

Original Article

Loss of Newborn Neuron Function Promotes A β Accumulation in an Alzheimer's Disease Mouse Model

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[Received June 6, 2026; Revised September 3, 2026; Accepted September 11, 2026]

ABSTRACT: Whether the loss of adult hippocampal newborn neuron (NBN) function contributes to Alzheimer's disease (AD) amyloid pathology remains unclear. Adult NBNs regulate dentate gyrus (DG) circuitry, and their decline has been observed during AD progression. To investigate their role, we generated NBN-APP mice by crossing APPswe/PS1dE9 mice with NBN-TetX mice. Importantly, *in vivo* microdialysis in the DG molecular layer prior to plaque formation demonstrated that the loss of NBN function for 4 weeks, starting at 3 months of age (early phase), elevated soluble A β levels ($p < 0.001$). At this early phase, we also observed increased A β deposition in the lateral entorhinal cortex ($p < 0.001$). Furthermore, to evaluate chronic cumulative pathology, we extended the loss of NBN function to 8 weeks starting at 5 months of age (late phase), which resulted in enhanced A β plaque deposition in the DG molecular layer, widespread glial activation and inflammatory responses throughout the hippocampus, alongside cognitive alterations. Together, these findings demonstrate that the loss of NBN function consistently induces A β accumulation across both early and late phases, and is associated with glial activation, highlighting its contribution to AD pathology.

Keywords: adult hippocampal neurogenesis, Alzheimer's disease, Amyloid β

INTRODUCTION

A β accumulation is a central pathological hallmark of Alzheimer's disease (AD), resulting from an imbalance between A β production and clearance [1]. Previous studies have shown that A β pathology itself can alter neuronal excitability and network activity, while neuronal activity also regulates extracellular A β release and amyloid deposition [2-4]. Aberrant neuronal hyperactivity and hippocampal network instability are increasingly recognized as early features of AD in both clinical and experimental studies [5, 6]. Importantly, increasing evidence suggests that neuronal hyperactivity and

network dysfunction occur at early stages of AD and may contribute to the progression of A β pathology through activity-dependent A β release mechanisms [7-9].

Adult hippocampal newborn neurons (NBNs) are a unique neuronal population that contributes to the regulation of dentate gyrus (DG) circuit activity and the maintenance of sparse neuronal activity within the hippocampus [10, 11]. Notably, impairments in NBNs have been reported in AD patients and may occur prior to overt A β accumulation and other pathological features [12, 13]. Alterations in NBNs have also been observed in several AD mouse models [14-16], suggesting that the

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disruption of NBN homeostasis is an early pathological feature of AD.

We previously investigated DG neuronal activity using the NBN-TeTX mouse model, a loss of NBN function model in which conditional expression of tetanus toxin selectively cleaves VAMP2 in NBNs and suppresses neurotransmitter release without altering cell numbers [17]. The loss of NBN function for 4 weeks in this model increased hippocampal activity and neuronal activity within the DG, including mossy cells, accompanied by the disruption of E/I balance in the DG circuit. Although NBN alterations have been observed in AD and neuronal activity is known to influence A β dynamics, whether DG circuit alterations induced by the loss of NBN function contribute to A β pathology remains unclear.

Here, we investigated whether the loss of NBN function contributes to A β pathology by generating NBN-APP mice through crossing NBN-TeTX mice with APP^{swe}/PS1dE9 mice. The loss of NBN function for 4 weeks in the early phase (starting at 3 months of age) increased soluble A β _{1–42} levels in the DG molecular layer (ML), accompanied by enhanced A β plaque deposition in the lateral entorhinal cortex (LEC). Region-specific glial reactivity and a postsynaptic reduction in granule cells (GCs) were also observed, as evidenced by a decrease in PSD95-positive puncta in the ML. When the duration of NBN function loss was extended to 8 weeks, A β deposition was significantly increased in the ML, and disease-associated glial changes and inflammatory responses were observed within the hippocampus. Together, these findings suggest that the loss of NBN function contributes to hippocampal A β accumulation.

MATERIALS AND METHODS

Mice

The NBN-TeTX (newborn neuron-tetanus toxin expressing) mouse model was generated by combining multiple transgenic systems: the *Nestin-CreERT2* system and the *TetO-TeTX* system [17] (Fig. 1A). *CreERT2* is inserted under the control of the *Nestin* promoter, which is active in neural progenitor cells [18]. In addition, under the control of the *α CaMKII* promoter, a stop codon flanked by loxP sites is inserted upstream of tTA, allowing tTA expression only after Cre-mediated recombination. Following Cre-mediated recombination, tTA expression drives TeTX expression under the control of the *tet* operator. TeTX cleaves VAMP2 and suppresses neurotransmitter release from newborn neurons [19, 20].

APP^{swe}/PS1dE9 (APP) mice (strain name: B6C3-Tg(APP^{swe}, PSEN1dE9) 85Dbo/J, stock number:

004462) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). NBN-APP mice were generated by crossbreeding NBN-TeTX mice with APP^{swe}/PS1dE9 mice in our laboratory. Depending on the breeding strategy, APP mice carried NBN-related transgenes (*α CaMKII-tTA* and *TetO-TeTX*) but lacked functional TeTX induction in newborn neurons. Only mice exhibiting inducible TeTX expression in newborn neurons were classified as NBN-APP mice. Before pooling the data from both sexes, we performed preliminary analyses using two-way RM ANOVA or unpaired *t*-tests, as appropriate, to evaluate potential sex effects. Because these analyses revealed no significant main effects of sex or sex-interaction effects ($p > 0.05$), male and female data were pooled for the final analyses. All mice were genotyped using standard procedures and housed under a 12-hour light/dark cycle with free access to food and water. All animal procedures were approved by the Ethics Committee of the University of Tokyo (C-20-2) and were conducted according to the guidelines for animal experimentation.

Tamoxifen administration

Tamoxifen was used to induce Cre recombinase activity in CreERT2-expressing cells. Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA, T5648) was dissolved in a mixture of corn oil and 100% ethanol (9:1 ratio) to a final concentration of 30 mg/mL. The solution was incubated at 53 °C for 1 hour with periodic agitation to ensure complete dissolution of tamoxifen. Mice were intraperitoneally administered tamoxifen at a daily dose of 180 mg/kg for three consecutive days.

Experimental plan

APP and NBN-APP mice were used for histological, biochemical, behavioral, and transcriptomic analyses. Tamoxifen was administered to all mice used in this study (both APP and NBN-APP mice). To assess soluble A β dynamics following the loss of NBN function in the early phase, tamoxifen was administered at 3 months of age, and *in vivo* microdialysis was performed 4 weeks later. For the microdialysis experiments, we used a total of 6 APP mice (4 males, 2 females) and 6 NBN-APP mice (4 males, 2 females). For the following assessment of A β accumulation and glial alteration, 4 APP mice (2 males, 2 females) and 4 NBN-APP mice (2 males, 2 females) were used. To evaluate the prolonged effects of an 8-week loss of NBN function on hippocampal A β accumulation, DG alterations, and behavioral changes in the later phase, tamoxifen was administered at 5 months of age, and mice were sacrificed 8 weeks later for tissue collection and

subsequent analyses. For the assessment of A β accumulation and glial alterations, 6 APP mice (4 males, 2 females) and 6 NBN-APP mice (4 males, 2 females) were used; for the ELISA quantification of APP, 7 APP mice (4 males, 3 females) and 7 NBN-APP mice (4 males, 3 females) were used; for the ELISA quantification of TNF- α , 10 APP mice (5 males, 5 females) and 10 NBN-APP mice (4 males, 6 females) were used; for the ELISA quantification of IL-6, 9 APP mice (4 males, 5 females) and 10 NBN-APP mice (4 males, 6 females) were used; for the MWM test, 7 APP mice (4 males, 3 females) and 10 NBN-APP mice (5 males, 5 females) were used; and for the CFC test, 10 APP mice (6 males, 4 females) and 10 NBN-APP mice (5 males, 5 females) were used.

Brain sample preparation

Under deep anesthesia, mice were perfused through the left ventricle with 20 mL of 1 $\times$ PBS. Brains were rapidly removed, and one hemibrain was fixed overnight in 4% paraformaldehyde at 4 °C. The hippocampus from the other hemibrain was dissected immediately and stored at –80 °C for biochemical analyses. The following day, the fixed hemibrain was sequentially immersed in 10% sucrose in 1 $\times$ PBS, 20% sucrose in 1 $\times$ PBS, and 30% sucrose in 1 $\times$ PBS. Hemibrains were then embedded in O.C.T. Compound (Sakura, Tokyo, Japan) and frozen. Coronal sections were cut at a thickness of 20 μ m using a cryostat (Thermo Fisher Scientific, Waltham, MA, USA) set to –25 °C. Sections from the hippocampal region (spanning bregma –1.34 to –3.50 mm) were collected, immersed in cryoprotectant solution, and stored at –30 °C until use.

Immunohistochemistry

To validate antibody specificity and distinguish genuine target staining from non-specific background, negative controls were performed by omitting the primary antibodies and incubating the sections with the corresponding secondary antibodies only. Free-floating brain sections were removed from cryoprotectant solution and washed in Tris-buffered saline (TBS). After blocking, the sections were incubated with primary antibodies at 4 °C overnight with agitation. The sections were then washed in TBS and incubated with appropriate secondary antibodies at room temperature for 2 h. After secondary antibody incubation, the sections were washed in TBS and mounted for imaging.

Primary antibodies used were anti-Iba1 (goat, 1:1000, Fujifilm Wako, Osaka, Japan, 011-27991), anti-GFAP (mouse, 1:1000, Sigma-Aldrich, G3893), anti-A β (clone BAN50; mouse, 1:1000, Fujifilm Wako, 013-26873),

anti-PSD95 (rabbit, 1:500, Thermo Fisher Scientific, 51-6900), and anti-Vglut1 (guinea-pig, 1:1000, Sigma-Aldrich, AB5905). The secondary antibodies used were anti-goat IgG Alexa Fluor 488 (1:1000, Thermo Fisher Scientific, A11055), anti-mouse IgG Alexa Fluor 647 (1:1000, Thermo Fisher Scientific, A31571), anti-mouse IgG Alexa Fluor 488 (1:1000, Thermo Fisher Scientific, A21202), anti-goat IgG CyTM3 (1:200, Jackson ImmunoResearch, West Grove, PA, USA, 705-165-147), anti-rabbit IgG Alexa Fluor 568 (1:1000, Thermo Fisher Scientific, A10042), and anti-guinea-pig IgG CyTM5 (1:200, Jackson ImmunoResearch, 706-175-148).

Image acquisition and analysis

All images were acquired using a confocal laser scanning microscope (FLUOVIEW FV3000, Olympus, Tokyo, Japan). Image processing and quantitative analysis were performed using ImageJ software by an investigator blinded to genotype. To measure the A β plaque area, BAN50 antibody-positive A β plaques were segmented using a constant intensity threshold that selectively identified plaque deposits while excluding diffuse background immunoreactivity. The plaque area was quantified as the percentage of the area occupied by thresholded BAN50-positive plaques within the ROI. To analyze astrocyte and microglia morphology, Z-stack images were acquired and processed using the Skeleton plugin in ImageJ to quantify the number of branches per astrocyte and the soma area per microglial cell. For PSD95 and Vglut1 puncta analyses, fluorescence images were converted to 8-bit grayscale, and a fixed threshold was applied to all images within each experiment. Positive puncta were identified and quantified using the Analyze Particles function under identical analysis parameters for all samples. Colocalization of PSD95 and Vglut1 was determined using the Image Calculator function (AND operation window function) in ImageJ.

snRNA-seq analysis

Two mice of each genotype (APP and NBN-APP; 1 male and 1 female each) were used for snRNA-seq. This snRNA-seq experiment was designed to identify cell-type-specific transcriptomic alterations associated with loss of NBN function for 8 weeks in the APP background. Because non-APP NBN-TetX mice were not included, this dataset does not allow discrimination between transcriptional effects attributable to NBN dysfunction alone and those specific to the APP/A β pathological context. After perfusion with 20 mL of 1 $\times$ PBS under deep anesthesia, brains were rapidly removed and sectioned into 500 μ m coronal slices in cold PBS using a vibratome.

The DG region was micro-dissected under a stereomicroscope, and the collected tissue was incubated in lysis buffer and gently homogenized. The lysate was centrifuged, and the resulting pellet was resuspended in Nuclei Wash and Resuspension Buffer (Sigma-Aldrich, NUC201). Intact nuclei were isolated using a sucrose gradient centrifugation method.

Library preparation and sequencing were performed in collaboration with Dr. Masashide Seki using the Chromium Single Cell 3' Gene Expression v3.1 kit (10x Genomics) according to the manufacturer's protocol. Raw sequencing data were processed using Cell Ranger (v9.1.0, 10x Genomics). Barcode processing and UMI counting were performed using the Cell Ranger pipeline. Reads were aligned to the mouse reference genome (GRCm39/mm39) using a pre-mRNA reference to include intronic reads. Libraries were sequenced on an Element AVITI platform (Element Biosciences, San Diego, CA, USA). After quality control and filtering, approximately 5779 nuclei were retained for downstream analysis, with an average of 1.2×10^5 reads per nucleus and ~49% uniquely mapped reads.

Data processing and analysis were performed using Seurat (v5.3.0) in R (v4.3.2). Nuclei expressing fewer than 1000 genes, more than 5000 genes (potential doublets), or exhibiting a mitochondrial gene fraction exceeding 5% were excluded. Following quality control filtering, the remaining nuclei were normalized, scaled, and clustered based on principal component analysis (PCA). Cell-type annotation was performed using representative marker genes characteristic of each cluster [21–23]. For downstream analyses, the exact number of nuclei recovered per individual sample for the specific clusters was as follows: GCs (APP: 222, NBN-APP: 1159), microglia (APP: 38, NBN-APP: 89), and astrocytes (APP: 116, NBN-APP: 1982). Differentially expressed genes between APP and NBN-APP mice were identified using a pseudobulk approach followed by DESeq2 (version 1.42.1). Genes with an adjusted *p*-value < 0.05 and an absolute log₂ fold change ≥ 0.25 were subjected to Gene Ontology (GO) enrichment analysis using ShinyGO and SynGO platforms, respectively. For the module score calculation, given the study design of *n* = 2 mice per group, statistical comparisons at the animal level lacked sufficient statistical power. Therefore, comparisons of module scores were not subjected to statistical testing, and these results are strictly considered exploratory.

Preparation of hippocampal soluble fractions

Frozen hippocampal tissue was homogenized in RIPA lysis buffer containing Protease Inhibitor Cocktail Set 1 (Fujifilm Wako, 165-26021) and centrifuged at 10,000 $\times$

g at 4 °C for 1 h. The resulting supernatant was collected as the soluble fraction. Protein concentrations were determined using the Bradford Protein Assay Kit (Takara Bio, Kusatsu, Japan, T9310A).

ELISA quantification of TNF- α and IL-6

Soluble fractions were used to quantify TNF- α levels using the LBIS Mouse TNF- α ELISA Kit (Fujifilm Wako, 296-88201) and IL-6 levels using the AssayMax Mouse IL-6 ELISA Kit (Assaypro LLC, St. Charles, MO, USA, EMI1006-1), according to the manufacturers' instructions.

Western blotting of full-length APP

Soluble fractions were used for Western blot analysis of full-length APP expression. Samples were mixed with sample buffer, heated at 95 °C for 5 min, and separated on 4–20% TGX polyacrylamide gels (Bio-Rad, Hercules, CA, USA, 4561096DC) by SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes (Bio-Rad, 1620113). Membranes were blocked and incubated overnight at 4 °C with primary antibodies, followed by incubation with secondary antibodies for 1 h at room temperature. Full-length APP signals (~100 kDa) were quantified and normalized to β -actin levels. Primary antibodies used were as follows: anti- β -actin (mouse, 1:5000, Proteintech, Rosemont, IL, USA, 66009-1-Ig) and anti-APP (rabbit, 1:5000, Abcam, Cambridge, UK, ab32136). Secondary antibodies used were HRP-conjugated anti-mouse IgG (1:5000, Proteintech, SA00001-1) and HRP-conjugated anti-rabbit IgG (1:5000, Proteintech, SA00001-2).

In vivo microdialysis and ELISA quantification of A β 1–42

In vivo microdialysis was performed to measure extracellular A β 1–42 levels in the hippocampal DG. Mice were implanted with a guide cannula at 3 months of age, corresponding to a stage prior to overt hippocampal plaque accumulation. After recovery from surgery, baseline microdialysis sampling was performed before tamoxifen administration, followed by a second sampling session 4 weeks after tamoxifen administration. A guide cannula (PEG-8; Eicom, Kyoto, Japan) was stereotactically implanted into the DG at the following coordinates relative to bregma: AP –2.3 mm, ML 1.7 mm, and DV –1.2 mm. The microdialysis probe (PEP-8-02; Eicom) and tubing were filled with artificial cerebrospinal fluid (aCSF) containing 0.15% BSA. The probe was inserted through the guide cannula, and aCSF was

circulated at a rate of 1.0 μL/min. After a 2 h equilibration period, samples were collected for 1 h. Each collected sample was immediately mixed with an equal volume of 1 M guanidine hydrochloride and incubated for 30 min at

room temperature to solubilize Aβ. The concentration of Aβ1–42 was quantified using the Human/Rat β amyloid (1–42) ELISA kit High Sensitive (Fujifilm Wako, 296-64401), according to the manufacturer’s instructions.

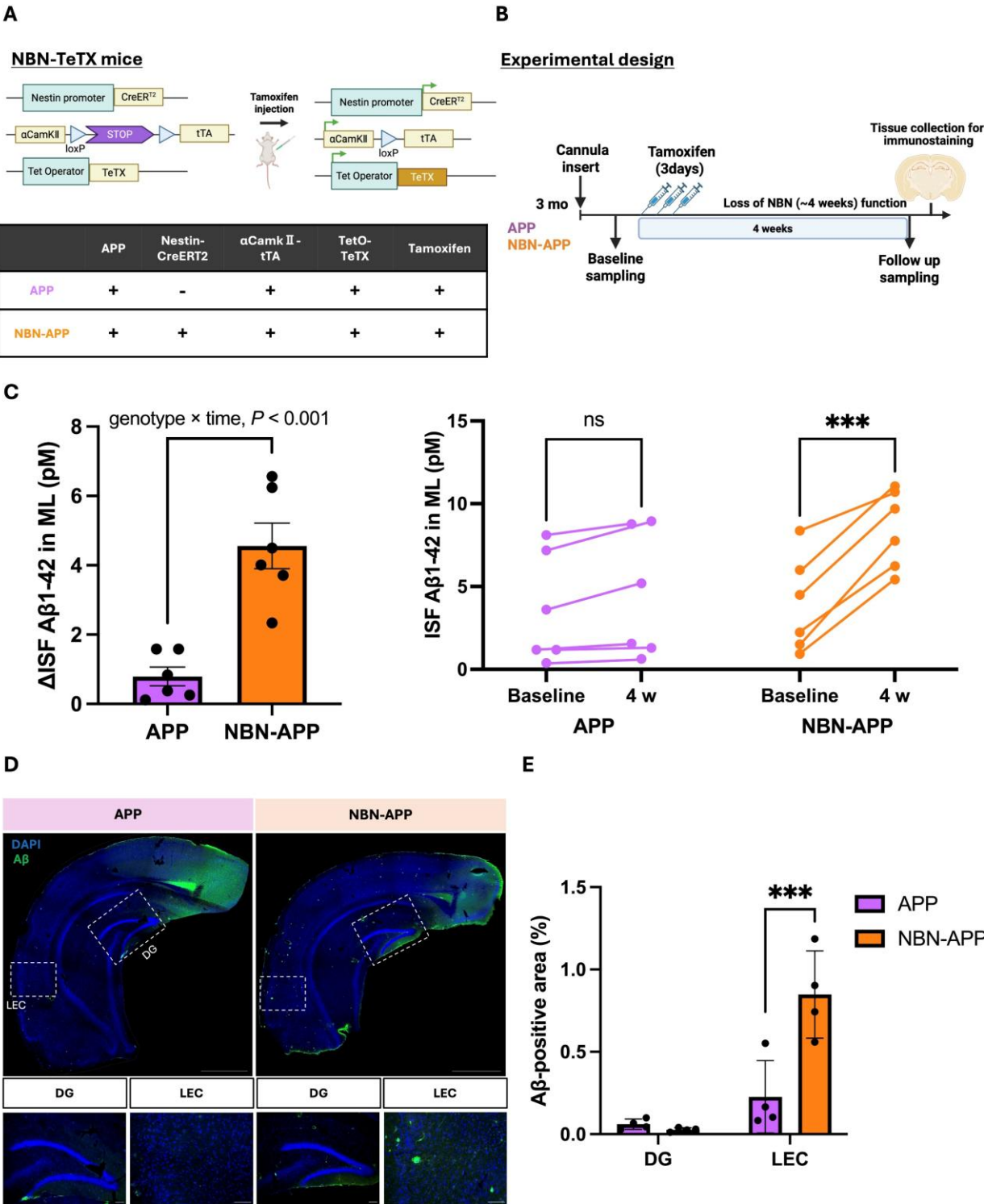


Figure 1. Loss of NBN function for 4 weeks in the early phase promotes Aβ pathology in APP mice. (A) Schematic of the NBN-TeTX mouse line carrying three transgenes. Tamoxifen-inducible *Nestin-CreERT2* activates the *αCamKII-tTA* transgene, which in turn drives TeTX expression under the control of the *tet* operator. (B) Experimental design for *in vivo* microdialysis of Aβ. Baseline samples were collected before tamoxifen administration at 3 months of age, and follow-up samples were collected 4 weeks

later. (C) ISF A β 1–42 levels in the DG ML measured by ELISA at baseline and 4 weeks after tamoxifen administration (two-way RM ANOVA, genotype: $F(1, 10) = 1.525, p = 0.245, \eta^2 = 0.132$; time: $F(1, 10) = 57.2, p < 0.0001, \eta^2 = 0.851$; interaction: $F(1, 10) = 28.3, p = 0.0003, \eta^2 = 0.739$; APP: $n = 6$, NBN-APP: $n = 6$; mean $\pm$ SEM). *** $p < 0.001$, Bonferroni's *post hoc* test. (D) Representative A β immunostaining images of coronal sections (scale bar: 1 mm), and magnified images of the DG (scale bar: 100 μ m) and the LEC (scale bar: 100 μ m) from APP and NBN-APP mice used for microdialysis. (E) Quantification of A β -positive plaque area in the DG and LEC of APP and NBN-APP mice (two-way RM ANOVA, genotype: $F(1, 6) = 11.7, p = 0.0141, \eta^2 = 0.661$; region: $F(1, 6) = 32.3, p = 0.0013, \eta^2 = 0.843$; interaction: $F(1, 6) = 14.2, p = 0.0093, \eta^2 = 0.703$; APP: $n = 4$, NBN-APP: $n = 4$; mean $\pm$ SEM). *** $p < 0.001$, Bonferroni's *post hoc* test.

Morris water maze

To assess hippocampus-dependent spatial learning and cognitive flexibility, the Morris water maze (MWM) test with a reversal learning phase was performed. A 10-cm-diameter platform was placed 1 cm below the water surface in one of four quadrants (NE, SW, SE, NW) within the pool as an escape platform. The water temperature was maintained at 22 ± 2 °C, and white non-toxic paint was added to render the water opaque, preventing visual detection of the platform. Distinct visual cues (heart, star, circle, and square) were placed on the walls in four directions of the room to provide spatial references. Experiments were conducted under dim yellow lighting. For both acquisition and reversal learning phases, mice were randomly released into one of the four quadrants and allowed to swim for a maximum of 60 s to reach the platform. Escape latency (time taken to reach the platform) was recorded as a measure of spatial learning. Upon reaching the platform, mice were allowed to remain there for 15 s. If a mouse failed to reach the platform within 60 s, it was gently guided to the platform and allowed to remain for 15 s.

The acquisition learning test was conducted in 4 trials per day for 5 consecutive days (days 1–5). This was followed by the reversal learning test (days 6–10), during which the platform was relocated to the opposite quadrant. Each of the 4 daily trials was performed with a 30-min inter-trial interval. Behavioral data were recorded and analyzed using SMART 3.0 (Panlab, Harvard Apparatus, Holliston, MA, USA). Swimming trajectories were exported as XY coordinate data and further analyzed using Pathfinder software [24]. Learning strategies were classified into seven categories: direct path, focal search, directed search, indirect search, scanning, thigmotaxis, and random search. These strategies were subsequently used for quantitative analysis of learning performance.

Contextual fear conditioning

To assess hippocampus-dependent contextual memory and cognitive flexibility, the Contextual fear conditioning (CFC) test was performed. The chamber was cleaned and scented with 70% isopropanol between sessions. During the acquisition (training) phase, mice underwent three

conditioning trials within a 370-s session. Each trial consisted of an electric shock (1 s, 0.75 mA) paired with an auditory cue (85 dB, 5000 Hz, 10 s) through the grid floor during the final second of the tone. The tone was presented at 128 s, 212 s, and 296 s after the start of the session. Twenty-four hours later, mice were returned to the same chamber for 512 s to assess contextual fear memory. No auditory cues or foot shocks were presented during this phase. Freezing behavior was recorded throughout the session to evaluate contextual fear (Med Associates, Inc., St. Albans, VT, USA). Seven days after the contextual memory test, mice were placed in the same chamber for 30 min to evaluate cognitive flexibility. Freezing behavior during this session was measured to assess the ability to extinguish acquired fear memory.

Statistical analysis

Statistical analyses for immunofluorescence staining, biochemical, and behavioral experiments were conducted based on the experimental design and sample size. Data normality was evaluated using the Shapiro–Wilk test. For two-group comparisons, a two-tailed unpaired *t*-test was used for datasets with normal distribution, while a two-tailed Mann–Whitney *U* test was applied for non-normally distributed data. To assess the interaction between time and genotype, as well as between region and genotype, a two-way repeated-measures ANOVA followed by Bonferroni's multiple comparison test was utilized. All histological and behavioral analyses were conducted independently to test distinct, prespecified *a priori* hypotheses. For behavioral evaluations across multiple timepoints or learning phases, family-wise error rates were controlled using the Bonferroni correction for pre-planned comparisons. For the transcriptomic comparisons of snRNA-seq data, statistical significance was determined using adjusted *p*-values based on the False Discovery Rate (FDR) for multiple-comparison correction, in accordance with standard bioinformatics pipelines. All statistical analyses were performed using GraphPad Prism 10.4.1 (GraphPad Software, Boston, MA, USA). Data are presented as the mean $\pm$ SEM, and $p < 0.05$ was considered statistically significant unless otherwise stated.

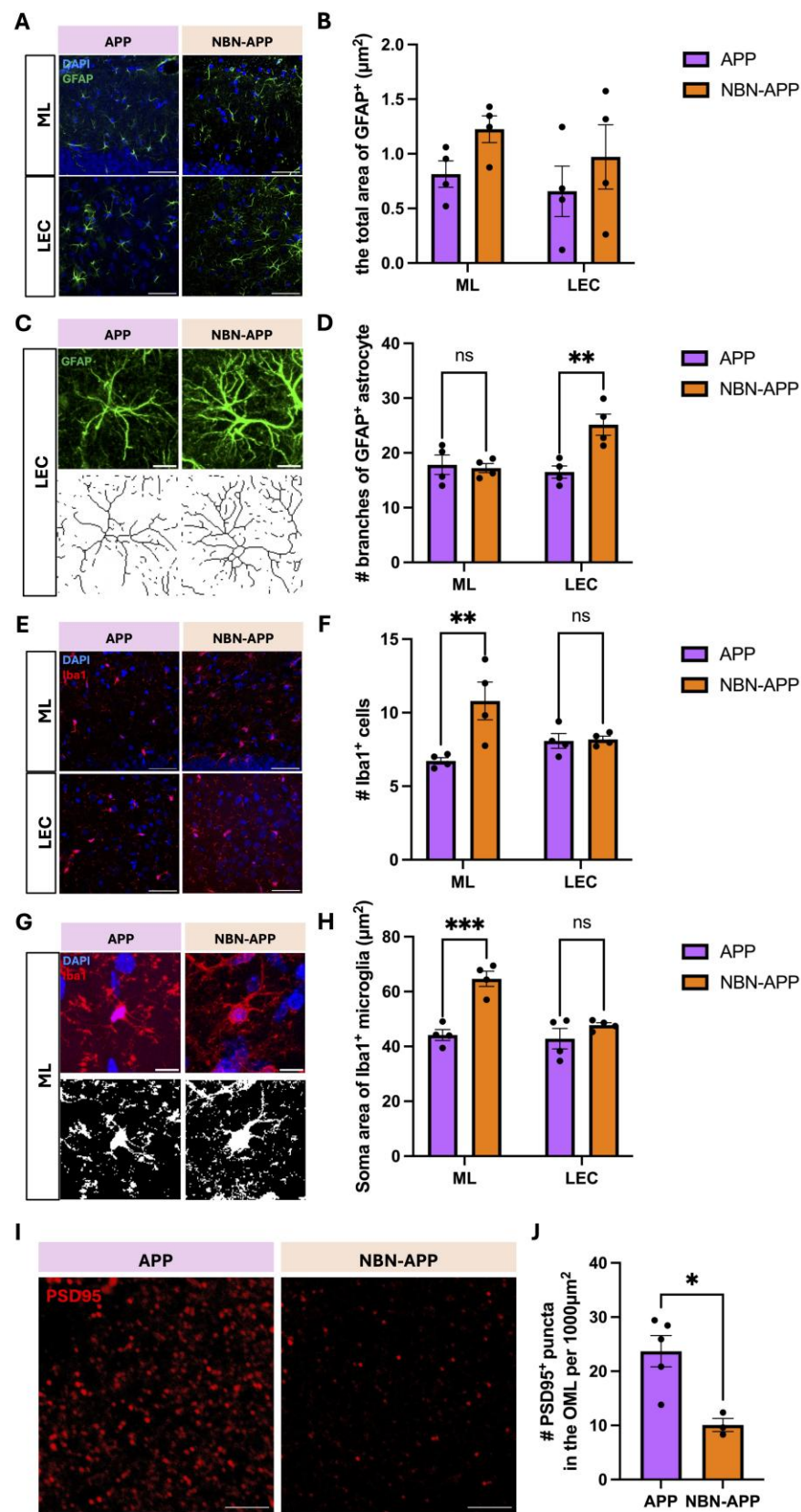


Figure 2. Loss of NBN function induces glial and synaptic alterations in APP mice. (A) Representative images of GFAP immunofluorescence in the ML and LEC (scale bar: 50 μ m). (B) Quantification of GFAP⁺ area in the ML and LEC (two-way RM ANOVA, genotype: $F(1, 6) = 2.64, p = 0.156, \eta^2 = 0.305$; region: $F(1, 6) = 1.20, p = 0.315, \eta^2 = 0.170$; interaction: $F(1, 6) = 0.0661, p = 0.806, \eta^2 = 0.0109$; APP: $n = 4$, NBN-APP: $n = 4$; mean $\pm$ SEM). (C) Representative GFAP⁺ astrocytes from APP and NBN-APP mice in the LEC, shown as original Z-stack images (top; scale bar: 10 μ m) and corresponding skeletonized images generated using ImageJ (bottom). (D) Quantification of the number of branches per GFAP⁺ astrocyte, analyzed from approximately 60 cells per mouse in the ML and LEC (two-way RM ANOVA, genotype: $F(1, 6) = 5.61, p = 0.056, \eta^2 = 0.483$; region: $F(1, 6) = 7.01, p = 0.038, \eta^2 = 0.539$; interaction: $F(1, 6) = 13.7, p = 0.0101, \eta^2 = 0.695$; APP: $n = 4$, NBN-APP: $n = 4$; mean $\pm$ SEM). $**p = 0.0029$, Bonferroni's *post hoc* test. (E) Representative images of Iba1 immunofluorescence in the ML and LEC (scale bar: 50 μ m). (F) Quantification of the number of Iba1⁺ microglial cells in the ML and LEC (two-way RM ANOVA, genotype: $F(1, 6) = 7.01, p = 0.0382, \eta^2 = 0.539$; region: $F(1, 6) = 1.06, p = 0.343, \eta^2 = 0.150$; interaction: $F(1, 6) = 10.7, p = 0.0171, \eta^2 = 0.641$; APP: $n = 4$, NBN-APP: $n = 4$; mean $\pm$ SEM). $**p = 0.003$, Bonferroni's *post hoc* test. (G) Representative Iba1⁺ microglia from APP and NBN-APP mice in the ML, shown as original Z-stack images (top; scale bar: 10 μ m) and corresponding ImageJ-generated binarized images (bottom). (H) Quantification of Iba1⁺ microglial soma size, analyzed from approximately 60 cells per mouse in the ML and LEC (two-way RM ANOVA, genotype: $F(1, 6) = 37.1, p = 0.0009, \eta^2 = 0.861$; region: $F(1, 6) = 9.53, p = 0.022, \eta^2 = 0.614$; interaction: $F(1, 6) = 6.94, p = 0.039, \eta^2 = 0.536$; APP: $n = 4$, NBN-APP: $n = 4$; mean $\pm$ SEM). $***p < 0.001$, Bonferroni's *post hoc* test. (I) Representative images of PSD95 immunostaining in the OML of the DG (scale bar: 10 μ m). (J) Quantification of the number of PSD95⁺ puncta in the DG OML per 1000 μ m² (two-tailed unpaired *t*-test, APP: $n = 5$, NBN-APP: $n = 3$; mean $\pm$ SEM). $*p = 0.0136$.

RESULTS

Loss of NBN function for 4 weeks in the early phase increases soluble A β levels and accelerates A β deposition in APP mice

To investigate whether the loss of NBN function influences A β pathology in APP mice, we generated NBN-APP mice by crossing APPswe/PS1dE9 mice with NBN-TetX mice, a tamoxifen-inducible model of loss of NBN function (Fig. 1A). Using *in vivo* microdialysis in the DG ML, we examined soluble A β dynamics following the loss of NBN function. Because our aim was to examine soluble A β dynamics before overt plaque accumulation, tamoxifen was administered at 3 months of age, and microdialysis samples were collected 4 weeks later (Fig. 1B). A two-way repeated-measures (RM) ANOVA revealed a significant time $\times$ genotype interaction in ISF A β 1–42 levels (Fig. 1C). *Post hoc* analyses showed a significant increase in ISF A β 1–42 levels over time in NBN-APP mice, whereas no significant increase was detected in APP mice. At this stage, analysis of A β deposition across the hippocampus and LEC revealed a significant region $\times$ genotype interaction (Fig. 1E). Both APP and NBN-APP mice exhibited little A β deposition in the hippocampus (Fig. 1D). In contrast, in the LEC, which is known to show physiological abnormalities and A β deposition at the earliest phase of AD [25, 26], *post hoc* analyses showed that NBN-APP mice exhibited significantly increased A β plaque deposition compared with APP mice (Fig. 1E).

Next, we evaluated glial alterations between APP and NBN-APP mice. Quantitative analyses showed no significant differences in the GFAP-positive area within the ML and LEC between the two groups (Fig. 2A, B). Because astrocyte morphology, particularly process thickness and branching complexity, is closely associated

with reactive states [27], we quantified the number of astrocytic branches. This analysis revealed a significant region $\times$ genotype interaction, and *post hoc* analyses showed an increase in branch number in the LEC of NBN-APP mice (Fig. 2C, D). Regarding microglial changes, quantification of the number of Iba1-positive microglia revealed a significant region $\times$ genotype interaction, with *post hoc* analyses showing an increase in the ML of NBN-APP mice compared with APP mice (Fig. 2E, F). Because microglial soma enlargement is associated with an activated state [28, 29], we subsequently measured microglial soma size. This analysis revealed a significant region $\times$ genotype interaction, and *post hoc* analyses showed an increase in microglial soma size in the ML of NBN-APP mice compared with APP mice (Fig. 2G, H). To further assess synaptic alterations associated with the loss of NBN function, we then examined postsynaptic structures of granule cells (GCs) using PSD95 immunostaining in the DG outer molecular layer (OML). PSD95-positive puncta in the DG OML were significantly reduced in NBN-APP mice compared with APP mice (Fig. 2I, J). Together, these findings suggest that the loss of NBN function for 4 weeks in APP mice promotes early soluble A β elevation in the ML and A β deposition in the LEC.

Loss of NBN function for 8 weeks in the later phase promotes A β deposition in APP mice

Because we observed elevated soluble ISF A β 1–42 levels following a 4-week loss of NBN function in APP mice, we next examined whether this early soluble A β elevation was associated with enhanced hippocampal A β deposition in the later phase. To this end, tamoxifen was administered at 5 months of age, and mice were sacrificed 8 weeks later for tissue collection and subsequent analyses (Fig. 3A). This time point was selected to capture the

pathological phase in APP mice, when initial Aβ deposition emerges in the hippocampus [30]. We quantified Aβ plaque area across hippocampal subregions and the LEC. A two-way RM ANOVA revealed significant main effects of genotype, region, and a genotype × region interaction. *Post hoc* analyses further revealed that this increase was particularly pronounced in the DG ML and LEC (Fig. 3B, C). To determine whether

Aβ plaque deposition in the ML was accompanied by altered APP expression, we assessed full-length APP levels in hippocampal tissue by Western blotting. However, there was no significant difference between APP and NBN-APP mice (Fig. 3D, E). Together, these findings suggest that the loss of NBN function for 8 weeks during the plaque deposition stage in APP mice promotes Aβ deposition in the DG ML and LEC.

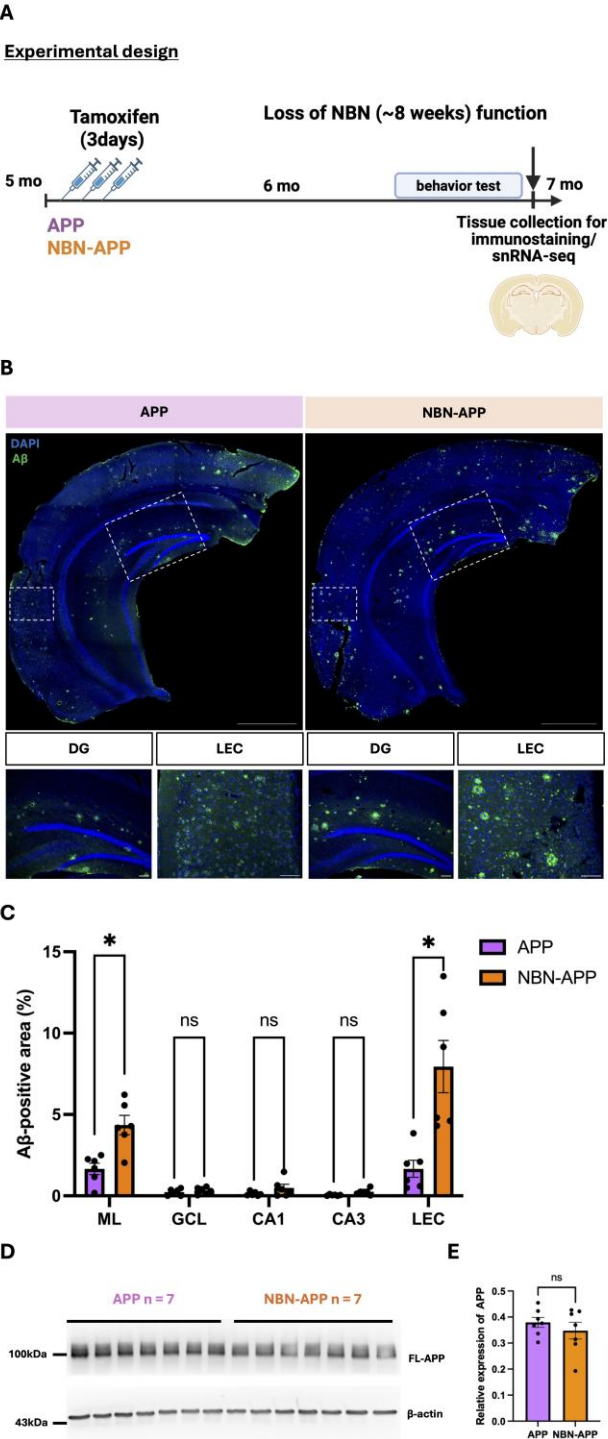


Figure 3. Loss of NBN function for 8 weeks increases A β deposition in the ML and LEC of APP mice. (A) Experimental schedule for behavioral tests, A β immunostaining and snRNA-seq, in which tamoxifen was administrated at 5 months of age, and mice were sacrificed 8 weeks later. **(B)** Representative A β immunostaining images of coronal sections (scale bar: 1 mm), and magnified images of the DG (scale bar: 100 μ m) and the LEC (scale bar: 100 μ m) from APP and NBN-APP mice. **(C)** Quantification of A β -positive plaque area across the hippocampus and the LEC in APP and NBN-APP mice (two-way RM ANOVA, genotype: $F(1, 10) = 15.3$, $p = 0.0029$, $\eta^2 = 0.605$; region: $F(4, 40) = 32.2$, $p < 0.0001$, $\eta^2 = 0.763$; interaction: $F(4, 40) = 12.9$, $p < 0.0001$, $\eta^2 = 0.563$; APP: $n = 6$, NBN-APP: $n = 6$; mean $\pm$ SEM). $*p < 0.05$, Bonferroni's *post hoc* test. **(D)** Representative immunoblots of full-length APP in whole hippocampal lysates from APP and NBN-APP mice, with β -actin as a loading control. **(E)** Quantification of APP protein levels normalized to β -actin (two-tailed unpaired *t*-test, APP: $n = 7$, NBN-APP: $n = 7$; mean $\pm$ SEM; $p = 0.418$).

Loss of NBN function for 8 weeks in the later phase promotes glial reactivity and neuroinflammatory responses in APP mice

To further characterize DG-associated neuronal and glial alterations accompanied by enhanced A β deposition following the loss of NBN function in APP mice, we performed single-nucleus RNA sequencing (snRNA-seq)

on the DG of APP and NBN-APP mice, a region in which NBNs contribute to local circuit regulation. Library preparation and sequencing were performed using the 10x Genomics Chromium platform (Fig. S1A). Cell-type annotation based on established marker genes enabled the identification of major hippocampal populations, including granule cells, astrocytes, and microglia (Fig. S1B).

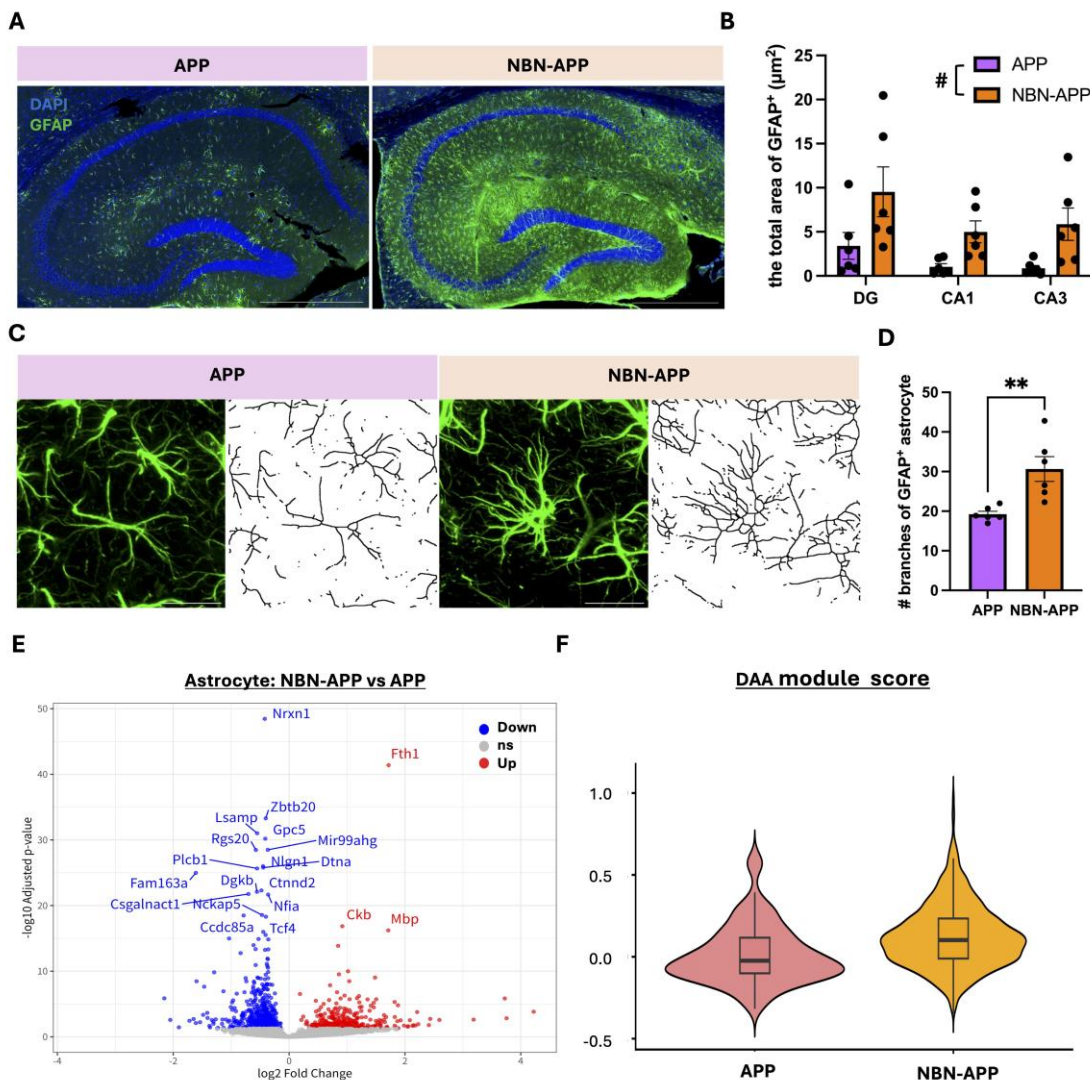


Figure 4. Loss of NBN function for 8 weeks in the later phase promotes astrocytic reactivity in APP mice. (A) Representative low-magnification images of GFAP immunofluorescence in the hippocampus (scale bar: 500 μ m). (B) Quantification of GFAP⁺ area across hippocampal subregions (two-way RM ANOVA, genotype: $F(1, 10) = 7.37$, $p = 0.0218$, $\eta^2 = 0.424$; region: $F(2, 20) = 12.7$, $p = 0.0003$, $\eta^2 = 0.559$; interaction: $F(2, 20) = 3.48$, $p = 0.0505$, $\eta^2 = 0.258$; APP: $n = 6$, NBN-APP: $n = 6$; mean $\pm$ SEM). # $p < 0.05$, main effect of genotype. (C) Representative GFAP⁺ astrocytes from APP and NBN-APP mice in the hippocampus, shown as original Z-stack images (left; scale bar: 20 μ m) and corresponding skeletonized images generated using ImageJ (right). (D) Quantification of the number of branches per GFAP⁺ astrocyte, analyzed from approximately 60 cells per mouse (two-tailed unpaired t -test, APP: $n = 6$, NBN-APP: $n = 6$; mean $\pm$ SEM). ** $p = 0.0052$. (E) Volcano plot showing differentially expressed genes in astrocytes from NBN-APP mice compared with APP mice. The top 20 genes ranked by adjusted p -value are labeled. (F) Violin plot showing the module score for DAA-associated genes, calculated using the AddModuleScore function in Seurat, based on previously published DAA signatures (median: APP = -0.0239 , NBN-APP = 0.0102).

Although a subset of cells expressing immature granule cell markers, including *Bcl11b*, *Prox1*, and *Tbr1*, was identified in GCs and neuroblast clusters (Fig. S1C) [31, 32], the number of these cells was limited, and their transcriptomic profiles did not allow

us to distinguish newly generated immature GCs from developmentally immature or transitional GC populations. Transcriptomic differences between NBN-APP and APP mice were then analyzed for each cluster using a pseudobulk approach (Fig. S1D).

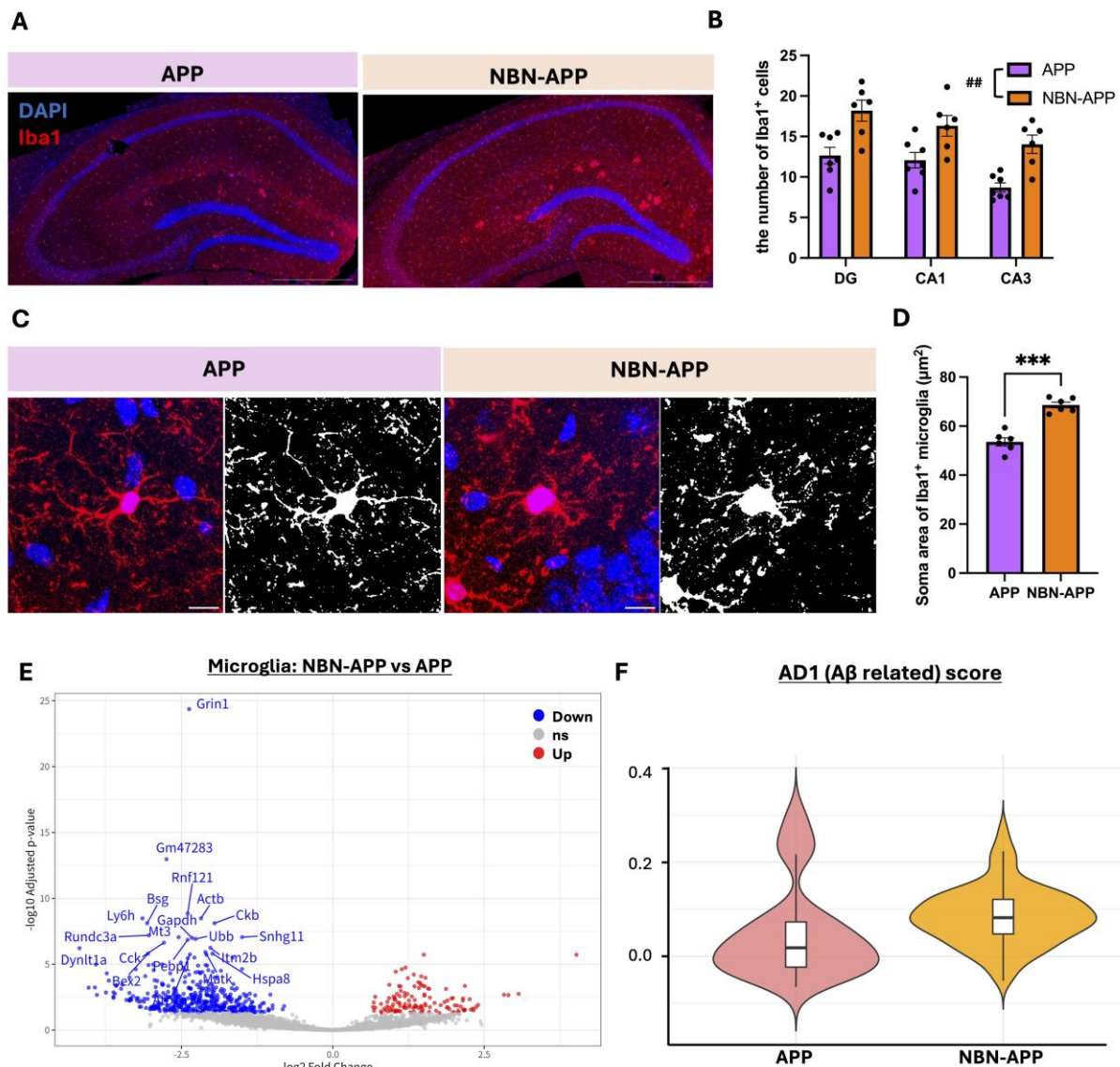


Figure 5. Loss of NBN function for 8 weeks in the later phase promotes microglial activation in APP mice. (A) Representative low-magnification images of Iba1 immunofluorescence in the hippocampus (scale bar: 500 μ m). (B) Quantification of the number of Iba1⁺ microglial cells across hippocampal subregions (two-way RM ANOVA, genotype: $F(1, 11) = 12.8$, $p = 0.0044$, $\eta^2 = 0.538$; region: $F(2, 22) = 57.6$, $p < 0.0001$, $\eta^2 = 0.840$; interaction: $F(2, 22) = 1.60$, $p = 0.225$, $\eta^2 = 0.127$; APP: $n = 7$, NBN-APP: $n = 6$; mean $\pm$ SEM). $##p < 0.01$, main effect of genotype. (C) Representative Iba1⁺ microglia from APP and NBN-APP mice in the hippocampus shown as original Z-stack images (left; scale bar: 20 μ m) and corresponding ImageJ-generated binarized images (right). (D) Quantification of Iba1⁺ microglial soma size, analyzed from approximately 60 cells per mouse (two-tailed unpaired t -test, APP: $n = 6$, NBN-APP: $n = 6$; mean $\pm$ SEM). $***p < 0.001$. (E) Volcano plot showing differentially expressed genes in microglia from NBN-APP mice compared with APP mice. The top 20 genes ranked by adjusted p -value are labeled. (F) Violin plot showing the module score for AD1-associated genes, calculated using the AddModuleScore function in Seurat, based on previously published AD1 signatures (median: APP = 0.018, NBN-APP = 0.082).

To assess astrocytic alterations in NBN-APP mice in the later phase, we quantified the GFAP-positive area in the hippocampus and observed an increase across hippocampal regions in NBN-APP mice compared with APP mice (Fig. 4A, B). Morphological assessment of GFAP-positive astrocytes revealed that NBN-APP mice exhibited an increase in the number of astrocytic branches compared with APP mice (Fig. 4C, D). Differential gene expression analysis revealed downregulation of genes associated with neuron–glia interactions and astrocytic

homeostatic functions, including *Nrxn1* (Fig. 4E) [33, 34]. To further assess the astrocytic phenotype, we subsequently evaluated disease-associated astrocyte (DAA) signatures [35]. As an exploratory analysis, calculation of a DAA module score based on the DAA gene set demonstrated a trend toward an increase in NBN-APP mice compared with APP mice (Fig. 4F). These findings suggest that astrocytic reactivity is increased in NBN-APP mice, as evidenced by both transcriptional and morphological alterations.

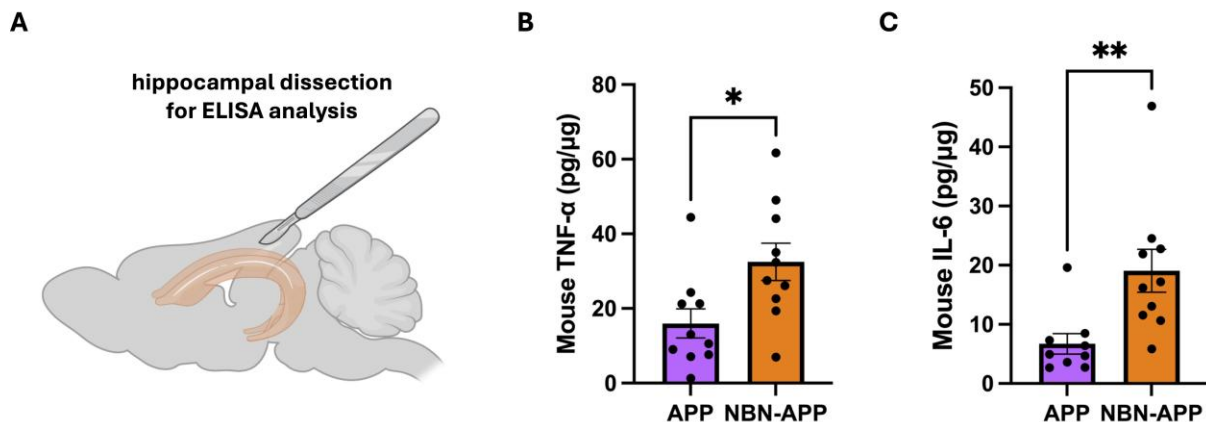


Figure 6. Loss of NBN function for 8 weeks in the later phase increases inflammatory cytokine levels in the hippocampus of APP mice. (A) Experimental schematic of dissection of the whole hippocampus for cytokine ELISA analysis. (B) Quantification of hippocampal TNF- α levels in APP and NBN-APP mice (two-tailed unpaired t -test, APP: $n = 10$, NBN-APP: $n = 10$; mean $\pm$ SEM). $*p = 0.0184$. (C) Quantification of hippocampal IL-6 levels in APP and NBN-APP mice (Mann-Whitney U test, APP: $n = 9$, NBN-APP: $n = 10$; mean $\pm$ SEM). $**p = 0.0021$.

Next, we quantified Iba1-positive microglial density across hippocampal regions and observed a significant increase in NBN-APP mice relative to APP mice (Fig. 5A, B). Additionally, NBN-APP mice exhibited an increase in microglial soma size compared with APP mice (Fig. 5C, D). Differential gene expression analysis revealed downregulation of genes related to protein homeostasis, cellular metabolism, and neuron–microglia interactions in microglia from NBN-APP mice (Fig. 5E). Notably, the most downregulated gene, *Grin1*, has been reported to be expressed in microglia in a context-dependent manner and to modulate inflammatory signaling [36]. To assess the disease relevance of these transcriptional changes, we evaluated A β -associated microglial (AD1) signatures

[37]. As an exploratory analysis, the AD1-related microglial score showed a trend toward an increase in NBN-APP mice compared with APP mice (Fig. 5F). These findings suggest that microglia in NBN-APP mice exhibit increased cell density, accompanied by AD-associated transcriptional changes and increased soma size, which are consistent with an activated microglial state.

Given that glial activation is known to be accompanied by inflammatory responses, we measured pro-inflammatory cytokine levels in hippocampal tissue. ELISA analyses demonstrated elevated levels of TNF- α and IL-6 in NBN-APP mice compared with APP mice (Fig. 6). Together, these findings suggest that NBN-APP

mice exhibit astrocytic and microglial reactivity, along with enhanced neuroinflammatory responses in the hippocampus during the Aβ plaque deposition stage.

Loss of NBN function for 8 weeks in the later phase impairs granule cell synapses and hippocampus-dependent cognitive flexibility in APP mice

Given the reduction in PSD95 puncta in GCs observed at the soluble Aβ elevation stage, we next examined whether

similar synaptic alterations persisted during the Aβ plaque deposition stage. PSD95-positive puncta in the OML of the DG were significantly reduced in NBN-APP mice compared with APP mice (Fig. 7A, B). Moreover, quantification of the percentage of Vglut1-positive puncta colocalized with PSD95, reflecting presynaptic–postsynaptic contacts in the OML, revealed a significant reduction in NBN-APP mice compared with APP mice (Fig. 7A, B).

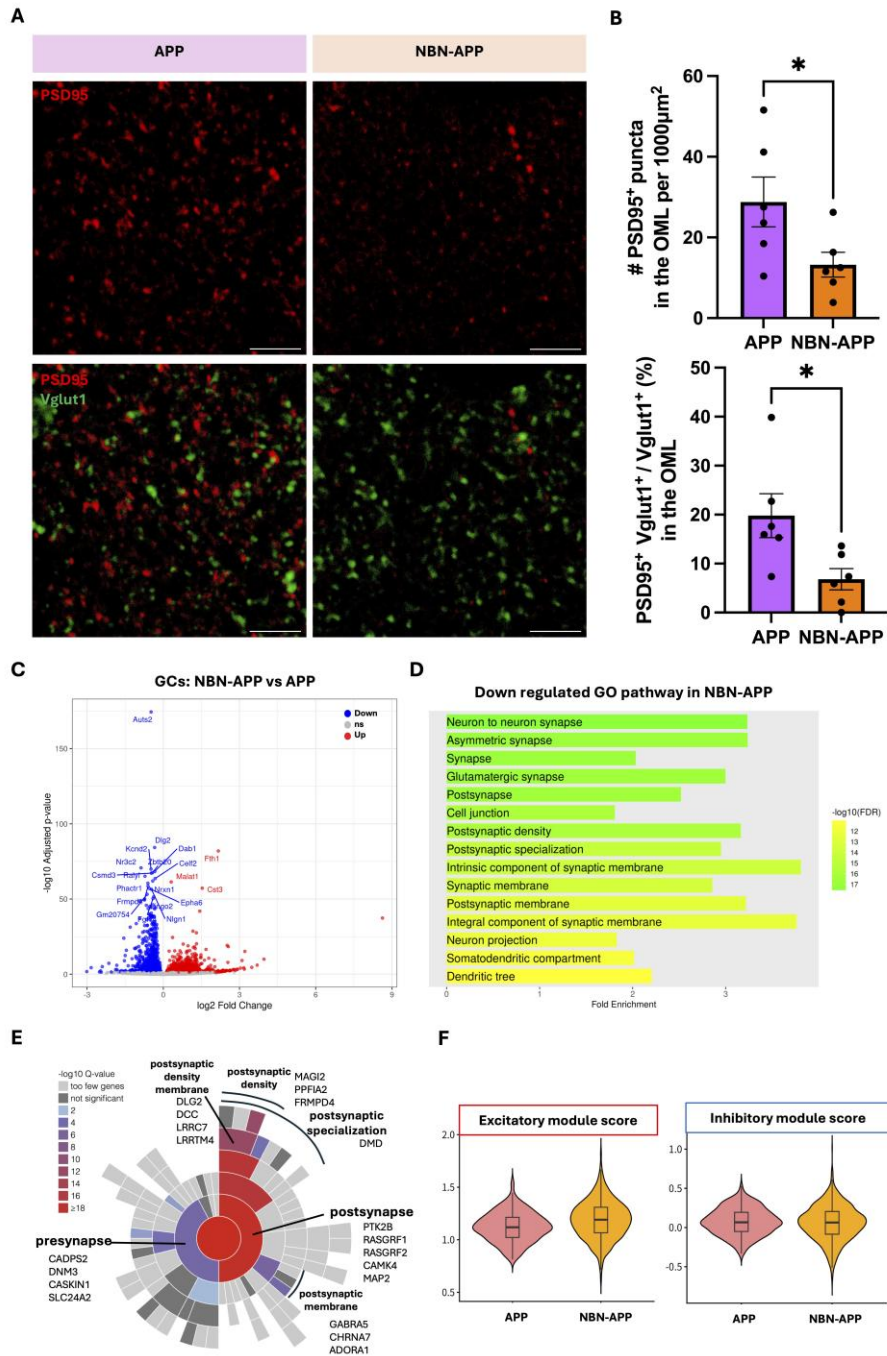


Figure 7. Loss of NBN function for 8 weeks in the later phase induces granule cell synaptic alterations in APP mice. (A) Representative images of PSD95 and Vglut1 immunostaining in the OML of the DG (scale bar: 10 μ m). (B) Quantification of the number of PSD95⁺ puncta (* p = 0.0482) (top) and the percentage of PSD95⁺ Vglut1⁺ / Vglut1⁺ (* p = 0.0263) (bottom) in the DG OML per 1000 μ m² (two-tailed unpaired t -test, APP: n = 6, NBN-APP: n = 6; mean $\pm$ SEM). (C) Volcano plot showing differentially expressed genes in GCs from NBN-APP mice compared with APP mice. The top 20 genes ranked by adjusted p -value are labeled. (D) GO cellular component (CC) enrichment analysis of genes downregulated in GCs from NBN-APP mice. GO terms are sorted by FDR from top to bottom. (E) SynGO enrichment analysis of downregulated genes in GCs from NBN-APP mice. The color gradient represents $-\log_{10}$ Q-values (FDR-corrected), with deeper red indicating greater enrichment (FDR: synapse 1.09×10^{-20} ; presynapse 2.03×10^{-5} ; postsynapse 5.52×10^{-20}). Representative differentially expressed genes in each category are shown. (F) (left) Excitatory module score distribution plot calculated from glutamatergic receptor-associated genes using the AddModuleScore function in Seurat (median: APP = 1.12, NBN-APP = 1.19). (right) Inhibitory module scores distribution plot calculated from GABAergic receptor-associated genes using the AddModuleScore function in Seurat.

Differential gene expression analysis revealed that upregulated genes in GCs from NBN-APP mice were associated with A β metabolism and neuronal stress responses, including *Fth1*, *Cst3*, and *Malat1* [38–40] (Fig. 7C). In contrast, downregulated genes were implicated in synaptic organization, neuronal signaling, and neuronal development. Notably, we observed a marked reduction in *Auts2* expression, a regulator known to suppress excitatory synaptic input in DG GCs [41]. We performed Gene Ontology (GO) enrichment analyses to characterize the downregulated transcriptional changes in GCs, and pathway terms were mainly associated with neuronal

compartments, particularly synaptic structures (Fig. 7D). We subsequently performed SynGO analysis, a synapse-specific annotation approach, to further characterize the affected synaptic pathways. This analysis revealed strong enrichment of genes associated with synaptic structures, with greater enrichment in postsynaptic components (Fig. 7E). As an exploratory analysis, to determine whether these transcriptomic changes were associated with the excitation and inhibition balance in GCs, we calculated the excitatory and inhibitory module scores of GCs based on the expression of glutamatergic receptor- and GABAergic receptor-associated genes.

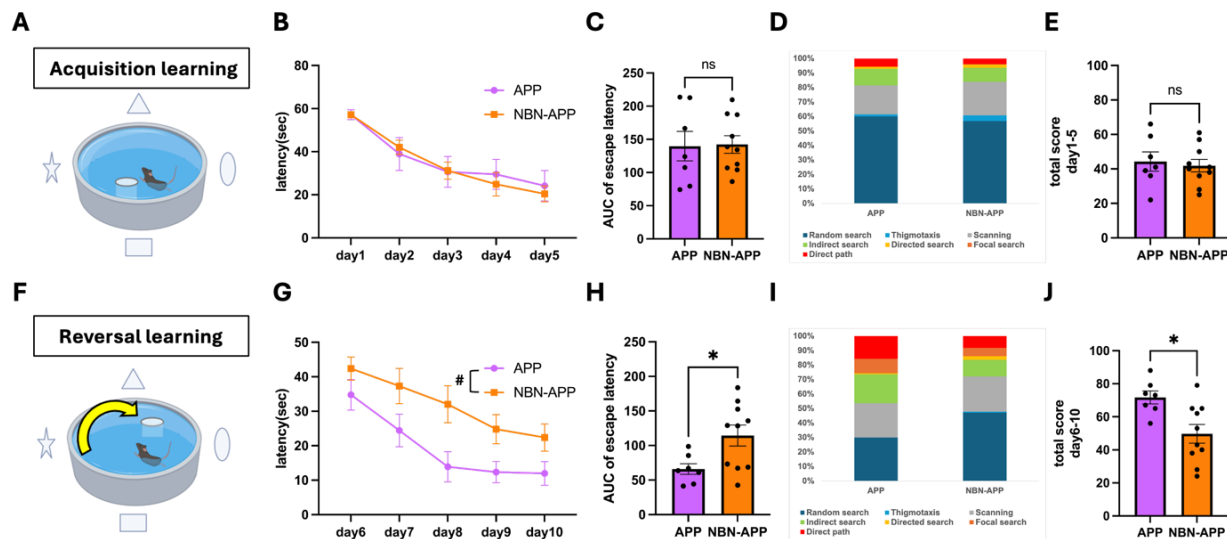


Figure 8. Loss of NBN function for 8 weeks in the later phase impairs reversal learning in the Morris water maze in APP mice. (A) MWM experimental setup schematic during acquisition learning. (B) Quantification of escape latency during acquisition learning (two-way RM ANOVA, genotype: $F(1, 15) < 0.001$, p = 0.997, $\eta^2 < 0.001$; day: $F(4, 60) = 19.4$, $p < 0.0001$, $\eta^2 = 0.564$; interaction: $F(4, 60) = 0.413$, p = 0.799, $\eta^2 = 0.0268$; mean $\pm$ SEM). (C) AUC of escape latency during acquisition learning (two-tailed unpaired t -test, p = 0.917). (D) Bar graph showing the group differences in the distribution of learning strategies during acquisition learning. (E) Calculation of the strategy score based on the score list (Fig. S2B) during acquisition learning (two-tailed unpaired t -test, p = 0.713). (F) MWM experimental setup schematic during reversal learning. The hidden platform was relocated to the opposite quadrant. (G) Quantification of escape latency during reversal learning (two-way RM ANOVA, genotype: $F(1, 15) = 4.94$, p = 0.042, $\eta^2 = 0.248$; day: $F(4, 60) = 11.5$, $p < 0.0001$, $\eta^2 = 0.434$; interaction: $F(4, 60) = 0.662$, p = 0.621, $\eta^2 = 0.0422$; mean $\pm$ SEM). # $p < 0.05$, main effect of genotype. (H) AUC of escape latency during reversal learning (two-tailed unpaired t -test, p = 0.0244, adjusted p = 0.0488 with Bonferroni correction). *adjusted- $p < 0.05$. (I) Bar graph showing the group differences in the distribution of learning strategies during reversal learning. (J) Calculation of strategy score based on score list (Fig. S2B) during reversal learning (two-tailed unpaired t -test, p = 0.0108, adjusted p = 0.0216 with Bonferroni correction). *adjusted- $p < 0.05$. For all MWM analyses: APP: n = 7, NBN-APP: n = 10.

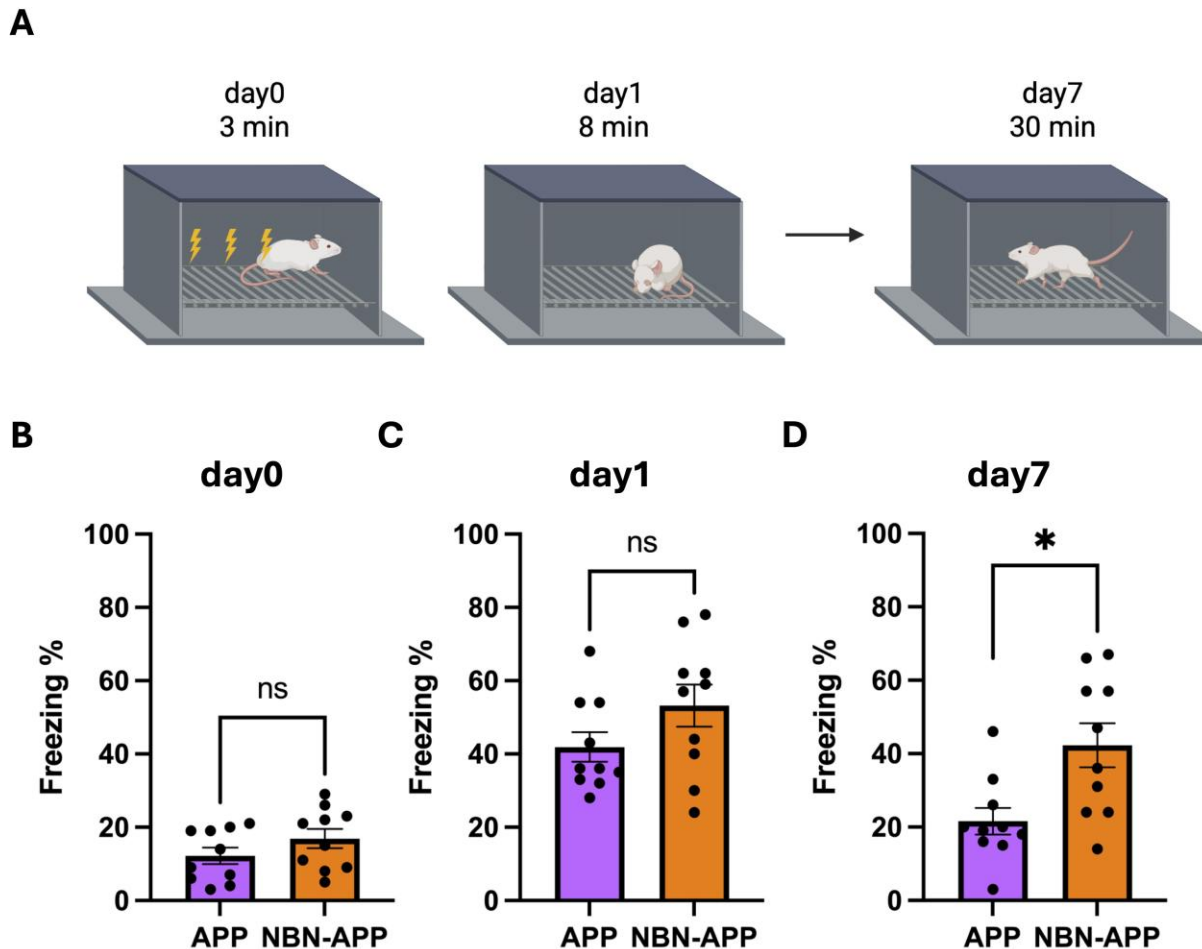


Figure 9. Loss of NBN function for 8 weeks in the later phase increases freezing behavior 7 days after contextual fear conditioning in APP mice. (A) Schematic of the CFC experimental protocol. An additional test was conducted on day 7 to assess flexibility in updating a previously fear-associated context. (B) Quantification of freezing percentage on conditioning day (day 0) (two-tailed unpaired *t*-test, $p = 0.194$). (C) Quantification of freezing percentage on day 1 (two-tailed unpaired *t*-test, $p = 0.126$). (D) Quantification of freezing percentage on day 7 (two-tailed unpaired *t*-test, $p = 0.0087$, adjusted $p = 0.0261$ with Bonferroni correction). *adjusted- $p < 0.05$. For all CFC analyses: APP: $n = 10$, NBN-APP: $n = 10$.

The excitatory module score showed a trend toward an increase in NBN-APP mice compared with APP mice, whereas the inhibitory module score remained unchanged (Fig. 7F). These findings suggest that NBN-APP mice exhibit synaptic alterations in GCs at both the histological and transcriptional levels.

To assess hippocampus-dependent spatial learning and cognitive flexibility during the A β plaque deposition stage, we performed the Morris water maze (MWM) task with a reversal learning phase in which the hidden platform was moved to the opposite quadrant (Fig. 8A, F). Throughout the learning phase, swim speed did not differ between APP and NBN-APP mice (Fig. S2A). No significant differences in escape latency were observed during acquisition learning (Fig. 8B, C). In contrast, during reversal learning, a significant main effect of genotype on escape latency was detected (Fig. 8G), and

an area under the curve (AUC) analysis of escape latency further confirmed impaired reversal learning performance in NBN-APP mice compared with APP mice (Fig. 8H). To further assess hippocampus-dependent learning strategies, MWM trajectories were analyzed using Pathfinder software based on XY coordinate data [24]. Learning strategies were classified into seven categories and subsequently grouped into hippocampus-dependent (direct path, focal search, directed search, indirect search) or hippocampus-independent (scanning, thigmotaxis, random search) strategies (Fig. S2B). The distribution of learning strategies did not differ during acquisition learning (Fig. 8D). However, during reversal learning, NBN-APP mice showed increased reliance on hippocampus-independent learning strategies (Fig. 8I). Consistent with this observation, quantitative scoring of hippocampus-dependent learning strategies revealed no

group differences during acquisition learning, whereas NBN-APP mice displayed significantly reduced hippocampus-dependent learning strategy scores during reversal learning compared with APP mice (Fig. 8E, J).

To further evaluate hippocampus-dependent contextual associative memory, we performed the contextual fear conditioning (CFC) test. An additional session was incorporated on day 7, during which mice were re-exposed to the same chamber for 30 min without an aversive stimulus, allowing the evaluation of cognitive flexibility (Fig. 9A). APP and NBN-APP mice showed the same levels of fear conditioning on day 0 and day 1 (Fig. 9B, C). However, NBN-APP mice exhibited significantly higher freezing levels than APP mice on day 7 (Fig. 9D). Together, these findings suggest that NBN-APP mice exhibit deficits in GC synapses and hippocampus-dependent cognitive flexibility.

DISCUSSION

In the present study, we found that the loss of NBN function in APP mice promotes hippocampal A β accumulation not only in the later phase, but even in the early pathological phase (Fig. 1). Using longitudinal microdialysis in the same animals and region-specific histological analyses, we show for the first time that a 4-week loss of NBN function induces elevated soluble A β levels in the DG molecular layer (ML) and enhanced A β deposition in the LEC during the early pathological phase in 4-month-old mice. While NBN numbers begin to decline from the early phase rather than being completely lost, leaving a persistent subpopulation during disease progression [12, 13, 16], several studies have shown an increase in the number of NBNs in some AD mouse models [42, 43]. Because our intervention targeted NBNs during this early phase, even prior to the robust emergence of A β plaques, we observed exacerbated A β accumulation in the DG ML, which resulted primarily from the functional blockade of ~4-week-old NBNs. These findings, in turn, suggest that enhancement of NBN function may contribute to suppressing hippocampal A β accumulation during AD progression.

At this early pathological stage, we observed not only increased A β plaque deposition but also astrocytic morphological alterations in the LEC (Fig. 2D), following a 4-week loss of function in NBNs up to 4 weeks post-differentiation. The LEC is known to be a highly vulnerable region in the early phase of AD and to exhibit several pathological changes such as functional alteration and A β deposition [25, 44]. Astrocytic morphological changes are known to occur in response to neural activity or neurotransmitters [45]. Our results suggest that astrocytic morphological changes associated with reactivity in the LEC may indicate elevated LEC activity

following the loss of NBN function. Although we have not directly demonstrated a link between the loss of NBN function and elevated LEC activity, these findings provide evidence of pathological alterations in the LEC of NBN-APP mice. A very recent transcriptomic study showed that enhancing NBNs in an AD mouse model restored the entorhinal cortex (EC) environment at the transcriptional level [46], suggesting a link between NBN function and EC function in AD. While it remains to be clarified how NBN loss affects EC neuronal function, these results underscore the importance of the EC in the study of AD pathogenesis.

Regarding the link between our observed A β deposition in the LEC and soluble A β elevation in the DG ML, previous studies have demonstrated that amyloidogenic proteins, such as APP and A β , which serve as seeds for A β accumulation, are transported from the EC and released into the DG ML via the perforant pathway [47-50]. Indeed, a study in an AD mouse model has demonstrated a correlation in A β load between the LEC and the ML [26]. In the DG ML, we observed microglial soma enlargement (Fig. 2H) during the early phase, along with elevated soluble A β accumulation. Microglia are known to play an important role in the clearance of soluble A β [51], and enlargement of the microglial soma is associated with an activated state [28, 29]. Such abnormally activated microglia lose their ability to clear A β [52, 53]. The reduction of PSD95 puncta in the ML may be the consequence of soluble A β toxicity, since several synaptic studies have shown that postsynaptic structures are particularly vulnerable to A β -associated synaptic alterations, including PSD95 reduction [54, 55]. Taken together, our findings raise the hypothesis that the loss of NBN function induces LEC pathology and increases soluble A β in the DG ML, mediated by microglial alterations.

The present report demonstrates that the loss of function in immature NBNs accelerates AD pathology progression, specifically within a narrow window of up to 4 weeks post-differentiation. Among adult hippocampal NBNs, particularly immature NBNs fire easily in response to weak inputs and have high plasticity [56-58], and importantly immature NBNs form synaptic contacts within the hilus and CA3 region as early as one week of age [59]. Therefore, these findings highlight that immature NBNs exert a significant impact on early-phase AD pathology. When tamoxifen was administered at 5 months of age and the mice were analyzed 8 weeks later (corresponding to the late phase), the loss of NBN function affected NBNs at a more mature stage (up to ~8 weeks old), although this population also included immature NBNs (~4 weeks old). We hypothesize that the prominent A β plaque accumulation observed in the ML in the later phase is an interactive consequence of the loss of

function in both immature and mature NBNs combined with the advanced amyloid accumulation of the older APP mice. We also showed no significant difference in full-length APP protein levels in the hippocampus in the later phase, which suggests that APP expression itself did not differ between APP and NBN-APP mice. However, other potential factors—such as increased APP processing, altered synaptic release, reduced enzymatic degradation, or impairments in clearance pathways—must also be considered. Ultimately, the precise mechanisms driving the increase in A β plaque deposition remain unresolved, and further studies are required to fully elucidate these dynamics.

In the later phase corresponding to elevated A β deposition in the ML of NBN-APP mice, we observed elevated levels of TNF- α and IL-6 in the hippocampus compared with APP mice. At the same stage, we also observed an increase in the GFAP-positive area and higher microglial density spreading across the DG, CA1, and CA3 regions, representing a substantially more pronounced and widespread glial response. These findings suggest that a widespread inflammatory response develops throughout the hippocampus after an 8-week loss of NBN function in the later phase. We cannot determine whether the inflammatory responses at this later stage are caused directly by the loss of NBN function. Instead, it is highly likely that this widespread inflammatory response at the later stage reflects a secondary reaction to the enhanced amyloid pathology. At the neuronal level, NBN-APP mice exhibited synaptic alterations in GCs. At the behavioral level, NBN-APP mice exhibited impairments in reversal learning in the MWM test, indicating deficits in hippocampus-dependent cognitive flexibility during the later phase. NBN-APP mice also exhibited higher freezing levels on day 7 in the CFC test. However, this result could be interpreted in multiple ways. This phenotype may reflect not just impaired fear extinction, but also enhanced fear memory consolidation, altered anxiety levels, or reduced general exploratory behavior. Nevertheless, the concurrent impairment observed in the reversal learning task strongly supports our primary interpretation of a deficit in cognitive flexibility.

Despite these findings, several limitations should be considered. First, neuronal and glial alterations were assessed exclusively in APP and NBN-APP mice. Consequently, we cannot definitively determine whether these changes reflect the loss of NBN function alone or interactions between the loss of NBN function and A β pathology. Additionally, although our experimental design minimizes confounding factors, potential subtle effects of the complex transgenic system itself cannot be definitively excluded. Furthermore, due to the limited number of biological replicates in our snRNA-seq

analysis, the transcriptomic findings should be viewed primarily as exploratory and hypothesis-generating. Finally, while we pooled male and female mice based on our preliminary analyses showing no sex differences in the current metrics, it is well documented that sex differences exist in APP/PS1 pathology, neurogenesis, and neuroinflammation. Due to sample size constraints, we were unable to include sex as a statistical covariate in our final analyses. Therefore, future studies with larger cohorts powered to detect sex-specific effects will be needed to fully elucidate the role of NBNs in a sex-dependent manner.

Taken together, our findings suggest a model in which the loss of NBN function promotes hippocampal A β accumulation in APP mice. Notably, recent gain-of-function studies enhancing adult hippocampal NBNs reported amelioration of A β pathology in an AD mouse model, providing complementary evidence that the modulation of NBN function influences A β dynamics [60]. Future studies will be important to clarify the precise circuit mechanisms and neuronal pathways through which the loss of NBN function alters A β dynamics to promote hippocampal A β accumulation.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research 21H02152, DSTEP (Data Scientist Training / Education Program), and JST SPRING, Grant Number JPMJSP2108. We thank Dr. Tadafumi Hashimoto for technical advice and guidance regarding *in vivo* microdialysis for A β measurement. We also thank Dr. Tatsunori Seki for valuable advice and discussion related to this project. Some figures were created using BioRender.com.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0713

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