Involvement of Granulin in Estrogen-Induced Neurogenesis in the Adult Rat Hippocampus

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Abstract. Recent studies have demonstrated the presence of neurogenesis in the adult mammalian hippocampus, and it has been suggested that estrogen and various growth factors influence the processes of adult neurogenesis. The present study assessed cell proliferation in the dentate gyrus and the mRNA expression levels of granulin, insulin-like growth factor-I (IGF-I), and brain-derived neurotrophic factor (BDNF) in the hippocampus 4 h after treatment with estradiol benzoate (EB) in 3- and 12-month old ovariectomized rats. At 3 months of age, mRNA expression of granulin precursor and cell proliferation were increased by EB treatment, although the mRNA expressions of IGF-I and BDNF remained unchanged. At 12 months of age, however, neither mRNA expression of the three genes nor cell proliferation in the dentate gyrus were affected by EB treatment. In addition, 17β-estradiol enhanced the proliferation of neural progenitor cells derived from hippocampal tissue of 3-month-old female rats in vitro; this was inhibited by neutralization of granulin with specific antibody. These results suggest that estrogen induces granulin gene expression in the hippocampus and that the product of this gene is involved in the mitogenic effects of estrogen in the dentate gyrus, although the responses to estrogen decline with age.

Key words: Aging, Estrogen, Granulin, Hippocampus, Neurogenesis, Rat

The brain has long been considered to be an organ that is not capable of regeneration. However, recent studies have shown the presence of active neurogenesis even in the brains of adult mammals [1]. Adult neurogenesis in the brain takes place principally in two distinct regions: the subventricular zone (SVZ) and the dentate gyrus of the hippocampus [2]. In the hippocampus, neural precursor cells are located in the subgranular zone (SGZ), which is the border region between the granule cell layer and the hilus in the dentate gyrus, and these precursor cells proliferate and produce daughter cells that are capable of differentiation into mature granule neurons [3]. Recent studies suggest that adult neurogenesis in the dentate gyrus is involved with learning [4, 5] and the behavioral effects of antidepressants [6], and a variety of factors such as aging [7], exercise [8], environment [9], growth factors [10–12], and hormones [3] influence this phenomenon.

Among the factors regulating neurogenesis, more attention has been paid to estrogen since it is known to enhance cell proliferation and increase the number of immature neurons in the adult dentate gyrus [13–15]. In addition, a recent study indicated the presence of estrogen receptors (ERs) in neural stem cells in the SVZ and SGZ [16]. Estrogen also affects learning tasks [17, 18] and the
state of depression in animals [19]. Therefore, enhanced neurogenesis is thought to be one of the routes through which estrogen exerts its effect on cognitive functions [20].

ERs function as transcription factors and modulate the mRNA levels of various genes, including growth factors [20]. For example, estrogen regulates the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus [21]. Estrogen also increases the mRNA level of insulin-like growth factor-I (IGF-I) in immortalized hippocampal cells [22] and interacts with IGF-I signaling, thus protecting hippocampal neurons from kainic acid-induced degeneration [23]. Collectively, these growth factor-mediated neurotrophic effects of estrogen are considered to support the growth and survival of neurons.

Recently, we found an increase in the mRNA level of granulin precursor in the hypothalamus of pups neonatally treated with estradiol [24, 25]. Granulins are 6 kDa polypeptides with unique cysteine motifs, and granulins are capable of promoting or inhibiting epithelial cell proliferation in vitro [26]. Estrogen treatment also increases the mRNA level of the granulin precursor in human breast cancer cells, and the granulin precursor itself can mediate the mitogenic effect of estrogen in these cells [27]. The mRNA encoding granulin is widely distributed in vivo and is also expressed in the adult hippocampal pyramidal and granule neurons [28, 29]. Recently, mutations in grn has been found in patients with frontotemporal dementia linked to chromosome 17, and these mutations lead to neurodegeneration [30, 31]. To date, however, the precise functions of granulins in the adult brain remain unknown.

Aging is another factor that influences adult neurogenesis. Neurogenesis in the dentate gyrus declines with age [7], and environmental enrichment [32] or replenishment of growth factors [33, 34] are reported to partly recover the decline in the number of new neurons in the dentate gyrus. IGF-I levels also decline with age in parallel with decreased hippocampal neurogenesis, although the proliferative capability of neural precursors in the dentate gyrus still remains responsive to IGF-I treatment [35], suggesting that the age-related decline in the number of new neurons in the brain is due to insufficient supply of growth factors rather than loss of responsiveness to the stimulatory effect of growth factors. The secretion of estrogens and progesterin from the ovaries decreases in middle-aged women (known as "menopause"), and some studies indicate that postmenopausal women receiving hormone replacement therapy experience recovery of cognitive functions [36]. Therefore, the decreased neurogenesis seen in middle age may arise from the decreased production of neurotrophic growth factors, primarily caused by the decline in estrogen levels after menopause.

From the currently reported observations described above, we hypothesized that the above-mentioned IGF-I and/or other growth factors induced by estrogen may stimulate cell proliferation in the dentate gyrus and that estrogen depletion in middle age may be the cause of the decrease in growth factor expressions, thus leading to a decline in hippocampal neurogenesis. In order to examine this hypothesis in the present study, we first investigated the possible correlation between cell proliferation and the increase in the precursor mRNA expressions of growth factors, such as granulin, BDNF and IGF-I, in the hippocampus of young (3-month-old) and middle-aged (12-month-old) ovariectomized (OVX) rats treated with estrogen or vehicle alone. Then, we investigated the possible involvement of granulin in the effect of estrogen on neural progenitor cell proliferation in vitro.

Materials and Methods

Animals and treatments

All experiments in this study were conducted according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agricultural and Life Sciences, The University of Tokyo. Female Wistar-Iramichi rats were housed and maintained under controlled lighting and temperature conditions (lights on 0500–1900 h; 23 ± 1 C). A laboratory diet and water were available ad libitum. Vaginal smears of female rats were observed microscopically to examine estrous cyclicity. The female rats were ovariectomized (OVX) under light ether anesthesia at the age of 3- or 12-months. Two weeks after the surgery, the OVX rats were subcutaneously injected with estradiol benzoate (EB; Sigma, St. Louis, MO, USA; 10 µg in 50 µl of sesame oil) or control sesame oil at 1200 h. Two hours after EB treatment, the rats (n=
5–7 for each group) were administered bromodeoxyuridine (BrdU; Wako, Osaka, Japan; 200 mg/kg BW, i.p.). After 2 h of survival, BrdU-treated rats were deeply anaesthetized with an overdose of sodium pentobarbital, and blood samples (5 ml) were collected. The rats were then immediately perfused transcardially with saline, followed by 4% paraformaldehyde in phosphate buffered saline (PBS). Some (n=3) of the rats were anesthetized 2 weeks after BrdU-treatment and perfused as described above. The brains were collected, post-fixed with 4% paraformaldehyde for 24 h, and then immersed in 30% sucrose/PBS for 3 days at 4 C. The post-fixed brains were embedded with OCT compound (Sakura Finetek Japan, Tokyo, Japan) and stored at –80 C until being processed for immunostaining for BrdU. For examination of the gene expression of growth factors, rats (n=5–6 at each group) were sacrificed 4 hours after EB treatment, and the whole hippocampus was collected and frozen immediately with liquid nitrogen until processing for gene expression analysis. The serum concentrations of estradiol were measured using an Estradiol EIA Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Neural progenitor cell culture

Neural progenitor cell culture was prepared as reported by Suzuki et al. [37]. Freshly isolated hippocampal tissue from 3-month-old female rats were mechanically chopped with a surgical blade, dissociated with trypsin-ethylenediaminetetraacetic acid (EDTA), and seeded into T25 flasks at a density of 200,000 cells/ml of growth medium [DMEM/Ham's F-12 (Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin (1%; Invitrogen) and supplemented with B27 (2%; Invitrogen), epidermal growth factor (EGF; 20 mg/ml; Sigma), and heparin (5 µg/ml; Sigma)]. All cultures were maintained in a humidified incubator (37 C; 5% CO₂ in the air), and half the medium was replenished every 3–4 days. The resulting neurospheres, which consisted of neural progenitor cells, were passaged every 14 days by trypsinization and seeded into fresh growth medium at a density of 100,000 cells/ml in 96-well plates, and treated with 17β-estradiol (10⁻⁸–10⁻⁴ M; Wako) and anti-granulin antibody (anti-acrogranin N-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 µg/ml) 4 h after a seeding. Twenty-four hours after treatment, cell numbers were assayed using a WST-1 Cell Counting Kit (Wako) according to the procedure suggested by the manufacturer.

For differentiation analysis, neural progenitor cells were cultured in differentiation medium [DMEM/F12 (7:3) supplemented with B27] for one week on coverslips coated with poly-L-lysine (Sigma) and laminin (Sigma).

Immunohistochemical study

Brain sections (30 µm-thick) were prepared with a cryostat throughout the entire dentate gyrus, and one out of every 12 slices were used. Fixed sections were mounted, incubated in 0.01 M citric acid buffer (pH 6.0) for 10 min at 90 C, and rinsed with 0.01 M PBS for 10 min. The sections were then incubated with 0.3% hydrogen peroxide in methanol for 30 min, digested in 0.05% trypsin 250 (Difco Laboratories, Detroit, MI, USA) in Tris-HCl (pH 8.0) containing 0.1% calcium chloride for 10 min at room temperature, rinsed with PBS, and then treated with 2 N HCl in PBS for 30 min at 37 C. After rinsing with PBS, the sections were blocked in 25% Block Ace (Snow Brand Milk Products, Sapporo, Japan) in PBS containing 1.5% normal goat serum for 2 h. The blocked sections were incubated with anti-BrdU antibody (mouse monoclonal; 1:200; Novocastra, Newcastle Upon Tyne, UK) diluted with 1% BSA/PBS for 24 h at 4 C. Then, the sections were rinsed with PBS three times and incubated with horseradish-peroxidase conjugated secondary antibody (Histofine Simple Stain Rat MAX PO; Nichirei, Tokyo, Japan) for 3 h. The sections were rinsed three times with PBS and incubated with 0.5 mg/ml dianimonobenzidine tetrahydro-chloride (Sigma) in Tris-HCl (pH 8.0) with 0.01% hydrogen peroxide for 2–5 min to visualize the signals. The sections were treated with graded ethanol and xylene (for 10 min each) and coverslipped with Diatex (Matsunami Glass, Osaka, Japan).

For double-label fluorescence study, mounted sections were incubated with 0.01M citric acid buffer (pH 6.0) for 2 h at 55 C and rinsed with PBS for 10 min. The sections were then incubated with 2 N HCl for 30 min at 37 C and rinsed with PBS for 10 min three times. After incubation with 5% normal
goat serum for 2 h, the sections were incubated with anti-BrdU antibody (rat monoclonal; 1:200; Serotec, Raleigh, NC, USA) and anti-NeuN antibody (mouse monoclonal; 1:500; Chemicon, Temecula, CA, USA) for 16 h at 4 °C. Then, the sections were rinsed with PBS for 10 min three times and incubated with Alexa 488 anti-mouse IgG (1:400; Invitrogen) and Alexa 594 anti-rat IgG (1:400; Invitrogen) for 3 h. The sections were rinsed with PBS and coverslipped with Gel/Mount (Biomeda, Foster City, CA, USA).

**Immunocytochemical study**

Cells cultured on coverslips were fixed with ice-cold methanol for 20 min and rinsed with PBS 3 times. The fixed cells were blocked with 5% normal goat serum plus 0.2% Triton X-100 (Sigma) in PBS for 30 min and incubated with anti-glia fibrillary acidic protein antibody (GFAP, 1:1,000; Dako Japan, Kyoto, Japan) and anti-β tubulin type III antibody (Tuj1, mouse monoclonal, 1:250; Babco, Berkeley, CA, USA) for 16 h at 4 °C. After rinsing with PBS for 5 min three times, the cells were incubated with Alexa 488 anti-mouse IgG (1:400; Invitrogen) and Alexa 594 anti-rabbit IgG (1:400; Invitrogen). Then, the cells were washed with PBS and incubated in 0.5 μg/ml Hoechst 33258 for 5 min and mounted.

**Gene expression analysis**

Total RNA was isolated from hippocampal samples or neurospheres using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (2 μg) from each sample was reverse-transcribed using oligo (dT)16 primer (Perkin-Elmer, Boston, MA, USA) and Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ, USA) in a total volume of 33 μl according to the manufacturer’s instructions. A Light Cycler (Roche Diagnostics, Mannheim, Germany) and a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) were used for real-time PCR analysis. The PCR primers used to amplify the mRNAs for the precursors of granulin, IGF-I, BDNF and ribosomal protein S29 (RPS29) are listed in Table 1. Dilutions (1:10, 1:100, 1:1000) of cDNA from each sample were used to construct a relative standard curve for each primer set. The linear relationships between the log concentrations of template and cycle numbers were obtained (r=-1, error <0.2 for all genes). The amplification program consisted of 1 cycle of 95 °C for 15 min followed by 40 cycles of 95 °C for 15 sec, 65 °C for 20 sec, and 72 °C for 15 sec. Acquisition was conducted at the end of a 15 sec hold at 72 °C. After the final elongation, melting curve analyses were performed. Both non-reverse transcribed samples and water were run as negative controls to assess the PCR specificity for constructing standard curves. The level of mRNA expression was normalized to RPS 29 because it is considered to be a stable housekeeping gene and was detected at the same level in all the samples used. In regard to the cDNA derived from neurospheres, PCR was carried out using αTag (Bionex, Seoul, Korea) according to the procedure suggested by the manufacturer. The PCR primers were confirmed to amplify the mRNA of β-actin, ERα and β, androgen receptor in our previous study [38]. The amplification program consisted of 1 cycle of 95 °C for 2 min followed by 35 cycles of 95 °C for 1 min, 55 °C for 20 sec, and 72 °C for 1 min. PCR products were electrophoresed on 1% agarose gel in 1× TAE buffer and DNA bands were detected by ethidium bromide staining for 7 min. The bands were photographed and scanned using a FAS-III

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward</th>
<th>Accession No.</th>
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<tr>
<td>Grn</td>
<td>5'-AGTTCGAATGCTCCTGACTCCGCA-3'</td>
<td>X62322</td>
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<tr>
<td>IGF-I</td>
<td>5'-TGTTGCTGGCTGAAAGCTACAAAG-3'</td>
<td>M15480</td>
</tr>
<tr>
<td>BDNF</td>
<td>5'-AAGGAGCAAAAGGATACTTGGGCT-3'</td>
<td>M61175</td>
</tr>
<tr>
<td>RPS29</td>
<td>5'-TGAGGGCGAAGATGGGTCAACGCAGC-3'</td>
<td>X59051</td>
</tr>
</tbody>
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Grn, granulin; IGF-I, insulin-like growth factor I; BDNF, brain-derived neurotrophic factor; RPS29, ribosomal protein S29.
(Toyobo, Tokyo, Japan).

Data analysis

All slides were coded and observed in random order at ×400 magnification under a microscope (BX-51; Olympus, Tokyo, Japan). BrdU-labeled cells in SVZ were counted for sections 1.8 and 2.2 mm caudal from the Bregma. BrdU-labeled cells in the SGZ were counted for every 12 sections throughout the dentate gyrus. The SGZ was defined as a 50 µm-wide zone from the inner edge of the granule cell layer. Cell numbers, gene expression levels in the hippocampus, and serum estradiol levels were analyzed using Student’s t-test, one-way ANOVAs followed by Holm or Dunnet post hoc comparisons, or Spearman’s rank correlation analysis with the R software (R Foundation for Statistical Computing, Vienna, Austria). Differences were considered significant at P<0.05.

Results

Effect of estrogen on cell proliferation in the hippocampus

BrdU-labeled cells were observed in the SVZ (Fig. 1A) and the dentate gyrus (Fig. 1B) of brain sections prepared from the 3-month-old vehicle- and EB-treated OVX rats, which all showed regular estrous cycles before OVX. In the dentate gyrus, large numbers of BrdU-labeled cells were located in the SGZ, the border region between the granule cell layer and the hilus. Although EB treatment did not affect the number of BrdU-labeled cells in the SVZ of the 3-month-old rats (Fig. 1C), it did significantly increase the BrdU-labeled cells in the SGZ (Fig. 1D). This EB treatment increased the serum estradiol levels from 8.6 ± 2.6 pg/ml (mean ± SEM, vehicle-treated rats) to 75.0 ± 16.8 pg/ml.

At 12 months of age, the intact female rats had irregular estrous cycles with extended estrus or diestrus, indicating the occurrence of atrophy of ovarian function. The number of BrdU-labeled cells decreased significantly in the 12-month-old OVX rats to approximately 32% and 13% of the levels in the 3-month-old rats in the SVZ (Fig. 1C) and SGZ (Fig. 1D), respectively. At 12 months of age, EB treatment did not affect the number of BrdU-labeled cells in either the SVZ and SGZ. Two weeks after BrdU treatment, approximately 30% of the BrdU-labeled cells were observed in the granule cell layer and were expressing mature neuron marker NeuN (Fig. 1E), indicating that some of the BrdU-labeled cells in the SGZ were neural progenitor cells.

Effect of estrogen on gene expression in the hippocampus

The mRNA levels of granulin, IGF-I, and BDNF, in the hippocampus were quantitatively analyzed using real-time RT-PCR. At 3 months of age, the expression level of granulin precursor mRNA was 4 times higher in the EB-treated rats than in those treated with vehicle (Fig. 2A). On the other hand, EB treatment had no effect on the mRNA expression levels of IGF-I and BDNF. At 12 months of age, EB treatment failed to alter the expression levels of the mRNA of granulin, IGF-I and BDNF in the hippocampus (Fig. 2B).

Effect of estrogen on neural progenitor cell culture

Neural progenitor cells derived from the hippocampi of female rats spontaneously formed neurospheres during culture for 3 weeks in the growth medium (Fig. 3A). Neural progenitor cells cultured in differentiation medium expressed β-tubulin type III (neuron marker, Fig. 3B) or GFAP (glial marker, Fig. 3C). Estradiol dose-dependently increased the number of cultured neural progenitor cells (Fig. 4A), and there was a significant correlation between the number of cells and the concentration of estradiol (r=0.49, P<0.05). Neural progenitor cells grew most vigorously under 10⁻⁵ M estradiol, while the effect of estradiol vanished when anti-granulin antibody was applied concomitantly with 10⁻⁵ M estradiol (Fig. 4B). RT-PCR analysis demonstrated the presence of the mRNA of β-actin, androgen receptor, and granulin in the cells consisting in neurospheres, although mRNA of classical estrogen receptors (ERα and β) was not detected using the present method (Fig. 4C).

Discussion

In the present study, estrogen stimulated cell proliferation in the SGZ of the hippocampus, but not in the SVZ at 3 months of age, which was consistent with previous reports [13–15]. Among the three genes examined in the hippocampus at
Fig. 1. BrdU-labeled cells (arrowheads) in the SVZ (A) and SGZ (B) 2 h after BrdU treatment, and the effect of EB on the number of BrdU-labeled cells in the SVZ (C) and SGZ (D) at 3 and 12 months of age. BrdU-labeled cells expressing mature neuron marker NeuN (arrowhead) in the granule cell layer of the dentate gyrus 2 weeks after BrdU treatment are shown in (C). The BrdU-labeled cell number is expressed in terms of the mean number of BrdU-labeled cells in one section. Each column and vertical bar represent the mean ± SEM, respectively (n=5–7). GCL: granule cell layer. LV: lateral ventricle. SGZ: subgranular zone. SVZ: subventricular zone. Scale bar: 200 µm. *: P<0.05 vs. vehicle. †P<0.05 vs. 3-month-old.
this age, only granulin precursor mRNA expression was increased by EB treatment for 4 h. The granulin gene has been isolated from the rat neonatal hypothalamic cDNA library by the cDNA subtraction method as a sex steroid-inducible gene [24], and has been suggested to be involved in sexual differentiation of the brain [39, 40]. We identified several half-sites of estrogen response element in the 5′ untranslated region of the granulin precursor gene (unpublished data), which

Fig. 2. Effects of EB on the mRNA expression levels of growth factors in the hippocampus at 3 (A) and 12 months (B) of age. Each column and vertical bar represent the mean ± SEM, respectively (n=5–6). Grn: granulin. IGF-I: insulin-like growth factor-I. BDNF: brain-derived neurotrophic factor. *: P<0.05 vs. vehicle.

Fig. 3. Neural progenitor cells derived from the hippocampus of a female rat forming neurospheres (A) in growth medium, and cells expressing neural marker β-tubulin type III (Tuj1, B) and astrocyte marker (C) after 1-week culture in differentiation medium. GFAP: glial fibrillary acidic protein. Scale bar: 100 µm (A) and 30 µm (B and C).
may be related to the estrogen-dependent increase in its gene expression, as was shown in our previous report [25]. Moreover, granulin precursor protein is known to stimulate cell proliferation [41–43] and mediate the mitogenic effect of estrogen in cancers [27, 44]. Taken together, it is probable that the granulin precursor induced by estrogen is involved in stimulating proliferation of neural progenitor cells in the hippocampi of 3-month-old rats.

Proliferation of neural progenitor cells separated from the hippocampi of 3-month old rats was also enhanced by estrogen in vitro in a dose-dependent manner, although the significant effect of estrogen was observed at a relatively high concentration (10^{-5} M). Since aromatase, an estradiol-synthesizing enzyme, is expressed in the cell of the dentate gyrus [45] and estrogen can increase aromatase expression [46], it is possible that systemically-administered estrogen increases hippocampal aromatase and that locally-produced estrogen in the hippocampus in turn participates in enhancing progenitor cell proliferation. Observation that the antibody against granulin precursor effectively blocked the stimulatory effect of estrogen on proliferation of progenitors further supports the above-mentioned notion that granulin is involved in estrogenic action on cell proliferation. Granulin precursor mRNA has been shown to be located in the pyramidal cell layer of the cornu ammonis (CA) and the granule cell layer of the dentate gyrus in an in situ hybridization study [29]. The present study also demonstrated the expression of granulin precursor mRNA in neural progenitors derived from the hippocampus. These observations suggest that granulin participates in neural progenitor proliferation in an, at least partially, autocrine/paracrine manner.

Fig. 4. Effects of estradiol on neural progenitor cell growth in neurospheres from the hippocampus in vitro (A), and the interference with the effect by anti-granulin antibody (B). Expression of mRNAs for sex-steroid receptors and granulin in the neural progenitor cells is shown in (C). Each column and vertical bar represent the mean ± SEM, respectively (n=4). CO: control. β-Act: β-actin. ER: estrogen receptor. AR: androgen receptor. Grn: granulin. RT: reverse transcription. *: P<0.05 vs. control (CO).

ERs have been demonstrated in neural stem cells derived from the ventricular zone or hippocampus [16], proliferating cells in the hippocampus [47], and progenitors in the adult SVZ and SGZ in an in situ hybridization study [48]. In the present study, however, the mRNAs of both ERα and β were not detected in neural progenitor cells derived from the hippocampus, suggesting that neural progenitors are heterogeneous in respect to classical ER expression and that neural progenitors capable of forming neurospheres might be devoid of both ERα and β. Despite the lack of classical ER, estrogen stimulated growth of neural progenitor cells in vitro in this study, and this phenomenon may imply existence of a non-classical estrogen receptor, such as G-protein coupled receptor 30 [49], in these cells. Further studies are needed to clarify the mechanisms underlying the estrogen effect, but the in vivo effects of estrogen on proliferation of neural progenitors may involve both classical and non-classical ERs. Although EB treatment for 4 h did not increase the mRNA expression for BDNF and IGF-I in the present study, estrogen has been shown to stimulate the expression of these genes at least 48 h after administration [21, 22]. Estrogen may induce various growth factors though various ERs with different time courses to maintain the proliferation of neural progenitor cells.

In the present study, 12-month-old rats with
irregular estrous cycles and extended estrus and diestrus did not respond to EB treatment in terms of granulin precursor, BDNF, or IGF-I mRNA expression. Granule cells in dentate gyrus are shown to display morphological changes in response to estrogen in age-dependent manner [50]. The loss of responsiveness to estrogen has been also shown in BDNF production in the olfactory bulb and its forebrain afferent in aged OVX rats [51]. These findings suggest that the middle-aged brain loses its ability to respond to estrogen. And the expression of ERα changes with age in a region-specific manner and ERα is even upregulated in several nuclei of the hypothalamus with age [52]. Although age-related changes in ERα expression in the hippocampus have not so far been reported, the downregulation of ERs in this region in accordance with age, if any, may explain incapability of estrogen in stimulating granulin precursor mRNA expression and in turn cell proliferation in the hippocampus.

If the neurotrophic effects of estrogen disappear with age, reconsideration of the effectiveness of estrogen replacement therapy on cognitive function would be required. Many women at or after menopause receive hormone replacement therapy to replenish declined estrogen and progesterone levels, and some studies have demonstrated that estrogen replacement for menopausal women ameliorates cognitive functioning [53]. On the contrary, the results of the Women’s Health Initiative Memory Study (a randomized, placebo-controlled clinical trial) showed that women who received hormone replacement therapy were more likely to develop probable dementia [54]. This inconsistency among reports should be resolved by clarifying the precise mechanism that leads to loss of responsiveness to estrogen in the brain with age, and the recovery of response to estrogen rather than mere estrogen treatment should be considered as one of the possible new strategies to improve the effectiveness of estrogen replacement therapy.

In conclusion, the present study demonstrated that estrogen enhanced the proliferation of neural progenitor cells in the hippocampus of young adult rats in both the in vivo and in vitro experimental models and suggests that granulin plays an important role in mediating estrogen actions on these cells. In middle-aged rats with irregular estrous cycles, however, the stimulatory effects of estrogen on both cell proliferation and granulin gene expression were no longer discernible. The processes through which the brain loses its ability to respond to estrogen remain to be elucidated.

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