Hippocampal neurons in direct contact with astrocytes exposed to amyloid β25-35 exhibit reduced excitatory synaptic transmission

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ABSTRACT

Amyloid β protein (Aβ) is closely related to the progression of Alzheimer's disease because senile plaques consisting of Aβ cause synaptic depression and synaptic abnormalities. In the central nervous system, astrocytes are a major glial cell type that contribute to the modulation of synaptic transmission and synaptogenesis. In this study, we examined whether astrocytes exposed to Aβ fragment 25-35 (Aβ25-35) affect synaptic transmission. We show that synaptic transmission by hippocampal neurons was inhibited by astrocytes exposed to Aβ25-35. The Aβ25-35-exposed astrocytes lowered excitatory postsynaptic release and the size of the readily releasable synaptic pool. The number of excitatory synapses was also reduced. However, the number of excitatory synapses was unchanged unless there was direct contact between Aβ25-35-exposed astrocytes and hippocampal neurons. These data indicate that direct contact between Aβ25-35-exposed astrocytes and neurons is critical for inhibiting synaptic transmission in the progression of Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease that involves memory impairment and behavioral disorders. AD pathology is characterized by two main features: senile plaques associated with aggregated amyloid β (Aβ) and neurofibrillary tangles consisting of hyperphosphorylated tau (Scheltens et al., 2016). These abnormalities lead to neuronal dysfunction and neuronal death. Aβ is thought to play a central role in AD (Hardy and Selkoe, 2002) and deposition of Aβ fibrils occurs in the early stages of the disease (Roth et al., 1966; Pike et al., 1994; Rama Rao and Kielian, 2015). Aβ is thought to contribute to AD pathogenesis by causing synaptic dysfunction and synaptic loss (Koffie et al., 2011; Hefti et al., 2013; Tu et al., 2014; Viola and Klein, 2015). In addition, Aβ affects not only neurons but also astrocytes, causing abnormalities in calcium signaling and glutamate release (Talantova et al., 2013; Lee et al., 2014).

Astrocytes are a major type of glial cell in the brain and play important roles in synapse formation and in modulation of synaptic function (Chung et al., 2015; Durkee and Araque, 2019; García-Cáceres et al., 2019). In the absence of astrocytes, the number of synapses decreases and synaptic transmission weakens (Hama et al., 2004). Thus, astrocytes and synapses are intimately involved in construction of an information network in the brain. Astrocyte dysfunction has been implicated in the development of several neurodegenerative diseases, including autism spectrum disorder (ASD), amyotrophic lateral sclerosis (ALS) and Rett syndrome (Laurence and Fatemi, 2005; Fatemi et al., 2008; Sloan and Barres, 2014). Astrocytes are therefore also speculated to be involved in the pathogenesis of AD (Gómez-Gonzalo et al., 2017).

In our previous study, we chronically exposed cultured cortical astrocytes to Aβ peptide fragment 1–40 (Aβ1–40), and then co-cultured these Aβ-exposed astrocytes with hippocampal naïve neurons (Kawano et al., 2017). This timing strategy ensured that only astrocytes were exposed to Aβ1–40, which is impossible *in vivo*. Using this neuronal culture system, we found that Aβ1–40-exposed astrocytes led to reduced glutamatergic synaptic transmission at the individual level. Meanwhile, in this study, we used another Aβ peptide fragment, Aβ25-35, which is a synthetic peptide of 11 amino acids that corresponds to a Aβ fragment and also forms a β-sheet structure (Pike et al., 1995). This fragment had similar effects on neuronal death and axonal atrophy as a fragment of full-length Aβ peptide (Kaminsky et al., 2010). Therefore, the goal of this study was to assess whether astrocytes exposed to Aβ25-35 affect synaptic transmission. Synaptic transmission among naïve hippocampal

neurons was measured in co-cultures with $A\beta_{25-35}$-exposed astrocytes. We show that direct contact between $A\beta_{25-35}$-exposed astrocytes and neurons is critical for aberrant synaptic transmission and synaptogenesis.

Experimental procedures

Animals

All procedures on animals were performed in strict accordance with the rules of the Experimental Animal Care and Welfare Committee of Fukuoka University, following approval of the experimental protocol (Permit Numbers: 1602907 and 1712128). Timed-pregnant Jcl:ICR mice (Catalogue ID: Jcl:ICR, CLEA Japan, Inc., Tokyo, Japan) were purchased at gestational day 15 from Kyudo (Tosu, Japan). Fifteen to seventeen-week-old pregnant Jcl:ICR mice were used. The body weight of pregnant mice was not recorded. Pregnant mice were housed individually in plastic cages in temperature-controlled rooms ($23 \pm 2\,^{\circ}C$) at our animal facility with a 12-h light-dark cycle. Food (CLEA Rodent Diet, CE-2, CLEA Japan, Inc.) and water were provided *ad libitum*.

Astrocyte culture

Cortical astrocyte cultures were prepared as reported previously (Bekkers and Stevens, 1991; Kawano et al., 2017). Cerebral cortices of newborn ICR mice were separated from the brain in ice-cold Hank's balanced saline solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and dissociated with 0.05% trypsin-EDTA (FUJIFILM Wako Pure Chemical Corporation). Cells were then plated in $75\,cm^2$ culture flasks (Corning Inc., Corning, NY, USA) in plating medium composed of Dulbecco's Modified Eagle's Medium with GlutaMAX™ and pyruvate (DMEM, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 0.1% MITO + Serum Extender (BD Biosciences, San Jose, CA, USA). The next day, culture flasks were gently rinsed once with fresh plating medium to remove non-adherent cells. When the culture reached confluence after two weeks, the microglia and other small cells were discarded by tapping the culture flask several times. Adherent cells were then resuspended and replated at a density of $6000\,cells/cm^2$ for mass and microisland cultures.

For the mass culture of astrocytes, 6-well plates (TPP, Switzerland) were uniformly coated with a 1:1 mixture of rat-tail collagen (final concentration 1.0 mg/mL, BD Biosciences) and poly-D-lysine (final concentration 0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). For microisland cultures, 300-μm square islands of collagen/poly-D-lysine mixture were stamped onto glass coverslips (thickness No. 1; Matsunami, Osaka, Japan) coated with 0.5% agarose.

Autaptic and sandwich neuron cultures

Hippocampi were separated from the brains of newborn ICR mice and neurons were enzymatically dissociated in DMEM containing papain (2 U/mL, Worthington, Columbus, OH, USA), for 60 min at 37 °C. Before plating neurons, the conditioned medium of the microisland and mass astrocyte cultures was replaced with Neurobasal-A medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 2% B27 supplement (Thermo Fisher Scientific, Waltham, MA, USA) and 1% GlutaMAX-I supplement (Invitrogen). For autaptic neuron culture, cells were plated at a density of $1500\,cells/cm^2$ onto the astrocyte microislands. For sandwich neuron culture, cells were plated at a density of $1500\,cells/cm^2$ onto glass coverslips stamped in the same way as for the microisland astrocytes. The glass coverslips also had four dots of paraffin wax on the stamped side to enable suspension of the coverslip above the cultured astrocytes. The coverslips were flipped over onto mass astrocyte cultures after one day.

Aβ preparation and treatment

A stock solution of $A\beta_{25-35}$ was prepared by dissolving $A\beta_{25-35}$ (Sigma-Aldrich) in sterile water at a concentration of 2 mM and incubating at 37 °C for 4 days. Astrocytes were exposed to $A\beta_{25-35}$ for 72 h prior to plating with neurons. After 72 h of $A\beta_{25-35}$ treatment, cells were rinsed three times with astrocyte plating medium to remove $A\beta_{25-35}$ from the astrocyte culture. To assess the effect of $A\beta_{25-35}$ on astrocytes, the number of astrocytes was counted by staining with NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Immunocytochemistry

Hippocampal neurons cultured for 14 days were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, and then blocked and permeabilized with PBS containing 5% normal goat serum and 0.1% Triton X-100 for 30 min. Samples were then incubated overnight at 4 °C with the following primary antibodies: anti-microtubule-associated protein 2 (MAP2, guinea-pig polyclonal, antiserum, Synaptic Systems, 1:1000 dilution) and anti-vesicular glutamate transporter 1 (VGLUT1, rabbit polyclonal, affinity purified, Synaptic Systems, 1:2000 dilution). Autaptic neurons were incubated with appropriate species-specific fluorochrome-conjugated goat secondary antibodies (Alexa Fluor 488 for MAP2, and Alexa Fluor 594 for VGLUT1, 1:400 dilutions, Invitrogen) for 1 h at room temperature. Double immunocytochemical staining was performed using a combination of MAP2 and VGLUT1 antibodies. Autaptic neurons were visualized by counterstaining with DAPI contained in the mounting medium (ProLongH Gold antifade mounting reagent, Invitrogen).

Solutions

The standard extracellular solution for patch-clamp experiments was (in mM) NaCl 140, KCl 2.4, HEPES 10, glucose 10, $CaCl_2$ 2, $MgCl_2$ 1, pH 7.4, with an adjusted osmotic pressure of 315–320 mOsm. Patch pipettes were filled with an intracellular solution composed of (in mM) K-gluconate 146.3, $MgCl_2$ 0.6, $ATP-Na_2$ 4, $GTP-Na_2$ 0.3, creatine phosphokinase 50 U/ml, phosphocreatine 12, EGTA 1, HEPES 17.8, pH 7.4. Hypertonic solutions for determining the size of the readily releasable pool of synaptic vesicles (RRP) were prepared by adding 0.5 M sucrose to the standard extracellular solution. The extracellular solutions were applied using a fast-flow application system (SF-77B, Warner Instruments, Hamden, CT, USA). Each flow pipe had a large diameter (430 μm), ensuring that the solution was applied to all parts of an autaptic neuron on an astrocytic microisland (300×300 μm squares). This experimental configuration is necessary when the application of sucrose or tetrodotoxin induces synaptic responses from all nerve terminals of the single neuron being recorded. All chemicals were purchased from Sigma-Aldrich except where otherwise specified.

Autaptic neuron culture electrophysiology

Autaptic neuronal cultures were used for synaptic recordings. Recordings were performed on 13–18 days *in vitro* (DIV) cultures to ensure that synaptic responses were stable with reliable space clamping. Synaptic responses were recorded using a patch clamp amplifier (MultiClamp 700B, Molecular Devices, Sunnyvale, CA, USA), in the whole-cell configuration under the voltage-clamp mode, at a holding potential (Vh) of –70 mV, and at room temperature in all cases. Patch-pipette resistance was 4–5 MΩ, and 70–90% of access resistance was compensated. Autaptic neurons showed synaptic transmission in response to an action potential elicited by a brief (2 ms) somatic depolarization pulse (to 0 mV) from the patch pipette. The synaptic responses were recorded at a sampling rate of 20 kHz and were filtered at 10 kHz. Data were excluded from analysis if a leak current of > 300 pA was observed. The data were analyzed offline using AxoGraph X 1.2

software (AxoGraph Scientific, Sydney, Australia). mEPSCs with an amplitude threshold of 5 pA were detected.

Image acquisition and quantification

Specimens were observed under an inverted microscope (Eclipse-TiE, Nikon, Japan) with a 40× objective lens (Plan Apoλ, NA 0.95, Nikon). Sixteen-bit images were acquired using a scientific CMOS camera (pco.edge 4.2, pco, Germany). Ten images were taken of each sample and a normalized average intensity picture of the 10 images was used for analysis. A Gaussian blur filter was applied to remove background noise (Iwabuchi et al., 2014). VGLUT1 puncta were detected with a size threshold ≥5 pixel using ImageJ, and the branching dendrites were analyzed by Sholl analysis (Sholl, 1953) using Plug-in for ImageJ (Kawano et al., 2012;).

Statistical analysis

Data are expressed as the mean ± SEM. Two groups were compared using Student's unpaired t-test. Statistical significance was considered when $p < 0.05$.

Results

To determine the adequate concentration of Aβ$_{25-35}$ on astrocytes, mass cultured astrocytes were treated with Aβ$_{25-35}$ at different concentrations (1, 3, and 10 μM) for 72 h. The number of astrocytes was counted by staining with NucBlue™ Live ReadyProbes™ Reagent 14 days after treatment with Aβ$_{25-35}$. As shown in Supplementary Fig. S1, astrocyte density significantly decreased at 3 and 10 μM compared with controls (control, 145 ± 13.3; 1 μM, 149 ± 9.58; 3 μM, 113 ± 6.62; 10 μM, 81.6 ± 9.32; Fig. S1A). Next, we observed dotted astrocytes at 14 days after treatment with Aβ$_{25-35}$. Configuration of the dotted astrocyte layer was maintained at 1 μM Aβ$_{25-35}$, but visibly collapsed at 3 and 10 μM (Fig. S1B). These data indicate that 1 μM concentration of Aβ$_{25-35}$ is physiologically suitable to examine the effect of Aβ$_{25-35}$ on synaptic transmission without cell death.

Excitatory synaptic transmission is attenuated by Aβ$_{25-35}$-exposed astrocytes

To assess effects on synaptic transmission in neurons co-cultured with Aβ$_{25-35}$-exposed astrocytes, we performed whole-cell patch clamp recording in autaptic neuron cultures under voltage clamp conditions. Importantly, neurons were not exposed to Aβ$_{25-35}$, but the co-cultured astrocytes were previously exposed to 1 μM Aβ$_{25-35}$. Therefore, this configuration makes it possible to evaluate whether Aβ$_{25-35}$ affects the influence of astrocytes on neurons.

First we recorded excitatory synaptic transmission in hippocampal neurons (Fig. 1A). The amplitude of the excitatory postsynaptic current (EPSC) by action potential stimulation was significantly smaller in hippocampal neurons co-cultured with Aβ$_{25-35}$-exposed astrocytes (control, 8.82 ± 0.79 nA; Aβ, 6.37 ± 0.65 nA; Fig. 1B). The evoked EPSC was completely blocked by 10 μM CNQX (data not shown) and is, therefore, mediated by AMPA receptors. Next, we measured spontaneous miniature EPSCs in the presence of 1 μM tetrodotoxin, a Na$^+$ channel blocker (Fig. 1C). Although the amplitude of mEPSCs was similar (control, 26.00 ± 1.39 pA; Aβ, 26.11 ± 1.43 pA; Fig. 1D), the frequency of mEPSCs was significantly decreased in hippocampal neurons co-cultured with Aβ$_{25-35}$-exposed astrocytes (control, 8.07 ± 0.80 Hz; Aβ, 5.72 ± 0.69 Hz; Fig. 1E). These data indicate that postsynaptic AMPA receptors were not affected by Aβ$_{25-35}$-exposed astrocytes. Therefore, the decrease in the evoked excitatory synaptic transmission by action potential stimulation is caused by a presynaptic action.

The size of the readily releasable pool of synaptic vesicles is decreased by Aβ$_{25-35}$-exposed astrocytes

To evaluate presynaptic actions in detail, we investigated the readily releasable pool of synaptic vesicles (RRP) in autaptic neuron cultures by measuring responses to 0.5 M sucrose (Rosenmund and Stevens, 1996; Fig. 2A). Neurons co-cultured with Aβ$_{25-35}$-exposed astrocytes showed a significant decrease in RRP size (control, 2.01 ± 0.27 nC; Aβ, 1.21 ± 0.15 nC; Fig. 2B). We then calculated the number of synaptic vesicles in the RRP by dividing the RRP charge by the averaged EPSC charge (Kawano et al., 2012). The number of synaptic vesicles was also significantly decreased in neurons co-cultured with Aβ$_{25-35}$-exposed astrocytes (control, 14934.93 ± 1854.36; Aβ, 9309.53 ± 1139.86; Fig. 2C). These results indicate changes in the release function. Therefore, we computationally estimated the vesicular release probability (Pvr) from the EPSC area and the sucrose response in the same recorded neuron. This is because the probability of action potential-evoked release originates from a portion of the RRP. The Pvr was hence calculated by dividing the action potential-induced EPSC charge by the sucrose-induced transient EPSC charge. Accordingly, the Pvr was unchanged by Aβ$_{25-35}$-exposed astrocytes (control, 7.05 ± 0.84%; Aβ, 5.92 ± 0.73%; Fig. 2D). Next, we measured the paired-pulse ratio (PPR) as another way of assessing release probability. This parameter indicates the sensitivity of the release mechanism to Ca^{2+} influx following an action potential (Xu-Friedman and Regehr, 2004). Autaptic cultures generally exhibit short-term plasticity such as paired-pulse depression (PPD) or paired-pulse facilitation (PPF) at individual synapses. Further it is likely that short-term plasticity emerges with subsequent stimulation in autaptic cultures (Pyott and Rosenmund, 2002). Thus, paired-pulse stimulation with 50 ms intervals, namely, 20 Hz stimulation is used to examine release probability in autaptic cultures. Based on this rationale, PPR was defined as the ratio of the amplitude of a second evoked EPSC to that of a first evoked EPSC when two EPSCs are evoked by action potentials separated by a short interval (50 ms). There was clearly no difference in the PPR between the two groups (control, 1.03 ± 0.03; Aβ, 1.02 ± 0.03; Fig. 2E). Although we did not observe PPD or PPF with a double-pulse of 50 ms interval (Fig. 2E), this protocol would be supportive for assessing release probability. Thus, the fact that the Pvr and the PPR were unchanged indicates that the synaptic release mechanism remains unchanged in neurons co-cultured with Aβ$_{25-35}$-exposed astrocytes.

The number of excitatory synapses is reduced by contact between Aβ$_{25-35}$-exposed astrocytes and neurons

We showed above that the release function was not associated with the reduction of RRP size; therefore, we speculated that the attenuated synaptic transmission caused by Aβ$_{25-35}$-exposed astrocytes may be caused by a decrease in the number of excitatory synapses. The excitatory synapses in hippocampal neurons possess high numbers of vesicular glutamate transporter 1 (VGLUT1) (Wojcik et al., 2004). Therefore, we regarded synaptic puncta labeled by a VGLUT1 antibody as glutamatergic excitatory synapses. The number of VGLUT1-labeled puncta was significantly decreased by Aβ$_{25-35}$-exposed astrocytes (control, 350.17 ± 32.85; Aβ, 203.91 ± 18.56; Fig. 3B). Sholl analysis was applied to assess dendritic morphology (Sholl, 1953). Sholl analysis is a common way to estimate neurite branching by counting the number of branch intersections in a series of concentric circles (e.g. at 20 μm intervals) centered at the soma of a single neuron. As shown in Fig. 3C, the number of dendritic branches declined evenly up to 200 μm from the soma in neurons co-cultured with and without Aβ$_{25-35}$-exposed astrocytes. However, the total number of branch intersections was significantly decreased in the Aβ$_{25-35}$-exposed astrocyte cultured neurons compared with the control neurons (control, 181.64 ± 13.50; Aβ, 134.82 ± 7.64; Fig. 3D).

We next considered the relationship between synaptogenesis and

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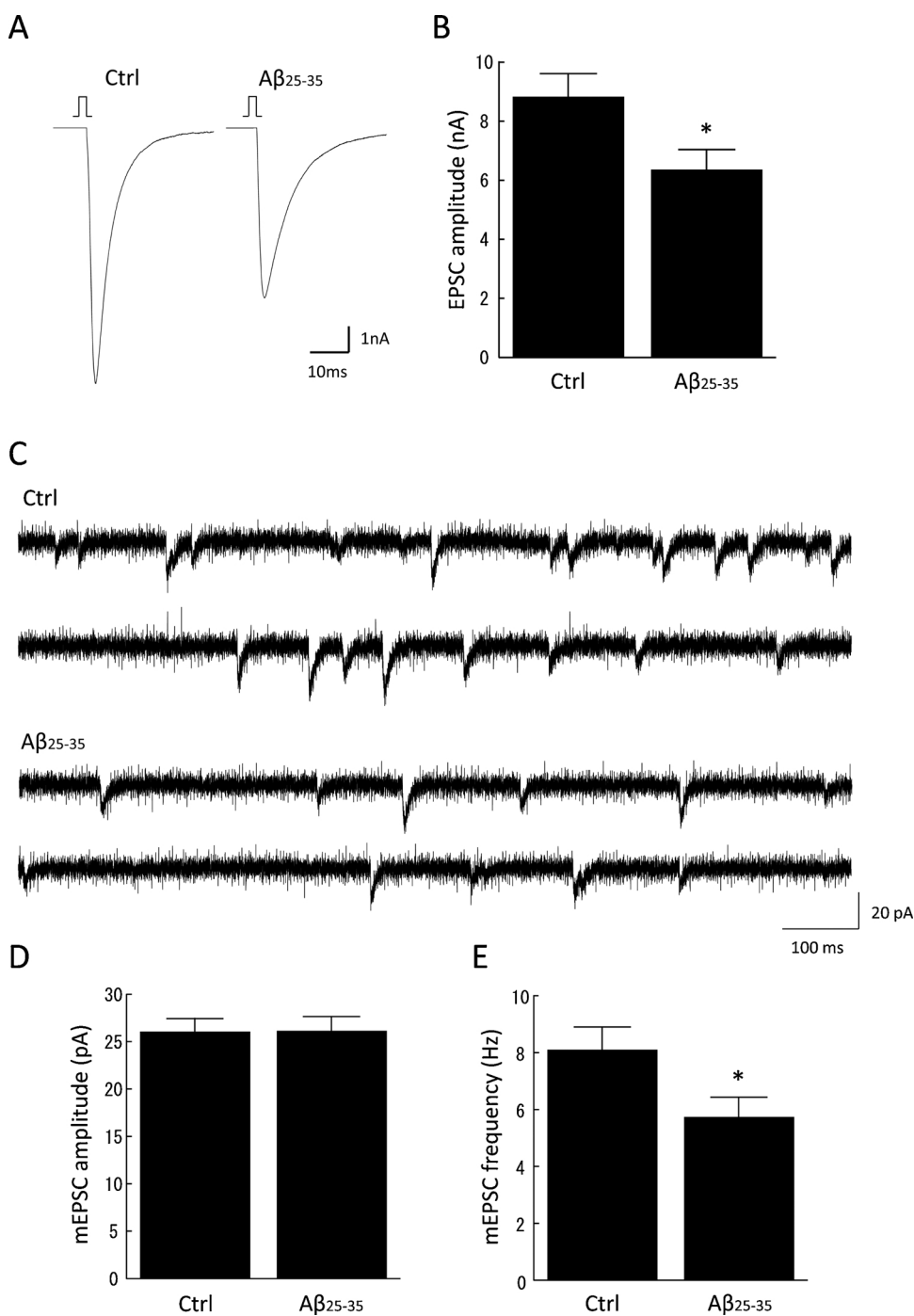

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Fig. 1. Aβ_{25-35}-exposed astrocytes decrease excitatory synaptic transmission. (A) Representative traces of EPSCs recorded from autaptic neurons (control and Aβ_{25-35}). Depolarization artifacts caused by generated action potential currents have been removed for clarity. (B) Average amplitude of evoked EPSCs in autaptic neurons co-cultured with control astrocytes (n = 50 from 12 cultures) or Aβ_{25-35}-exposed astrocytes (n = 49 from 12 cultures), *p < 0.05. (C) Representative traces of miniature mEPSCs in neurons co-cultured with control astrocytes (n = 50 from 12 cultures) or Aβ_{25-35}-exposed astrocytes (n = 49 from 12 cultures). (D) Amplitude of mEPSCs in autaptic neurons co-cultured with control astrocytes (n = 50 from 12 cultures) or Aβ_{25-35}-exposed astrocytes (n = 49 from 12 cultures). (E) Frequency of mEPSCs in autaptic neurons co-cultured with control astrocytes or Aβ_{25-35}-exposed astrocytes.

astrocytes. There appear to be two different factors in the relationship: diffusible astrocyte-derived factors and direct contact between the astrocytes and neurons. Synaptogenesis is promoted by molecules released from astrocytes (Mauch et al., 2001; Christopherson et al., 2005; Kucukdereli et al., 2011); however, Aβ reduces the secretion of diffusible factors from astrocytes, such as glial derived neurotrophic factor (GDNF) and thrombospondins (Tseng et al., 2012). Contact between astrocytes and neurons promotes synapse formation (Hama et al., 2004; Garrett and Weiner, 2009); however, it is not clear whether contact between Aβ_{25-35}-exposed astrocytes and neurons contributes to synapse formation. Therefore, we focused on astrocyte-neuronal contacts and assessed synapse number using sandwich cultures, prepared according to a previous study (Kaech and Banker, 2006). In this technique, hippocampal neurons grow under a coverslip suspended above a layer of

astrocytes. The astrocytes and neurons do not come into direct contact, and membrane-bound astrocyte-derived factors cannot affect the neurons. Therefore, if the number of synapses does not change under these experimental conditions, it is likely that membrane-bound astrocyte-derived factors rather than diffusible astrocyte-derived factors inhibited synapse formation.

We evaluated dendritic morphology and the number of VGLUT1-labeled puncta in the sandwich culture preparation. The number of excitatory synapses was not changed by Aβ_{25-35}-exposed astrocytes (control, 213.38 ± 24.49; Aβ, 221.00 ± 24.52; Fig. 4B). Additionally, there was no change in dendrite morphology (Fig. 4C). Statistically, the number of total branch intersections was not different between groups (control, 157.44 ± 15.14; Aβ, 164.27 ± 14.69; Fig. 4D). Therefore, it is possible to maintain synaptogenesis when Aβ_{25-35}-exposed astrocytes

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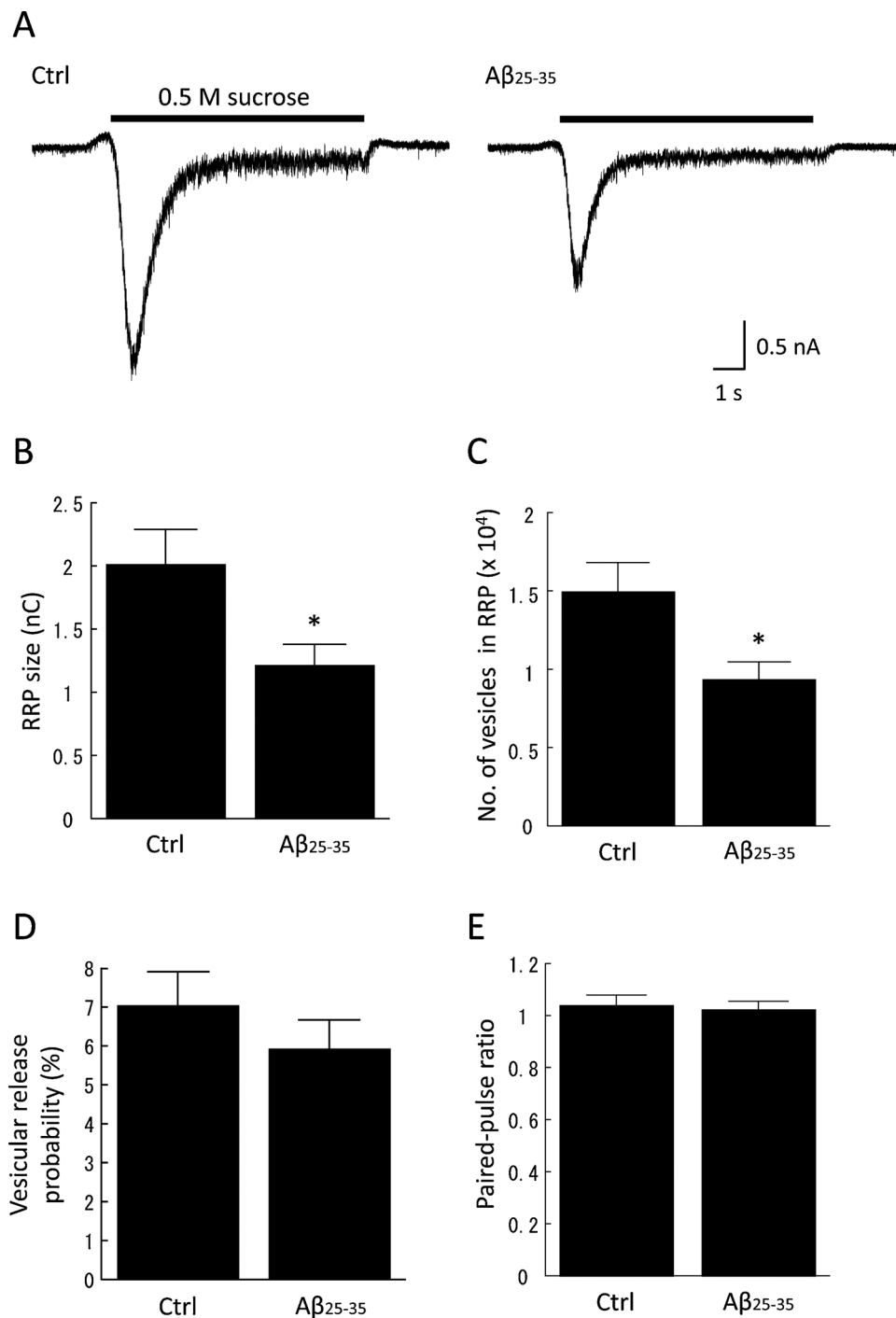
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Fig. 2. The readily releasable pool of synaptic vesicles (RRP) is reduced by Aβ$_{25-35}$-exposed astrocytes. (A) Representative traces of the response to 0.5 M sucrose solution (8 s) in autaptic neurons co-cultured with control astrocytes or Aβ$_{25-35}$-exposed astrocytes. (B) Average RRP size in autaptic neurons co-cultured with control astrocytes (n = 50 from 12 cultures) or Aβ$_{25-35}$-exposed astrocytes (n = 49 from 12 cultures), *p < 0.05. (C) The number of synaptic vesicles in the RRP. Data were obtained from the same neurons examined in (B), *p < 0.05. (D) Vesicular release probability (Pvr) in autaptic neurons co-cultured with control astrocytes (n = 50 from 12 cultures) or Aβ$_{25-35}$-exposed astrocytes (n = 49 from 12 cultures). (E) Paired-pulse ratio (EPSC$_2$/EPSC$_1$) in autaptic neurons co-cultured with control astrocytes (n = 50 from 12 cultures) or Aβ$_{25-35}$-exposed astrocytes (n = 49 from 12 cultures).

do not directly contact neurons.

Discussion

It is well known that Aβ causes defective synaptic transmission in AD (Cullen et al., 1996; Small et al., 2001; Selkoe, 2002). Aβ generally effects not only neurons but also astrocytes. Astrocytes are required for synaptogenesis and for functional synaptic transmission. For example, neurons cultured *in vitro* without astrocytes barely form synapses and the number of active synapses is small, indicating abnormal spontaneous synaptic activity and synaptic transmission. However, in neurons co-cultured with abundant astrocytes, the number of synapses increases dramatically, along with increased frequency and amplitude of spontaneous postsynaptic currents; *i.e.* the synaptic transmission is normal (Pfrieger and Barres, 1997). Therefore, we suggest that disruption of astrocytes by Aβ-exposure is sufficient to cause impaired synaptic transmission in AD.

It is experimentally difficult to only expose astrocytes to Aβ in a mixed cell type culture or tissue; therefore, we co-cultured single hippocampal neurons with astrocytes that had been previously chronically exposed to Aβ. Excitatory synaptic transmission was significantly reduced by Aβ$_{25-35}$-exposed astrocytes (Fig. 1B). In addition, a decreased number of excitatory synapses (Fig. 3B) led to a decrease in the frequency of mEPSCs (Fig. 1E) and the RRP size (Fig. 2B). Aβ can cause AMPA receptor dysfunction (Hsieh et al., 2006); however, there was no change in the amplitude of mEPSCs in this study (Fig. 1D).
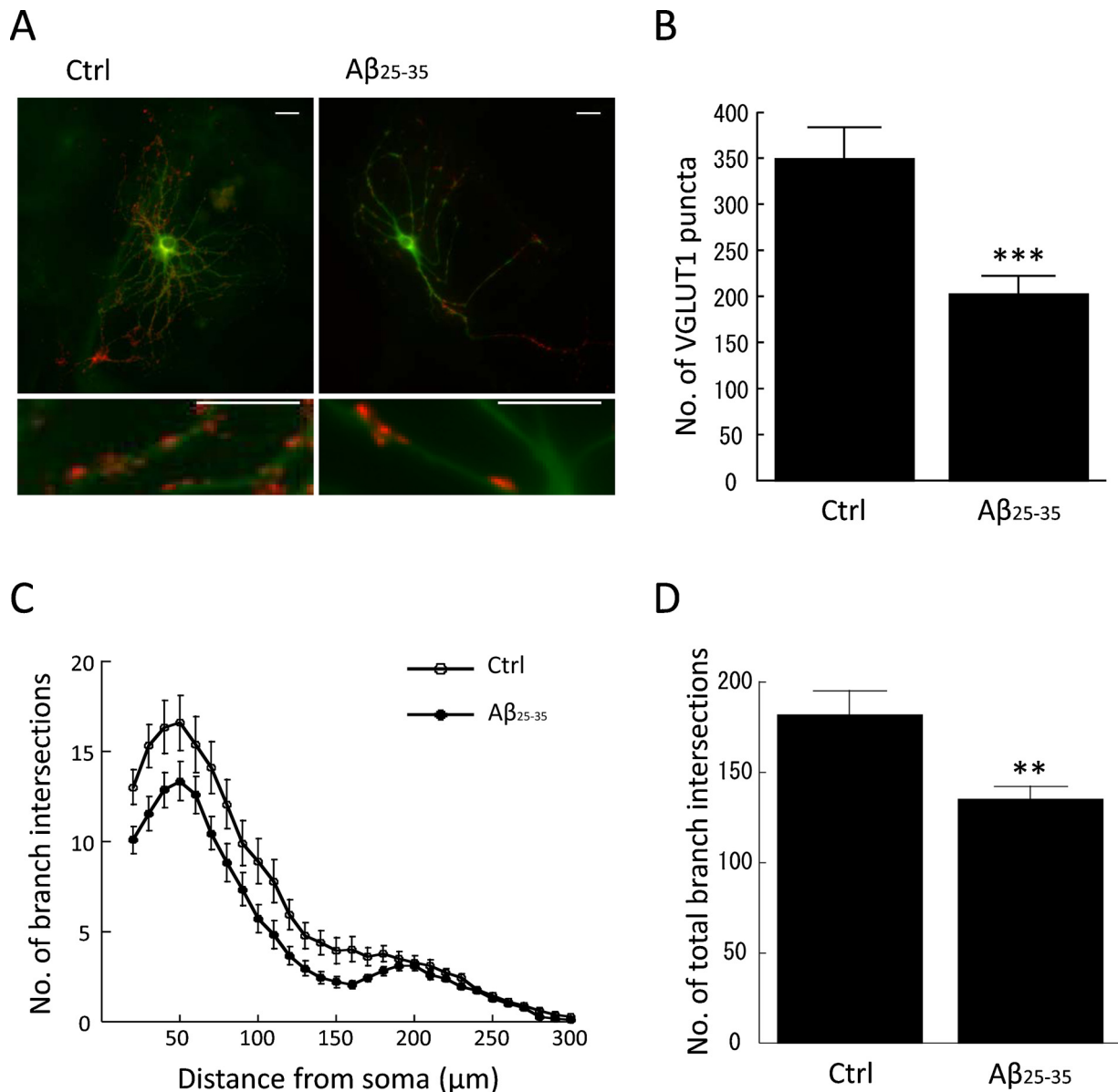
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Fig. 3. Excitatory synapse formation is inhibited by Aβ25-35-exposed astrocytes. (A) Representative images of autaptic neurons immunostained for the dendritic marker, microtubule-associated protein 2 (MAP2) (in green) and the excitatory synapse marker, vesicular glutamate transporter 1 (VGLUT1) (in red). Parts of the images in the top row (scale bars = 20 μm) are enlarged in the bottom row (scale bars = 10 μm). (B) The number of VGLUT1 puncta in autaptic neurons co-cultured with control astrocytes (n = 39 from 4 cultures) and Aβ25-35-exposed astrocytes (n = 35 from 4 cultures), ***p < 0.001. (C) Sholl analysis of dendrites. Data were obtained from the cultures analyzed in (B). (D) The total number of branch intersections. Data were obtained from the cultures analyzed in (B), **p < 0.01.

Hippocampal neurons co-cultured with astrocytes exposed to a different Aβ fragment, Aβ1–40, (Kawano et al., 2017) also showed impaired excitatory synaptic transmission, similar to the results in the present study; therefore, we speculate that Aβ-exposed astrocytes do not affect the properties of AMPA receptors in neurons.

Direct contact of astrocytes with hippocampal neurons activates PKC signaling and promotes synaptogenesis and synaptic transmission (Hama et al., 2004). Furthermore, decreased expression of N-cadherin, a transmembrane protein, in hippocampal astrocytes suppresses neurite outgrowth (Kanemaru et al., 2007). In addition, diffusible astrocyte-derived factors are thought to be important for synaptogenesis. For example, Tseng et al. (2012) reported that diffusible astrocyte-derived factors that promote synaptogenesis were decreased by Aβ. Furthermore, depletion of neurolysine 2, an enzyme present in cortical astrocytes, inhibits excitatory synapse formation (Stogsdill et al., 2017). However, our data showed that the number of excitatory synapses was

not decreased in sandwich cultures, in which neurons and astrocytes were not in direct contact (Fig. 4B). These data indicate that a direct adhesion factor between neurons and astrocytes is necessary for normal neuronal morphology and synaptogenesis (Allen and Eroglu, 2017). Therefore, disruption of this astrocyte-mediated adhesion by exposure to Aβ25-35 would result in a decrease in synapse number. Further investigations are required to explore these adhesion factors in detail.

Our study shows that exposure of astrocytes to Aβ25-35 is associated with abnormal synaptogenesis and synaptic transmission. This indicates that astrocyte dysfunction resulting from Aβ accumulation is associated with synaptic abnormalities in AD. Specifically, our data are more directly related to synapse formation of developing neurons. As discussed in our previous paper (Kawano et al., 2017), neurons co-cultured with astrocytes exposed to Aβ fail to mature completely. Considering that neurons are already mature at a time when astrocytes are exposed to Aβ during the aging process, it is not yet clear how Aβ-exposed astrocytes
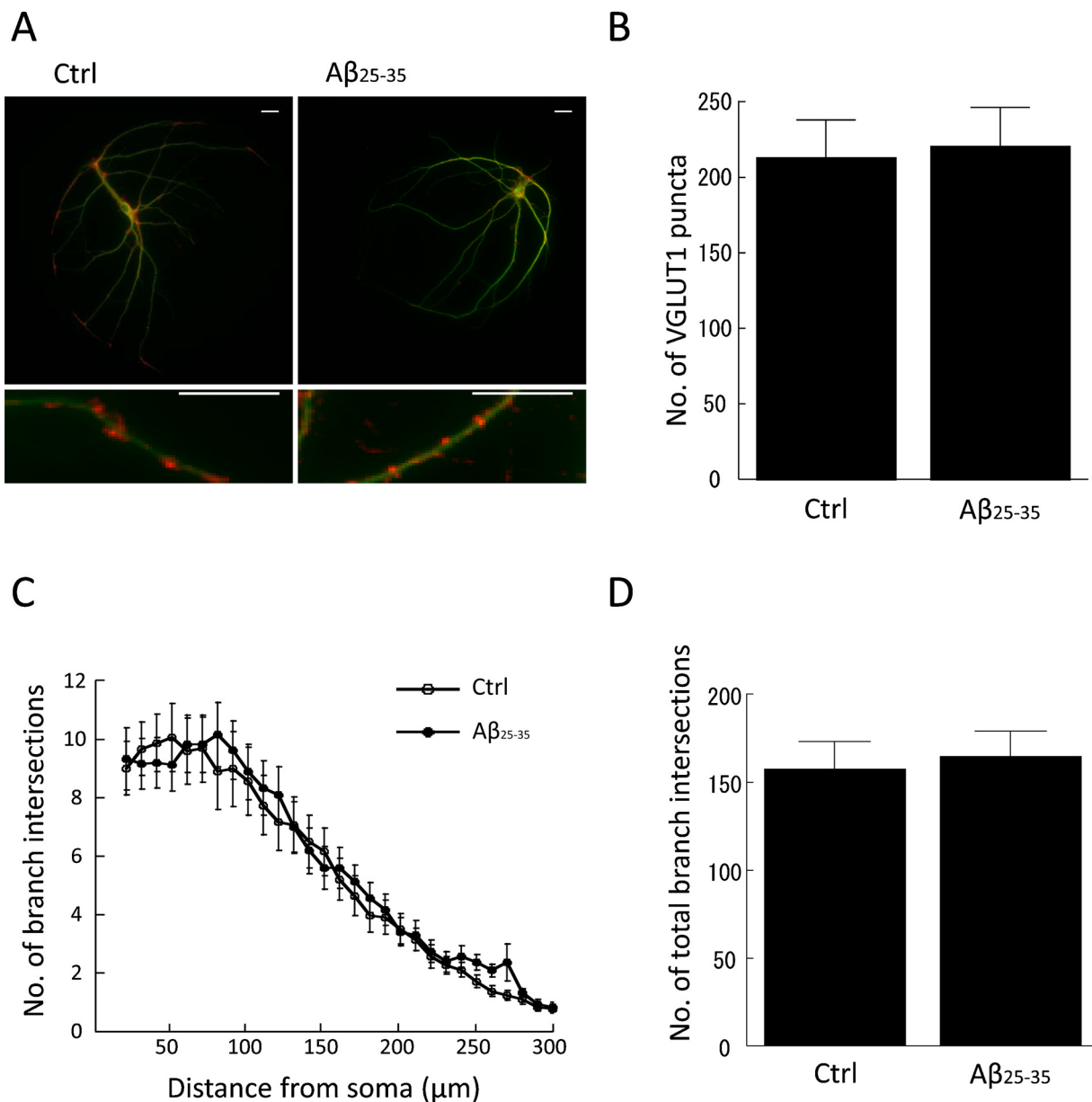
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Fig. 4. Excitatory synapse formation is not changed by Aβ$_{25-35}$-exposed astrocytes. (A) Representative images of autaptic neurons immunostained for the dendritic marker, MAP2 (in green) and the excitatory synapse marker, VGLUT1 (in red). Parts of the images in the top panel (scale bars 20 = μm) are enlarged in the bottom panel (scale bars = 10 μm). (B) The number of VGLUT1 puncta in autaptic neurons co-cultured with control astrocytes (n = 26 from 4 cultures) and Aβ$_{25-35}$ = exposed astrocytes (n = 31 from 4 cultures) (C) Sholl analysis of dendrites. Data were obtained from the specimens analyzed in (B). (D) The total number of branch intersections. Data were obtained from the cultures analyzed in (B).

affect "mature neurons" in AD brain. Therefore, if possible, further experiments using aged neurons should be performed under these circumstances.

In *in vitro* cultured cortical neurons, neurotoxicity and impairment of synaptic activity caused by Aβ were reversible (Lee et al., 2013; Tanokashira et al., 2017). In AD mice, rapid inhibition of transgenic APP alleviates cognitive dysfunction, and this effect results from a reduction of Aβ (Fowler et al., 2014). Furthermore, immunotherapy targeting Aβ reduces Aβ toxicity and ameliorates synaptic dysfunction and cognitive impairment (Lord et al., 2009; Wisniewski and Goñi, 2015). Taken together, these studies indicate that a reduction or elimination of Aβ from astrocytes might be effective in treating neuronal disorders.

In addition to the direct effects of Aβ on neurons in AD pathology, we propose that there is also an astrocyte-mediated effect of Aβ. Therefore, treatments that target astrocytes as well as neurons may contribute to the establishment of a new AD therapy.

Conflict of interest

None.

Ethical statement

All experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and the Japan Society for the Promotion of Science guidelines.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ibror.2019.07.1719.

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