## Title

# Response of ERß and aromatase expression in the monkey hippocampal formation to ovariectomy and menopause

Sayuri Higaki[a], Ken Takumi[a, b], Mariko Itoh[c], Gen Watanabe[d, e], Kazuyoshi Taya[d, e], Keiko Shimizu[a, f], Motoharu Hayashi[a, g, h], Takao Oishi[a]*

[a] Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Aichi 484-8506, Japan

[b] Department of Anatomy and Neurobiology, Graduate School of Medicine, Nippon Medical School, Tokyo 113-8602, Japan

[c] Futuristic Environmental Simulation Center, Research Institute of Environmental Medicine, Nagoya University, Aichi 464-8601, Japan

[d] Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

[e] Department of Basic Veterinary Science, The United School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

[f] Department of Zoology, Faculty of Science, Okayama University of

Science, Okayama 700-0005, Japan

[g] Division of Applied System Neuroscience, Advanced Medical Science Course, Nihon University School of Medicine, Tokyo 173-8610, Japan

[h] Department of Physiology, Nippon Medical School, Tokyo 113-8602, Japan

* Corresponding author at: Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Aichi 484-8506, Japan

Tel.: +81-568-63-0573; Fax: +81-568-63-0416.

E-mail address: toishi@pri.kyoto-u.ac.jp (T. Oishi).

## Abstract

Changes in the expression of estrogen-related substances in monkeys' brains at the menopausal transition, when estrogen deficit starts to occur, have not yet been examined thoroughly. In the present study, we immunohistochemically investigated the expression levels of estrogen receptor beta (ERß) and aromatase (local estrogen synthesizing enzyme) in the hippocampal formation of premenopausal, menopausal, and ovariectomized premenoposal monkeys. In all monkeys tested, ERß immunoreactivity was observed in interneurons located in the subiculum and the Ammon's horn, and most of these ERß-immunoreactive neurons coexpressed a GABAergic neuron marker, parvalbumin. In the menopausal monkeys who exhibited a decline in estrogen concentration, hippocampal ERß was highly upregulated, while aromatase expression was not markedly changed. By contrast, aromatase in the ovariectomized monkeys was significantly upregulated, while ERß expression was not changed. In the brains of ovariectomized and menopausal monkeys, depletion of ovary-derived estrogen brought about different reactions which may be attributed to the senescence of brain aging.

Keywords: estrogen receptor beta, aging, hippocampus, macaque

## 1. Introduction

Estrogen homeostatically keeps exerting influences on the brain, as well as on peripheral organs, at regular cycles over almost half of women's life until menopause. A relationship between estrogen and cognitive functions has been clinically documented indicating that menopausal women are at a higher risk of developing Alzheimer's disease (AD) and depression (Launer et al., 1999; Weissman et al., 1993) although there remains some controversy over the sex difference (Hebert et al., 2001). The aged hippocampus is particularly vulnerable to age-related neurodegenerative disorders presenting as atrophy (De Leon et al., 1997), increased levels of neurofilament protein (Vickers et al., 1994), senile plaques and neurofibrillary tangles (Hof, 1997; Rapp et al., 2006), and synapse loss (West et al., 1994). The neural circuits between the hippocampus and the neocortex are highly degenerated in AD patients whose episodic memory is often affected even at an early stage of AD (Morrison and Hof, 1997). Studies of aged monkeys have demonstrated that age-related cognitive impairment reflects the vulnerability of these circuits and that the synaptic alterations occurring in pyramidal cells can

be restored by estrogen administration (Hof, 1997). Although the estrogen replacement therapy (ERT) for menopausal women is expected to have protective effects on the prefrontal cortex and the hippocampus to protect against AD-related cognitive declines (Gibbs and Aggarwal, 1998), there is thought to be a critical window for the initiation of ERT (Henderson et al., 2003). The menopausal transition, a limited period following the loss of ovary function, is considered to be highly susceptible to hormone treatment (Gibbs and Gabor, 2003). Macaques, including Japanese monkeys and rhesus monkeys, enter menopause at around 25 years old, and have a postreproductive life span of 5 or more years (Pavelka and Fedigan, 1999; Walker, 1995). Aged monkeys can be an appropriate model in terms of sharing similar endocrinological and histological senescence of the ovary with humans (Gilardi et al., 1997; Nichols et al., 2005; Nozaki et al., 1995).

In broad areas of the brain, estradiol binds almost equally to two different subtypes of estrogen receptors, ERα and ERβ (Kuiper et al., 1997). Previous studies have demonstrated that ERβ mRNA (Gundlah et al., 2000) and protein (Blurton-Jones et al., 1999) were more abundant than those of ERα in the monkey hippocampus. Selective ERβ agonists enhance

synaptic plasticity in the hippocampus and cognitive performance, which was confirmed by absence of the effects in ERβ knockout mice (Liu et al., 2008; Walf et al., 2008). ERβ is suggested to colocalize with GABAergic neuron marker, parvalbumin and indirectly upregulate brain-derived neurotrophic factor in the rat (Blurton-Jones and Tuszynski, 2002; 2006). However, the primate hippocampus has not been analyzed in terms of colocalization of ERβ and GABAergic neuron markers, such as calcium binding proteins.

Estrogen is derived not only from endocrine glands, but also from the brain. Aromatase contributes to the conversion of androgens into estrogens, the last step of estrogen synthesis. Previous immunohistochemical studies have shown the presence of de novo synthesized neurosteroids in the monkey (Wehrenberg et al., 2001; Yague et al., 2008) and human (Webber et al., 2006; Yague et al., 2010) hippocampus. In order to study menopause-related changes in the brain, it is necessary to consider local estrogen synthesis in the brains of young and aged females, whose peripheral hormone levels are utterly different. In the present study, we attempted to elucidate the changes of ERβ and aromatase expression in the

monkey hippocampus under the diminished blood levels of estrogen which were caused by natural menopause and surgical ovariectomy.

## 2. Materials and methods

### *2.1 Animals*

Twelve female Japanese monkeys (*Macaca fuscata*) used in this study were housed individually in indoor cages. Based on the age and daily menstruation records, nine monkeys were assigned to one of three categories: premenopausal (n=3; age 9 yr, 11 yr and 13 yr; regular monthly menstruation), perimenopausal (n=3; age 25, 26 and 26 yr; irregular menstruation within past 1 year), and postmenopausal (n=3; aged 29, 30 and 31 yr; no episodes of menstrual bleeding for at least 2 years). The cycling premenopausal monkeys were sacrificed on the second day (D2) (monkey ID, Mff1538 and Mff1564) or D5 (monkey ID, Mff1729) of menses (i.e. in the early follicular phase). The remaining three premenopausal monkeys were bilaterally ovariectomized (n=3; age 9, 13 and 18 yr; regular monthly menstruation) on D20 (monkey ID, Mff1512) or D24 (monkey ID, Mff1283 and Mff1736) of menses (i.e. in the luteal phase). Then, they were sacrificed two months after the operation, as a perimenopausal model in endocrinological aspects. To verify circulating hormone levels of all

monkeys, blood samples at the point of sacrifice were assayed for 17β-estradiol, progesterone and luteinizing hormone (LH). Additionally, in several premenopausal, perimenopausal and ovariectomized monkeys, the hormonal rhythmicity was monitored by analyzing blood samples collected every 2 - 3 days during more than 120 days. All animals were pretreated with 10 mg/kg ketamine hydrochloride (Sankyo Yell Yakuhin Co., Ltd., Tokyo, Japan) and deeply anesthetized with 30 mg/kg pentobarbital sodium (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Monkeys were perfused through the heart with the prewash solution, and then perfused with 4% paraformaldehyde. After perfusion, the brains were immediately removed and then immersed in 2% PFA and 5% sucrose in phosphate-buffered saline (PBS) for 24 hours, followed by successive immersions in 10%, 20% and 30% sucrose in PBS. The brain blocks cut into the coronal plane at 5-mm thickness were embedded in Tissue-Tek OCT compound (Miles Inc, Elkhart, IN, USA) followed by rapid freezing in a dry-ice acetone bath. All experimental procedures were planned and executed in accordance with The Guide for the Care and Use of Laboratory Primates (2002) established by the Primate Research Institute of Kyoto

University and NIH Guide for the Care and Use of Laboratory Animals (1996).

*2.2 Measurement of hormone levels by radioimmunoassay (RIA)*

The blood samples were analyzed for LH levels using a heterologous RIA system described previously (Watanabe et al., 1990). The iodinated preparation was NIDDK-rat LH-I-5. The antiserum was anti-ovine LH (YM#18; Dr. Y. Mori, University of Tokyo, Tokyo, Japan). Concentrations of 17β-estradiol and progesterone were measured using double-antibody RIA systems with 125I-labeled radioligands, as previously described (Taya et al., 1985) using antiserum against 17β-estradiol (GDN#244; Dr. G. D. Niswender, Animal Production and Biotechnology, Colorado State University, Fort Collins, CO, USA) and against progesterone (GDN#337; Dr. G. D. Niswender, Animal Production and Biotechnology, Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 4.4% and 16.1% for LH, 4.7% and 13.3% for 17β-estradiol, and 5.5% and 16.8% for progesterone, respectively.

*2.3 Immunohistochemistry*

The coronal sections were cut at 40-μm thickness with a cryostat (HM500-M, Micron, Waldorf, Germany) and stored in cryoprotectant at -20°C until the time of processing (Watson et al., 1986). Immunohistochemistry of monkey brains was performed as described elsewhere (Hayashi et al., 2001). The sections were washed in PBS, processed with additional antigen retrieval by boiling for 30 min in citrate buffer (pH 6.8) for anti-aromatase staining, and then soaked in 0.3% H2O2 in methanol for 30 min to inactivate endogenous peroxidase. They were preincubated with PBS containing 0.5% TritonX-100, 4% goat or donkey serum and 10% fish gelatin for 1 h at room temperature.

For the single-label staining, the sections were incubated with antibody against human ERß (N-19, 1:50, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or human placental aromatase (R-8-1, 1:1000, generously supplied by Dr. Y. Osawa) for 48 h at 4°C. The specificities of these antibodies were shown in the following studies: ERß (Kruijver et al., 2003) and aromatase (Ishimura et al., 1989) antibodies. After brief washes with PBS, sections were treated with a biotinylated second antibody for 24

h at 4°C, and then processed with the avidin-biotin complex peroxidase method (ABC elite kit, Vector Laboratories, Burlingame, CA, USA). The immunoreactive sites were visualized with a solution of 0.003% H2O2, 0.6 mg/ml 3, 3'-diaminobenzidine tetrahydrochloride (DAB-buffer tablets, Merck, Darmstadt, Germany). The adjacent sections were stained by the Nissl-staining method with cresyl violet (Merck, Darmstadt, Germany) for cytoarchitectonic reference.

For the double-label immunofluorescence to identify colocalization of ERß with one of the calcium binding proteins, parvalbumin (PV) , calretinin (CR) or calbindin (CB), ERß immunoreactivity was detected using a tyramide signal amplification kit (TSA Perkin Elmer, Boston, MA, USA) after endogenous peroxidase activity was reduced with 3% $H_2O_2$ in PBS for 30 min. Then, the sections were incubated with diluted antibody for PV (clone PARV-19, 1:2000, Sigma, Saint Louis, MO, USA), CR (clone 6B3, 1:2000, Swant, Bellinzona, Switzerland) or CB (clone 300, 1:2000, Swant, Bellinzona, Switzerland) for 48 h at 4°C and immunoreactivities were visualized with second antibody conjugated to AlexaFluor-546 (1:200, Molecular Probes, Eugene, OR, USA). Immunolabeled images were directly

captured by an Olympus BX-50 fluorescence microscope connected to a digital camera Olympus DP20 (Olympus Corporation, Tokyo, Japan). Confocal images were obtained with a LSM5 Pascal laser-scanning microscope (Carl Zeiss, Jena, Germany).

*2.4 Semi-quantitative image analysis*

The degree of immunoreactivity for ERß and aromatase in the subiculum was expressed as the integrated optical density (IOD; the product of optical density and area) by using computer-assisted image analysis (NIH ImageJ; http://rsb.info.nih.gov/nih-image/). We identified the subiculum from adjacent Nissl-stained images. The specified subicular region on the gray scale image stained for ERß was processed with thresholding filter (maximum entropy) to select ERß-immunostained pixels for IOD calculation. The number of ERß-immunoreactive cells was also counted manually in each subicular region. These analyses for ERß were performed in five sections of each animal. For the aromatase immunoreactivity, two different fields from a section, three sections from each animal, including pyramidal neurons homogeneously in the

subiculum were analyzed. Unlike ERß immunoreactivity, aromatase immunoreactivity showed uniform cellular and subcellular localization in all the four monkey groups. In order to precisely evaluate the intensity of aromatase immunostaining in individual cells, 30 pyramidal cells with relatively large cross-section (minor axis length of the soma > 9 μm) were selected in a field and delineated manually. The mean IOD value of the selected cells in an image was calculated. ERß-immunoreactive neurons colocalizing with PV were counted manually in two different fields within the merged fluoroscopic subiculum area. The analysis for ERß/PV colocalization was performed in three sections of each animal. All results were statistically analyzed with one-way analysis of variance followed by the Games-Howell test for multiple comparisons. Differences were considered statistically significant if the $p$ value was less than 0.05.

## 3. Results

### *3.1 Hormonal profile at each reproductive stage*

The premenopausal monkeys exhibited regular menstruation and estrogen, progesterone and LH surges until they were sacrificed (Fig. 1). In ovariectomized monkeys, the mean circulating estrogen level dropped drastically to below one-sixth the pre-ovariectomy level within 4 days and the LH level became elevated to over six-fold within 8 days after the operation. Like the ovariectomized monkeys at postoperation, the perimenopausal monkeys exhibited low estrogen and high LH levels with no menstrual bleeding, in spite of having shown irregular menstruation during the previous year. Individual endocrine backgrounds at the time of sacrifice confirmed the reproductive stage of each monkey.

### *3.2 ERβ and aromatase immunoreactivity localizations*

We found conspicuous ERβ immunoreactivity in the hippocampal formation of all monkey groups (Fig. 2a). Within the hippocampal formation, ERβ was confined to the plasma membrane and perikaryon, but

not to the nucleus. Membranous ERß immunoreactivity was most intensely observed throughout the subiculum (Fig. 2c). These subicular ERß-immunoreactive neurons were found to consist of interneurons of fusiform or multipolar type that could be distinguished from typical pyramidal neurons. In addition, ERß-immunoreactive neurons were located in the stratum oriens of the Ammon's horn (Fig. 2e). To characterize these ERß-immunoreactive neurons, double immunofluorescence histochemistry was carried out for calcium-binding proteins: PV, CR and CB. A large population of ERß-immunoreactive neurons exhibited PV immunoreactivity in the hippocampal formation (Fig. 3a, b). Within the subiculum, approximately 70 to 85% of the total ERß-immunoreactive neurons were co-localized with PV in each of the premenopausal, ovariectomized, perimenopausal and postmenopausal monkey groups, and about 75 to 80% of the total PV-immunoreactive neurons were co-localized with ERß. Throughout the hippocampal formation, no ERß-immunoreactivity was found to be co-localized with CR or CB in a cell (Fig. 3c, d).

The immunoreactivity of aromatase, the enzyme responsible for local estrogen biosynthesis, was present homogeneously in pyramidal neurons and interneurons extending from Ammon's horn to the subiculum (Fig. 2b, d, f).

*3.3 Menopause-related changes in ERß and aromatase immunoreactivities*

In the ovariectomized monkeys, our quantitative analysis showed that the intensity of ERß immunoreactivity was similar to that in the premenopausal group (Fig.4a). In the premenopausal and ovariectomized monkeys, ERß immunoreactivity was localized primarily in somatic membrane sites and, to a lesser extent, in proximal dendrites. By contrast, the intensity of aromatase immunoreactivity in the subiculum was significantly higher in the ovariectomized monkeys than in the premenopausal monkeys ($p < 0.01$, Fig. 4b). Upregulation after ovariectomy and a lack of age-dependent change in aromatase expression were also observed in other subregions of the hippocampus, such as CA1.

In both the perimenopausal and postmenopausal monkeys, on the other hand, the ERß levels were highly elevated as compared to those in

the premenopausal monkeys ($p < 0.05$, Fig. 4a). The number of ERß-immunoreactive neurons, however, was not altered with menopause (the mean cell number ± standard error per $mm^2$ subicular area, premenopausal: 365.5 ± 19.0, ovariectomized: 338.6 ± 18.6, perimenopausal: 362.5 ± 21.9, postmenopausal: 345.0 ± 13.9; one-way analysis of variance, $p = 0.708$). Upregulation of ERß immunoreactivity of subicular interneurons in perimenopausal and postmenopausal monkeys was observed in somatic membranes and dendrites (both proximal and distal) compared with that in premenopausal monkeys. Meanwhile, aromatase immunoreactivity in the subiculum of the perimenopausal and the postmenopausal monkeys was similar to that of the premenopausal monkeys (Fig. 4b). Size of aromatase immunoreactive soma in the postmenopausal monkeys was significantly smaller than that of premenopausal and ovariectomized monkeys (the mean soma sizes ± standard error ($\mu m^2$), premenopausal: 183.81 ± 4.59, ovariectomized: 194.57 ± 4.24, perimenopausal: 184.01 ± 5.53, postmenopausal: 167.00 ± 3.13).

## 4. Discussion

Here we report that ERß expression is prominent in the subiculum of the hippocampal formation of menopausal monkeys. In the present study, ERß immunoreactivity in the hippocampus was identified at the plasma membrane of non-pyramidal neurons. The estrogen receptors are classically well known to exert genomic actions through translocation into the cell nucleus from the cytoplasm when they bind to 17ß-estradiol (McKenna and O'Malley, 2002). Both ERα and ERß also exert non-genomic rapid action to regulate cell signaling pathways, including calcium ion channels and kinase signaling pathways (Raz et al., 2008). ERß expression at the plasma membrane has been convincingly confirmed by electron microscopy and time lapse microscopy (Milner et al., 2005; Sheldahl et al., 2008). Rapid action of membranous ERß was confirmed with the study using membrane-impermeable estradiol conjugated to bovine serum albumin and selective ERß agonist (Wu et al., 2011). Such rapid action may be exerted in the monkey hippocampus.

Our finding that many of the ERß-immunoreactive neurons co-expressed PV in the subiculum of monkey hippocampal formation was

consistent with the study in the rat (Blurton-Jones and Tuszynski, 2002). It has been well-known that estrogen affects the mRNA expression of a GABA transporter and a GABA-synthesizing enzyme, glutamic acid decarboxylase (Herbison et al., 1995; McCarthy et al., 1995). In the CA1 of hippocampus, estrogen reduces GABA synthesis in inhibitory interneurons that regulates pyramidal neurons (Murphy et al., 1998). Our study suggests that ERβ is concerned with regulation of GABAergic systems in the subiculum, which sends excitatory output signals from the hippocampus to widespread areas.

Aromatase localized in hippocampal neurons synthesize 17β-estradiol (Hojo et al., 2004). Aromatase expression in the hippocampus is confirmed immunohistochemically in both cycling and ovariectomized monkeys (Yague et al., 2008) and in menopausal humans (Ishunina et al., 2007). We confirmed aromatase expression in all groups examined.

Our study showed that ovariectomized monkeys retained an ERβ level in the subiculum equivalent to that of premenopausal monkeys. Alternately, immunoreactivity for aromatase was upregulated in the subiculum of ovariectomized monkeys. In contrast to ovariectomized

monkeys, perimenopausal and postmenopausal monkeys exhibited obvious upregulation of ERß with unaltered aromatase expression in the subiculum of hippocampal formation. Though both ovariectomized and menopausal monkeys experienced peripheral estrogen depletion, reactive regulation in the expression of aromatase and ERß was quite different. Though difference in the senescence of the brain may cause these differences, molecular mechanism remains unclear.

In contrast to our results, aged rats showed significant downregulation of ERß in the hippocampus (Mehra et al., 2005). Middle aged rats exhibit the opposite endocrinological status (i.e. high estrogen level and low LH level) to perimenopausal macaques (low estrogen level and high LH level), though prolonged cycles and gradual cessation of estrus of middle aged rats are similar to characteristics in human or macaque menopausal transition (Chakraborty and Gore, 2004). In aged rats, high estrogen level from ovary may bring about the decrease of ERß expression in the hippocampus. Thus, in both aged monkeys and rats, ERß expression in the hippocampus seems to keep homeostasis in response to age-related changes in the level of ovary-derived estrogen.

It has been reported that postmortem brains developing AD showed apparent upregulation of ERß in the CA1-4 regions of the hippocampus compared to age-matched control patients (Savaskan et al., 2001). The upregulation of ERß in the hippocampus of AD patients and our menopausal monkeys may be induced to enhance the neuroprotective effect of estrogen. In the hippocampus of healthy menopausal women, aromatase immunoreactivity were upregulated (Ishunina et al., 2007), which is similar to our finding in the hippocampus of ovariectomized monkeys rather than that of menopausal monkeys. Similarity in the response of aromatase against estrogen depletion between healthy menopausal humans and ovariectomized premenopausal monkeys is consistent with a hypothesis that healthy aging of the brain is one of the features of brain evolution in hominids (Allen et al., 2005). We assume that adequate level of estrogen actively supplied with aromatase in the hippocampus may be crucial for the aged women to keep brains healthy for the longest life expectancy.

Acknowledgments

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Figure Legends

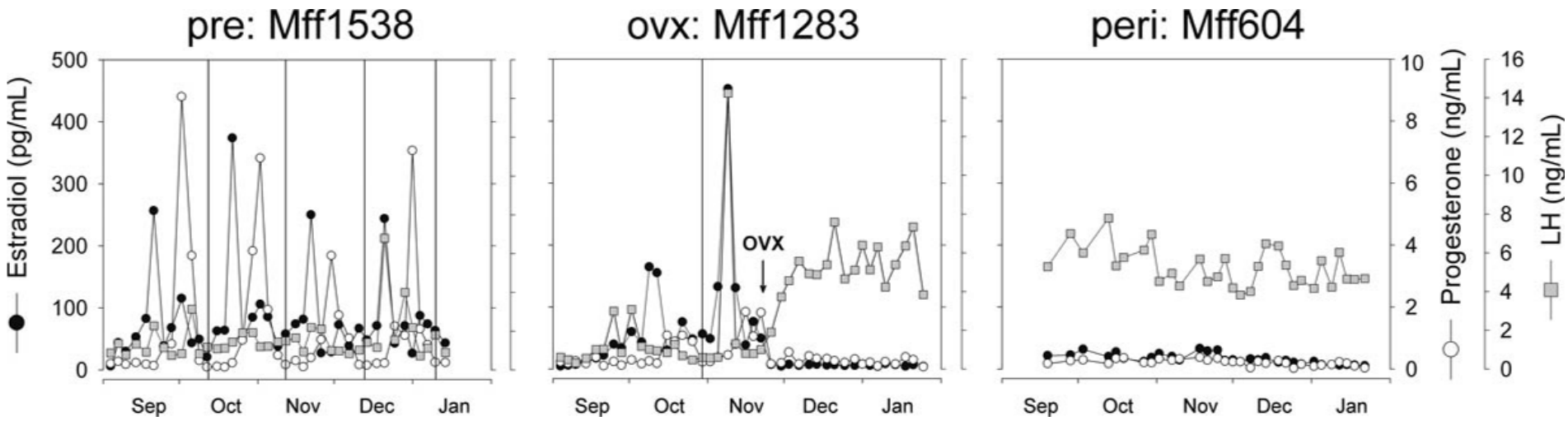
Fig.1. Representative hormonal profiles of premenopausal (pre), ovariectomized (ovx), and perimenopausal (peri) monkeys from September to January. Solid vertical bars indicate the first day of menstruation, and an arrow indicates the day of ovariectomy. The hormonal profile of the ovx monkey after the operation was similar to that of the perimenopausal monkey. Filled circles: estradiol, open circles: progesterone, gray squares: luteinizing hormone (LH).

Fig. 2. ERß-immunoreactivity (ir; a, c, e) and Aromatase (Arom)-ir (b, d, f) in the hippocampal formation. Membranous ERß-ir was observed in fusiform and multipolar interneurons in the subiculum (Sub; c) and in the stratum oriens of CA1 (e) of the hippocampus.. Aromatase (Arom)-ir was also observed in the Sub (d) and CA1 (f). ERß-ir and Arom-ir were present in the hippocampus of all the examined groups. Bars = 500 µm (a, b) and 50 µm (c-f), respectively.

Fig. 3. Colocalization of ERß and calcium binding proteins in single cells are confirmed with z-projection of the confocal image stacks. Nearly all of ERß (green) was colocalized with parvalbumin (PV, red) in the subiculum (a, b). Double-immunofluorescence for ERß (green) and calretinin (CR, red in c) or calbindin (CB, red in d) in the subiculum failed to demonstrate colocalization. Bars = 20 μm (a, c, d), 50 μm (b)

Fig.4. Integrated optical density (IOD) for semi-quantified ERß (a) and Arom (b)- ir in the subiculum of ovariectomized (ovx), perimenopausal (peri) and postmenopausal (post) monkeys is represented as the ratio to that of premenopausal (pre) monkeys. Note the ERß-ir was prominently elevated in peri and post monkeys, and Arom-ir was elevated in ovx monkeys compared with pre monkeys. Each column indicates an index ± standard error. Bars with different superscripts differ significantly (a : b, $p < 0.05$). Photographs are representative clipped images used for the image analysis at each reproductive status. Bars = 50 μm

Fig. 1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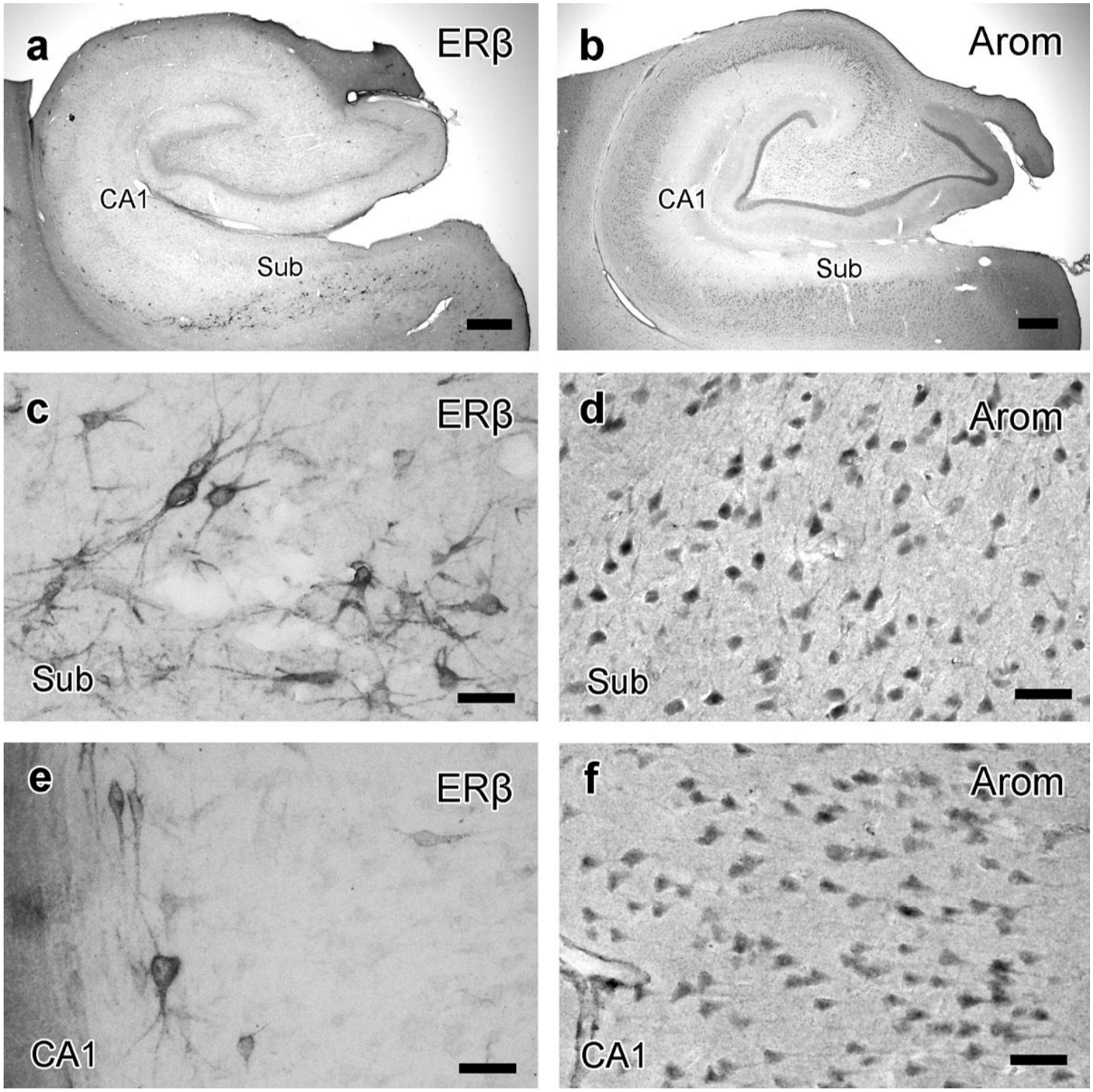
Progesterone (ng/mL)
LH (ng/mL)
OVX
Sep
Oct
Nov
Dec
Jan

Fig. 2


a
ERβ
CA1
Sub
b
Arom
CA1
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c
ERβ
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d
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e
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CA1
f
Arom
CA1

Fig. 3

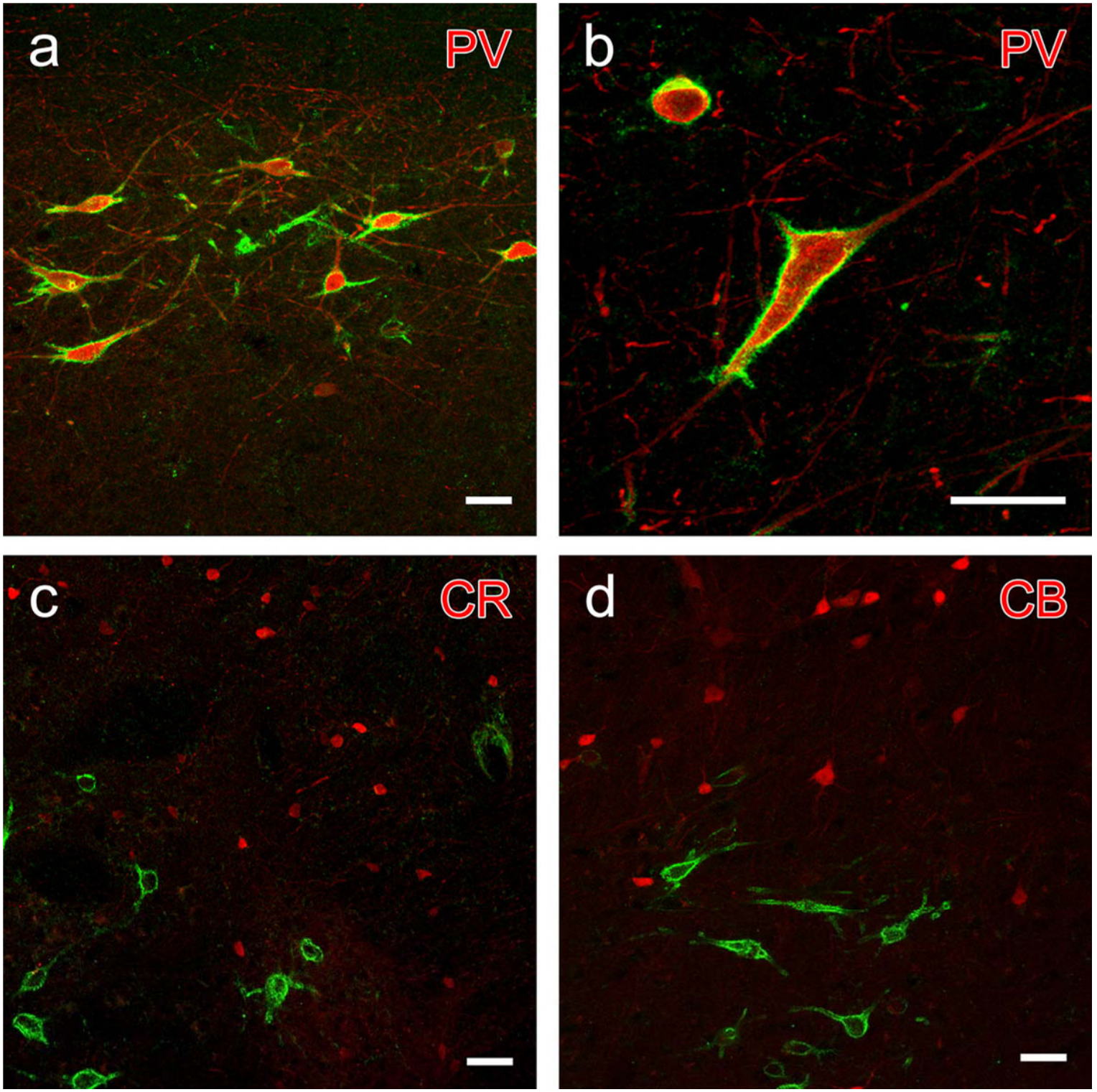
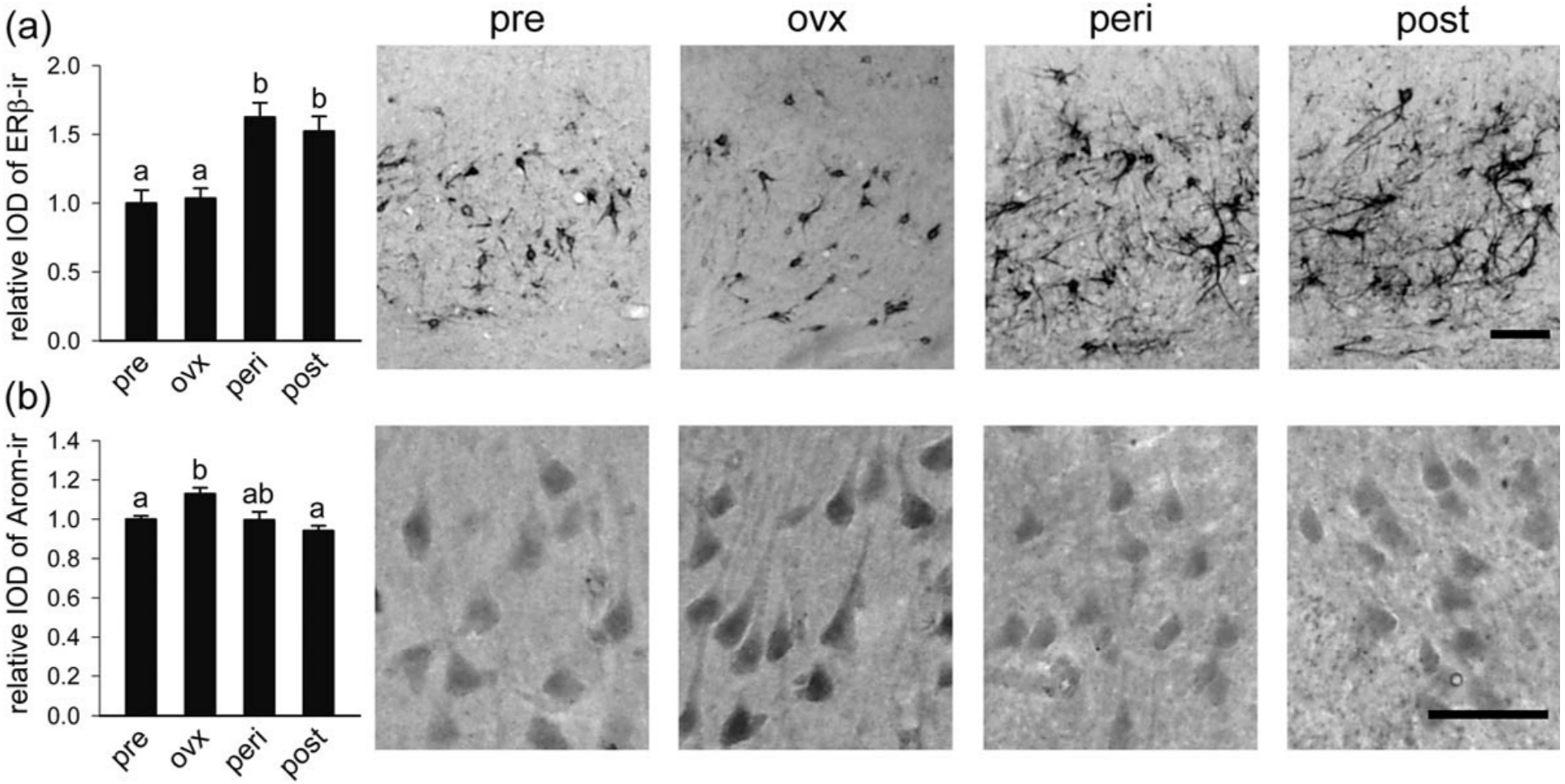


Fig. 4


(a)
relative IOD of ERβ-ir
2.0
1.5
1.0
0.5
0.0
a
a
b
b
pre
ovx
peri
post
(b)
relative IOD of Arom-ir
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
ab
a
pre
ovx
peri
post

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