice. Statistical test: two-sided t-test. **d** Left: Sholl analysis of

periplaque microglia. Right: Sholl analysis of distal microglia and WT microglia. N = 83 (WT), 152 (APP/PS1-periplaque), 224 (APP/PS1:Cx43$^{mgl\text{-}cKO}$-periplaque), 77 (APP/PS1-distal), 79 (APP/PS1:Cx43$^{mgl\text{-}cKO}$-distal) cells. Statistical test: two-way ANOVA. **e** Representative images of dye uptake experiment performed on 9-month-old APP/PS1 acute brain slices. Cx43-specific channel blocker GAP26 and pan gap junction/hemichannel blocker CBX were added to 9-month-old APP/PS1 acute brain slices. Scale bar: 10 μm. **f** Heatmap shows differentially expressed genes across WT, APP/PS1, and APP/PS1:Cx43$^{mgl\text{-}cKO}$ microglia. Right: functional annotation analysis of APP/PS1 or APP/PS1:Cx43$^{mgl\text{-}cKO}$ module, i.e., genes significantly enriched in APP/PS1 or APP/PS1:Cx43$^{mgl\text{-}cKO}$ microglia. N = 3 mice. **g** Representative IBA1, ARG1, and Aβ staining. Scale bar: 10 μm. Arg1+ area in microglia was quantified and normalized to APP/PS1. N = 5 mice. Statistical test: two-sided t-test. **h** Representative IBA1, CLEC7A, and Aβ staining. Scale bar: 50 μm and 10 μm (magnification). CLEC7A+ area in microglia was quantified and normalized to WT. N = 5 mice. Statistical test: two-sided t-test. **i** Representative image of IBA1, GRN, and Aβ staining. Scale bar: 50 μm and 10 μm (magnification). GRN+ area in microglia was quantified and normalized to WT. N = 5 mice. Statistical test: two-sided t-test. **j** Dye uptake experiment on acute brain slices counterstained with GFAP to analyze the astrocytic hemichannel opening. Scale bar: 10 μm. EtBr intensity was normalized to the WT group for each experiment. N = 5 mice. Statistical test: one-way ANOVA. **k** D-serine level in the supernatant of acute brain slices measured was normalized to the APP/PS1 group. N = 4 mice. Statistical test: paired t-test. Dot plots show mean ± SEM, each data point, and *p*-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided as a Source Data file.

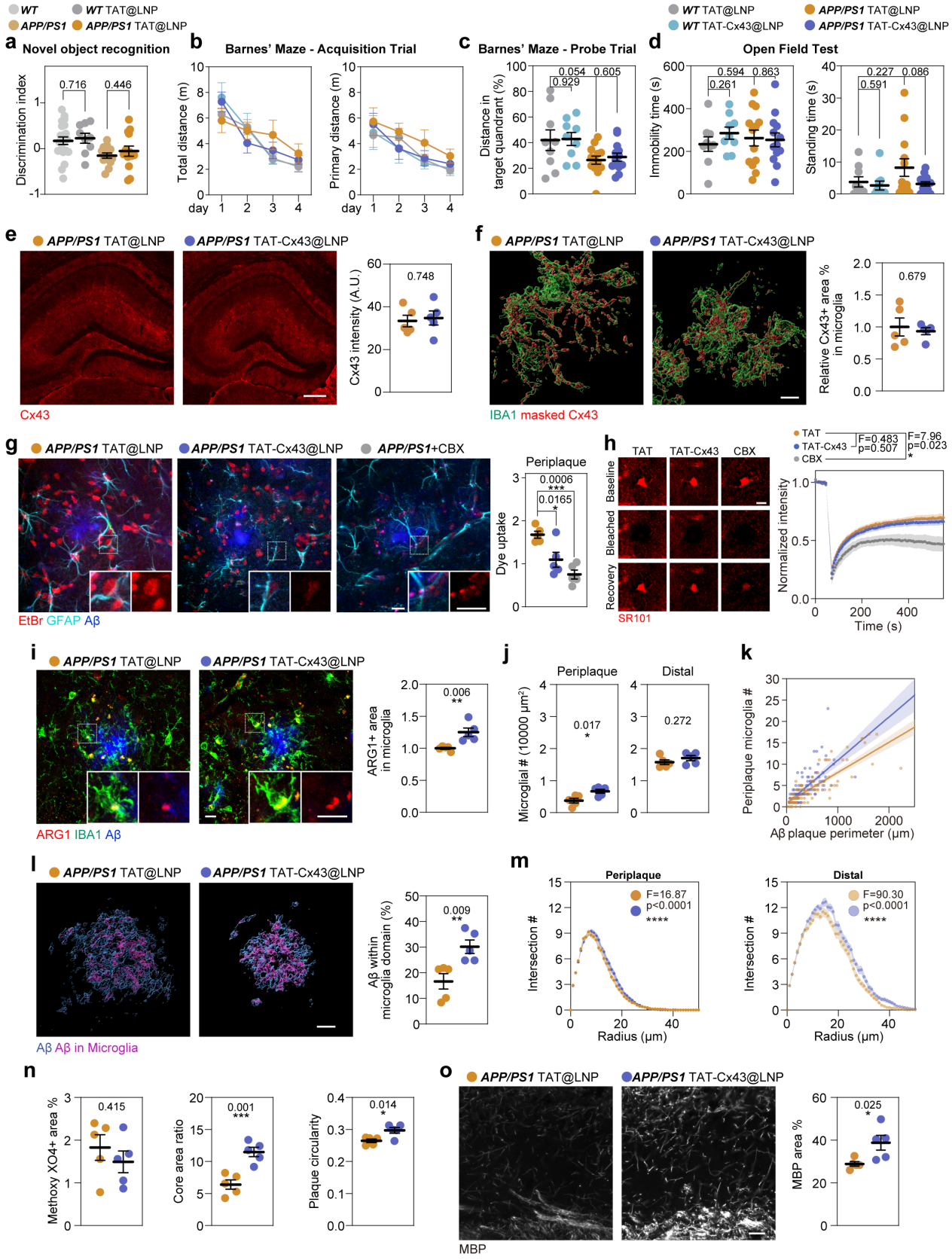
Supplementary Fig. 5: Evaluation of TAT-CX43@LNP.



a-b Primary microglia were treated with 50 μM TAT or TAT-Cx43$_{266-283}$ peptide and with or without

1 μM $A\beta_{25-35}$ for 3 days. Images show staining of ARG1 or iNOS with CD11b and DAPI. Scale bar, 5 μm. ARG1 or iNOS intensity was quantified and normalized to TAT-treated control. N = 3 experiments. Statistical test: two-sided t-test. **c** Dye uptake experiment on APP/PS1 acute brain slices and counterstained with GFAP. EtBr intensity of GFAP+ astrocytes was quantified. N = 4 experiments. Statistical test: one-way ANOVA. **d** gap-FRAP experiment using SR101 dye on acute brain slices from APP/PS1 mice to examine the astrocytic gap junction function. Scale bar, 10 μm. N = 25 (TAT), 33 (TAT-$Cx43_{266-283}$), 12 (CBX) cells. Statistical test: two-way ANOVA. **e** Cy5.5 fluorophore-conjugated TAT or TAT-$Cx43_{266-283}$ peptide were applied to acute brain slices, followed by counterstaining of Cx43 (secondary antibody, Alexa Fluor 555 conjugated). Scale bar: 10 μm and 2 μm (magnification and orthogonal view). Disaggregation of channels in white circle was shown. Orthogonal view of TAT-$Cx43_{266-283}$-Cy5.5 group showcase the true colocalization of Cy5.5 and Cx43 signal. **f** Quantification of Cy5.5 signal intensity in brain slices. The Cy5.5 intensity in Cx43+ volume was further quantified. N = 26 (control), 16 (TAT-Cy5.5), 19 (TAT-$Cx43_{266-283}$-Cy5.5) images. Statistical test: two-sided t-test (between treatments), or paired t-test (between total and Cx43+ volume). **g** Mice were intraperitoneally injected with TAT-Cx43-Cy5.5@LNP. Acute brain slices were analyzed on day 1 post injection with Cx43 counterstaining. Scale bar: 10 μm and 2 μm (magnification). White circles highlight examples of Cy5.5 enrichment at Cx43+ puncta. **h** Quantification of Cy5.5 intensity in control and TAT-Cx43-Cy5.5@LNP treated mouse brain slices. N = 16 (control), 21 (TAT-$Cx43_{266-283}$-Cy5.5) images. Statistical test: two-sided t-test (between treatments), or paired t-test (between total and Cx43+ volume). **i** Representative Cx43 and Cy5.5 colocalization in periplaque IBA1+ microglia. Scale bar: 10 μm and 2 μm (orthogonal view). **j** Stability of DOTAP-NLG lipid nanoparticles in serum-containing buffer. Lipid nanoparticles were incubated in PBS and 1640:10%FBS, sampled at different time points for size measurement. N = 3 experiments. **k** Tissue lysates were collected 1 day post-injection for microplate reader analysis of Cy5.5 intensity. N = 4 experiments. **l** Mice were weighed once a week during prolonged TAT@LNP or TAT-Cx43@LNP treatment. N = 7~9 mice. **m** Representative electrocardiogram of mice after 6-week treatment and quantification of electrocardiogram parameters before and after treatment. N = 7 mice. **n** Heatmap showing serum biochemistry parameters measured before and after TAT@LNP or TAT-Cx43@LNP treatments. Available reference ranges are listed, accompanied by the association between parameters and vital organs. ALB, albumin. TP, total protein. GLB, globulin. TBIL, total bilirubin. GGT, γ-glutamyl transpeptidase. AST, aspartate aminotransferase. ALT, alanine aminotransferase. ALP, alkaline phosphatase. TBA, total bile acids. AMS, serum amylase. LPS, lipase. LDH, lactate dehydrogenase. CK, creatine kinase. Cr, creatinine. UA, uric acid. BUN, blood urea nitrogen. GLU, glucose. TC, total cholesterol. TG, triglyceride. CO2, carbon dioxide. Ca, Calcium. P, phosphate. N = 3 mice. Dot plots show mean ± SEM, each data point, and *p*-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided as a Source Data file.

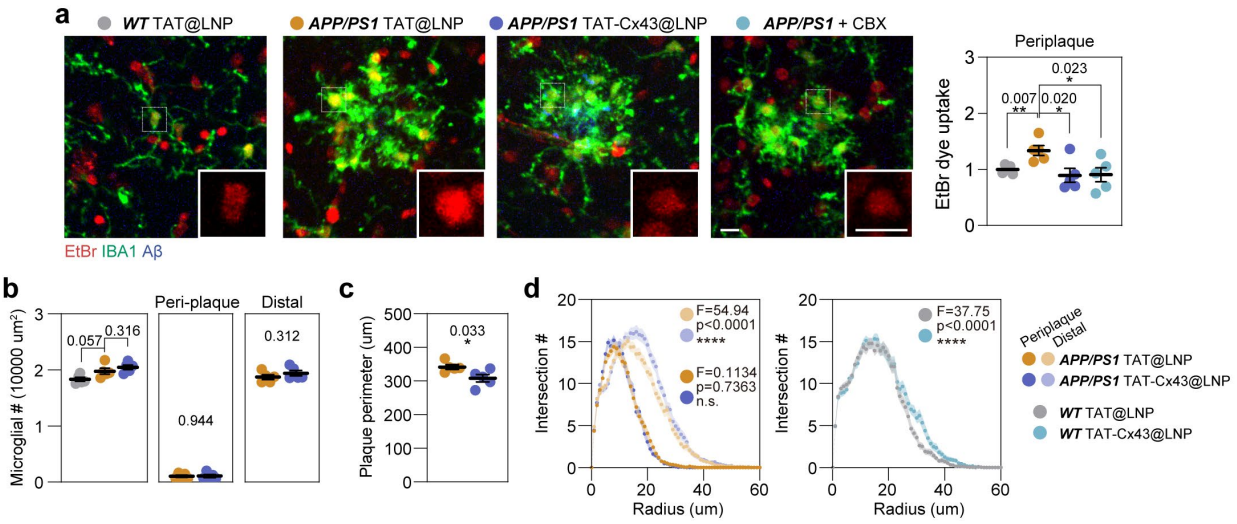
Supplementary Fig. 6: Effect of TAT-CX43@LNP on APP/PS1 mice.



a Comparison of novel object recognition index between untreated mice and TAT@LNP treated mice. See Fig. 3g and Fig. 6d. Statistical test: two-sided t-test. **b** Quantification of total distance, and

primary distance to the target hole during Barnes' maze acquisition trial. **c** Quantification of distance spent in the target quadrant during Barnes' maze probe trial. **d** Quantification of immobility time and standing time during the open field test. For all behavioral tests, N = 9 (WT TAT and WT TAT-CX43 groups) or 13 (APP/PS1 TAT and APP/PS1 TAT-CX43 groups) mice. Statistical test: two-sided t-test. **e** Representative image of Cx43 staining of hippocampus and quantification of Cx43 intensity. Scale bar: 100 μm. N = 5 mice. Statistical test: two-sided t-test. **f** 3D rendering of IBA1 staining and Cx43 signal within IBA1+ domain. Scale bar: 10 μm. The microglial Cx43 signal was quantified, N = 5 mice. Statistical test: two-sided t-test. **g** Dye uptake experiment performed on acute brain slice followed by GFAP staining, Scale bar: 10 μm. The EtBr intensity of GFAP+ astrocyte was quantified. N = 5 mice. Statistical test: one-way ANOVA. **h** Representative image of gap-FRAP experiments and quantification of SR101 dye intensity. Scale bar: 10 μm. N = 5 mice. Statistical test: two-way ANOVA. **i** Representative image of IBA1, ARG1, and Aβ staining. Scale bar: 10 μm. Arg1+ area in microglia was quantified and normalized to the mean of APP/PS1. N = 5 mice. Statistical test: two-sided t-test. **j** Quantification of periplaque microglia and distal microglia normalized to the area. N = 5 mice. Statistical test: two-sided t-test. **k** the number of periplaque microglia was plotted against the plaque perimeter. The linear regression analysis was performed. **l** 3D rendering of Aβ plaque and Aβ plaque within IBA1+ microglial domain. Scale bar: 10 μm. The fraction of Aβ plaque within IBA1+ microglial domain was quantified. N = 5 mice. Statistical test: two-sided t-test. **m** Sholl analysis of periplaque and distal microglia. N = 174 (APP/PS1-TAT-periplaque), 161 (APP/PS1-TAT-C43-periplaque) 69 (APP/PS1-TAT-distal) 79 (APP/PS1-TAT-Cx43-distal). Statistical test: two-way ANOVA. **n** Quantification of Methoxy X04+ Aβ plaque area, plaque core area ratio, and plaque circularity. N = 5 mice. Statistical test: two-sided t-test. **o** Representative image of MBP staining of hippocampal CA1 region. Scale bar: 10 μm. MBP+ area in the hippocampus was quantified. N = 5 mice. Statistical test: two-sided t-test. Dot plots show mean ± SEM, each data point, and *p*-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided as a Source Data file.

Supplementary Fig. 7: Early intervention with TAT-CX43@LNPs in APP/PS1 mice.



a Dye uptake experiment was performed on APP/PS1 acute brain slices and counterstained with IBA1. Scale bar: 10 µm. EtBr intensity of IBA1+ microglia was quantified and normalized to WT. N = 5 mice. Statistical test: one-way ANOVA. **b** Quantification of the number of total microglia, periplaque microglia, and distal microglia normalized to the area. N = 5 mice. Statistical test: two-sided t-test. **c** Quantification of Plaque perimeters. N = 5 mice. Statistical test: two-sided t-test. **d** Sholl analysis of periplaque and distal microglia in APP/PS1 mice as well as WT microglia under TAT or TAT-CX43@LNP treatment. For periplaque microglia, N = 65 (TAT) and 72 (TAT-CX43) cells. For distal microglia, N = 50 (TAT) and 62 (TAT-CX43) cells. For WT, N = 42 (TAT) and 45 (TAT-CX43) cells. Statistical test: two-way ANOVA. Dot plots show mean ± SEM, each data point, and *p*-value. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided as a Source Data file.