Biochemical and Biophysical Research Communications 379 (2009) 480–484

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Social isolation stimulates hippocampal estradiol synthesis

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ARTICLE INFO

Article history:
Received 16 December 2008
Available online 27 December 2008

Keywords:
Estradiol
Hippocampus
Neurosteroid
Social isolation
P450arom
StAR
Real-time RT-PCR
Molecular psychology

ABSTRACT

17β-Estradiol is synthesized *de novo* in the rat hippocampus. However, the regulatory mechanism of hippocampal estradiol synthesis has remained unclear. We investigated the effects of social isolation on rat hippocampal estradiol synthesis. Rats were divided into two groups: social isolation and pair housed group. Socially isolated rats were housed individually while pair housed rats were housed two per cage for 8 weeks. Social isolation activated the transcription of neurosteroidogenic molecules, including steroidogenic acute regulatory protein (StAR) and CYP19 (cytochrome P450arom). These two molecules are involved in the regulatory step for steroidogenesis and final step of estradiol synthesis. In contrast, the mRNA levels were not affected in rat olfactory bulb. The hippocampal estradiol content was increased in accordance with the increased mRNA levels. The hippocampal estradiol content exhibited correlations with the StAR and P450arom mRNA levels. These data suggest that social isolation may enhance *de novo* estradiol synthesis in the hippocampus.

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Neurosteroids are synthesized *de novo* from cholesterol in the central and peripheral nervous systems through mechanisms that are independent of peripheral steroidogenic organs, such as the adrenal glands and gonads [1]. Neurosteroid synthesis has been reported in various brain regions, including the hippocampus [2]. The adult rat hippocampus possess the active steroidogenic enzymes and proteins required for *de novo* synthesis of 17β-estradiol (estradiol) and 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), such as steroidogenic acute regulatory protein (StAR), peripheral-type benzodiazepine receptor (PBR), CYP11A1, P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD), CYP17, cytochrome P450 17α-hydroxylase/c17,20-lyase (P450(17α)), 17β-hydroxysteroid dehydrogenase (17β-HSD), CYP19, cytochrome P450arom (P450arom), steroid 5α-reductase (5α-reductase), 3α-hydroxysteroid dehydrogenase (3α-HSD) [3,4]. Fig. 1 shows the pathway of estrogen and allopregnanolone synthesis in the hippocampus. Kretz et al. [5] reported estradiol synthesis in the cultured rat hippocampus. The estradiol content in the rat hippocampus is much higher than that in plasma, indicating *de novo* estradiol synthesis in this organ [4]. However, there is little information regarding the regulatory mechanism of estradiol synthesis in the hippocampus.

Estradiol is widely accepted to be a sex steroid that acts on the brain and regulates reproductive behavior. On the other hand, there is evidence of estrogen actions in non-reproductive functions in the developing, adult and aging brain. Using adult rat hippocampal slices, estradiol was found to rapidly enhance long-term depression in CA1, CA3, and the dentate gyrus, as well as the density of spines of the pyramidal neurons in CA1 [4]. Estradiol regulates the morphology of astrocytes and the expression of brain-derived neurotrophic factor (BDNF) [6]. In addition, estradiol appears to protect the brain against injury because it reduces cell death in response to various noxious stimuli, such as oxidative stress and kainic acid treatment [7].

Hippocampal estradiol is synthesized not only from circulating testosterone but also from cholesterol [1]. Hippocampus-derived estradiol plays important roles in the hippocampus. Treatment with letrozole, a P450arom inhibitor, results in differential expression of estrogen receptor α and β [8]. Spines, spine synapses, and synaptic proteins are downregulated in the CA1 region of the rat hippocampus in response to letrozole [5]. These data suggest a possible involvement of hippocampus-derived estradiol in the maintenance of hippocampal functions.

Rats deprived of social contact with other rats at a young age experience a form of prolonged stress that leads to long-lasting alterations in their behavioral profiles [9]. Isolated rats are aggres-

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doi:10.1016/j.bbrc.2008.12.076

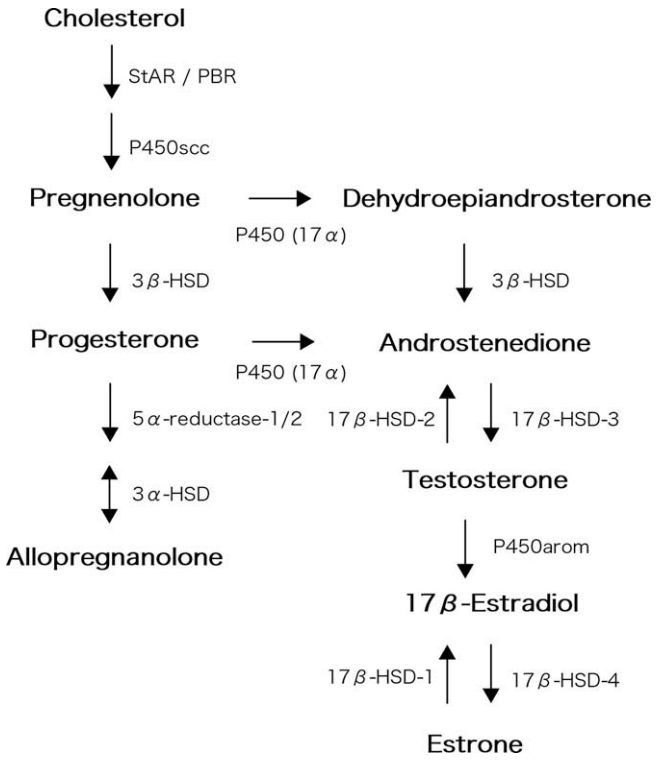

Fig. 1. Pathway of estrogen and allopregnanolone synthesis in the rat hippocampus. The arrows indicate the specific steps mediated by individual enzymes and proteins.

sive and neophobic. These rats also exhibit impaired learning in the Morris water maze, a hippocampus-dependent task [9]. Social isolation causes not only behavioral but also neurochemical and morphological changes [9]. In particular, the hippocampus was reported to be sensitive to social isolation stress [10]. Social isolation reduces the spine density of pyramidal neurons, BDNF expression, and cell survival in the hippocampus [11,12]. Pibiri et al. [13] reported that social isolation decreased allopregnanolone level in the mouse olfactory bulb. They have also reported that this housing condition induced the decrease in 5α-reductase-1 mRNA level in the mouse hippocampus [14]. These data suggest that neurosteroidogenesis is affected by housing conditions. To date, however, most studies on the effects of social isolation on neurosteroidogenesis have focused on allopregnanolone synthesis. No reports have appeared concerning the effects of social isolation on estradiol synthesis, despite the fact that estradiol plays important roles in various brain regions such as hippocampus.

The purpose of the present study was to analyze the effects of social isolation on estradiol synthesis in the rat hippocampus. First, we analyzed the mRNA levels of enzymes and proteins involved in estradiol synthesis in the hippocampus and olfactory bulb, in which neurosteroidogenic enzymes have been detected [2]. In addition, since social isolation affects allopregnanolone contents in the mouse hippocampus and olfactory bulb [13], we analyzed mRNA levels of 5α-reductase and 3α-HSD in both brain regions in rats (Fig. 1). Finally, we quantified the estradiol contents in the rat hippocampus after social isolation. Our results clearly demonstrate that hippocampal estradiol synthesis is significantly upregulated by social isolation.

Materials and methods

Animals. Wistar male rats (Japan SLC Inc., Shizuoka, Japan) were maintained under standard laboratory conditions on a 12-h light/12-h dark cycle, with tap water and regular rat chow available *ad libitum*. This study was carried out after receiving approval from the Committee of Animal Experimentation at Hiroshima University.

Isolation rearing. Wistar rats on postnatal day 28 were randomly divided into two groups: social isolation and pair housed group. Socially isolated rats were housed individually in clear plastic cages (26.5 × 42 × 18 cm) while pair housed rats were housed two per cage in the same type of cage for 8 weeks. Although the rats reared under both conditions could observe ongoing activity in other cages, the socially isolated rats received no physical contact with other animals.

Real-time RT-PCR. Hippocampus and olfactory bulb were isolated from rats after rearing. The weights of the whole brain, hippocampus, and olfactory bulb were measured. The protein contents were determined using the BCA assay. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). After incubation of the isolated total RNA with DNase I (RNase-free; TaKaRa, Tokyo, Japan), cDNAs were prepared from 5 μg of total RNA following the ReverTra Ace protocol (Toyobo, Tokyo, Japan) with a random primer (9-mer; TaKaRa).

Real-time PCR analysis was performed as previously described [15], using Light cycler (Roche Diagnostics, Mannheim, Germany), SYBR Green Master Mix (Toyobo), 100–600 ng hippocampal cDNA and 2.5–10 pmol of each of the primers. The primers used were as follows: GAPDH, 5′-AACGACCCCTTCATTGACCTC-3′ (forward) and 5′-CCTTGACTGTGCCGTTGAACT-3′ (reverse); StAR, 5′-GCAAAGCGG TGTCATCAG-3′ (forward) and 5′-GGCGAACTCTATCTGGGTCT-3′ (reverse); PBR, 5′-GTATTCGGCCATGGGGTA-3′ (forward) and 5′-GTAG AGACCCAAGGGAACCAT-3′ (reverse); P450scc, 5′-TATTCCGCTTTG CCTTTGAG-3′ (forward) and 5′-CACGATCTCCTCCAACATCC-3′ (reverse); 3β-HSD, 5′-AGGCCTGTGTCCAAGCTAGTGT-3′ (forward) and 5′-CTCGGCCATCTTTTTGCTGTAT-3′ (reverse); P450(17α), 5′-CATC CCCCACAAGGCTAAC-3′ (forward) and 5′-TGTGTCCTTGGGGACA GTAAA-3′ (reverse); P450arom, 5′-ATCGGAAGAATGCACAGG-3′ (forward) and 5′-AGTGTAACCAGGACAACTTT-3′ (reverse); 17β-HSD-1, 5′-CTGAATTGGGATGGTCTGC-3′ (forward) and 5′-GGCTAC ATAGTGAAACCTTGTC-3′ (reverse); 17β-HSD-2, 5′-GCTGGGGTC TTGCACTTTCC-3′ (forward) and 5′-GGCGGCCACCATCTGAAAT-3′ (reverse); 17β-HSD-3, 5′-CTGACTTGGACAACACCAT-3′ (forward) and 5′-CTGTCAACATGGAACCG-3′ (reverse); 17β-HSD-4, 5′-GACGCC TCAAGGATGTTGG-3′ (forward) and 5′-AGCCGTTCTTCAGGTCAAT-3′ (reverse); 5α-reductase-1, 5′-ACTGGGCAACCTGCCTAAC-3′ (forward) and 5′-ATCAGAACCGGGAAAACCA-3′ (reverse); 5α-reductase-2, 5′-CAGGAAGCCTGGAGAAGTCA-3′ (forward) and 5′-CAAT AATCTCGCCCAGGAAA-3′ (reverse); 3α-HSD, 5′-GCACTCAACTGG ACTATGTGGA-3′ (forward) and 5′-GCTCATCTCGTGGGAAAAAT-3′ (reverse).

After denaturation at 95 °C for 2 min, 50 cycles of 94 °C for 30 s, annealing at 55–60 °C for 5–20 s and elongation at 65 °C for 8–20 s were performed. The negative controls showed no amplified products. The sequences of the recovered PCR products were confirmed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) after subcloning into pTA2 using a TArget Clone -Plus- kit (Toyobo). Real-time PCR results were normalized by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. In some experiments, the mRNA contents of StAR and P450arom in the hippocampus were determined by comparing the threshold cycle (C_t) values of each sample with those of known amounts of StAR and P450arom mRNA. The C_t value refers to the PCR cycle number at which a sample reaches the threshold level of fluorescence. Standard curves were generated by plotting known amounts of StAR and P450arom mRNAs versus the C_t values obtained in the real-time PCR amplifications. The amounts were calculated as the molecules of mRNA/mg protein in the hippocampus.

Enzyme immunoassay (EIA) of estradiol. After rearing, the hippocampus of the socially isolated and pair housed rats were homogenized and their protein contents were determined using the BCA assay. The homogenates were incubated with 1000 cpm of [2,4,6,7,16,17-^{3}H]estradiol (NEN, Boston, MA) for determination of the extraction recoveries. Estradiol was extracted from the homogenates using ethyl acetate:hexane (3:2, v/v). The extracts were subjected to solid-phase extraction with a C18 Amprep mini column (GE Healthcare, Buckinghamshire, UK) [16]. The eluates were dried and the concentrations of estradiol were determined using an estradiol EIA kit (Cayman Chemical, Ann Arbor, MI). The antiserum used in this assay cross-reacted with estrone at 4%, estriol at 0.57%, testosterone at 0.1%, 5α-dihydrotestosterone at 0.1% and 17α-estradiol at <0.1%. The interassay coefficient of variation was <10% and the least detectable amount was 0.033 pmol/ml. The radioactivity of [^{3}H] estradiol was analyzed with Clearsol (Nacalai Tesque, Kyoto, Japan) in a scintillation counter (LSC-6100; Aloka, Tokyo, Japan). The recovery during the extraction procedure was 70–80%.

Statistical analysis. Two-way mixed-typed measures analysis of variance (ANOVAs) were performed on the body weight for the factors of housing conditions (socially isolated and pair housed) and weeks (8 weeks). The Greenhouse-Geisser ε correction for violation of sphericity was applied. Multiple comparisons were carried out by the Bonferroni procedure. Differences in the weights (whole brain, hippocampus, and olfactory bulb), amounts of each mRNA and amounts of estradiol between the socially isolated and paired housed rats were examined using two-tailed unpaired *t*-tests. Pearson's coefficients of correlation were calculated for the amounts of estradiol and the mRNA levels of StAR and P450arom. The criterion for significance was $p < 0.05$. All results were expressed as means ± SEM.

Results

Weight changes in the body, whole brain, hippocampus, and olfactory bulb

Fig. 2 shows the weights of the body, whole brain, olfactory bulb and hippocampus in the socially isolated and pair housed rats during the 8-week housing period. The body weights showed a significant group × week interaction (F (7,105) = 3.96, $p < 0.001$, $\varepsilon = 0.45$). Multiple comparisons revealed that the effect of the week did not differ between weeks 7 and 8 ($p > 0.05$). No significant differences were observed in the whole brain, hippocampus and olfactory bulb weights between the two groups ($p > 0.05$ for each).

Effects of social isolation on mRNA levels of neurosteroidogenic enzymes and proteins in the rat hippocampus and olfactory bulb

We carried out comparative analyses by quantitative real-time RT-PCR to examine the changes in gene expression of neurosteroidogenic enzymes and proteins following social isolation for 8 weeks. In the hippocampus, the mRNA levels for StAR (t (15) = 2.76, $p < 0.05$, 2-fold), P450(17α) (t (15) = 8.89, $p < 0.001$, 1.9-fold), 17β-HSD-1 (t (15) = 3.77, $p < 0.05$, 1.4-fold) and 17β-HSD-4 (t (15) = 2.59, $p < 0.05$, 1.2-fold) were increased by social isolation. Surprisingly, the mRNA level for P450arom, the key enzyme of estradiol synthesis, was increased more than 8-fold in socially isolated rats (t (12) = 6.40, $p < 0.001$). The other mRNA levels remained unchanged ($p > 0.05$ for each) (Fig. 3). These data clearly show that social isolation activates the transcription of enzymes and proteins involved in hippocampal estradiol synthesis. On the other hand, in the rat olfactory bulb, 5α-reductase-2 and 3α-HSD mRNA levels were decreased by 27% (t (15) = 2.41, $p < 0.05$) and 26% (t (15) = 2.68, $p < 0.05$), respectively, by social isolation (Fig. 3). The other mRNA levels in the olfactory bulb were not affected ($p > 0.05$ for each).

Effect of social isolation on the hippocampal estradiol contents

Since social isolation was found to drastically activate the transcription of neurosteroidogenic molecules involved in estradiol synthesis, we examined the hippocampal estradiol levels in socially isolated and pair housed rats. The amounts of estradiol in socially isolated and pair housed rats were 126.3 ± 11.3 and 84.4 ± 11.9 fmol/mg protein, respectively. The estradiol level was 1.5-fold higher in the hippocampus of the socially isolated rat (t (15) = 2.55, $p < 0.05$). To verify that the increased hippocampal estradiol level is due to transcriptional activation of neurosteroidogenic molecules, we carried out correlation analyses. In these analyses, we determined the neurosteroidogenic mRNA contents and hippocampal estradiol levels in individual rat hippocampus from socially isolated and pair housed rats. The Pearson's coefficient of correlation method revealed significant correlations between the estradiol level and the mRNA level of StAR and P450arom (Fig. 4).

Discussion

Our data indicate that social isolation increases the mRNA expression levels of StAR, P450(17α), P450arom, 17β-HSD-1, and 17β-HSD-4 in the hippocampus, but not in the olfactory bulb. These mRNAs encoding neurosteroidogenic enzymes and proteins are involved in *de novo* hippocampal estrogen synthesis. In particular, it should be noted that the hippocampal mRNA level of P450arom was >8-fold higher in socially isolated rats compared with pair housed rats. Since the hippocampus was reported to be sensitive to social isolation [17], this housing condition may affect

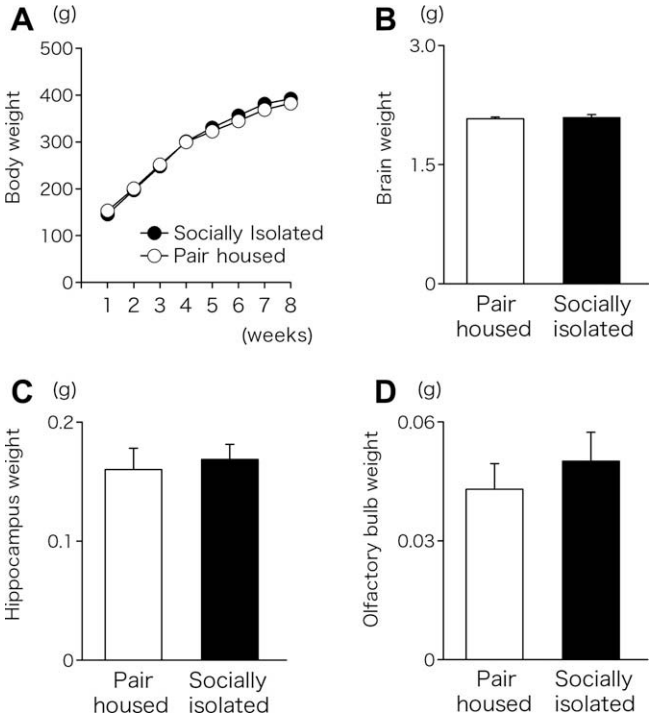

Fig. 2. Effects of social isolation on the weights of the body, whole brain, hippocampus, and olfactory bulb. (A) Body weights are presented as the means ± SEM of animals at each week for the duration of the rearing period (8 weeks in total). (B–D) After the rearing period, the weights of the whole brain (B), hippocampus (C), and olfactory bulb (D) were measured.

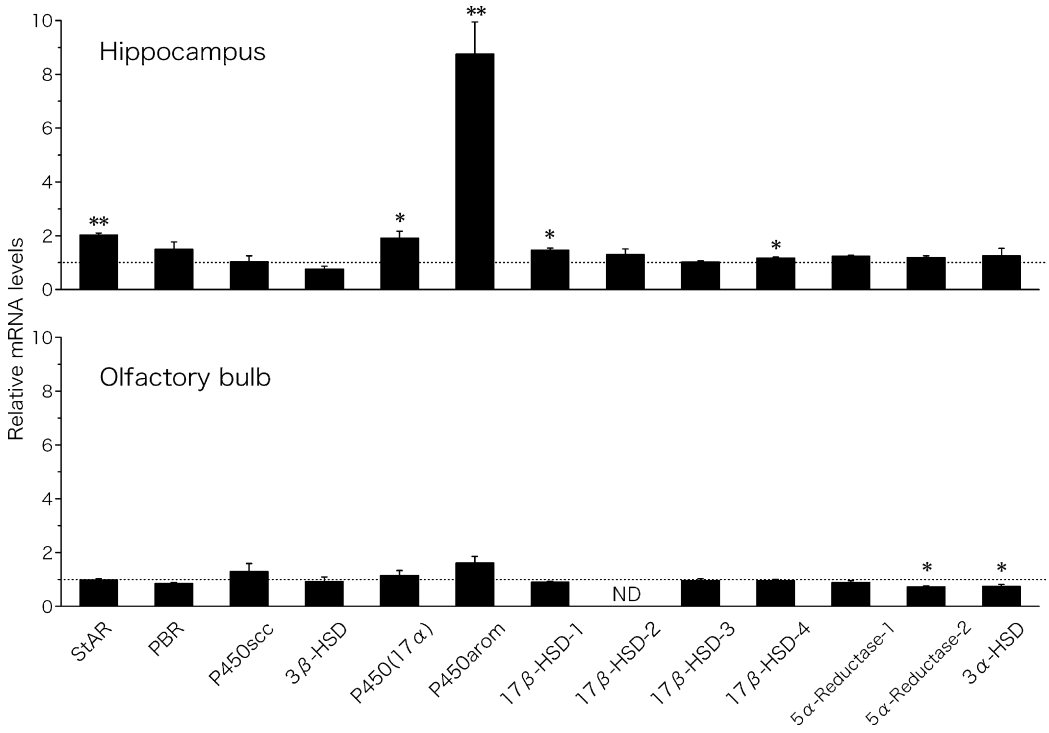

Fig. 3. Real-time PCR analysis of the mRNA levels of neurosteroidogenic enzymes and proteins in the rat hippocampus (upper) and olfactory bulb (lower). The amounts of mRNA in socially isolated rats are indicated as relative values to those in the corresponding organs of pair housed rats (dashed line). mRNA levels of StAR, PBR, 17β-HSD-1, 17β-HSD-4, 5α-reductase-1, 5α-reductase-2, and 3α-HSD in the olfactory bulb were 3- to 5-fold higher than those in the hippocampus ($p < 0.05$ for each). Amount of P450scc mRNA level in the olfactory bulb was 50-fold lower than that in the hippocampus ($p < 0.05$). mRNA expression of 17β-HSD-2 was not detected in the olfactory bulb. The columns and error bars represent the means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.0001$ versus pair housed rats. ND, not detected.

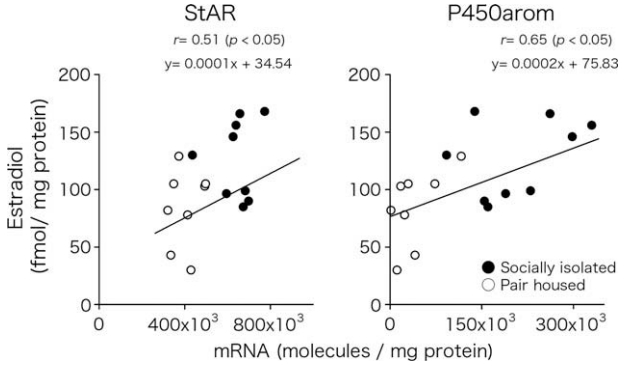

Fig. 4. Relationship between the StAR mRNA level and the estradiol concentration (left). Relationship between the P450arom mRNA level and the estradiol concentration (right). The Pearson's coefficients of correlation between these mRNA levels and the amount of estradiol (vertical axis) were calculated. The solid lines indicate the regression lines.

the mRNA levels encoding molecules involved in estradiol synthesis in the hippocampus, but not in the olfactory bulb.

The drastic upregulation of P450arom mRNA appeared to lead to increased hippocampal estradiol levels. Hippocampal estradiol is synthesized not only by *de novo* synthesis from cholesterol but also by synthesis from circulating testosterone [1]. Therefore, it remains unclear whether social isolation stimulated *de novo* hippocampal estradiol synthesis. However, a significant correlation was observed between the hippocampal estradiol level and the amount of StAR mRNA in this study. Since StAR is involved in the first step of *de novo* estradiol synthesis, the observed increase in the hippocampal estradiol level by social isolation may be at least partly due to the upregulation of StAR mRNA expression. Taken together, it is likely that social isolation enhanced *de novo* estradiol synthesis in the hippocampus.

In our experiment, mRNA levels of 5α-reductase-2 and 3α-HSD were decreased in the rat olfactory bulb after social isolation for 8 weeks, in accordance with the previous report that social isolation decreases allopregnanolone synthesis in the mouse olfactory bulb [13]. Although it has been reported that social isolation decreased mRNA level of 5α-reductase-1 in the mouse hippocampus [14], the present study shows that social isolation hardly affected on expression levels of 5α-reductase-1 in the rat hippocampus. This might be due to the differential effects of social isolation on rats and mice or due to the differences in housing conditions between them.

It has been demonstrated that estradiol plays important roles in processes such as synapse formation and neuroprotection in the hippocampus [18]. Administration of estradiol increases neuronal survival after different forms of insults [7,19]. Recently, Rune et al. [20,21] reported that endogenous estradiol synthesized in the hippocampus has neuroprotective effects. Specifically, inhibition of P450arom induces apoptosis while hippocampus-derived estradiol prevents neuronal loss by excitotoxic damage, further demonstrating the importance of hippocampus-derived estradiol in neuroprotection. On the other hand, social isolation was reported to increase circulating corticosterone levels [22]. Elevated corticosterone levels have harmful effects on the hippocampus such as decreasing the BDNF expression levels and inducing apoptosis [23,24]. However, estradiol can attenuate corticosterone-induced neuronal damage [25]. Furthermore, the hippocampus was reported to be vulnerable to elevated corticosterone levels [26]. Therefore, the increase in hippocampal estradiol synthesis induced by social isolation may counteract the harmful effects of social isolation. Similar to the effects of social isolation, brain injury induces the expression of P450arom, thereby leading to increased levels of estradiol in the brain [27,28]. Lavaque et al. [29] reported that tran-

scription of StAR and P450scc was activated by brain injury. Therefore, activation of estradiol synthesis appears to be a general stress response mechanism. In the present study, we have shown that social isolation upregulates the mRNA expression levels of several steroidogenic enzymes and proteins, leading to increased estradiol synthesis. The nervous system may respond to damage with increased steroidogenesis via transcriptional activation of the enzyme that catalyzes the aromatization of steroid precursors to estradiol. This effect may represent a cellular mechanism activated in the brain to avoid the harmful effects of social isolation.

The social isolation paradigm has been proposed as an animal model for developmental neuropsychological disorders [9]. In the present study, we have reported a relationship between hippocampal estradiol synthesis and social isolation for the first time. Of particular interest is the finding that the mRNA level of P450arom was elevated by >8-fold in socially isolated rats compared with pair housed rats. Since real-time PCR is a convenient method that yields highly quantitative and reproducible results, quantification of hippocampal P450arom mRNA may be useful for psychological analysis as a molecular probe for quantifying the effects of social isolation.

Acknowledgments

This work was supported in part by the Training Program for Research Managers in Integrated Arts and Sciences, Hiroshima University, and the Sumitomo Foundation.

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