



# Induced neuronal activity does not attenuate amyloid beta-induced synaptic loss in vitro

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## Abstract

**Aim:** The accumulation of amyloid beta ( $A\beta$ ) is one of the characteristics of Alzheimer's disease. The excessive accumulation of  $A\beta$  has been suggested to result in a decrease in the number of synapses. Although the number of synapses is generally modulated by neuronal activity, whether neuronal activity affects  $A\beta$ -induced synapse loss remains unknown. Therefore, we addressed this question using a primary culture of hippocampal neurons.

**Method:** The neuronal activity of cultured hippocampal neurons from mouse pups was increased using the chemogenetic technique designer receptors exclusively activated by designer drugs (DREADD). The cultured neurons were treated with  $A\beta$ , and synapse density was assessed by immunocytochemistry.

**Results:**  $A\beta$  decreased the synapse density probably by decreasing postsynapse. On the other hand, enhanced neuronal activity did not affect the synapse density significantly. However, there was a trend that enhanced neuronal activity increased especially presynapse density.

**Conclusion:** We found that enhanced neuronal activity did not affect  $A\beta$ -induced synapse loss in vitro.

## KEYWORDS

Alzheimer's disease, amyloid beta, hippocampus, neuronal activity, synapse

## 1 | INTRODUCTION

Alzheimer's disease (AD), which is the most common cause of dementia, is a progressive neurodegenerative disorder that significantly affects patients' cognitive function and their quality of life. In the brains of AD patients, accumulated amyloid beta ( $A\beta$ ) is one of the major pathological characteristics of AD.<sup>1</sup>  $A\beta$  has been linked to cognitive dysfunction in AD patients<sup>2</sup> partly because  $A\beta$  induces abnormalities in synaptic function and structure, including the loss of synapses.<sup>3,4</sup> In the healthy brain, specifically during development, neuronal activity plays a key role in regulating the formation and elimination of synapses.<sup>5</sup> It has also

been suggested that, in the AD brain, synapse density is influenced by neuronal activity.<sup>6</sup> However, confirmation of a direct link between neuronal activity and  $A\beta$ -induced synapse loss is lacking. Here, we directly investigated whether neuronal activity modulates  $A\beta$ -induced synapse loss using primary cultures of hippocampal neurons.

## 2 | METHODS

Hippocampal neuronal cultures were prepared from postnatal day 1 B57BL6/J mice (SLC, Shizuoka, Japan) as previously described.<sup>7</sup>

Rena Kono and Gyu Li Kim equally contributed to this study.

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Briefly, hippocampi were removed from 3 to 6 mice and minced in warmed Hank's balanced salt solution (HBSS). After trypsinization, the dissociated neurons were plated onto 12-mm cover slips coated with poly-D-lysine at a cell density of  $7.1 \times 10^5$  cells/cm<sup>2</sup> in culture medium (neurobasal medium containing B27 supplement [1/50 mL], 0.5 mmol/L glutamine, 25  $\mu$ mol/L glutamate, penicillin (10 000 units/mL)/streptomycin (10 000  $\mu$ g/mL) [250  $\mu$ L/50 mL], 1 mmol/L HEPES, and 10% heat-inactivated donor horse serum) in 24-well plates and incubated at 37°C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere. The medium was changed after 1 day in vitro (DIV) and every 3 days thereafter.

After 6 DIV, the neurons were infected with AAV<sub>dj</sub>-hSyn-hM3Dq-EGFP at a multiplicity of infection (MOI) of 8000 viral genomes per cell. The medium was replaced with dimethyl sulfoxide (DMSO, 0.1%)- or clozapine N-oxide (CNO, 20  $\mu$ mol/L)-containing medium at 9 DIV. After 24 hours, the neurons were fixed with paraformaldehyde. In some cases, fluorescently labeled A $\beta$ <sub>1-42</sub> (AS-60480-01, AnaSpec, 200 nmol/L) was suspended in the medium together with DMSO or CNO. For c-Fos immunostaining, neurons were fixed after 6 hours of DMSO or CNO application.

For immunocytochemistry, fixed neurons were treated with blocking solution (10% goat serum and 0.3% Triton X-100 in phosphate-buffered saline (PBS)) for 1 hour at room temperature, followed by incubation with primary antibodies for 16 hours at 4°C. The neurons were then incubated with a secondary antibody solution for 4 hours at room temperature. The primary antibodies used were as follows: mouse anti-Map2 (microtubule-associated protein 2) (1:1000, Merck Millipore), chicken anti-GFP (green fluorescent protein) (1:500, Abcam), rabbit anti-c-Fos (1:5000, Synaptic Systems), guinea pig anti-Vglut1 (1:1000, Synaptic Systems), and rabbit anti-Homer 1 (1:500; Synaptic Systems). The secondary antibodies used were as follows: Alexa 594-labeled anti-mouse IgG (1:500; Thermo Fisher Scientific), Alexa 488-labeled anti-chicken IgY (1:500; Thermo Fisher Scientific), Alexa 594-labeled anti-rabbit IgG (1:500; Thermo Fisher Scientific), Alexa 647-labeled anti-guinea pig IgG (1:500; Thermo Fisher Scientific), and Alexa 405-labeled anti-rabbit IgG (1:500; Thermo Fisher Scientific).

The samples were mounted with Fluoro-KEEPER Antifade Reagent with DAPI (Nacalai Tesque, 12745-74) or PermaFluor (Thermo Fisher Scientific, TA-030-FM) and observed with an FV1200 (Olympus) confocal system with 20 $\times$  (NA = 0.75), 40 $\times$  (NA = 0.95), 60 $\times$  (NA = 1.35) and 100 $\times$  (NA = 1.40) objectives. Z-series images were collected at 0.33  $\mu$ m intervals. The stacked images were prepared and analyzed using ImageJ. Synapse puncta quantification was performed as previously described using ImageJ with modifications.<sup>8</sup> Briefly, Vglut1 channel and Homer 1 channel images were thresholded in a blinded manner so that the background signals were excluded. Then, the colocalization of thresholded images of Vglut1 and Homer 1 were prepared using Image Calculator command in the process menu. Finally, the number of colocalized puncta was counted using particle analysis command. For analyzing the density of EGFP<sup>+</sup> neurons, the number of cell body that included nucleus was manually counted.

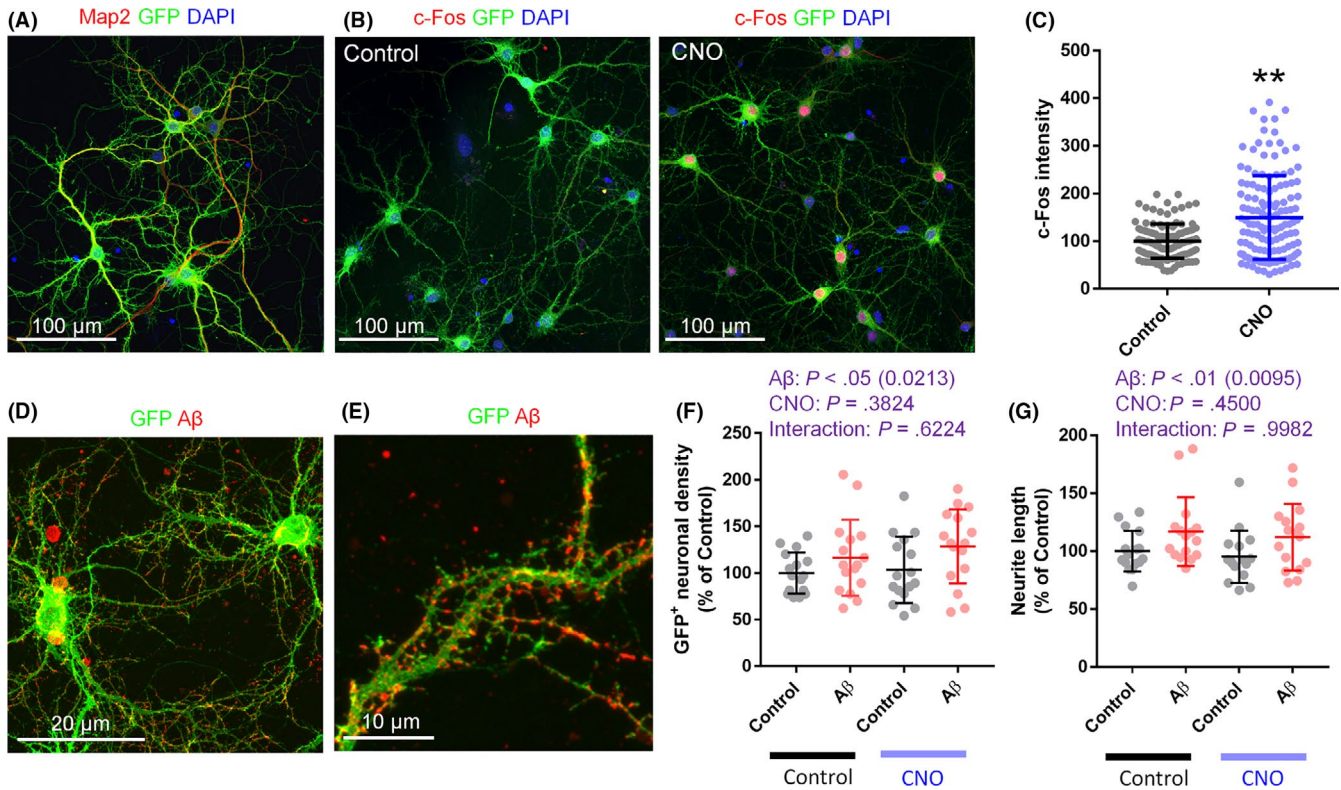
The data are represented as the mean  $\pm$  standard deviation (SD) with scatter dot plots and were pooled from at least 3 independent experiments. The data were collected and statistically analyzed independently by 2 researchers in a blinded manner. Student's t test or two-way analysis of variance (ANOVA) was performed for statistical analysis. All raw data are disclosed in Data S1.

### 3 | RESULTS AND DISCUSSION

To increase the activity of the cultured neurons, we used the chemogenetic technique designer receptors exclusively activated by designer drugs (DREADD), a method for the remote and transient manipulation of the activity of cells that express "designer receptors," namely mutated human muscarinic receptors, including hM3Dq, that are exclusively activated by the "designer drug" clozapine N-oxide (CNO).<sup>9</sup> hM3Dq stands for a DREADD (D) primarily derived from the human (h) M3 muscarinic receptor (M3) and couples preferentially with G<sub>q</sub> (q). CNO activates G<sub>q</sub> protein-coupled receptor signaling, and thus releasing intracellular calcium stores, which results in the enhancement of neuronal excitation. In order to induce hM3Dq expression to neurons, we used AAV<sub>dj</sub>-hSyn-hM3Dq-EGFP. The human synapsin 1 gene promoter hSyn is known to confer highly neuron-specific long-term transgene expression. We transfected cultured neurons with hM3Dq in combination with EGFP and found that almost all MAP2-positive neurons expressed EGFP (95%, n = 76 cells from 10 images) 72 hours after infection with AAV<sub>dj</sub>-hSyn-hM3Dq-EGFP (Figure 1A). Furthermore, we confirmed with c-Fos immunoreactivity that hM3Dq-expressing neurons exhibited increased activity in response to a 6-hour CNO application compared to that in control neurons (Figure 1B,C).

Next, we examined the effects of A $\beta$  and neuronal activity on the density of GFP<sup>+</sup> neurons. A $\beta$  labeled with red fluorescence and CNO were applied to the cultured medium at 9 DIV for 24 hours. The applied A $\beta$  was interspersed mostly in a uniform manner but sometimes formed aggregated puncta on cultured neurons (Figure 1D), including their neurites (Figure 1E). Statistical analysis of GFP<sup>+</sup> neuronal density by two-way ANOVA showed that there was no interaction between A $\beta$  and CNO. Therefore, we examined the main effect by either A $\beta$  or CNO, not by multiple comparison. As a result, we found that A $\beta$ , but not CNO, increased the density of GFP<sup>+</sup> neurons (Figure 1F), suggesting that a 24-hour application of A $\beta$  and enhanced neuronal activity did not induce the significant death of GFP<sup>+</sup> neurons. Similarly, neurite length was also increased by A $\beta$ , and there was no main effect of CNO or interaction (Figure 1G).

Next, we performed immunocytochemistry to examine the effects of A $\beta$  and neuronal activity on synapse density (Figure 2A). Presynapses and postsynapses were labeled with Vglut1 and Homer 1, respectively, and colocalized puncta were considered synapses (Figure 2A). We applied A $\beta$  and CNO to neuronal culture for 24 hours at 9 DIV. Neither A $\beta$  nor CNO affected the density of Vglut1 puncta, though there was a slight trend that CNO increased the density of Vglut1 (Figure 2B). On the other hand, A $\beta$  significantly decreased the



**FIGURE 1** Experimental system in the present study. A, A representative image of AAV<sub>dj</sub>-hSyn-hM3Dq-EGFP-infected neurons at 10 DIV. The samples were immunostained for the neuronal marker MAP2 (red) and GFP (green) and stained with the nuclei marker DAPI (blue). B, Representative images of AAV<sub>dj</sub>-hSyn-hM3Dq-EGFP-infected cultured neurons 6 hours after DMSO or CNO treatment at 9 DIV. The samples were immunostained for c-Fos (red) and GFP (green) and stained with DAPI (blue). C, Quantification of c-Fos intensity of each neuron in DMSO-treated control and CNO-treated cultures.  $**P < 0.01$ , Student's *t* test,  $n = 122$  (Control) and  $137$  (CNO) cells. Mean  $\pm$  SD are as follows:  $100 \pm 36.129$  (Control) and  $149.576 \pm 87.666$  (CNO). D, E, Representative images of GFP<sup>+</sup> neurons cultured with Aβ (red) (D) and their dendrites (E) at 10 DIV. The samples were immunostained for GFP (green). F, GFP<sup>+</sup> neuronal density in each group. Two-way ANOVA,  $n = 16$  images for each group. Mean  $\pm$  SD are follows: Control-control,  $100 \pm 21.900$ ; Control-Aβ,  $116.505 \pm 40.681$ ; CNO-control,  $103.398 \pm 35.589$ ; CNO-Aβ,  $128.641 \pm 39.804$ . G, The lengths of neurites in each group. Two-way ANOVA,  $n = 16$  images for each group. Mean  $\pm$  SD are follows: Control-control,  $100 \pm 17.530$ ; Control-Aβ,  $116.843 \pm 29.682$ ; CNO-control,  $95.203 \pm 22.600$ ; CNO-Aβ,  $112.075 \pm 28.857$

density of Homer 1 puncta, while CNO did not affect it (Figure 2C). We further found that the density of colocalized puncta of Vglut1 and Homer 1, that is synapse density, was decreased significantly by Aβ but was not affected by CNO (Figure 2D). Finally, there was no interaction between Aβ and CNO on puncta density. Together, we found that Aβ decreased the synapse density likely affecting postsynapses and that increased neuronal activity affect neither pre- nor postsynaptic density.

Our findings suggest that CNO-induced neuronal activity does not affect Aβ-induced synapse loss in our experimental conditions. A previous research has reported that Aβ reduces the spine density in a dose-dependent manner from picomolar to ten nanomolar order in the primary culture of hippocampal neurons.<sup>10</sup> Thus, Aβ at higher nanomolar concentration also might decrease synapse density. However, because micromolar concentration of Aβ induces neuronal death,<sup>11</sup> it will be difficult to investigate the effect of Aβ on synapses without considering neuronal death.

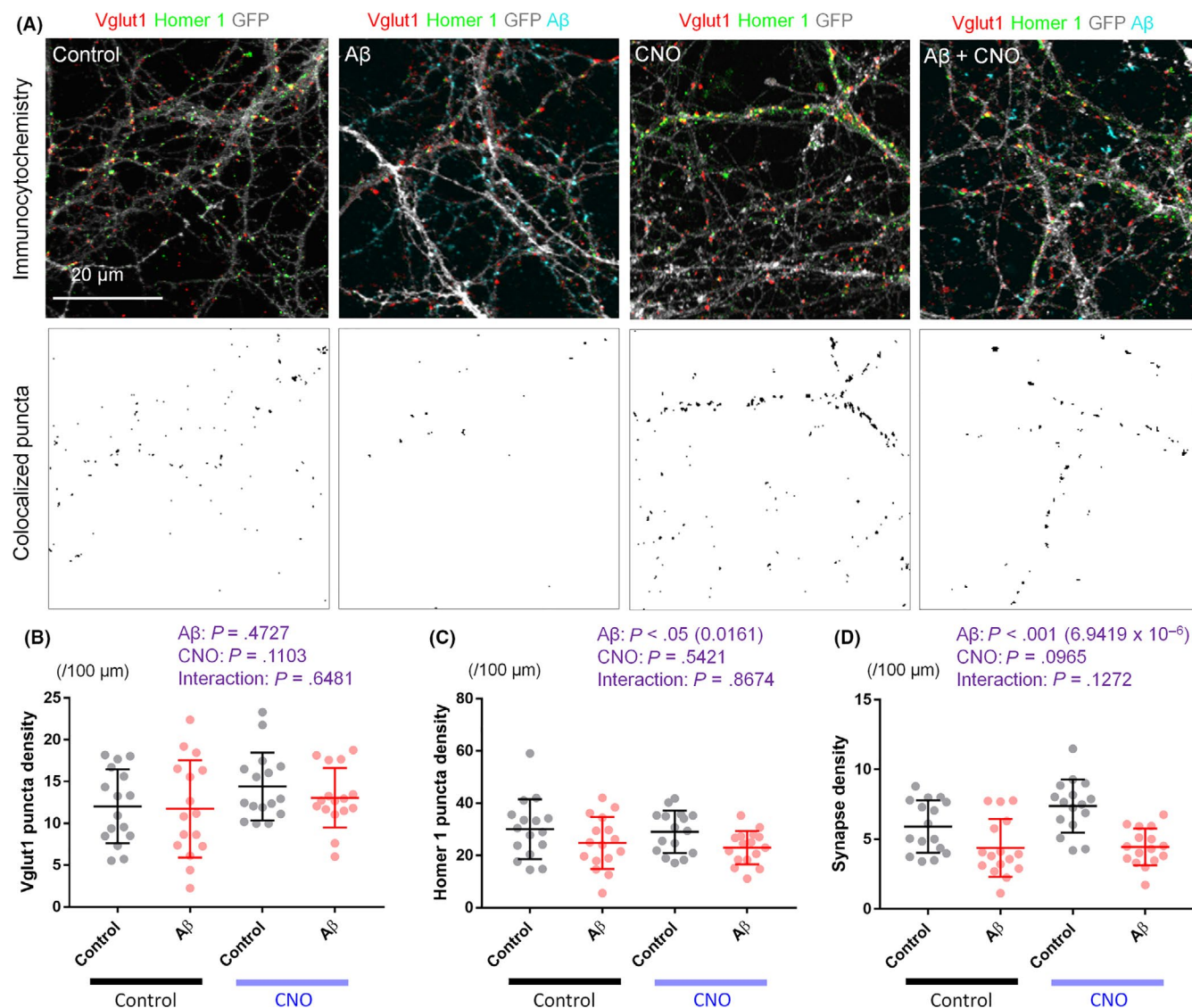
In addition, it has been reported that 20 μmol/L CNO results in faster increase in intracellular calcium levels compared to 1 μmol/L

CNO in primary hippocampal neurons and that CNO at more than 20 μmol/L would not result in more enhanced neuronal activity.<sup>12</sup>

Finally, it should be also noted that we examined the effects of neuronal activity and Aβ in cultured neurons prepared from postnatal mice at around 1 to 2 weeks in vitro, which may not be directly extrapolated to the situation in aged human brain with AD.

We found that Aβ increased the neuronal density significantly. A previous report has indicated that application of Aβ<sub>40</sub> is both neurotrophic and neurotoxic depending on the condition of Aβ concentration or the stage of neuronal differentiation in rat hippocampal neuronal culture.<sup>13</sup> Further, the functional domain of Aβ required for neurotrophic effect has been suggested to be contained in the Aβ<sub>25-35</sub> sequences.<sup>13</sup> Though our experimental system is different from this previous report, it is possible that our findings might reflect the similar neurotrophic effect of Aβ against cell death. In addition, we found that Aβ significantly increased the neurite length. Considering that Aβ decreased the density of synapses, it is possible that neurons elongate the neurite to maintain the total amount of synapses to receive.





**FIGURE 2** The effects of A $\beta$  and enhanced neuronal activity on synapse density. A, (upper) Representative images of AAV<sub>dij</sub>-hSyn-hM3Dq-EGFP-infected neurons at 10 DIV, which was 24 hours after the treatment of Control (DMSO), A $\beta$  (DMSO + A $\beta$ ), CNO, or A $\beta$  + CNO. The samples were immunostained for the presynaptic marker Vglut1 (red), the postsynaptic marker Homer 1 (green), and GFP (white). A $\beta$  is shown in cyan. (lower) Vglut1 and Homer 1-colocalized puncta are extracted and shown in the lower images. B-D, The density of Vglut1 puncta (B), Homer 1 puncta (C) and synapse (D) at 10 DIV (24 hours after treatment with the reagents). Two-way ANOVA,  $n = 16$  images for each group. Data represent the mean  $\pm$  SD. Mean  $\pm$  SD are follows: (B), Control-control,  $12.036 \pm 4.414$ ; Control-A $\beta$ ,  $11.737 \pm 5.825$ ; CNO-control,  $14.398 \pm 4.053$ ; CNO-A $\beta$ ,  $13.056 \pm 3.565$ . (C) Control-control,  $30.051 \pm 11.479$ ; Control- A $\beta$ ,  $24.750 \pm 9.980$ ; CNO-control,  $29.028 \pm 8.101$ ; CNO- A $\beta$ ,  $22.956 \pm 6.373$ . (D) Control-control,  $5.909 \pm 1.874$ ; Control- A $\beta$ ,  $4.376 \pm 2.064$ ; CNO-control,  $7.376 \pm 1.908$ ; CNO- A $\beta$ ,  $4.441 \pm 1.323$

It has also been reported that neuronal activity modulates synaptic properties through microglial activation both in physiological and pathological conditions.<sup>14,15</sup> Our neuronal culture includes few microglia, thus, it can be assumed that neuronal activity directly affects synapse density. Some previous studies have shown that neuronal activity modulates synapses in vitro without microglia.<sup>16,17</sup> Several mechanisms have been suggested to explain the phenomena. For example, NMDA receptor and MAPK signaling in neurons are involved in activity-dependent morphological plasticity of spines in cultured hippocampal neurons.<sup>17</sup>

Enhanced neuronal activity induced the formation and secretion of A $\beta$  in mice overexpressing A $\beta$  precursor protein.<sup>3</sup> In addition, the attenuation of neuronal activity decreased A $\beta$  deposition and neurotoxicity *in vivo*.<sup>18</sup> Moreover, the overproduction of A $\beta$  from neurites reduced the spine density at nearby dendrites, but blockade of the action potential inhibited spine loss.<sup>19</sup> These results suggest that enhanced neuronal activity may exacerbate AD pathology. However, in physiological conditions, enhanced neuronal activity contributed to the stabilization of synapses or the induction of synaptogenesis.<sup>20</sup> In addition, synaptic activity reduced intraneuronal A $\beta$  and protected against A $\beta$ -related synaptic



alterations.<sup>6</sup> Thus, whether enhanced neuronal activity attenuates synapse loss and improves pathology in AD remains unclear. In the present study, we found that enhanced neuronal activity did not attenuate A $\beta$ -induced synapse loss in vitro, though there was a trend that enhanced neuronal activity increased synapse density. Future studies are necessary to clarify the underlying mechanisms of this limited effect. It is also important to further examine how to modulate neuronal activity in the AD brain in vivo and whether it modifies the pathogenesis of AD.

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## CONFLICT OF INTEREST

Authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Re.K. conducted experiments, analyzed the experimental data, and wrote the manuscript. G.L.K. prepared neuronal cultures and performed immunocytochemistry. H.N. designed and prepared AAV. Ry.K. designed and planned the project and wrote the manuscript. Y.I. discussed the results and commented on the manuscript.

## DATA REPOSITORY

We have made our data publicly available in Data S1.

## APPROVAL OF THE RESEARCH PROTOCOL BY AN INSTITUTIONAL REVIEWER BOARD

N/A.

## INFORMED CONSENT

N/A.

## REGISTRY AND THE REGISTRATION NO. OF THE STUDY/TRIAL

N/A.

## ANIMAL STUDIES

All experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo and according to the University of Tokyo's guidelines for the care and use of laboratory animals.

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## SUPPORTING INFORMATION

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