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—Original Article—

Lack of estrogen receptor α in astrocytes of progranulin-deficient mice

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Abstract. Progranulin (PGRN) is a multifunctional growth factor with functions in neuroprotection, anti-inflammation, and neural progenitor cell proliferation. These functions largely overlap with the actions of estrogen in the brain. Indeed, we have previously shown that PGRN mediates the functions of estrogen, such as masculinizing the rodent brain and promoting adult neurogenesis. To evaluate the underlying mechanism of PGRN in mediating the actions of estrogen, the localization of estrogen receptor α (ERα) in the brains of wild-type (WT) and PGRN-deficient (KO) mice was investigated. First, double-labeling immunofluorescence was performed for ERα with neuronal nuclei (NeuN), ionized calcium-binding adaptor molecule 1 (Iba1), and glial fibrillary acidic protein (GFAP), as markers for neurons, microglia, and astrocytes, respectively, in female mice in diestrous and estrous stages. ERα-immunoreactive (IR) cells were widespread and co-localized with NeuN in brain sections analyzed (bregma –1.06 to –3.16 mm) of both WT and KO mice. In contrast, expression of ERα was not observed in Iba1-IR cells from both genotypes. Interestingly, although ERα was co-localized with GFAP in WT mice, virtually no ERα expression was discernible in GFAP-IR cells in KO mice. Next, the brains of ovariectomized adult female, adult male, and immature female mice were subjected to immunostaining for ERα and GFAP. Again, co-localization of ERα with GFAP was observed in WT mice, whereas this co-localization was not detected in KO mice. These results suggest that PGRN plays a crucial role in the expression of ERα in astrocytes regardless of the estrous cycle stage, sex, and maturity.

Key words: Astrocyte, Estrogen receptor α, Progranulin

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Progranulin (PGRN) is a glycoprotein that works as a growth factor with multiple functions, such as neuroprotection, anti-inflammation, and proliferation of neural progenitor cells [1–3]. In the rodent brain, PGRN is mainly expressed in the cingulate and piriform cortices, the pyramidal cell layer and dentate gyrus of the hippocampus, the amygdala, the ventromedial and arcuate nuclei of the hypothalamus, and the Purkinje cell layer in the cerebellum [4, 5]. We have demonstrated that PGRN is predominantly expressed in neurons, and after brain injury, PGRN is also expressed in activated microglia but not in astrocytes [2, 6]. In 2006, a heterozygous mutation of the PGRN gene was reported as one of the major factors causing frontotemporal lobar degeneration (FTLD) [7, 8]. Subsequently, PGRN deficiency was shown to be involved in the development of other neurodegenerative diseases such as Alzheimer's disease [9], amyotrophic lateral sclerosis [10], and neuronal ceroid lipofuscinosis (NCL) [11].

We have reported that PGRN is involved in sexual differentiation of the rodent brain during the neonatal period [12, 13] and adult neurogenesis in the dentate gyrus of the hippocampus [6, 14], using rats and PGRN-deficient mice that we have generated. PGRN deficiency results in a decrease in ejaculation incidence, an increase in aggression, and anxiety in males, with histological changes in the brain including a larger volume of the locus coeruleus [15] and a thicker density of Purkinje cell dendrites [16]. In addition, we recently demonstrated that PGRN deficiency exacerbates neuronal damage, neuroinflammation, and lysosomal biogenesis in activated microglia following traumatic brain injury (TBI) [2, 17]. We have also shown that aged PGRN-deficient mice present NCL-like pathology, as well as TAR DNA binding protein-43 aggregates, a characteristic feature of FTLD, and that these pathological changes are the result of lysosomal dysfunction [18]. The expression of PGRN in the brain was reported to be upregulated by injury [2, 3], exercise [6], and estrogen treatment [14, 19].

It is well known that estrogen has a potent neuroprotective function, as well as functions to induce sex differences of the brain during the neonatal period and sexual behaviors in adulthood. For example, estrogen protects neurons by reducing astrogliosis in a TBI model [20] and improves locomotor functions after spinal cord injury [21]. In addition, estrogen promotes neurogenesis after ischemia [22, 23] and enhances proliferation of neural progenitor cells [24]. Thus, PGRN and estrogen have common neuroprotective properties in the brain. Indeed, we have previously demonstrated that PGRN mediates at least some of the actions of estrogen, such as masculinization of the rodent brain [12, 13] and induction of hippocampal adult neurogenesis [14, 19]. In the present study, to evaluate the underlying mechanism of the role of PGRN in mediating the function of estrogen, the localization of estrogen receptor α (ERα), a classic well-studied subtype of estrogen receptors, in the brain of PGRN-deficient mice was assessed using immunohistochemistry, and interesting cell type-specific suppression of ERα expression

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was observed in astrocytes of PGRN-deficient mice.

Materials and Methods

Animals

Adult female (8- to 11-week old), male (9-week old), and immature female (5-week old) C57BL/6J wild-type (WT) and PGRN-deficient (KO) mice [13] of the same genetic background, bred in our laboratory, were used. Food and water were available *ad libitum*, and the animals were housed in a controlled temperature of 23 ± 1°C and under a 12 h light-dark cycle (lights on at 0700 h). Vaginal smears were taken from adult female mice to monitor estrous cycles, and mice in diestrous or estrous stages were used. Some female mice were ovariectomized (OVX) 1 week before being used. In all experimental groups, three animals were used, except for WT females in the estrous stage (n = 4). All experiments were conducted following the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Histological analysis

Brains of the mice were sampled after decapitation and immersed in 4% paraformaldehyde (Wako, Tokyo, Japan), dissolved in phosphate-buffered saline (PBS) for 48 h, in 15% sucrose/PBS for 24 h, and 30% sucrose/PBS for 48 h, embedded in OTC compound (Sakura Finetek Japan, Tokyo, Japan), and stored at –80 °C until cut. Coronal sections (30-μm thick) from the bregma –1.06 to –3.16 mm of the brain were produced using a cryostat (Carl Zeiss, Oberkochen, Germany) and stored in anti-freezing buffer (0.1 M PBS, 30% ethylene glycol, 20% glycerol) at –20°C until use. Three sections (anterior, middle, and posterior portion of the bregma –1.06 to –3.16 mm) were washed in PBS for 10 min, incubated in Block Ace solution (4 mg/ml, Megmilk Snow Brand, Tokyo, Japan), and dissolved in 0.3% Triton X-100 in PBS (PBST) for 2 h at room temperature (r.t.). For double immunostaining, sections were incubated with primary antibodies for ERα (rabbit anti-ERα, C1355; 1:500, EMD Millipore Corporation, Billerica, MA) and each cell marker: mouse anti-neuronal nuclei (NeuN) as a marker of neurons (A60; 1:1000, EMD Millipore Corporation), goat anti-ionized calcium-binding adaptor molecule 1 (Iba1) as a marker of microglia (ab5076; 1:400, Abcam, Cambridge, UK), and goat anti-glial fibrillary acidic protein (GFAP) as a marker of astrocytes (ab53544; 1:2000, Abcam) for 16 h at 4°C. After washing in 0.03% PBST, sections were incubated with Alexa Fluor Dye-conjugated secondary antibodies (1:1000; Invitrogen, Carlsbad, CA); Alexa Fluor 594 goat anti-rabbit IgG (H + L) and Alexa Fluor 488 goat anti-mouse IgG (H + L) for double-staining of ERα and NeuN, and Alexa Fluor 594 donkey anti-rabbit IgG (H + L) and Alexa Fluor 488 donkey anti-goat IgG (H + L) for ERα and Iba1 or GFAP, in 0.3% PBST for 2 h at r.t., and washed in 0.03% PBST. Finally, sections were mounted on MAS-coated slides and sealed with Fluoromount (Diagnostic Biosystems, Pleasanton, CA) and coverslips (Matsunami Glass, Osaka, Japan). Images were obtained with a microscope (BX-53; Olympus, Tokyo, Japan) equipped with a CCD camera (DP73; Olympus) and then merged using Adobe Photoshop (version CC 2015; Adobe Systems, San Jose, CA).

Antibody absorption test

We synthesized a peptide with the same sequence as the antigen used to produce the ERα antibody used in the present study (amino acids 585–599 of the mouse ERα sequence; TYYIPPEAEGFPNTI, TORAY Research Center, Tokyo, Japan). The ERα peptide was dissolved in PBS and mixed with ERα antibody solution (20:1, molar ratio) at 4°C overnight. A mixture of antibody and peptide was used for the primary antibody.

Results

A double-labeling immunofluorescence study was performed to characterize ERα-immunoreactive (IR) cells in the brain of the mice sampled on the day of estrus (n = 4 for WT mice, n = 3 for KO mice) and diestrus (n = 3 for each genotype). ERα immunoreactivity was observed in brain sections containing the cortex, hippocampus, amygdala, thalamus, and hypothalamus of both WT and KO mice, and since the same results were obtained among these brain regions, representative images of the dentate gyrus (DG) in the hippocampus and the dorsomedial nucleus of the hypothalamus are shown in Fig. 1. These brain regions were selected because neurogenesis in the DG is promoted by estrogen [14], and the hypothalamus is the major target of estrogen [25, 26]. ERα and NeuN co-staining was often observed (Fig. 1A), whereas ERα-IR cells were never Iba1-positive (Fig. 1B) in the brains of both genotypes. In contrast, co-localization of ERα and GFAP was not discernible in five of six KO mice, whereas these two markers showed co-localization in WT mice (Fig. 1C). In one KO mouse at the estrous stage, ERα immunoreactivity was observed in GFAP-IR cells (data not shown). These results imply that PGRN plays an important role in ERα expression in astrocytes regardless of the estrous stage. Absorption controls showed no ERα-IR signals in the brains of both WT and KO mice (Supplementary Fig. 1: online only), confirming the specificity of the antibody used.

Subsequently, to evaluate the effect of endogenous estrogen on the possible regulation of ERα expression by PGRN in astrocytes, double-immunostaining of ERα and GFAP in the brains of adult OVX, adult male, and immature female mice (n = 3 for each group, Fig. 2) was performed. Co-localization of ERα and GFAP was not observed in the brains of all KO mice used, whereas these markers co-localized well in the brains of WT mice, suggesting that estrogen is not involved in the regulation of ERα expression by PGRN.

Discussion

The present study suggests that PGRN plays a crucial role in the expression of ERα in astrocytes regardless of estrous cycle, sex, and maturity. In the rodent brain, it has been reported that ERα is mainly localized to the cortex, hippocampus, amygdala, thalamus, and hypothalamus [27–29], and that both neurons and astrocytes express ERα [30–33]. Consistently, in the present study, ERα immunoreactivity was observed in neurons and astrocytes of these brain regions in WT mice. However, in the KO mice, ERα expression in astrocytes was not detected in practically all animals used, whereas all KO mice showed ERα expression in neurons. We have previously demonstrated that PGRN is expressed in neurons and microglia, but not in astrocytes [2, 6]. Astrocytes are reported to interact with

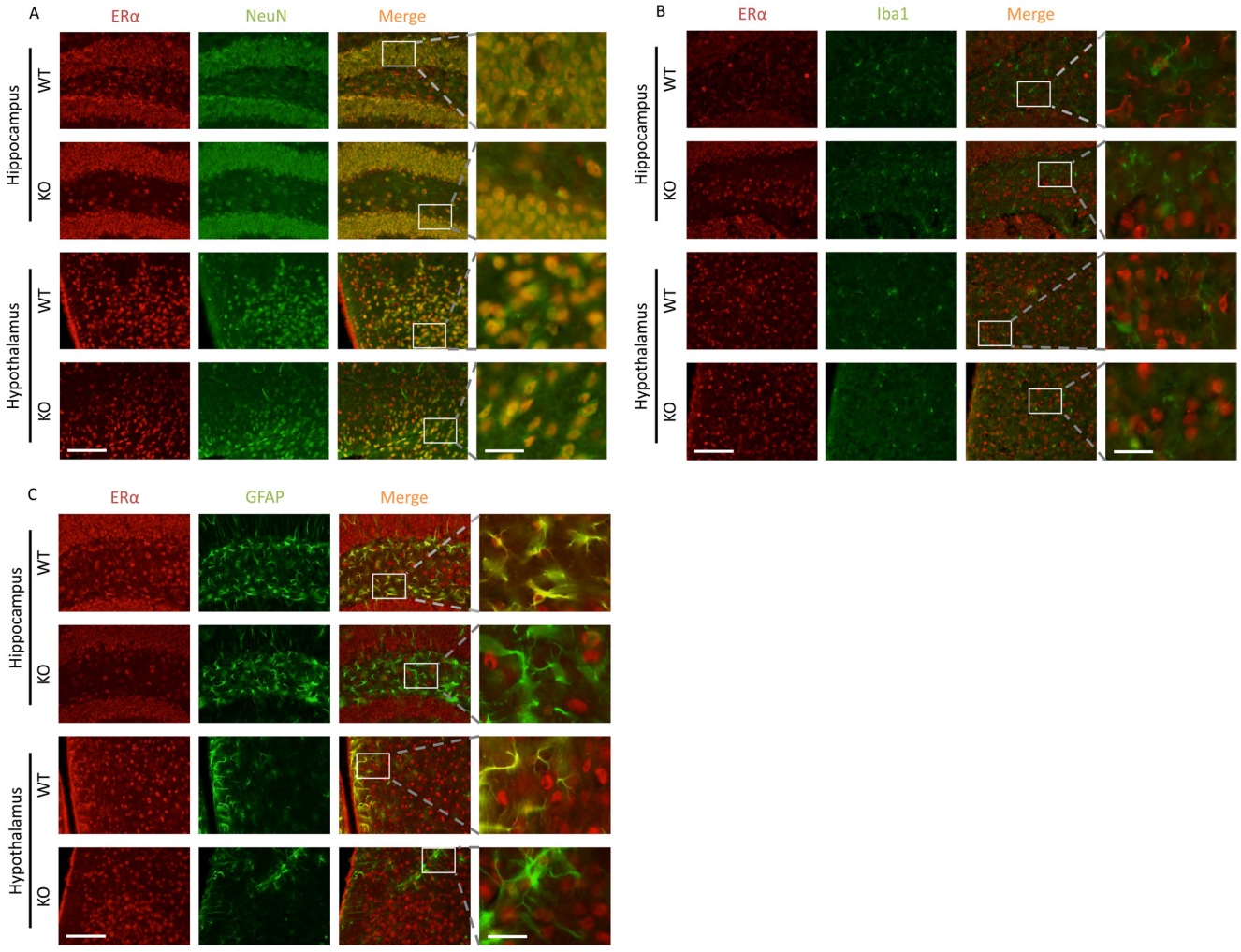

Fig. 1. Double immunostaining of ERα and NeuN (A), Iba1 (B), or GFAP (C) in the dentate gyrus of the hippocampus and the hypothalamus of intact adult female mice. ERα co-stained with NeuN, but not with Iba1 in both genotypes. ERα immunoreactivity was observed in GFAP-IR cells in WT mice, whereas no co-localization was discernible in KO mice. Scale bar = 100 μm. Right panels represent higher magnification images of merged images. Scale bar = 25 μm.

neurons through signal transduction via the synapse [34] and with microglia during brain inflammation [35]. Thus, PGRN possibly affects neurons or microglia to regulate ERα expression in astrocytes. Another possible mechanism is that PGRN is produced and secreted from neurons, and acts on astrocytes to control the expression of ERα, since PGRN is a secretory glycoprotein detected in cerebrospinal fluid [36]. In the present study, ERα immunoreactivity was observed in GFAP-IR cells of one adult female KO mouse of 15. The reason for this exceptional case is currently unknown, but the possibility that PGRN plays a role in the degradation of ERα rather than its synthesis cannot be ruled out, since we have previously suggested that PGRN is involved in the autophagy-lysosome system [17, 18].

Estrogen is reported to suppress TBI-induced neuroinflammatory responses by reducing astrogliosis [20]. In contrast, we have previously shown that activation of microglia and subsequent neuroinflammatory responses induced by TBI are enhanced by PGRN deficiency, implying that PGRN has anti-inflammatory and neuroprotective effects [17, 18]. Since activated astrocytes are reported to activate microglia [35], the deficiency of ERα in astrocytes could be a major factor causing hyperactivation of microglia and neuroinflammation in KO mice. These studies suggest that PGRN regulates the expression of ERα in astrocytes, which in turn mediates the anti-inflammatory function of estrogen in the brain. In addition, estrogen promotes the release of neuroprotective factors from astrocytes, such as glial cell-line derived neurotrophic factor [37, 38], nerve growth factor [38], vascular endothelial growth factor [39], and brain-derived neurotrophic factor [38]. Furthermore, estrogen was shown to decrease the expression of chemokines via ERα in astrocytes in an experimental autoimmune encephalomyelitis model [40], and astrocytes mediated the neurotrophic function of estrogen to prevent neural death induced by β-amyloid protein [41]. Thus, PGRN might be associated with these neuroprotective functions of estrogen in

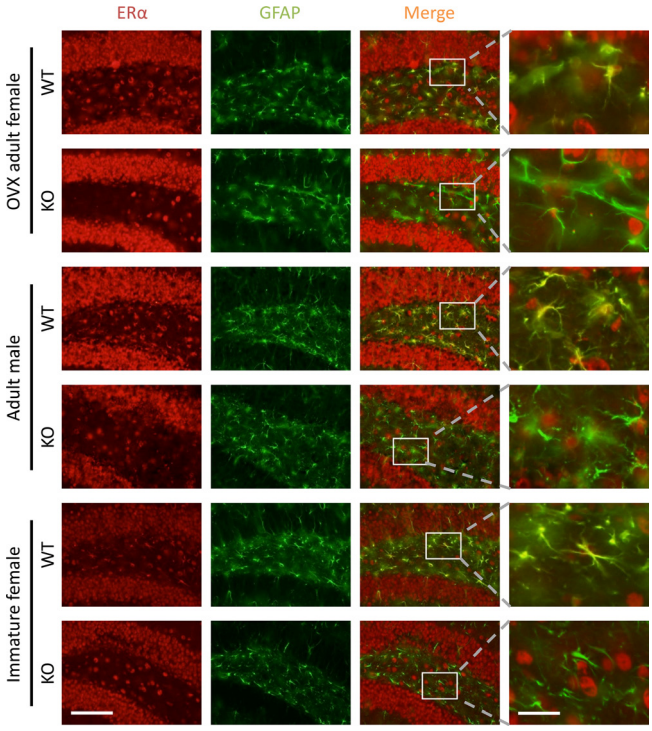

Fig. 2. Double immunostaining of ERα and GFAP in the dentate gyrus of the hippocampus of adult OVX, adult male, and immature female mice. Co-localization of ERα immunoreactivity and GFAP immunoreactivity was not observed in KO mice, whereas these markers co-localized in WT mice. Scale bar = 100 μm. Right panels represent higher magnification images of merged images. Scale bar = 25 μm.

astrocytes, and PGRN-deficient mice, used in the present study, might be a good experimental model to investigate the function of estrogen/ERα in astrocytes.

As another subtype of the estrogen receptor, estrogen receptor β (ERβ), was discovered in 1996 [42], the functions of estrogen via ERα and ERβ have been considered. These receptors are broadly expressed in the brain, but their distributions are different [25, 43, 44]. The expression of ERβ was reported in neurons, astrocytes, and even microglia [45]. The structure of these receptors is similar in their DNA-binding domains and ligand-binding domains [23, 42], and they share some common roles [46]. Recent studies show that ERβ also mediates the neuroprotective function of estrogen. For example, the function of estrogen to promote neurogenesis is mediated by both ERs, but its function in increasing neural progenitor cells is mediated by ERβ, but not by ERα [47]. To precisely clarify the role of PGRN in mediating the actions of estrogen, further studies on the localization of ERβ in PGRN-deficient mice might be required.

In conclusion, the present study suggests that PGRN produced in neurons and/or microglia acts on astrocytes to induce the expression of ERα, which probably mediates the actions of estrogen to suppress neuroinflammation after brain damage. To understand whether PGRN affects ERα expression in astrocytes at the level of transcription, translation, or degradation needs further investigation.

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