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COMBINATION OF CHRONIC STRESS AND OVARIECTOMY CAUSES CONDITIONED FEAR MEMORY DEFICITS AND HIPPOCAMPAL CHOLINERGIC NEURONAL LOSS IN MICE

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Abstract—We have recently found that the combination of ovariectomy (OVX) and chronic restraint stress (CS) causes hippocampal pyramidal cell loss and cognitive dysfunction in female rats and that estrogen replacement prevents the OVX/CS-induced morphological and behavioral changes. In this study, to clarify the mechanisms underlying the OVX/CS-mediated memory impairment further, we examined the roles of cholinergic systems in the OVX/CS-induced memory impairment in mice. Female Slc:ICR strain mice were randomly divided into two groups: OVX and sham-operated groups. Two weeks after the operation, the mice of each group were further assigned to CS (6 h/day) or non-stress group. Following the 3-week-stress period, all mice were subjected to contextual fear conditioning, and context- and tone-dependent memory tests were conducted 1 or 24 h after the conditioning. Overburden with 3 weeks of CS from 2 weeks after OVX impaired context- and tone-dependent freezing and the OVX/CS caused significant Nissl-stained neuron-like cell loss in the hippocampal CA3 region, although OVX and CS alone did not cause such behavioral and histological changes. Replacement of 17β-estradiol for 5 weeks after OVX suppressed OVX/CS-induced memory impairment and hippocampal Nissl-positive cell loss. Furthermore, the OVX/CS mice exhibited a significant decrease in choline acetyltransferase in the hippocampus compared with other groups. The cholinesterase inhibitors donepezil and galantamine ameliorated OVX/CS-induced memory impairment. These data suggest that cholinergic dysfunction may be involved in the OVX/CS-induced conditioned fear memory impairment. Overall, our findings suggest that the OVX/CS mouse model is useful to study the mechanisms underlying estrogen loss-induced memory deficits.

Key words: chronic stress, ovariectomy, contextual fear, hippocampus, cholinergic system, estrogen.

Clinical studies have reported that memory loss is a very frequent complaint during perimenopause and early postmenopause (Woods et al., 2000). As menopause is associated with reduced ovary functions, resulting in lower levels of ovarian hormones such as estrogens (Zapantis and Santoro, 2003), surgically ovariectomized (OVX) animals have experimentally been used to investigate the molecular mechanisms behind estrogen loss-induced memory deficits. However, they are not sufficient models for estrogen loss-induced memory deficits because clinical evidence has shown that postmenopausal women do not always suffer from memory loss.

From a different viewpoint in which the development of memory deficits in postmenopausal women requires additional factors besides ovarian hormone loss, we have recently found that an overburden of chronic restraint stress (CS) to OVX causes cognitive dysfunction in rats (Takuma et al., 2007a). In addition, we have revealed that OVX rats exposed to CS display pyramidal cell loss in the hippocampal CA3 region, and that OVX/CS-induced behavioral and morphological changes were suppressed by estrogen replacement (Takuma et al., 2007a) and long-term treatment with *Ginkgo bioba* extract EGb761 (Takuma et al., 2007b), which is clinically used for Alzheimer's disease (AD) therapy in Europe. Although we regard this evidence as proof of remarkable success in the establishment of animal models for estrogen loss-induced memory deficits, we have also come to the conclusion that mouse models are more valuable than rat ones after consideration of the recent

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Abbreviations: ABAD, Aβ-binding alcohol dehydrogenase; ACh, acetylcholine; AChE, acetylcholinesterase; Aβ, amyloid-β peptide; BACE1, β-site amyloid precursor protein-cleaving enzyme-1; BMD, bone mineral density; ChAT, choline acetyltransferase; ChE, cholinesterase; CS, chronic restraint stress; DG, dentate gyrus; DHBE, dihydro-β-erythroidine; EIA, enzyme immunoassay; ISI, inter-stimulus intervals; nAChR, nicotinic ACh receptor; OVX, ovariectomy or ovariectomized; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

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development of transgenic techniques to produce various mouse disease models.

In the present study, to establish a reliable mouse model of estrogen loss-related memory impairment, we first examined the well-known general effect of OVX on estrogen levels, body weight, bone mineral density (BMD), and uterus weight in female mice, and then we determined the combined effects of OVX and CS on conditioned fear memory and brain morphological changes of hippocampus with or without chronic estrogen treatment. In previous studies, we found that this mouse model displays a significant increase in β-site amyloid precursor protein-cleaving enzyme-1 (BACE1) activity in hippocampal CA1 and CA3 regions (Fukuzaki et al., 2008a), and that OVX mice exhibit an increase in serum amyloid-β peptide (Aβ) level and hippocampal Aβ-binding alcohol dehydrogenase (ABAD) level (Fukuzaki et al., 2008b). Both BACE1 and ABAD are considered as key molecules in the hippocampal cholinergic dysfunction (Devi and Ohno, 2010; Lustbader et al., 2004). Thus, to clarify the mechanisms underlying the OVX/CS-mediated memory impairment, we further determined the levels of choline acetyltransferase (ChAT), acetylcholinesterase (AChE) activity, and acetylcholine (ACh) release by membrane depolarization in the hippocampus, and evaluated the effects of cholinesterase (ChE) inhibitors, donepezil and galantamine, on OVX/CS-induced conditioned fear memory impairment.

EXPERIMENTAL PROCEDURES

Chemicals and drugs

Reagents were obtained from the following sources: 17β-estradiol [1,3,5(10)-estratriene 3,17-β-diol], methyllycaconitine citrate hydrate, dihydro-β-erythroidine (DHBE) hydrobromide, anti-β-actin (clone AC-15), acetylthiocholine iodide (ATCI), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), tetraisopropyl pyrophosphoramide (*iso*-OMPA), Sigma-Aldrich Co. (St. Louis, MO, USA); goat anti-ChAT antibody, Millipore Co. (Billerica, MA, USA); Alexa Fluor® 594 rabbit anti-goat IgG (H+L), Invitrogen Co. (Carlsbad, CA, USA); horseradish peroxidase (HRP)-conjugated anti-goat IgG, Santa Cruz Biotechnology (Santa Cruz, CA, USA); HRP-conjugated anti-mouse IgG, KPL (Gaithersburg, MD, USA). Donepezil hydrochloride and galantamine hydrobromide were gifts from Eisai Co., Ltd. (Tokyo, Japan) and Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (a division of Janssen Pharmaceutica N.V., Raritan, NJ, USA), respectively. All other chemicals used were of the highest purity commercially available.

Animals

Female Slc:ICR strain mice (Japan SLC Inc., Hamamatsu, Japan) were obtained at 8–9 weeks old and used for the experiments. They were housed four to six per cage under standard light–dark conditions (12-h light cycle starting at 8:45 h) at a constant temperature of 23±1 °C. The animals had free access to food and water and they were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Kanazawa University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

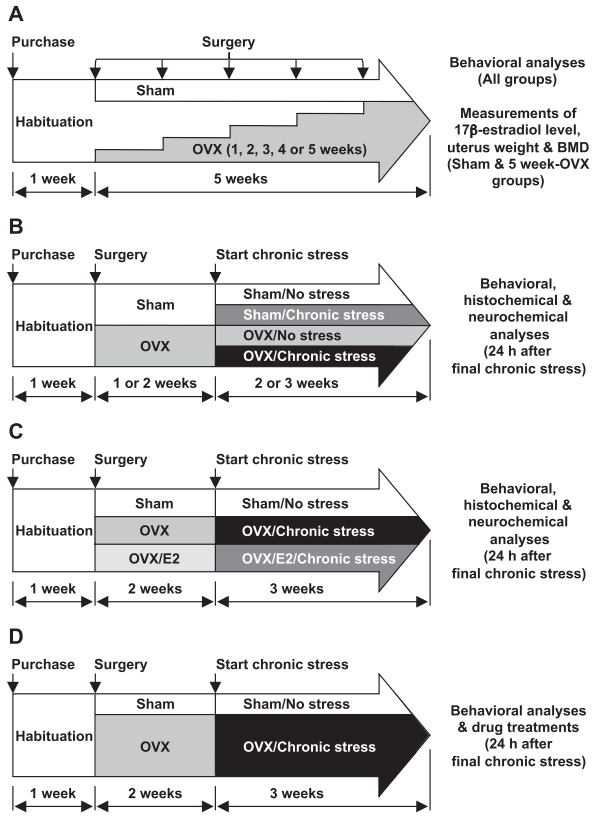

Fig. 1. Timeline of treatments and analyses. One to five weeks after arrival, all female Slc:ICR strain mice, at 9–13 wks old, were sham-operated or bilaterally ovariectomized (OVX) (A–D). The restraint stress (6 h between 9:00 AM and 3:00 PM) was repeated every day for 14 or 21 consecutive days, and then behavioral, histochemical, and neurochemical analyses were performed 24 h after the last stress session (B–D). (A) Experiment to determine the effects of OVX alone on fear learning and memory and biochemical changes in female mice. Six weeks after arrival, all mice were subjected to behavioral analysis, and then mice of sham-operated and 5-wk-OVX groups were killed for measurements of 17β-estradiol level, uterus weight, and bone mineral density (BMD). (B) Experiment to determine the effects of combination of OVX and CS on fear learning and memory, and histochemical and biochemical changes in female mice. One or two weeks after the operation, the mice of each group were further divided into a daily restraint stress or non-stress group. (C) Experiment to investigate the effects of estrogen replacement against the OVX/CS-induced impairment of conditioned fear memory in female mice. The OVX mice were subcutaneously treated with 17β-estradiol (E2) at 3 μg/d or vehicle once daily at 9:00 AM (immediately before the stress session) from one day after surgery to the final day of restraint stress. (D) Experiment to investigate the effects of ChE inhibitors and nAChR antagonists against the OVX/CS-induced impairment of conditioned fear memory in female mice. The ChE inhibitors and nAChR antagonists were administered to some animals before the retention session.

Treatments and drug administration

Timeline of each experiment is presented in Fig. 1. In all experiments, surgical operation was carried out under pentobarbital (40 mg/kg) anesthesia.

Experiment 1. One to five weeks after arrival, all experimental animals were sham-operated or bilaterally OVX. The body weight of each mouse was recorded every 3 days. Six weeks after arrival, all mice were subjected to behavioral analysis, and then mice of sham-operated and 5-week-OVX groups were killed for