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Original Article

Involvement of hippocampal excitability in amyloid β-induced behavioral and psychological symptoms of dementia

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ABSTRACT — In patients with Alzheimer's disease, in addition to the core symptoms, i.e., cognitive dysfunction, behavioral and psychological symptoms of dementia (BPSD) such as aggression, anxiety, and hallucinations are known to occur frequently. Because various environmental factors influence the onset and progression of Alzheimer's disease, in the present study, BPSD-like behavioral abnormality of Amyloid β (Aβ)1-42-injected mice was assessed under social isolation, which induces behavioral abnormality. Aβ protein (500 pmol) was injected into the lateral ventricle of mice, which were individually housed. Two and three weeks after injection into adult mice, the rate of mice that exhibited aggressive behavior, i.e., biting attacks and wrestling, to the total mice, was markedly increased by Aβ injection. Aβ-injected adult mice also showed anxiety-like behavior, in addition to cognitive decline. Serum corticosterone level was markedly increased by Aβ injection. When excitability of hippocampal neurons was checked using hippocampal slices, KCl-induced presynaptic activity was enhanced in hippocampal slices prepared from Aβ-injected mice. These results suggest that social isolation housing of Aβ1-42-injected adult mice induces BPSD-like behavioral abnormality in addition to cognitive decline. It is likely that behavioral abnormality of Aβ1-42-injected adult mice is associated with excitability of hippocampal glutamatergic neurons, which is associated with the elevated corticosterone level.

Key words: Amyloid-β, Alzheimer's disease, Aggression, Social isolation, Corticosterone, Hippocampus

INTRODUCTION

Alzheimer's disease is a progressive neurodegenerative disorder that is characterized by the presence of senile plaques and neurofibrillary tangles together with synaptic and neuronal loss (Terry *et al.*, 1991). The major component of senile plaques is amyloid β (Aβ) proteins, which comprises peptides of approximately 39-43 amino acid residues derived from the transmembrane amyloid precursor protein (APP) (Selkoe, 2002). Aβ can exist as monomers and form a variety of different aggregate morphologies including dimers, small soluble oligomers, protofibrils, diffuse plaques, and the fibrillar deposits seen in senile plaques. The aggregation of Aβ-peptide is widely considered to be the critical step in the pathology of Alzheimer's disease. Small, soluble Aβ oligomers have been shown to be more neurotoxic than large, insoluble aggregates and fibrils (Cleary *et al*., 2005; Lesné *et al*., 2006; Shankar *et al*., 2008). Aβ1-42 is the major component of amyloid deposits in senile plaques (Iwatsubo *et al*., 1994). On the other hand, postmortem studies suggest that the hippocampus and entorhinal cortex are the first brain regions to be affected (Hyman *et al*., 1984; Nestor *et al*., 2004).

In patients with Alzheimer's disease, not only core symptoms such as cognitive impairment, but also

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behavioral and psychological symptoms of dementia (BPSD) such as aggression, anxiety, and hallucinations often emerge. BPSD is a serious problem for caregivers, and because its severity and the care burden show a positive correlation, therapy for BPSD is considered to be as important as therapy for the core symptoms (Nagaratnam *et al*., 1998; Tanji *et al*., 2005). Among BPSD, agitation and aggression occur in over 60% of patients with dementia (Mirakhur *et al*., 2004) and are frequently the primary cause of hospitalization or institutionalization (Steele *et al*., 1990). Non-pharmacological interventions such as environmental changes are proposed as the first-line treatment for BPSD, although medical therapy is often required (Mizukami, 2008).

On the other hand, various environmental and genetic factors influence the onset and progression of Alzheimer's disease (Green *et al*., 2006); it is possible that environmental changes are closely linked to the pathophysiology of BPSD. Social isolation housing of rodents causes a variety of behavioral changes, including hyperlocomotion, anxiety, impulsivity, aggression, and learning and memory deficits (Koike *et al*., 2009). However, behavioral abnormality and pathophysiology induced with Aβ has been mainly studied focusing on core symptoms and the involvement of social isolation in pathogenesis of BPSD is poorly understood (Sekiguchi *et al*., 2011).

In the present study, BPSD-like behavioral abnormality was examined in mice after intracerebroventricular injection of Aβ1-42, which were housed individually.

MATERIALS AND METHODS

Chemicals

Synthetic Aβ1-42 for human was purchased from ChinaPeptides (Shanghai, China). Aβ1-42 was dissolved in saline and immediately used when the experiments were performed. SDS-PAGE showed that Aβ1-42 in saline prepared in the present study was mainly monomers and that dimers and a very small portion of trimers were also contained in the prepared solution as reported previously (Takeda *et al*., 2014).

FM4-64, an indicator of presynaptic activity, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent indicator was dissolved in dimethyl sulfoxide (DMSO) and then diluted to artificial cerebrospinal fluid (ACSF) containing containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, and 11 mM D-glucose (pH 7.3).

Experimental animals

Male ddY mice (3 and 10 weeks old) were purchased from Japan SLC (Shizuoka, Japan). They were housed (five mice per cage) under the standard laboratory conditions (23 ± 1°C, 55 ± 5% humidity) and had access to tap water and food *ad libitum*. One week later, mice were housed individually in transparent plastic cages (24 x 17 x 13 cm). The lights were automatically turned on at 8:00 and off at 20:00. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka that refer to American Association for Laboratory Animals Science and the guidelines laid down by the NIH (NIH Guide for the Care and Use of Laboratory Animals) in the USA. Newly prepared mice were used in each group in all experiments.

Intracerebroventricular injection of Aβ

One week after individual housing, mice (4- and 11-week-old) were anesthetized with chloral hydrate (30 mg/kg) and placed in a stereotaxic apparatus. A microinjection canula (AMI-6, Eicom Co., Kyoto, Japan) was positioned 0.5 mm posterior to the bregma, 1.0 mm lateral, 2.0-2.4 mm inferior to the dura for intracerebroventricular injection. Aβ1-42 in saline (25 μM) was injected via the microinjection canula at the rate of 0.5 μL/min for 40 min (500 pmol/mouse). The individual housing was continued for the following experiments, which were performed 2 and 3 weeks after intracerebroventricular injection of Aβ.

Open-field test

Behavior and locomotor activity of mice were examined in the open-field test. Each mouse was placed in an arena (67.5 x 58.0 x 28.5 cm) made of a black-colored wooden box, in which it had never been placed previously (n = 11). The room light was covered with a translucent black polyethylene sheet. Behavior of each mouse in the arena, which was recorded with a video camera, was observed for 5 min.

Resident-intruder test

In the resident-intruder test, intruder mice, which were housed in a group of five, were individually transferred to the cages of resident mice from their home cages. Behavior of resident and intruder mice was observed for 5 min with a video camera. Biting attacks and wrestling of resident mice were included in aggressive behavior and tail rattle, lateral threat and pursuit were excluded from it.

Elevated plus maze

Anxiety-related behavior was evaluated using the plus-maze test. The plus-maze consists of two oppos-

ing open arms with low side walls (20 x 5 x 2 cm) and two enclosed arms (20 x 5 x 15 cm) that were extended from a central platform (5 x 5 cm), elevated 40 cm above the floor. The room light was covered with a translucent black polyethylene sheet. Mice were placed individually on the central platform facing an enclosed arm and were allowed to freely explore the maze for 5 min. Behavior of each mouse was monitored using a video camera. The total times spent in the open arms and the closed arms were measured. The ratio (%) of time spent in the open arms to total time spent in any arm was calculated as the standard anxiety index.

Object recognition memory

Mice were placed for 10 min into an open field, which was a 30 x 40 cm arena surrounded by 35 cm high walls, made of a black-colored plastic. Twenty-four hours after open field exploration, mice were trained and tested in a novel object recognition task. Training in the object recognition task took place in the same area used for the open field exploration. The open field exploration was thus used as a context habituation trial for the recognition memory task. The object recognition test requires that the mice recall which of two earthenware objects they had been previously familiarized with. Twenty-four hours after arena exploration, training was conducted by placing individual mice into the field, in which two identical objects (objects A1 and A2) were positioned in two adjacent corners, 13 cm from the walls. Mice were left to explore the objects for 5 min. Mice were not used for the test when the total of the object exploration time was less than 20 sec. In the test given 1 hr after training, the mice explored the open field for 3 min in the presence of one familiar (A) and one novel (B) object. Behavior of mice was recorded with a video camera during the training and the test, and then two persons independently measured exploratory time and the averaged time was used. All objects presented similar textures, colors and sizes, but distinctive shapes. A recognition index calculated for each mouse was expressed by the ratio $T_B/(T_A+T_B)$ [T_A = time spent to explore the familiar object A; T_B = time spent to explore the novel object B]. Between trials the objects were washed with 70% ethanol solution. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. We confirmed that there was no preference for the objects used.

Serum corticosterone concentration

Blood samples were collected from the common carotid arteries of diethyl ether-anesthetized mice. The collection was performed in the morning (10-11 o'clock) and quickly finished within 2 min to avoid the artificial increase in serum corticosterone (Watanabe *et al*., 2010). Blood samples were kept on ice and centrifuged for 10 min (5,500 rpm, 4°C). Corticosterone concentration in the serum obtained was determined by a corticosterone [^{125}I] RIA kit (MP Biomedicals, Inc, Irvine, CA, USA).

Brain slice preparation

Mice were anesthetized with diethyl ether and decapitated. The brain was quickly removed and immersed in ice-cold choline-ACSF containing 124 mM choline chloride, 2.5 mM KCl, 2.5 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH 7.3) to suppress excessive neuronal excitation. Brain slices (400 μm horizontal section) were prepared using a vibratome ZERO-1 (Dosaka, Kyoto, Japan) in an ice-cold choline-ACSF. Slices were then maintained in ACSF at 25°C for at least 1 hr. All solutions used in the experiments were continuously bubbled with 95% O_2 and 5% CO_2.

Exocytosis

The brain slices were transferred to an incubation chamber filled with ACSF containing 5 μM FM4-64 and 45 mM KCl, allowed to stand at 25°C for 90 sec and transferred a chamber filled with ACSF to wash out extracellular FM4-64 for 180 sec. Fifteen minutes later, brain slices were transferred to a recording chamber filled with ACSF, in which 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist of AMPA/kainate receptors, is contained, to prevent recurrent activity. The basal fluorescence of FM4-64 (excitation, 543 nm; emission, 640 nm) was measured with a confocal laser-scanning microscopic system LSM 510 META at the rate of 1 Hz for 30 sec through a 10 × objective. Because FM4-64 fluorescence originates from vesicular membrane-bound FM4-64, FM4-64 fluorescence is attenuated by presynaptic activity (Klingauf *et al*., 1998, Zakharenko *et al*., 2001). To induce non-specific depolarization, KCl in ACSF was added to the brain slices (the final KCl concentration, 60 mM) and attenuation of FM 4-64 fluorescence (destaining) based on presynaptic activity was measured in the same manner for 240 sec. The activity-dependent component of FM4-64 fluorescence in the stratum radiatum of the CA1 (the Schaffer collateral/commissural pathway terminals) and the stratum lucidum of the CA3 (mossy fiber terminals) was measured for each punctum by subtracting its residual fluorescence intensity 240 sec after stimulation with 60 mM KCl. FM4-64 fluorescence was then normalized by the basal fluorescence intensity before the stimulation, which is expressed as 100%.

Statistical analysis

Student's *t*-test was used for comparison of the means of paired and unpaired data.

RESULTS

Aβ-induced behavioral abnormality

In the initial experiment, intracerebroventricular injection of Aβ1-42 was performed using isolated 4-week-old mice. When behavior and locomotor activity of mice were examined in the open-field test, line crossing, rearing behavior, and grooming behavior were almost the same between the control and Aβ-injected mice both 2 and 4 weeks after intracerebroventricular injection (data not shown). In the resident-intruder test, the rate of mice that exhibited aggressive behavior to the total mice was 0% 2 weeks after the injection (n = 7) and 16.7% 3 weeks after the injection (n = 4).

Because Aβ injection into young mice did not seem to facilitate aggressive behavior, we used isolated 11-week-old mice. The mean body weight was 41.6 ± 0.5 g (vehicle-injected mice, control) and 40.9 ± 0.4 g (Aβ-injected mice) 2 weeks after intracerebroventricular injection, and 41.9 ± 0.4 g (vehicle-injected mice) and 42.4 ± 0.4 g (Aβ-injected mice) 3 weeks after intracerebroventricular injection. When behavior and locomotor activity of mice were examined in the open-field test, line crossing, rearing behavior, and grooming behavior were almost the same between the control and Aβ-injected mice both 2 (Fig. 1A-1C) and 3 (1D-1F) weeks after intracerebroventricular injection.

In contrast, behavioral abnormality was observed in the resident-intruder test. The rate of mice that exhibited aggressive behavior, i.e., biting attacks and wrestling, to the total mice was significantly increased both 2 and 3 weeks after intracerebroventricular injection of Aβ (Fig. 2A and 2D). There was no significance difference in the rate of aggressive mice between 2 and 3 weeks. The latency time to start aggressive behavior (Fig. 2B and 2E) and the duration of aggressive behavior (Fig. 2C and 2F) were not significantly different between the control and Aβ-injected mice. When anxiety-related behavior was evaluated using the plus-maze test, time spent in open arms was not decreased 2 weeks after intracerebroventricular injection of Aβ (Fig. 3A) but significantly decreased 3 weeks after intracerebroventricular injection of Aβ (Fig. 3B).

Cognitive activity was assessed in object recognition test. Object recognition was impaired both 2 and 3 weeks after intracerebroventricular injection of Aβ (Fig. 3C and 3D).

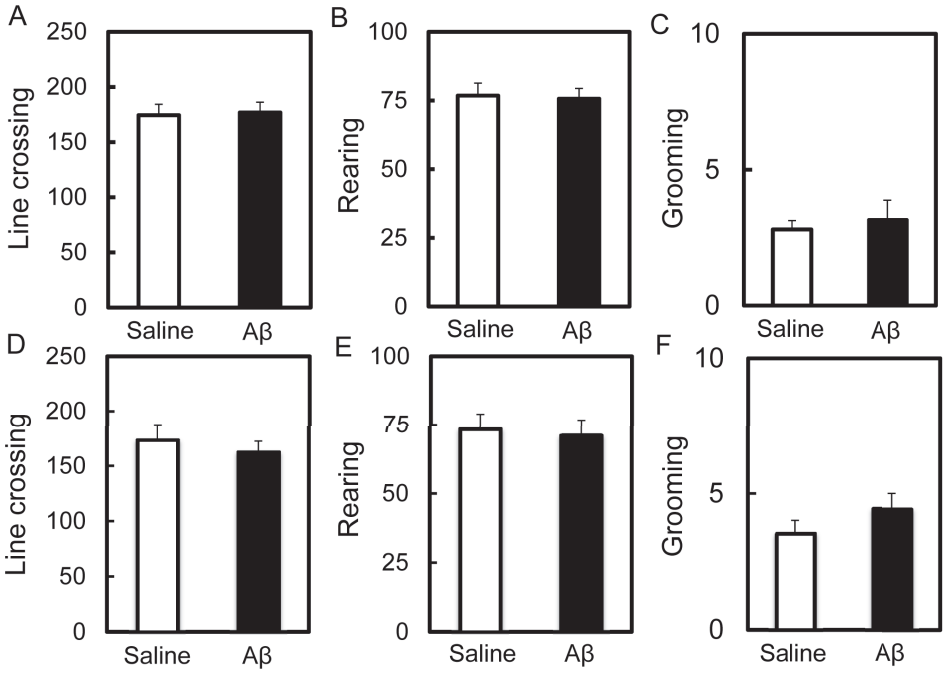

Fig. 1. Open-field test. The open-field test was performed 2 (A-C) and 3 (D-F) weeks after injection of saline and Aβ in saline into the lateral ventricle of 11-week-old mice. Line crossing (counts), rearing (counts), and grooming (sec) were measured. Each bar and line represents the mean ± S.E.M. (n = 17-18).

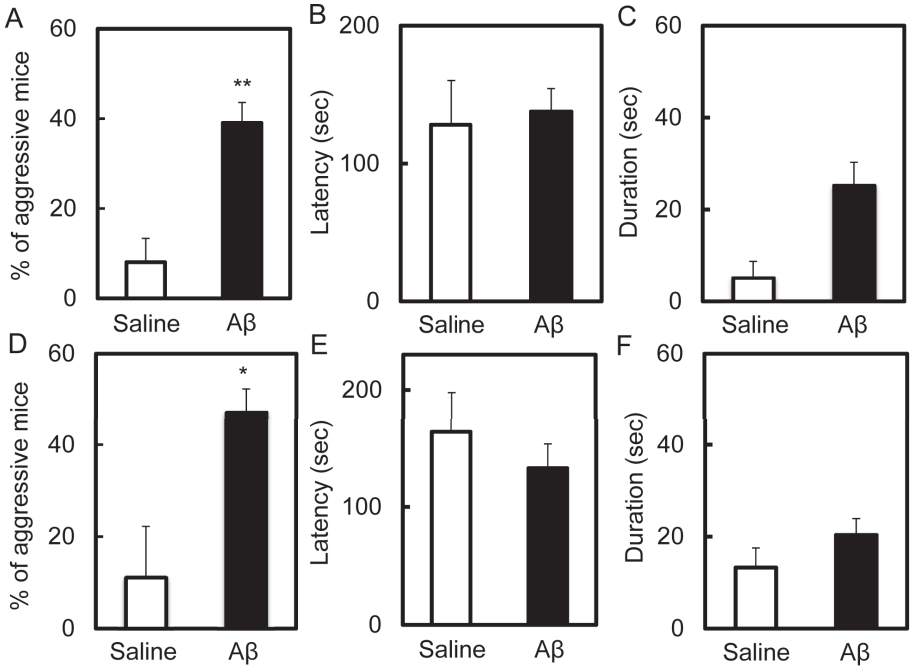

Fig. 2. Resident-intruder test. The resident-intruder test was performed 2 (A-C) and 3 (D-F) weeks after injection of saline and Aβ in saline into the lateral ventricle of 11-week-old mice. Each bar and line (mean ± S.E.M.) represents the mean of three experiments that were separately done (n = 7-11 per experiment). *, $P < 0.05$, **, $P < 0.01$ vs. saline (Student's t-test).

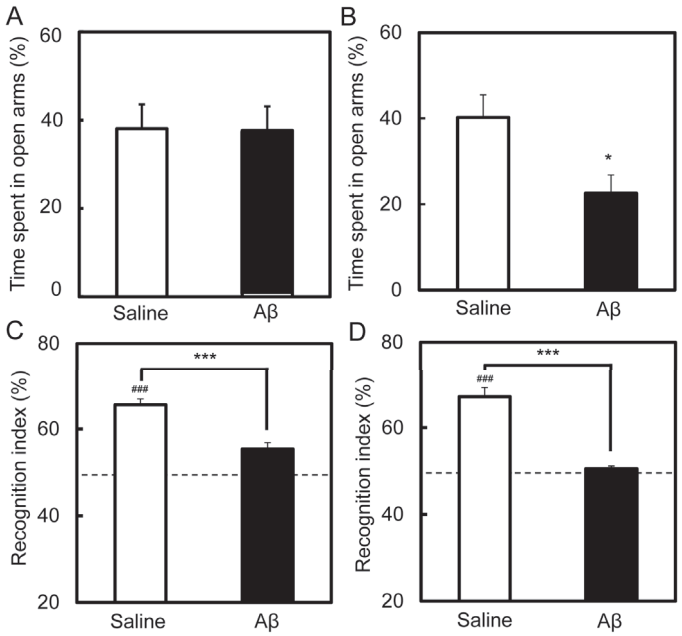

Fig. 3. Elevated plus maze and object recognition memory. Anxiety-like behavior in the elevated-plus maze was assessed 2 (A) and 3 (B) weeks after injection of saline or Aβ into the lateral ventricle. Each bar and line (mean ± S.E.M.) represents the time spent in open arms (n = 9-10). *, $P < 0.05$ vs. saline (Student's t-test). In another experiment, object recognition test was performed 2 (C) and 3 (D) weeks after injection of saline and Aβ in saline into the lateral ventricle. Each bar and line represents the mean ± S.E.M. (n = 18). ###, $P < 0.001$ vs. training (paired t-test), ***, $P < 0.001$ vs. saline (Student's t-test).

Aβ-induced neuronal excitability

Disruption of the hypothalamic-pituitary-adrenal (HPA) activity may be associated with Aβ-induced pathophysiology (Dong and Csernansky, 2009). When serum corticosterone concentration was checked, it was significantly higher in Aβ-injected mice than in the control mice (Fig. 4A). Because excess glucocorticoid secretion can enhance neuronal excitability in the hippocampus (Karst *et al*., 2005), presynaptic activity in the hippocampus was assessed using brain slices prepared from mice after intracerebroventricular injection of Aβ. Attenuation of FM 4-64 fluorescence based on presynaptic activity was significantly enhanced in the stratum radiatum where Schaffer collateral-CA1 pyramidal cell synapses exist (Figs. 4B and 4C) and also in the stratum lucidum where mossy fiber-CA3 pyramidal cell synapses exist (destaining: saline, 37.2 ± 5.7%; Aβ, 55.5 ± 5.8%, n = 7, $p < 0.05$).

DISCUSSION

It has been reported that glucocorticoids inhibit insulin-degrading enzyme activity, which is a candidate protease for the clearance of amyloid-beta peptide from the brain (Kulstad *et al*., 2005; Wang *et al*., 2011; Liu *et al*., 2012). Serum cortisol is associated with the clearance of amyloid-beta peptide and the progression in subjects with Alzheimer-type dementia. Correlations have been reported between increases in HPA axis activity and dementia severity or hippocampal volume loss in individuals with probable Alzheimer's disease (Csernansky *et al*., 2006). In Alzheimer's disease patients, moreover, core symptoms such as cognitive deficits and BPSD are associated with an early dysregulation of the HPA axis activity (Swanwick *et al*., 1998). The deregulation is also the most prevalent in stress-related disorders. Social isolation, which is stressful circumstances, also affects HPA axis function (Hawkley *et al*., 2012). In the present study, BPSD-like behavioral abnormality was assessed using mice, which were housed individually and intracerebroventricularly injected with Aβ1-42, because social isolation is potentially a factor to induce BPSD-like behavioral abnormality (Sekiguchi *et al*., 2009 and 2011)

The rate of mice that exhibited aggressive behavior was markedly increased 2 and 3 weeks after Aβ injection into adult (11-week-old) mice, but not after Aβ injection into young (4-week-old) mice. Aβ-injected adult mice also showed anxiety-like behavior and cognitive decline. Transgenic mice overexpressing the 695-amino acid isoform of human β-amyloid precursor protein (APP), a model of Alzheimer's disease, have normal learning and memory in spatial reference and alternation tasks at 3 months of age, while show impairments at 9 to 10 months of age (Hsiao *et al*., 1996). Aβ accumulates in the brain of transgenic mice overexpressing mutated human APP with aging (Hsiao *et al*., 1996; Ikarashi *et al*., 2004). Furthermore, not only core symptoms such as cognitive def-

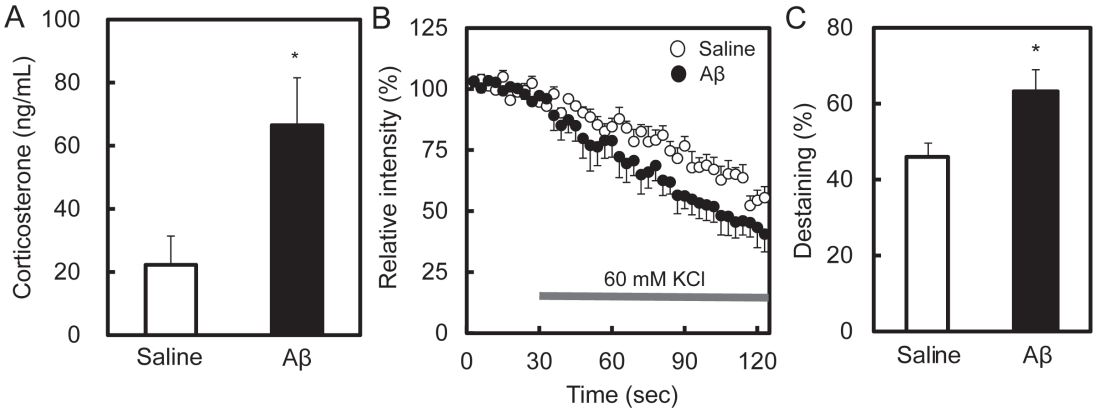

Fig. 4. Serum corticosterone and hippocampal exocytosis. Serum corticosterone concentration was determined 3 weeks after injection of saline and Aβ in saline into the lateral ventricle (A). Each bar and line represents the mean ± S.E.M. (n = 10-11) *, $P < 0.05$ vs. saline (Student's t-test). In another experiment, exocytosis was determined at Schaffer collateral terminals. Brain slices were prepared 2-3 weeks after injection of saline and Aβ in saline into the lateral ventricle, labeled with FM4-64, and stimulated with 60 mM KCl after measuring the basal FM4-64 fluorescence for 30 sec, as shown by a shaded bar. Region of interest (ROI) was set at the stratum radiatum of hippocampal CA1. Each point and line (mean ± S.E.M.) represents the decrease in FM4-64 fluorescence after stimulation with KCl (n = 6-7) (B). Each bar and line (the mean ± S.E.M.) represents the decreased FM4-64 fluorescence 120 sec after stimulation with KCl (C). *, $P < 0.05$ vs. saline (Student's t-test).

icits but also BPSD-like symptoms such as aggression, hyperactivity, and impulsive behavior have been observed in the transgenic mice (Lalonde *et al*., 2003; Stackman *et al*., 2003; Ognibene *et al*., 2005; Dong *et al*., 2005; Adriani *et al*., 2006; Quinn *et al*., 2007). These symptoms may age-dependently occur in transgenic mice (Hsiao *et al*., 1996; Moechars *et al*., 1999; Chen *et al*., 2000). It has been reported that the APP transgenic mice are valuable tools to develop new drugs for dementia and BPSD (Lalonde *et al*., 2012). The present data indicate that a single injection of Aβ1-42 (500 pmol/mouse) into the lateral ventricle of individually housing adult mice irreversibly induces cognitive decline and BPSD-like symptoms in a few weeks and may be a protocol to prepare a model for BPSD with dementia. On the other hand, a single injection of Aβ1-42 (25 pmol/rat) into the hippocampal dentate gyrus of rats transiently induces cognitive decline without appreciable changes in behavior and locomotor activity (Takeda *et al*., 2014), suggesting that the dose of Aβ1-42 and its injection site is critical for Aβ1-42 pathophysiology. Social isolation changes the HPA axis activity in the experimental animals (Serra *et al*., 2005), followed by behavioral abnormality such as aggression (Blanchard *et al*., 2001; Backström and Winberg, 2013). BPSD-like symptoms may be facilitated by social isolation in Aβ1-42-injected adult mice. Therefore, it is likely that the environmental factors such as individual housing are also critical for Aβ1-42 pathophysiology.

Brureau *et al*. (2013) report that serum corticosterone level increases in rats after intracerebroventricular injection of aggregated Aβ25-35 and that elevated glucocorticoids observed in Alzheimer's disease could be first a consequence of amyloid toxicity. In the present study, serum corticosterone level was markedly increased by injection of Aβ1-42, which is the major component of amyloid deposits (Iwatsubo *et al*., 1994). Corticosterone acts synergistically with glutamate in the hippocampus. Corticosterone-induced increase in extracellular glutamate levels occurs through the non-genomic action of membrane-associated mineralocorticoid receptors and/or glucocorticoid receptors in the hippocampus (Karst *et al*., 2005; Takeda and Tamano, 2014). Furthermore, it is possible that the persistent increase in corticosterone level modifies hippocampal excitability; glutamatergic neuron activity in the hippocampus is enhanced in zinc-deficient animals, in which serum corticosterone levels are persistently elevated (Takeda and Tamano, 2009). When excitability of hippocampal neurons was checked using brain slices, KCl-induced exocytosis (presynaptic activity) was enhanced in brain slices prepared from Aβ-injected mice. It is likely that glutamatergic neuron activity in the hippocampus is enhanced via high levels of corticosterone after Aβ injection into the mouse brain. Aβ-mediated increase in corticosterone might be involved in BPSD-like symptoms and cognitive decline of Aβ-injected mice.

In conclusion, the present study suggests that social isolation housing in Aβ-injected adult mice irreversibly induces BPSD-like behavioral abnormality. It is likely that the behavioral abnormality of Aβ-injected adult mice is associated with excitability of hippocampal glutamatergic neurons, which is associated with enhanced activity of the HPA axis. It is estimated that intracerebroventricularly injected Aβ is transported into the hippocampus, which is involved in the negative feedback mechanism of glucocorticoid secretion, and affects hippocampal function. To understand Aβ pathophysiology in individual housing, it is necessary to examine how the high dose (500 pmol Aβ/mouse) used in the present study changes glucocorticoid secretion.

Conflict of interest---- The authors declare that there is no conflict of interest.

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