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Enhanced activity of hippocampal BACE1 in a mouse model of postmenopausal memory deficits

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Abstract

Ovarian hormone decline after menopause may influence cognitive performance and increase the risk for Alzheimer's disease (AD) in women. We have recently demonstrated that a combination of ovariectomy and chronic stress (OVX/stress) causes hippocampus-associated cognitive dysfunction in mice. In this study, we examined whether OVX/stress could affect the levels of AD-related molecules in the mouse hippocampus. Female ICR mice were ovariectomized or sham-operated, and then randomly divided into a daily restraint stress (21 days, 6 h/day) or non-stress group. Although OVX or stress alone did not affect β-site amyloid precursor protein (APP)-cleaving enzyme-1 (BACE1) activity, OVX/stress increased activity in hippocampal CA1 and CA3 regions, compared with other groups. In contrast, OVX/stress did not affect γ-secretase activity, $A\beta_{1\text{-}40}$, and phosphorylated-tau levels in the hippocampus. These findings suggest that a stressful life after menopause can influence the levels of AD-related molecules and that BACE1 is the most sensitive molecule for such a situation.

Keywords: Ovariectomy; Alzheimer's disease; BACE; Amyloid β peptide; Phosphorylated-tau

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, and the histopathological hallmarks of the disease are senile plaques and neurofibrillary tangles, which consisted amyloid β (Aβ) peptide and hyperphosphorylated-tau protein, respectively [25]. Several clinical studies have demonstrated that the prevalence and incidence of AD are higher in postmenopausal women than men, even after adjusting for their differential survival [3,8,10], and that the high prevalence of AD in postmenopausal women might be associated with decreased ovarian hormones, such as estrogens [14]. Accordingly, there is much evidence showing that estrogen ameliorates AD-related neuronal dysfunction, especially Aβ-induced neurotoxicity, in animal studies [1,33]. In addition, estrogen has been shown to modulate the levels of Aβ peptides in the brain [22], and to decrease hyperphosphorylation of tau protein in rat cortical neurons [2]; however, it is still unclear whether estrogen decline after menopause can affect the activity and expression of AD-related molecules in *in vivo* levels.

We have recently found that a combination of ovariectomy (OVX) and chronic restraint stress causes cognitive dysfunction and reduces hippocampal CA3 neurons in rats [28] and mice [15], and that OVX/stress-induced behavioral and morphological changes were suppressed by estrogen replacement [28] and long-term treatment of *Ginkgo biloba* extract EGb761 [29], which is clinically used for AD therapy in Europe. The animal model should be beneficial for clarifying the mechanisms

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of the pathogenesis of AD, as well as postmenopausal memory deficits.

In the present study, we employed mice subjected to OVX and chronic restraint stress to investigate the effect of the postmenopausal condition on the activity and expression of AD-related molecules. We first examined the change in β-site amyloid precursor protein (APP)-cleaving enzyme-1 (BACE1) and γ-secretase activities, which are involved in Aβ generation, and then determined the levels of Aβ and hyperphosphorylated-tau in the hippocampus of this animal model.

Female ICR mice (Japan SLC Inc., Hamamatsu, Japan) were obtained at 8–9 weeks old and used for the experiments. They were housed under standard environmental conditions (23 ± 1 °C; 12-h light–dark cycle with lights on at 8:45 h; food and water ad libitum). The animals were handled according to the guidelines established by the Institutional Animal Care and Use Committee of Kanazawa University and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

One week after arrival, all animals were bilaterally OVX or sham-operated under pentobarbital (40 mg/kg) anesthesia. After recovery from operation for 2 weeks, chronic immobilization stress (6 h/day; starting at 9:00) using stainless steel mesh [20,28] was commenced for mice in their home cage. Some animals were not subjected to stress (no stress group). After the 3-week stress period, biochemical analysis was performed.

Animals were decapitated, and then the brains were quickly removed and cut into 1 mm-thick coronal slices using a Brain Matrix (BrainSience Idea Co., Ltd., Osaka, Japan). CA1 and CA3 regions, and the dentate gyrus (DG) in the hippocampus were isolated on ice under a microscope with a 10-fold magnification.

BACE1 and γ-secretase activities were measured using fluorescence-quenching substrates MOCAc-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Arg-Lys(Dnp)-Arg-Arg-NH_2 (Peptide Institute, Inc., Minoh, Japan) [9] and Nma-Gly-Gly-Val-Val-Ile-Ala-Thr-Val-Lys(Dnp)-D-Arg-D-Arg-D-Arg-NH_2 (Peptide Institute) [7], respectively. Briefly, the tissues from hippocampal subfields were lysed in 100 μl of extraction buffer (20 mM MES pH 6.0, 150 mM NaCl, 2 mM EDTA, 5 μg/ml leupeptin, 0.2 mM PMSF, and 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 0.5% Triton X-100) and disrupted with a handheld homogenizer. The homogenates were shaked at 300 rpm at 4 °C for 1 h, and further centrifuged at 16,000 × *g* for 20 min at 4 °C. The protein concentration in the resulting supernatant was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Then, 50 μl of lysates were incubated with three volumes of reaction buffer (BACE1: 20 mM sodium acetate, pH 8.4, 0.06% Triton X-100; γ-secretase: 50 mM Tris–HCl, pH 6.8, 2 mM EDTA, 0.25% CHAPS) containing 10 μM of each fluorogenic substrate at 37 °C for 1 h (BACE1) or 12 h (γ-secretase). Absorbance at 405 nm (BACE1) or 460 nm (γ-secretase) was measured on a Bio-Rad Model 680 microplate reader. A standard curve was created by measuring solutions of known concentrations of MOCAc-Pro-Leu-Gly (Peptide Institute, Inc.) (BACE1) or 1:1 mixture of FRETs-25-STD1 (Peptide Institute, Inc.) and FRETs-25-STD2 (Peptide Institute, Inc.) (γ-secretase). Data are expressed as nmol of the liberated MOCAc fragments (BACE1) or Nma fragments (γ-secretase) per mg protein.

$Aβ_{1-40}$ levels in the tissue were measured using a human/rat β amyloid (40) ELISA kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions, which can detect mouse $Aβ_{1-40}$ with high sensitivity (>0.25 pmol/l) and high specificity ($Aβ_{1-40}/Aβ_{1-42}$ = 685). The whole hippocampus was homogenized in 200 μl of ice-cold standard diluent with a handheld homogenizer, and centrifuged at 800 × *g* for 5 min at 4 °C. The protein concentration in the supernatant was determined using a Bio-Rad Protein Assay. The supernatants and standards were then put into the appropriate wells of microtiter plates coated with anti-$Aβ_{11-28}$ monoclonal antibody (clone BNT77). After incubation overnight at 4 °C, all wells were incubated with HRP-conjugated anti-$Aβ_{1-40}$ monoclonal antibody (clone BA27) for 60 min at 4 °C, and then reacted with TMB solution. Absorbance at 450 nm was measured on a Bio-Rad Model 680 microplate reader. Data are expressed as pmol of $Aβ_{1-40}$ per mg protein.

Phosphorylated-tau levels were determined as previously described [34]. Briefly, the whole hippocampus was homogenized in five volumes of ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM sodium orthovanadate, 50 mM NaF, 0.1% SDS, 1% Nonided P-40, 1% sodium deoxycholate, 10 mM sodium pyrophosphate decahydrate, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 1 mM PMSF, and 10 μg/ml pepstatin A, 0.5 mM dithiothreitol), sonicated and then centrifuged at 12,000 × *g* for 20 min. The protein concentration in the resulting supernatant was determined using a Bio-Rad Protein Assay. Samples containing 10 μg proteins were boiled at 95 °C for 5 min under reduced condition, electrophoretically separated by 10% polyacrylamide gel, and subsequently transferred to a PVDF membrane. The membranes were incubated with primary and secondary antibodies with the following combinations and dilution: rabbit phosphorylated-tau Ser396 antibody (1:1000; Sigma–Aldrich, St. Louis, MO) and horseradish peroxidase (HRP)-labeled anti-rabbit IgG (1:10000; GE Healthcare Bio-Sciences, Piscataway, NJ); mouse phosphorylated-tau (Ser202/Thr205) antibody (clone AT8; 1:200; Innogenetics, Ghent, Belgium) and HRP-labeled anti-mouse IgG (1:1000; KPL, Gaithersburg, MD). To quantify the relative amount of proteins, the membranes were stripped at 55 °C for 30 min and reprobed with BioSource™ mouse anti-tau (clone TAU-5; 1:1000; Invitrogen, Carlsbad, CA) or mouse anti-β-actin (clone AC-15; 1:5000; Sigma–Aldrich), followed by HRP-conjugated anti-mouse IgG (1:10000; KPL). The immune complexes were visualized using Amersham ECL Western Blotting Detection Reagents (GE Healthcare Bio-Sciences) and quantified using a Light-capture cooled CCD camera system for bio/chemiluminescence detection (AE-6972FC; Atto, Tokyo, Japan).

Statistical analysis of the experimental data was carried out using GraphPad Prism 4 for Macintosh (GraphPad Software, San Diego, CA). The significance of differences was determined by a one-way ANOVA, followed by the Tukey's multiple comparison test for multigroup comparisons. Unpaired *t*-test was used for

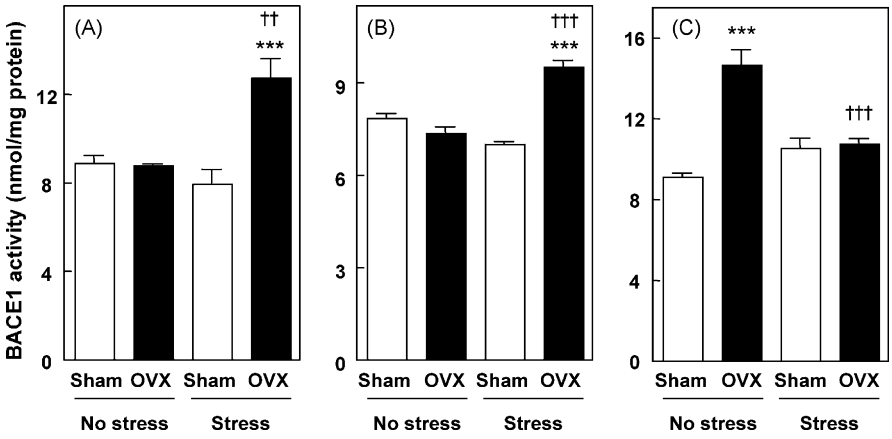

Fig. 1. Effects of OVX and restraint stress on BACE1 activity in hippocampal CA1 (A) and CA3 (B) regions, and DG (C) of mice. BACE1 activity in the indicated tissue extracts was assayed using fluorescence-quenching substrate MOCAc-SEVNLDAEFRK(Dnp)RR-NH_2. Values indicate the mean ± S.E. (sham/no stress: $n = 6$–7; OVX/no stress: $n = 4$–6; sham/stress: $n = 4$–5; OVX/stress: $n = 5$–7). $^{***}p < 0.001$, significantly different from the corresponding sham-operated group. $^{††}p < 0.01$, $^{†††}p < 0.001$, significantly different from the OVX/no stress group (ANOVA and post hoc Tukey's multiple comparison test). (A) $F_{3,18} = 12.53$, $p < 0.0001$; (B) $F_{3,16} = 32.57$, $p < 0.0001$; (C) $F_{3,18} = 22.55$, $p < 0.0001$.

two-group comparisons. The criterion for statistical significance was $p < 0.05$.

We have recently demonstrated that 3-week chronic stress after a 2-week recovery period from OVX in mice causes persistent impairment of hippocampus-dependent memory in a contextual fear conditioning test, while stress or ovariectomy alone does not affect the memory function [15]. In the present study, we first examined whether the combination of OVX and chronic stress could influence BACE1 activity in the mouse hippocampus. Although OVX for 5 weeks or stress for 3 weeks did not affect BACE1 activity in CA1 (Fig. 1A) and CA3 (Fig. 1B) regions of the hippocampus, their combination caused a significant increase in activity compared with other groups. In contrast, OVX for 5 weeks alone increased BACE1 activity in DG (Fig. 1C), which was attenuated by chronic stress (Fig. 1C).

Next, we examined the effects of OVX and chronic stress on the activity of γ-secretase, which acts as another essential APP-cleaving enzyme producing Aβ. OVX, chronic stress and their combination did not influence γ-secretase activity in CA1 (ANOVA: $F_{3,19} = 0.7602$, $p = 0.5302$), CA3 ($F_{3,21} = 1.367$, $p = 0.2801$) regions, and DG ($F_{3,21} = 1.734$, $p = 0.1908$) of the hippocampus.

We further examined the effects of OVX and chronic stress on Aβ levels in the mouse hippocampus. As it is generally known that the ratio of generation for two major isoforms of Aβ, 1-40 and 1-42 peptide fragments, is 5:1 [4], we measured $Aβ_{1\text{-}40}$ levels by ELISA in this study, which showed that OVX, chronic stress and their combination did not influence hippocampal $Aβ_{1\text{-}40}$ levels compared with sham-operated controls (Fig. 2).

It is reported that the tau in AD is abnormally phosphorylated at potent sites, such as Ser46, Ser199, Ser202, Ser235, Ser396, and Ser404, which are responsible for reducing the microtubule binding possibly involved in neuronal degeneration [11]. Moreover, a recent study demonstrated that cold-water stress caused an increase in tau phosphorylation, which was detected by antibodies against the phosphorylation sites, Ser199/202, Ser202/Thr205, and Ser396 [34]. Thus, we finally analyzed the effects of OVX and chronic stress on levels of Ser202/Thr205- and Ser396-phsophorylated-tau in the mouse hippocampus. As shown in the typical images of immunoblot analyses in Fig. 3, OVX and chronic stress did not influence hippocampal Ser202/Thr205- and Ser396-phsophorylated-tau levels compared with the sham-operated control (Ser202/Thr205: sham, 100 ± 5.6%, $n = 6$, OVX/stress, 111.6 ± 4.9%, $n = 5$, unpaired t-test, $p = 0.1635$; Ser396: sham, 100 ± 7.8%, $n = 6$, OVX/stress, 110.6 ± 5.8%, $n = 5$, unpaired t-test, $p = 0.3210$).

Our previous studies revealed that OVX/stress caused cognitive dysfunction and reduced hippocampal CA3 neurons in rats [28] and mice [15]. The present study aimed to clarify how the depletion of female sex hormones and environmental stress influenced the amount of AD-related molecules in the mouse hippocampus. As a result, we found that OVX/stress caused an increase in BACE1 activity in hippocampal CA1 and CA3

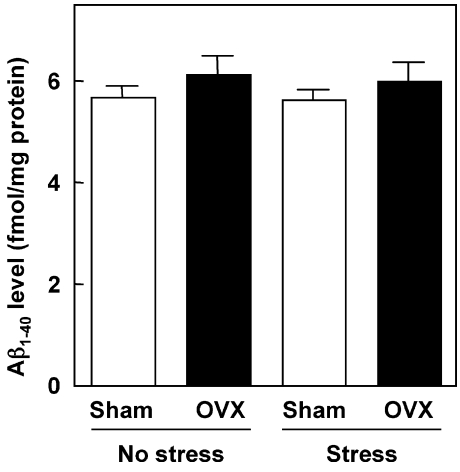

Fig. 2. Effects of OVX and restraint stress on $Aβ_{1\text{-}40}$ levels in the mouse hippocampus. The $Aβ_{1\text{-}40}$ content in hippocampal tissue homogenates was assayed by ELISA. Values indicate the mean ± S.E. (sham/no stress: $n = 5$; OVX/no stress: $n = 5$; sham/stress: $n = 5$; OVX/stress: $n = 5$). ANOVA: $F_{3,16} = 0.6400$, $p = 0.5610$.

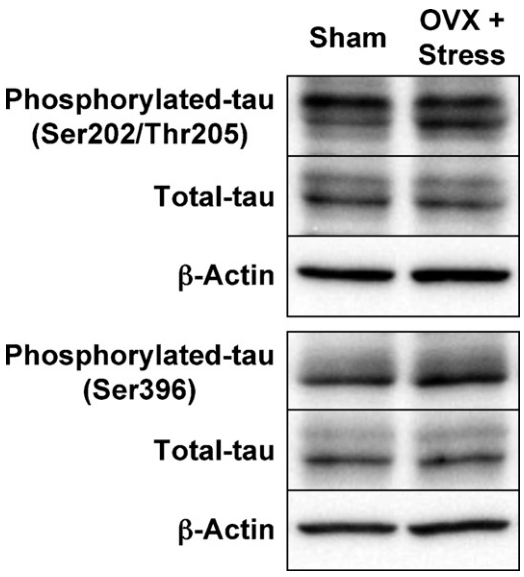

Fig. 3. Effects of OVX and restraint stress on protein levels of phosphorylated- and total-tau in the mouse hippocampus. Hippocampal homogenates containing 20 μg of proteins were subjected to 10% SDS-PAGE. Typical immunoblot images detected by antibodies against phospho-(Ser202/Thr205)-tau (upper) and phospho-(Ser396)-tau (lower) are shown from five independent experiments. Each membrane was further reprobed by total-tau and β-actin to quantify the relative amount of proteins.

regions, compared with other groups. We also observed that OVX alone increased BACE1 activity, which was attenuated by combination with the stress in DG. In addition, we demonstrated that OVX, stress and their combination did not affect γ-secretase activity, $A\beta_{1\text{-}40}$, and hyperphosphorylated-tau levels in the hippocampus. These results suggest that stress in postmenopause triggers the pathogenesis of AD by increasing BACE1 activity.

There is much evidence showing that both BACE1 protein levels and enzymatic activity are increased in the AD brain [9,27], and that increased BACE1 may be involved in late-onset sporadic AD; however, as recently reported, BACE1 gene promoter activity is complexly regulated by various factors in a cell type-specific manner [18]. This study also showed that ovarian hormones regulated hippocampal BACE1 expression in a region-specific manner, and DG was a more sensitive region to OVX although the cell types were not identified. More interestingly, this study further demonstrated that OVX/stress, by which hippocampus-associated memory impairment was evoked in mice, caused a significant increase in BACE1 activity in hippocampal CA1 and CA3 subregions. These subregions express higher levels of estrogen receptor α [26] and corticosteroid receptors [13], which are thought to complementarily regulate stress-activated protein kinases, such as c-Jun *N*-terminal kinase (JNK) and p38 mitogen-activated protein kinase in various cells [17,24]. In addition, recent study has shown that the up-regulation of BACE is mediated by JNK activation [30]. Thus, complex regulation of JNK activity by estrogen and corticosterone may be involved in the region-specific BACE expression. Furthermore, many studies indicate that CA1 and CA3 subregions play important roles in the acquisition and consolidation of memory [5,21]. Taken together, although the exact mechanisms of region-specific regulation in BACE expression are still unclear, we assume that OVX/stress-induced BACE1 expression in hippocampal CA1 and CA3 subregions may be strongly associated with memory loss in postmenopausal women.

It is widely accepted that excessive BACE1 expression causes overproduction of Aβ, leading to neuronal dysfunction. As Aβ deposits were randomly observed in the hippocampus of AD patients [32] and AD model transgenic mice [19], we assayed Aβ levels in the whole hippocampus. Contrary to our expectations, we could not detect a change in $A\beta_{1\text{-}40}$ levels in the hippocampus of OVX/stress mice. In this respect, our findings raise several possibilities. Aβ accumulation is known to depend on Aβ-degrading activities by such as neprilysin and insulin-degrading enzyme, as well as Aβ-generating activities by BACE1 and γ-secretase [6]. Thus, we first propose that the effects of OVX/stress on Aβ-degrading enzymes are weak or lacking, and the enzymes immediately remove Aβ from the hippocampus of OVX/stress mice, but this hypothesis cannot fully explain OVX/stress-induced neuronal loss in hippocampal CA3 regions.

On the other hand, recent studies reveal that APP, a transmembrane substrate of BACE1, plays important roles in several neural functions, such as neurite growth, synaptogenesis, and synaptic plasticity [12,23]. In addition, APP has been reported to regulate presynaptic localization and activity of the choline transporter [31]. From these findings, we raise the possibility that OVX/stress-induced BACE1 the hippocampal CA1 and CA3 subregions may cause synaptic dysfunction by reducing APP levels. Furthermore, a recent study demonstrating that BACE1 regulates voltage-gated sodium channels [16] implies another possibility that BACE1 may directly mediate neuronal activity. Given the results of this study showing that OVX/stress did not affect phosphorylated-tau levels, reflecting a late stage of neuronal death, these last two hypotheses may be reasonable to explain the molecular mechanism underlying BACE-mediated memory impairment in our animal model. In any case, further examination is required to elucidate the role of BACE in postmenopausal memory impairment.

In conclusion, we provide evidence that ovarian hormone complexly regulates BACE1 activity and that OVX/stress causes an increase in BACE1 activity in hippocampal CA1 and CA3 regions. Furthermore, we show that OVX/stress did not affect γ-secretase activity, $A\beta_{1\text{-}40}$, and hyperphosphorylated-tau levels in the hippocampus, while the condition evoked an impairment of hippocampus-dependent memory. Thus, we propose that OVX/stress-induced BACE1 in hippocampal CA1 and CA3 regions may be associated with cognitive dysfunction via an Aβ-independent mechanism. Further, the present study suggests that OVX/stress mice are useful as an animal model of postmenopausal memory deficits.

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