

Original Article

Specific knockdown of hippocampal astroglial EphB2 improves synaptic function via inhibition of D-serine secretion in APP/PS1 mice

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Abstract: Increasing evidence emphasizes the protective role of Eph receptors in synaptic function in the pathological development of Alzheimer's disease (AD); however, their roles in the regulation of hippocampal astrocytes remain largely unknown. Here, we directly investigated the function of astroglial EphB2 on synaptic plasticity in APP/PS1 mice. Using cell isolation and transgene technologies, we first isolated hippocampal astrocytes and evaluated the expression levels of ephrinB ligands and EphB receptors. Then, we stereotaxically injected EphB2-Flox-AAV into the hippocampus of GFAP-cre/APP/PS1 mice and further evaluated hippocampal synaptic plasticity and astroglial function. Interestingly, astrocytic EphB2 expression was significantly increased in APP/PS1 mice in contrast to its expression profile in neurons. Moreover, depressing this astroglial EphB2 upregulation enhanced hippocampal synaptic plasticity, which results from harmful D-serine release. These results provide evidence of the different expression profiles and function of EphB2 between astrocytes and neurons in AD pathology.

Keywords: Alzheimer's disease, astrocyte, D-serine, EphB, synaptic plasticity

Introduction

As the most prevalent form of dementia, Alzheimer's disease (AD) is a progressive brain disorder characterized by progressive memory decline and cognitive deficiency that affects more than 26 million people worldwide [1]. Although the underlying pathological mechanism remains largely unclear, extracellular amyloid- β (A β) plaques and intracellular neurofibrillary tangles are well established in synaptic deficits, including synaptic loss and synaptic structure impairment [2], which are correlated with cognitive declines [3, 4]. Therefore, exploring the pathological mechanisms of synaptic dysfunction in AD may contribute to its treatment.

EphrinB ligands and EphB receptors are receiving increasing attention for their participation in synaptic plasticity regulation [5, 6]. For example, during pathological development, A β oligo-

mers downregulate EphB2 expression and cause major loss of the N-methyl-D-aspartate receptor (NMDAR) [7, 8]. Thus, overexpression of EphB2 can counteract A β oligomer-induced neurotoxicity in hippocampal neurons and upregulate synaptic NMDAR expression [9], suggesting that EphB2 may be related to AD-induced synaptic dysfunction. Moreover, knockdown of EphB2 reduced NMDAR currents and impaired long-term potentiation in the dentate gyrus. Nevertheless, increasing EphB2 expression rescued NMDAR-dependent long-term potentiation deficits and ameliorated cognitive deficits in human amyloid precursor protein transgenic mice [10, 11]. The protective effect of EphB2 may be related to the AMPA-type glutamate receptor subunit GluA2, which can bind to the PDZ-binding motif of EphB2 via PDZ domain-containing proteins and contribute to the accumulation of NMDAR in membranes [12].

The effect of EphB2 on AD

Recent studies suggest that Eph receptors are also expressed in hippocampal astrocytes and mediate the release of gliotransmitters such as D-serine, which may have an important role in synaptic hippocampal transmission and plasticity [13]. Moreover, brain injury increases the release of D-serine from reactive astrocytes, contributing to synaptic damage in the hippocampus [14]. A β can also activate astrocytes and increased D-serine release [15], indicating that EphBs may affect synaptic function by regulating D-serine release from astrocytes in AD. However, the expression patterns and roles of ephrinB ligands and EphB receptors in astrocytes during the pathological development of AD are largely unknown.

In this study, to investigate the effects of ephrinB ligands and EphB receptors on hippocampal astrocytes in APP/PS1 mice, we investigated astrocyte activation and ephrinB ligand and EphB receptor expression in astrocytes in APP/PS1 mice. To estimate further the role of EphB2 expression in astrocytes, GFAP-cre/APP/PS1 mice injected with EphB2-Flox-AAV in the hippocampus were used to detect the effects of EphB2 on the synaptic plasticity of astrocytes. Furthermore, related mechanisms were explored.

Materials and methods

Animals

All mice (including C67BL/6J mice (strain: C67BL/6J mice, stock No.: 000664), APP/PS1 mice (strain: B6C3-Tg (APP^{swe}, PSEN1dE9) 85Dbo/Mmjax, stock No.: 34829), and GFAP-cre mice (train: B6.Cg-Tg (Gfap-cre) 77.6Mvs/2J, stock No.: 024098)) were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) housed in animal center of Xuzhou Medical University, kept under standard conditions of temperature (22°C \pm 2) and humidity (40-50%), a 12-h/12-h dark-light cycle environment, with free access to food and water. All animal tests were approved by Institutional Animal Care and Use Committee (IACUC) of the Xuzhou Medical University Experimental Animal Department.

Immunofluorescence staining

As previous described [16], 12 month-old WT and APP/PS1 mice were anesthetized (i.p., ketamine (100 mg/kg) and xylazine (10 mg/kg) in 0.9% saline) and transcardially perfused with

PBS and 4% paraformaldehyde (PFA) in PBS in order. Then brain were removed quickly and dehydrated gradient with 20% and 30% sucrose at 4°C, respectively. After cutting into 12 μ m on a cryostat (CM1950, Leica, Wetzlar, Germany), brain sections were incubated with blocking buffer (10% goat serum, 1.0% Triton X-100 in PBS) for 1 hour at room temperature, following incubated with primary antibodies (mouse anti-6E10 monoclonal antibody, 1:500, Covance, Princeton, NJ, USA; rabbit anti-GFAP polyclonal antibody, 1:300, Dako Chemicals, Japan; rabbit anti-SRR polyclonal antibody, 1:100, Abcam, Cambridge, UK) at 4°C overnight. then washed three times with PBS before incubated with second antibodies (Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, 1:500, Invitrogen; Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 633, 1:500, Invitrogen) for 1 hour in room temperature. The sections were washed with PBS three times, and then mounted in fluorescent mounting media. Fluorescent images were imaged by Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus Corporation, shinjuku-ku, Tokyo, Japan).

Astrocyte isolation and purity confirmation

Mice were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) in 0.9% saline and transcardially perfused with Hanks' Balanced Salt Solution (HBSS) without Ca²⁺ and Mg²⁺. Following removed and separated hippocampus of corresponding mice into cold HBSS buffer, single cells were prepared using Neural Tissue Dissociation Kit (T) (Miltenyibiotec, Germany) according to the manufacturer's protocol. Briefly, hippocampus tissue was cut into pieces and a pre-warmed enzyme mix is added to the tissue pieces and incubated with agitation at 37°C. GLAST positive cells were isolated by Anti-GLAST (ACSA-1) MicroBead Kit (Miltenyibiotec, Germany), cells were fluorescently stained with Anti-GLAST (ACSA-1)-APC and analyzed by flow cytometry using the MACSQuant® Analyzer. Cell debris and dead cells were excluded from the analysis based on scatter signals and propidium iodide fluorescence.

Western blot

Hippocampal tissues and hippocampal astrocytes were collected and total protein was extracted using RIPA Lysis Buffer (Beyotime,

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Table 1. The primers designed for RT-qPCR

| Gene | Forward primer | Reverse primer |
|-----------------|-------------------------|-------------------------|
| <i>Eaat2</i> | ACAATATGCCCAAGCAGGTAGA | CTTTGGCTCATCGGAGCTGA |
| <i>Glast1</i> | ACCAAAAGCAACGGAGAAGAG | GGCATTCCGAAACAGGTAAGTC |
| <i>Aqp4</i> | CTTTCTGGAAGGCAGTCTCAG | CCACACCGAGCAAAACAAAGAT |
| <i>Tgfb1</i> | CTCCCGTGGCTTCTAGTGC | GCCTTAGTTTGGACAGGATCTG |
| <i>Tgfb1</i> | TCTGCATTGCACTTATGCTGA | AAAGGGCGATCTAGTGATGGA |
| <i>Socs3</i> | ATGGTCACCCACAGCAAGTTT | TCCAGTAGAATCCGCTCTCCT |
| <i>Jak1</i> | CTCTCTGTCAACCTCTTCGC | TTGGTAAAGTAGAACCTCATGCG |
| <i>Cdkn2b</i> | CCCTGCCACCCTTACCAGA | CAGATACCTCGCAATGTCCAG |
| <i>Smad2</i> | ATGTCGTCCATCTTGCCATTC | AACCGTCTGTTTTCTTTAGCTT |
| <i>Smad3</i> | CACGCAGAACGTGAACACC | GGCAGTAGATAACGTGAGGGA |
| <i>Stat3</i> | CAATACCATTGACCTGCCGAT | GAGCGACTCAAAGTGCCT |
| <i>Il10r1</i> | CCCATTCTCGTCACGATCTC | TCAGACTGGTTTGGGATAGGTTT |
| <i>Il10r2</i> | ACCTGCTTTCCCAAAACGAA | TGAGAGAAGTCGCACTGAGTC |
| <i>Hevin</i> | GGCAATCCCGACAAGTACAAG | TGGTTTTCTATGTCTGCTGTAGC |
| <i>Sparc</i> | GTGGAAATGGGAGAATTTGAGGA | CTCACACACCTTGCCATGTTT |
| <i>Testican</i> | CGGCTGAGTGTGCATCAATTT | GGATGGGAAGAACCACGAA |
| <i>Smoc2</i> | CCCAAGCTCCCCTCAGAAG | GCCACACACCTGGACACAT |
| <i>Mertk</i> | CAGGGCCTTTACCAGGGAGA | TGTGTGCTGGATGTGATCTTC |
| <i>Megf10</i> | GAAGACCCCAACGTATGCAG | CGGTGCAGCTGTGTAGTAGA |
| <i>Il1α</i> | CGAAGACTACAGTTCTGCCATT | GACGTTTCAGAGTTCTCAGAG |
| <i>Il1b</i> | GCAACTGTTCTGAACTCAACT | ATCTTTTGGGGTCCGTCAACT |
| <i>Il1r1</i> | GTGCTACTGGGGCTCATTTGT | GGAGTAAGAGGACACTTGCGAAT |
| <i>Il1rn</i> | GCTCATTGCTGGGTACTTACAA | CCAGACTTGGCACAAGACAGG |
| <i>Il6</i> | TAGTCCTTCTACCCCAATTTCC | TTGGTCCTTAGCCACTCCTTC |
| <i>Il6ra</i> | CCTGAGACTCAAGCAGAAATGG | AGAAGGAAGGTCCGCTTCAGT |
| <i>Tnfa</i> | CCCTCACACTCAGATCATCTTCT | GCTACGACGTGGGCTACAG |
| <i>Tnfrsf1a</i> | CCGGGAGAAGAGGGATAGCTT | TCGGACAGTCACTACCAAGT |
| <i>Tnfrsf1b</i> | ACACCCTACAAACCGGAACC | AGCCTTCTGTCCATAGTATTCTT |
| <i>Ccl2</i> | TTAAAAACCTGGATCGGAACCAA | GCATTAGCTTCAGATTTACGGGT |
| <i>Ccl3</i> | TTCTCTGTACCATGACACTCTGC | CGTGGAACTTCCGGCTGTAG |
| <i>Ccl4</i> | TTCTCTGTGTTTCTTACACCT | CTGTCTGCCTCTTTTGGTCAG |
| <i>Ccl5</i> | GCTGCTTTGCCTACCTCTCC | TCGAGTGACAAACGACTGC |
| <i>Cxcl2</i> | CCAACCACAGGCTACAGG | GCGTCACACTCAAGCTCTG |
| <i>Cxcl10</i> | CCAAGTGTGCCGTCAATTTTC | GGCTCGCAGGGATGATTTCAA |
| <i>Casp1</i> | ACAAGGCACGGGACCTATG | TCCCAGTCAGTCTGGAAATG |
| <i>Tlr1</i> | TGAGGGTCTGATAATGTCTAC | AGAGGTCAAATGCTTGAGGC |
| <i>Tlr2</i> | GCAAACGCTGTTCTGCTCAG | AGGCGTCTCCCTCTATTGTATT |
| <i>Tlr4</i> | ATGGCATGGCTTACACCACC | GAGGCCAATTTTGTCTCCACA |
| <i>Cd14</i> | CTCTGTCTTAAAGCGGCTTAC | GTTGCGGAGGTTCAAGATGTT |
| <i>Gapdh</i> | AGGTCCGTGTGAACGGATTG | TGTAGACCATGTAGTTGAGGTCA |

with 5% (w/v) bovine serum albumin (BSA) in Tris-Buffered Saline with Tween 20 (0.1%) for 1 hour at room temperature. The membranes were incubated with primary antibodies (anti-GFAP polyclonal antibody, 1:1000, Cell signaling Technology, Beverly, MA, USA; anti-S100β monoclonal antibody, 1:2000, Abcam; anti-ephrinB1 Polyclonal antibody, 1:2000, Abcam; anti-ephrinB2 Polyclonal antibody, 1:2000, Abcam; anti-ephrinB3 Polyclonal antibody, 1:2000, Abcam; anti-EphB1 monoclonal antibody, 1:500, Santa Cruz, Dallas, Texas, USA; anti-EphB2 monoclonal antibody, 1:500, Santa Cruz; anti-EphB3 monoclonal antibody, 1:500, Santa Cruz; anti-PSD95 monoclonal antibody, 1:1000, Cell signaling Technology; anti-SYP monoclonal antibody, 1:1000, Abcam; anti-GluR1 polyclonal antibody, 1:1000, Abcam; anti-GluR2 polyclonal antibody, 1:1000, Abcam; anti-GluN1 polyclonal antibody, 1:1000, Abcam; anti-GluN2B polyclonal antibody, 1:1000, Abcam; anti-SRR monoclonal antibody, 1:1000, Cell signaling Technology; anti-β-actin monoclonal antibody, 1:500, Santa Cruz.) for 12 hours at 4°C. The membranes were incubated with secondary antibodies for 1 hour at room temperature, and the target proteins were detected by an enhanced chemiluminescence (ECL) detection system (Tanon, Shanghai, China) and quantified using Tanon Gel Image Analysis System subsequently.

Shanghai, China) on ice. Protein concentration was measured by NanoDrop 2000 ultramicrospectrophotometer (Thermo Scientific, Waltham, MA, USA). Total protein was separated by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked

Electrophysiological analysis

The experiment was performed according to previous study [16]. Briefly, Hippocampal slices (400 μm thick) were prepared in ice-cold, oxygenated (95% O₂/5% CO₂) dissection artificial

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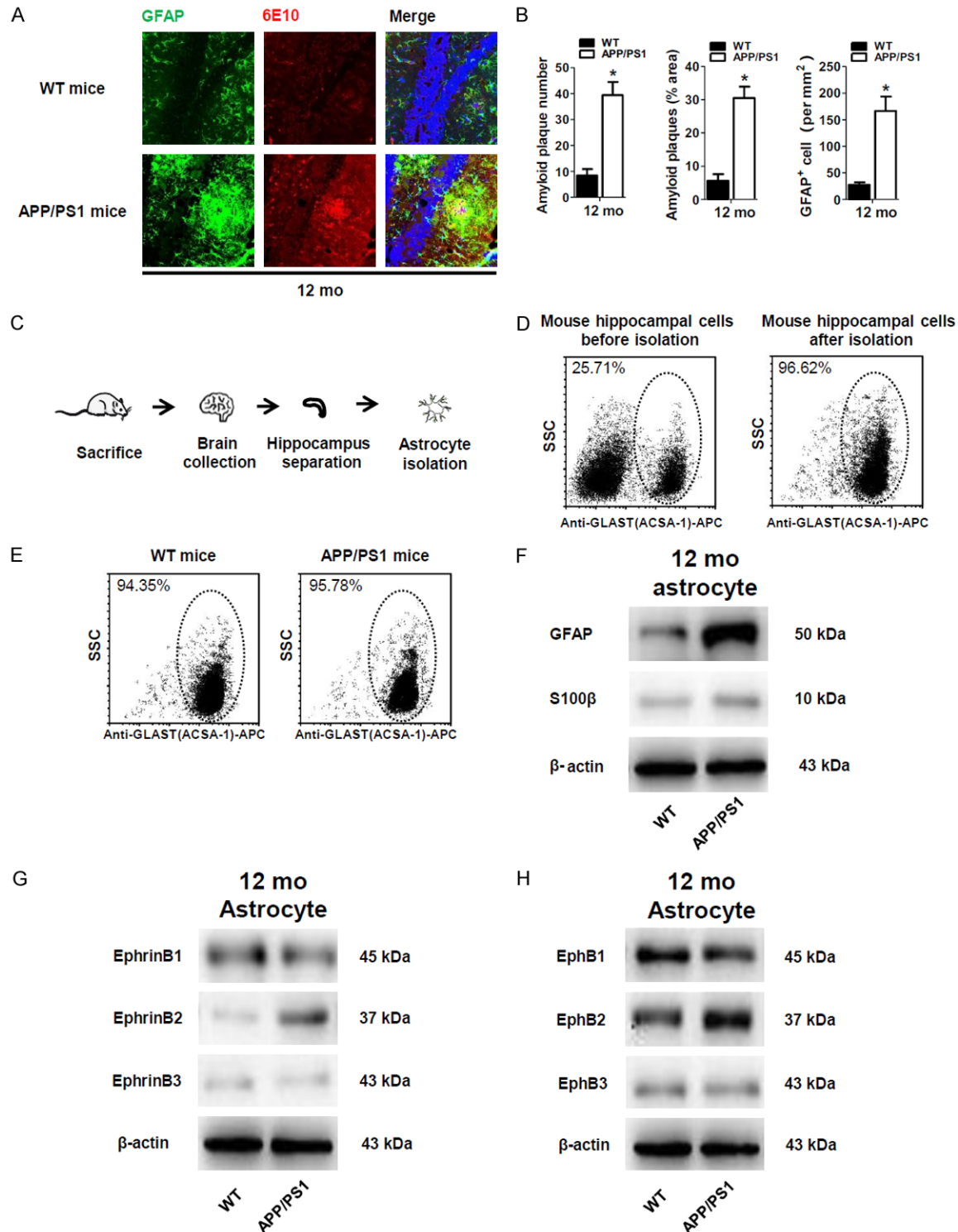


Figure 1. Expression profile of ephrinB ligands and EphB receptors in hippocampal astrocytes in APP/PS1 mice. (A) Astroglial activation and amyloid plaque deposition were increased by staining for GFAP and 6E10, respectively, and (B) calculating the amyloid plaque number, amyloid plaque area, and GFAP-positive cell number during AD pathological development. Then, we established the procedures for (C) hippocampal astrocyte isolation and (D) the separated astrocyte with cell purity over 90%. After (E) separating astrocytes from the hippocampus of WT and APP/PS1 mice, the expression of (F) proteins related to astroglial activation (GFAP and S100 β) were found to be upregulated, and ephrinB2 and EphB2 among (G) ephrinB ligands and (H) EphB receptors were detected to be upregulated. $n = 6$ per group. Data are presented as the mean \pm SEM. * $P < 0.05$ compared with WT mice.

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cerebrospinal fluid (ACSF: 120-mM NaCl, 3-mM KCl, 4-mM MgCl₂, 1-mM NaH₂PO₄, 26-mM NaHCO₃, and 10-mM glucose) from 12-month-old GFAP-cre/APP/PS1 mice, which were injected with EphB2-Flox-AAV or control-AAV. Slices were placed in ACSF for 45 min at room temperature for recover and then 45 min at 30°C prior to the recording. To record Field excitatory postsynaptic potentials (fEPSPs), a stimulating electrode and a recording electrode were placed in the stratum radiatum of CA1. Following baseline responses, Long-term potentiation (LTP) was induced with high frequency stimulation (HFS, 200 Hz/0.5 s stimulus trains). LTP values for the 1 hour time point were determined by averaging 5 minutes of normalized slope values at 55-60 minutes post-HFS. The initial fEPSP slope was measured by using Signal software (V4.08, Cambridge Electronic Design, Cambridge, UK).

RT-PCR analysis

Total RNA was extracted from Hippocampal astrocytes by total RNA extraction reagent (Vazyme, Nanjing, China) quickly, according to the manufacturer's suggested protocol to extract total RNA, and reverse transcribed total RNA (1 µg) into cDNA using HiScript Q RT SuperMix for qPCR kit. The oligonucleotide primer sequences were obtained from GenScript Biotechnology (Nanjing, China) and provided in the **Table 1**.

Microdialysis in vivo

The microdialysis procedure was performed as previous showed [18]. In short, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) in 0.9% saline and fixed on the stereotaxic frame. Guide cannulas were implanted into the hippocampus (from bregma, AP: -3.1 mm, ML: -2.5 mm, DV: -1.2 mm at 12° angle) and fixed with dental cement. Following one week recovery, a microdialysis probe (MAB 4.9.2.Cu, 2 mm membrane length, Microbiotech, Stockholm, Sweden) was inserted into the guide cannulas, which were implanted into the hippocampus previously, connected to a syringe pump and perfused with ACSF at constant flow rate (1 µL/min). Meanwhile, the microdialysate was collected and microdialysate ATP, Glutamate and D-serine level were examined using corresponding assay kit according to the manufacturer's instructions.

(ATP assay kit: Beyotime, Jiangsu, China; Glutamate Assay Kit: Biovision, San Francisco, USA; D-Serine Colorimetric Assay Kit, Cosmo Bio Co., Ltd, Japan).

Statistical analysis

Statistics were performed using Graph Pad Prism Software 6 (La Jolla, CA, USA). Data were reported as mean ± SEM. Student t test and one-way ANOVAs test were utilized for comparisons of the parameters between groups. *P* < 0.05 were considered statistically significant.

Results

Expression of ephrinBs and EphBs in APP/PS1 mouse hippocampal astrocytes

To investigate the role of astroglial activation in the pathological progression of AD, immunostaining of GFAP and 6E10 (**Figure 1A**) was performed on the hippocampus of 12-month-old wild-type (WT) and APP/PS1 mice. An increase in the number and area of amyloid plaques was accompanied by an increase in GFAP-positive cells in the hippocampus of APP/PS1 mice, compared with WT mice (**Figure 1B**). Studies have revealed that astroglial activation triggers specific protein expression in different regions; however, the precise role of astroglial activation during AD pathology in relation to ephrinB ligands and EphB receptors remains unclear. We separated hippocampal astrocytes via magnetic-activated cell-sorting (MACS; **Figure 1C**) and confirmed the purity using flow cytometry (**Figure 1D**). After MACS, the purity of astrocytes was above 95%, making them suitable for further research (**Figure 1E**). Furthermore, we detected the expression of astroglial markers (GFAP and S100β) and ephrinBs and EphBs in hippocampal astrocytes after isolation. The results revealed significant upregulation of GFAP, but no change in S100β (**Figures 1F** and **S1A**). Among the ephrinBs and EphBs, only ephrinB2 and its receptor EphB2 were significantly increased (**Figures 1G, 1F, S2B** and **S2C**).

Effect of specific hippocampal astroglial EphB2 knockdown on synaptic plasticity in APP/PS1 mice

Given that EphB2 levels were increased in 12-month-old APP/PS1 mouse hippocampal astrocytes, we speculated that astroglial eph-

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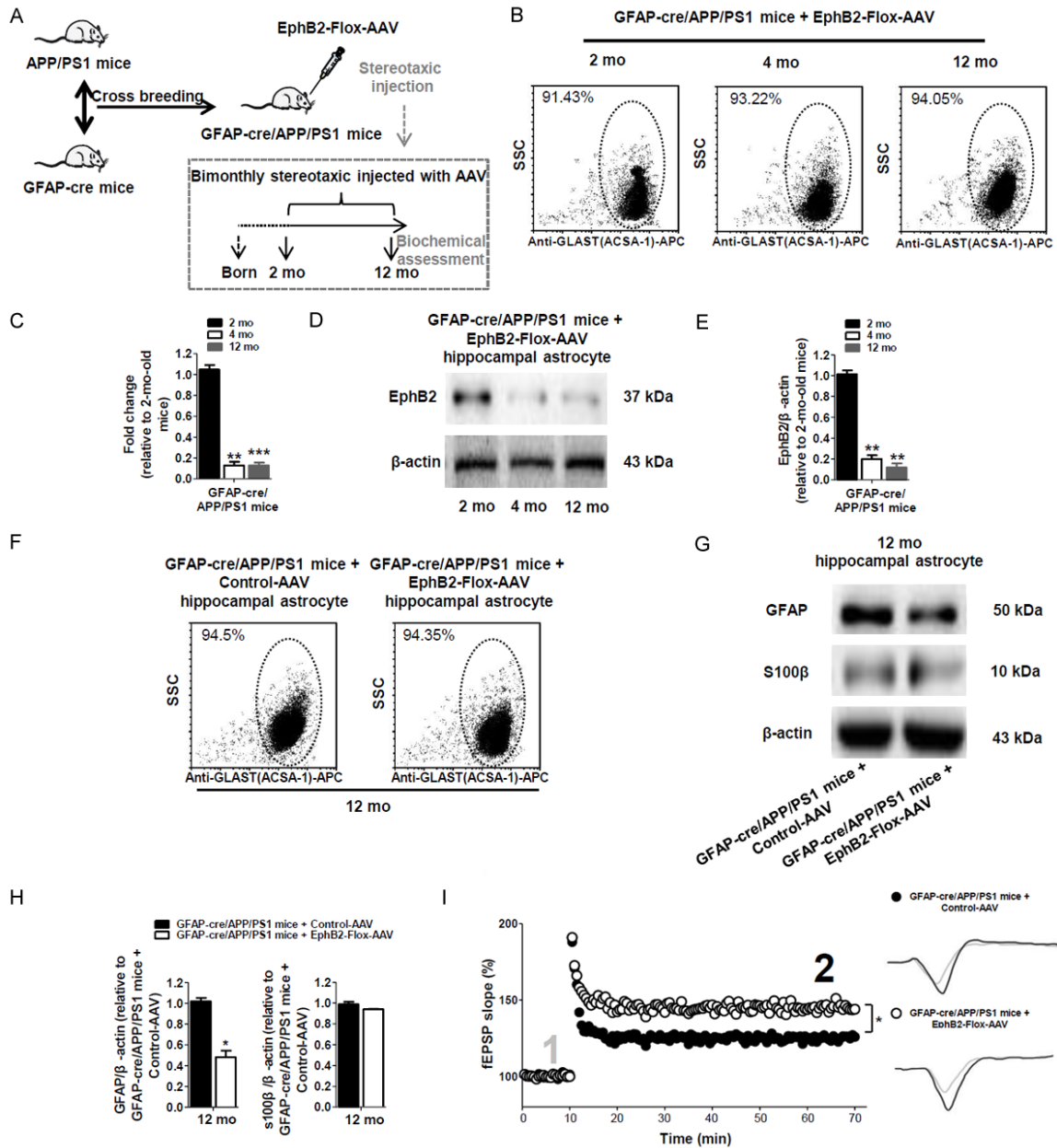


Figure 2. Specific hippocampal astroglial EphB2 knockdown-improved synaptic function in APP/PS1 mice. (A) The GFAP-cre/APP/PS1 mice were generated by crossbreeding GFAP-cre mice with APP/PS1 mice, and then stereotaxically injected EphB2 knockdown AAV into the hippocampus bimonthly from age 2 months to 12 months. In (B) isolated hippocampal astrocytes, the (C) mRNA and (D, E) protein levels of EphB2 were significantly reduced in 4-month-old, and 12-month-old GFAP-cre/APP/PS1+EphB2-Flox-AAV mice, compared to 2-month-old. Moreover, astroglial activation-related protein levels (GFAP and S100 β) also were notably reduced in hippocampal astrocytes (F) from GFAP-cre/APP/PS1+EphB2-Flox-AAV mice, compared to GFAP-cre/APP/PS1+Control-AAV mice (G, H) evaluated. Additionally, the field excitatory postsynaptic potential (fEPSP; calibration: vertical, 1 mV; horizontal, 5 ms) induced by high-frequency conditioning tetanus was recorded to maintain at a higher level in GFAP-cre/APP/PS1+EphB2-Flox-AAV mice. (C, E) $n = 6$ per group. Data are presented as the mean \pm SEM. ** $P < 0.01$, *** $P < 0.001$ compared with 2-month-old mice. (H) $n = 6$ per group and (I) $n = 12-14$ per group. Data are presented as the mean \pm SEM. * $P < 0.05$ compared with GFAP-cre/APP/PS1+Control-AAV mice.

rinB2/EphB2 signaling may be involved in pathological development. Therefore, we crossed APP/PS1 mice with GFAP-cre mice to obtain

GFAP-cre/APP/PS1 mice, then stereotaxically injected EphB2-Flox-AAV into the hippocampus of the GFAP-cre/APP/PS1 mice bimonthly from

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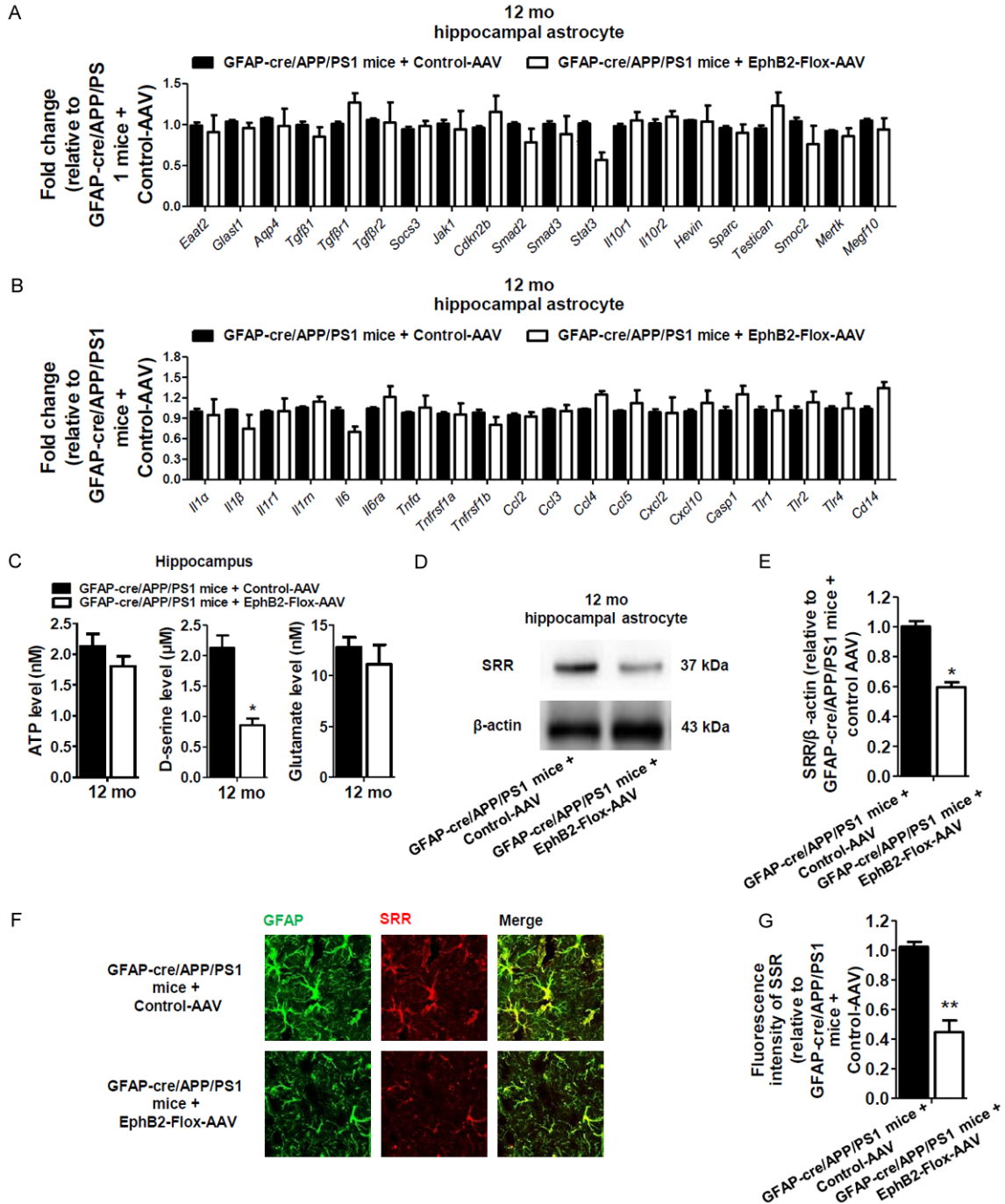


Figure 3. Specific hippocampal astroglial EphB2 knockdown-reduced astroglial D-serine secretion in APP/PS1 mice. A series of mRNA levels of (A) regulation- and growth-related and (B) inflammatory-related mediators in hippocampal astrocytes was detected. (C) D-serine concentration in the hippocampus was reduced after specific astroglial EphB2 knockdown, while ATP and glutamate concentration were not altered. After that, we confirmed the downregulation of SRR protein levels in hippocampal astrocytes by WB (D and E) and IF (F and G). $n = 6$ per group. Data are presented as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ compared with GFAP-cre/APP/PS1+Control-AAV mice.

age 2 months until 12 months to specifically delete hippocampal astroglial EphB2 in APP/PS1 mice during pathological development (Figure 2A). The purity of astrocytes after isola-

tion was above 95% (Figure 2B). Moreover, the mRNA and protein levels in hippocampal astrocytes were significantly depressed after AAV injection (Figure 2C-E).

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Next, we separated astrocytes from GFAP-cre/APP/PS1+Control-AAV mice and GFAP-cre/APP/PS1+EphB2-Flox-AAV mice and confirmed their suitability for subsequent analysis (**Figure 2F**). Specific astroglial EphB2 knockdown significantly reduced GFAP expression but did not alter S100 β expression (**Figure 2G** and **2H**), and long-term potentiation (LTP) induced by high-frequency conditioning tetanus was higher in GFAP-cre/APP/PS1+EphB2-Flox-AAV mice than in GFAP-cre/APP/PS1+Control-AAV mice (**Figure 2I**).

Underlying mechanisms of hippocampal astroglial EphB2 knockdown in promoting synaptic function

To identify the underlying mechanisms, we assessed proteins associated with synaptic functions, such as synaptic structure (SYP and PSD95; **Figure S2A** and **S2B**), the AMPA receptor (GluR1 and GluR2; **Figure S2C** and **S2D**), and the NMDAR (GluN1 and GluN2B; **Figure S2E** and **S2F**). Unexpectedly, not all proteins were significantly altered in GFAP-cre/APP/PS1+EphB2-Flox-AAV mice compared with GFAP-cre/APP/PS1+Control-AAV mice. The results indicated that the promotion of synaptic function induced by hippocampal astroglial EphB2 knockdown was not mediated via direct regulation of the expression of synaptic structure (SYP and PSD95) or postsynaptic transmission (AMPA and NMDA receptors) proteins. Therefore, we postulated that specific knockdown of astroglial EphB2 influenced astroglial function via enhanced synaptic function, and examined a battery of mRNA-level regulation- and growth-related (**Figure 3A**) and inflammatory-related (**Figure 3B**) mediators in hippocampal astrocytes isolated from GFAP-cre/APP/PS1+EphB2-Flox-AAV and GFAP-cre/APP/PS1+Control-AAV mice. The mRNA levels of most regulation- and growth-related and inflammatory-related mediators were unchanged, except stat3, which was significantly downregulated. Overall, this indicated that astroglial EphB2 knockdown in the hippocampus did not notably influence astroglial regulation-, growth-, and inflammatory-related regulator gene expression. In addition, synaptic function-related astroglial secretion was evaluated (including ATP, D-serine, and glutamate; **Figure 3C**). Surprisingly, secreted D-serine was significantly increased but ATP and glutamate concentrations were not markedly altered. Furthermore,

serine racemase (SRR, which converts L-serine into D-serine) was decreased in isolated hippocampal astrocytes in GFAP-cre/APP/PS1+EphB2-Flox-AAV mice compared with GFAP-cre/APP/PS1+Control-AAV mice (**Figure 3D** and **3E**). Therefore, we also detected the SRR expression in vivo, and IF data also revealed that EphB2 knockdown reduced GFAP and SRR expression in astrocyte (**Figure 3F** and **3G**). Taken together, enhanced synaptic function via specific knockdown of EphB2 might be regulated by astroglial D-serine release.

Discussion

Amyloid plaques and astrogliosis are major features in AD pathology, and increased amyloid plaques impair synaptic plasticity via NMDAR inhibition, which contributes to cognitive deficits [19-22]. Consistent with previous studies, the number and area of amyloid plaques and GFAP-positive astrocytes were increased in the hippocampus of 12-month-old APP/PS1 mice compared with WT mice, suggestive of amplified amyloid plaques and astrogliosis in the hippocampus of APP/PS1 mice, which contribute to cognitive deficits [23]. Meanwhile, cross-talk between neurons and glia has an important role in regulating synapse development and function [24]. Here, we established a process to obtain and identify hippocampal astrocytes. In hippocampal astrocytes from APP/PS1 mice, higher GFAP and S100 β protein levels indicated an augmentation of astrocyte proliferation.

Ephrin/EphB signaling is known to initiate bidirectional signaling between sets of pre- and post-synaptic proteins, which induces the formation of dendritic spines and excitatory synapses, controls NMDAR recruitment, localization and function, and impacts synaptic plasticity and learning function [25-30]. Impairment of synaptic plasticity and an imbalance between firing homeostasis and synaptic plasticity are among the earliest signatures of early-phase AD pathogenesis in AD patients and animal models [31, 32]. For example, the direct interaction between A β oligomers and the fibronectin domain of EphB2 induces EphB2 depletion by facilitating EphB2 degradation in neuronal cultures, and neuronal EphB2 knockdown in the hippocampus leads to impairment of cognitive functions, NMDAR functions, and LTP [33]. Numerous reports have focused on the func-

tions of EphrinBs and EphBs in neurons. By contrast, we observed that ephrinB2 and EphB2 were significantly upregulated in hippocampal astrocytes, unlike in neurons [7]. Thus, we further directly investigated the effects of hippocampal astroglial EphB2 on synaptic function in APP/PS1 mice during the pathological development of AD through knockdown of hippocampal astroglial EphB2 via AAV injection. Interestingly, specific knockdown of hippocampal astroglial EphB2 significantly improved synaptic function but did not alter the expression of proteins related to synaptic structures (SYP and PSD95), AMPA receptors (GluR1 and GluR2), and NMDARs (GluN1 and GluN2B).

Astrocytes secrete molecules that contribute to synaptic plasticity and neuronal homeostasis, such as apolipoprotein E, thrombospondins, and gliotransmitters [34, 35]. Reactive astrocytes localize surround amyloid plaques and elevate cytokine levels, which are neurotoxic and related to cognitive decline [36]. In AD, A β inhibits the function of glutamate transporter 1 and glutamate aspartate transporter, reduces astrocytic glutamate levels, and activates excitotoxicity in neurons [37, 38]. Moreover, astrocytes modulate synaptic transmission and plasticity via the release of gliotransmitters such as ATP, D-serine, and adenosine [39]. Most astroglial regulation-, growth-, and inflammatory-related genes were unchanged after specific knockdown of hippocampal astroglial EphB2 in APP/PS1 mice, although astroglial D-serine secretion was notably reduced, while ATP and glutamate secretion were not altered. D-serine released from astrocytes functions as an endogenous NMDAR co-agonist [40]; therefore, the D-serine reduction in hippocampal EphB2-knockout astrocytes contributed to the improvement in synaptic plasticity in the astrocytic EphB2-knockout APP/PS1 mice. In brief, we found that EphB2 protein levels were augmented in hippocampal astrocytes from APP/PS1 mice, and astrocytic EphB2 knockout reduced D-serine levels, which contributed to restore synaptic plasticity in the hippocampus.

In summary, this study demonstrated that the upregulation of EphB2 in hippocampal astrocytes in APP/PS1 mice induced synaptic dysfunction via the modulation of D-serine release from astrocytes, unlike in neurons, indicative of the complex role of ephrinB2/EphB2 signaling in different cell types during AD pathology.

Therefore, additional research should be conducted to consider ephrinB2/EphB2 signaling as a potential therapeutic target for AD.

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Disclosure of conflict of interest

None.

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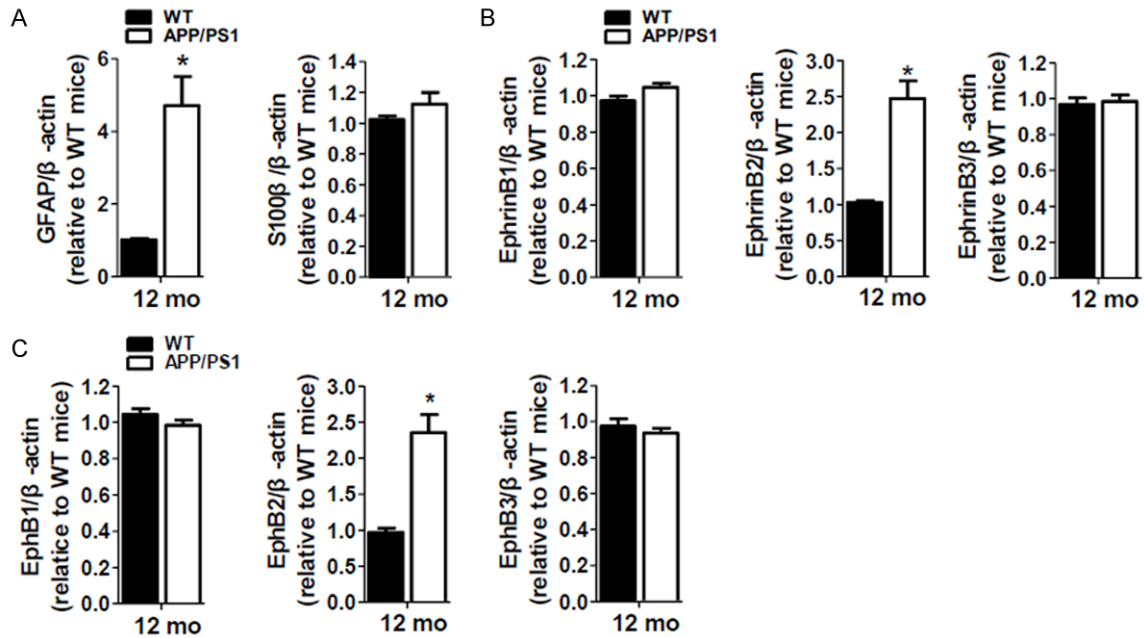


Figure S1. Quantification of the protein expression of markers of (A) astroglial activation (GFAP and S100 β), (B) ephrinB ligands (ephrinB1, ephrinB2, and ephrinB3), and (C) EphB receptors (EphB1, EphB2, and EphB3). $n = 6$ per group. Data are presented as the mean \pm SEM. * $P < 0.05$ compared with WT mice.

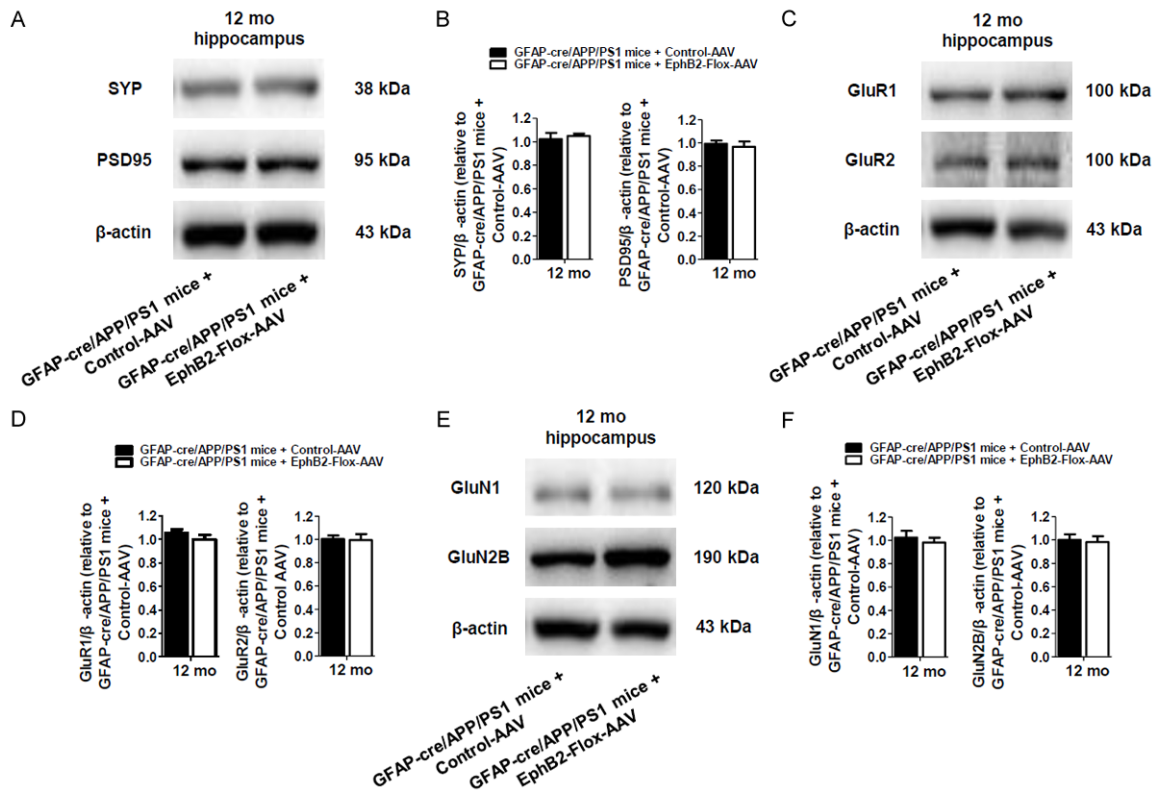


Figure S2. Levels of proteins associated with (A, B) synaptic structures (SYP and PSD95), (C, D) AMPA receptors (GluR1 and GluR2), and (E, F) NMDARs (GluN1 and GluN2B) were not altered after specific hippocampal astroglial EphB2 knockdown. $n = 6$ per group. Data are presented as the mean \pm SEM compared with GFAP-cre/APP/PS1+Control-AAV mice.