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## G-protein Coupled Estrogen Receptor, Estrogen Receptor $\alpha$ , and Progesterone Receptor Immunohistochemistry in the Hypothalamus of Aging Female Rhesus Macaques Given Long-Term Estradiol Treatment

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### Abstract

Steroid hormone receptors are widely and heterogeneously expressed in the brain, and are regulated by age and gonadal hormones. Our goal was to quantify effects of aging, long-term estradiol (E<sub>2</sub>) treatment, and their interactions, on expression of G protein-coupled estrogen receptor (GPER), estrogen receptor  $\alpha$  (ER $\alpha$ ) and progesterone receptor (PR) immunoreactivity in two hypothalamic regions, the arcuate (ARC) and the periventricular area (PERI) of rhesus monkeys as a model of menopause and hormone replacement. Ovariectomized (OVX) rhesus macaques were young (~11 years) or aged (~25 years), given oil (vehicle) or E<sub>2</sub> every 3 weeks for 2 years. Immunohistochemistry and stereologic analysis of ER $\alpha$ , PR, and GPER was performed. More effects were detected for GPER than the other two receptors. Specifically, GPER cell density in the ARC and PERI, and the percent of GPER-immunoreactive cells in the PERI, were greater in aged than in young monkeys. In addition, we mapped the qualitative distribution of GPER in the monkey hypothalamus and nearby regions. For ER $\alpha$ , E<sub>2</sub> treated monkeys tended to have higher cell density than vehicle monkeys in the ARC. The percent of PR density in the PERI tended to be higher in E<sub>2</sub> than vehicle monkeys of both ages. This study shows that the aged hypothalamus maintains expression of hormone receptors with age, and that long-term cyclic E<sub>2</sub> treatment has few effects on their expression, although GPER was affected more than ER $\alpha$  or PR. This result is surprising in light of evidence for E<sub>2</sub> regulation of the receptors studied here, and

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differences may be due to the selected regions, long-term nature of E<sub>2</sub> treatment, among other possibilities.

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Reproductive aging in females is highly divergent among mammalian species. Menopause is limited to those few species that menstruate (humans, great apes, and some non-human primates); it is a natural transition to reproductive senescence associated with decreased levels of the sex steroid hormones estradiol (E<sub>2</sub>) and progesterone (P<sub>4</sub>) (Trévoux et al., '86; Burger et al., 2002). Estrogens and progestins are not only critical for reproduction, but also play significant roles in the normal functioning of brain networks, cardiovascular systems, bone maintenance, and many others (Baulieu and Robel, '90; Inoue, 2002; McEwen, 2002). In women, the menopausal decline in circulating hormones is often accompanied by symptoms that have a dramatic negative impact on quality of life such as mood alterations, sleep disruptions, increased risk of osteoporosis and more. There are many available treatments for mitigation of menopausal symptoms, the most common being hormone replacement therapy (HRT) containing estrogens, or estrogens in combination with progestins. The risks versus benefits of health outcomes are highly controversial (Herrinton and Weiss, '93; Fitzpatrick et al., 2000; Rossouw et al., 2002; Canonico et al., 2008; Talboom et al., 2008; Prentice et al., 2009; Terauchi et al., 2012; Manson et al., 2013), with differential results due in part to variations in hormone formulations and timing/duration of hormone treatment relative to the menopausal transition.

Non-human primates undergo many similar neurobiological (functional and cellular) and physical (e.g., osteoporosis, metabolic) alterations with menopause as in women (Hao et al., 2003, 2007; Rapp et al., 2003; Maffucci and Gore, 2006). Furthermore, mammalian species that do not menstruate may also undergo a loss of reproductive capacity with aging, often very differently from primates due to those species' unique reproductive properties such as strong seasonal breeding period, estrous cycles or induced ovulation (as opposed to spontaneous ovulation and reproductive cycles), and other reproductive traits (Maffucci and Gore, 2006; Kermath and Gore, 2012). Although there may be variability, a conserved property across species is that reproductive senescence involves the three levels of the hypothalamic-pituitary-gonadal (HPG) axis. Declines in hypothalamic function precede ovarian failure in rodents and primates, although the ovary may play a more primary role in women (Wise, '84; Richardson et al., '87; Gougeon et al., '94; Gore et al., 2000; Gill et al., 2002a,b; Weiss et al., 2004; Downs and Wise, 2009). While age-related changes in positive and negative feedback on gonadotropin-releasing hormone (GnRH) and gonadotropin release clearly occur in rodents, the evidence is less clear for both non-human and human primates (Van Look et al., '77; Wise and Ratner, '80; Gore et al., 2000; Tsai et al., 2004; Hall, 2007; Downs and Wise, 2009; Rance, 2009; Shaw et al., 2011). The neurons that synthesize GnRH are modulated by ovarian hormonal feedback both directly and indirectly via steroid hormone receptors, including G protein-coupled estrogen receptor (GPER), estrogen receptor  $\alpha$  (ER $\alpha$ ), and progesterone receptor (PR), among others (Van Look et al., '77; Liu and Yen, '83; Sullivan et al., '95; Terasawa, '95; Skinner et al., '98; Wilson et al., 2002; Dorling et al., 2003; Petersen et al., 2003; Glidewell-Kenney et al., 2007). An important research gap is whether, and how, hormone feedback on the hypothalamus may change with aging, and on which cells these effects are mediated. The mechanism for these

changes is also unclear, although it may involve age-related change in expression or function of the steroid hormone receptors that mediate steroid hormone effects [reviewed in Chakraborty and Gore (2004)].

In the current study, we addressed this question in female monkeys as a translational model for the neurobiology of menopause in women (Gilardi et al., '97; Kaplan, 2004). Rhesus monkeys have 28-day menstrual cycles and undergo natural reproductive senescent changes that mirror the human menopausal transition, albeit much later in life (Krey et al., '75; Gilardi et al., '97; Archer, 2004; Gore et al., 2004). We focused our work on two sub-regions of the hypothalamus involved in HPG function in primates, the arcuate nucleus (ARC) and the periventricular region (PERI), which are integral to reproduction, growth, thermoregulation and metabolism (Wiegand and Terasawa, '82; Hofman, '97; Downs and Wise, 2009; Castellano et al., 2010; Mittelman-Smith et al., 2012). These hypothalamic areas also express high levels of steroid hormone receptors and are important targets of E<sub>2</sub> feedback in the regulation of the HPG axis (Bethea et al., '96; Skinner et al., '98; Blurton-Jones et al., '99; Mills et al., 2002; Petersen et al., 2003; Rapp et al., 2003; Tsai et al., 2004; Michael et al., 2005). To determine whether there are age-related changes in steroid hormone receptors, and altered responses of these receptors to E<sub>2</sub> feedback, we quantified the density and percentage of cells that express GPER, ER $\alpha$ , and PR in the ARC and PERI of young and aged macaques that were ovariectomized (OVX) and given E<sub>2</sub> or vehicle treatment for 2 years. Because relatively little is known about the distribution of GPER neurons in the adult brain, we also mapped their localization across the hypothalamus.

## MATERIALS AND METHODS

### Animals

Adult female rhesus macaques (*Macaca mulatta*) from the California National Primate Research Center at the University of California at Davis were used. These animals were part of a larger Program Project Grant collaboration on "Estrogen and the Aging Brain," and first described in (Hao et al., 2003). Table S1 shows animal demographics, age distributions, and age at euthanasia, with 21 young adults (mean age 11  $\pm$  4.4 years) and 15 aged adults (mean age 24.9  $\pm$  2.2 years). They were singly housed to enable collection of daily urine samples, with water available ad libitum, monkey chow provided in excess of nutritional needs, and regularly supplemented with fresh fruit. Candidate monkeys, classified as either pre- or perimenopausal by their reproductive history, had not been used in any invasive or pharmacological analyses before this study. They underwent behavioral assessment of learning and memory for companion studies (Hao et al., 2003, 2006, 2007; Rapp et al., 2003; Wang et al., 2010). All experimental procedures were approved by Institutional Animal Care and Use Committees at the University of California-Davis and the Mount Sinai School of Medicine and conformed to National Institutes of Health guidelines.

### Ovariectomy and Estradiol Replacement

All animals were bilaterally ovariectomized (OVX) and randomly assigned to vehicle or estradiol treatment groups. The post-OVX recovery period was ~3 months to ensure that all animals had a consistent estrogen-free baseline prior to experimentation, as verified by

urinary estradiol assays. After this 3-month recovery, treatment was initiated, with an intramuscular injection given every 3 weeks. Treatments were estradiol cypionate (E<sub>2</sub>C; 100 µg/ml in sterile peanut oil, Pharmacia, Peapack, NJ) in 13 young (YE) and 7 aged (AE) monkeys, or vehicle (peanut oil) in 8 young (YV) and 8 aged (AV) monkeys. Some additional animals (not included in Table S1) were excluded after the study started due to illnesses, sudden death or incomplete OVX. The efficacy of E<sub>2</sub>C injections was previously demonstrated by a rapid rise in circulating E<sub>2</sub> that peaked within 24 h, at levels comparable with the preovulatory surge in intact female monkeys and similar to women (Oriowo et al., '80; Rahimy et al., '99; Shideler et al., 2003; Hao et al., 2007). Treatment duration was 2–3 years with all injections coded and administered in a blind fashion.

### Perfusion and Tissue Processing

Twenty-four hours after the last E<sub>2</sub>C or vehicle injection, animals were deeply anesthetized and perfused as per (Hao et al., 2003). Fixative was 1% paraformaldehyde (PFA) for 1 min, then 4% PFA for 12 min in phosphate buffered saline (PBS) with post-fixation for 6 hr in 4% PFA. At the time of euthanasia, at 24 hr post-E<sub>2</sub> monkeys were expected to be in the negative feedback phase of steroid feedback onto the HPG axis (Oriowo et al., '80; Terasawa, '95; Rahimy et al., '99). Group assignment was validated at perfusion, resulting in the exclusion of two vehicle animals that had residual ovarian tissues. After perfusion, the hypothalamus was dissected, and shipped in PBS to the University of Texas at Austin. Other tissues were allocated to other investigators and collaborators. Hypothalamic tissues used for the current study were coronally sectioned with a vibrating microtome (VT 1000s; Leica Instruments, Nussloch, Germany) at 40 µm, cryoprotected, and stored at –20°C for subsequent immunohistochemistry and light microscopy studies.

### Immunohistochemistry

Immunohistochemistry was performed for each of three receptor proteins, using a 1:10 series sections through the ARC and PERI regions of each monkey, when available. Some of the hypothalamic blocks were damaged during dissection or sectioning, therefore, not all of the animals were represented for all three of the receptor analyses. On average, five and seven sections were used per monkey for the ARC and PERI, respectively. Final n's represent monkey numbers (not number of tissue sections) and are shown in group data graphs; they represent the maximum number of monkeys for each antibody for which a complete series could be collected. Attrition was due primarily to poor tissue quality, and was randomly distributed across groups. The same protocol was used to quantify each of the hormone receptors, with the exception of primary antibody concentration. All steps were carried out at room temperature with constant agitation, unless otherwise indicated. PBS washes were performed prior to all steps, with the exception of the primary antibody. The tissue was treated to quench endogenous peroxidase activity for 20 min (30% of 3:1 methanol: 3% H<sub>2</sub>O<sub>2</sub> in PBS) and to block non-specific binding for 1 hr (10% normal goat serum (NGS), S-1000, Vector Laboratories, Burlingame, CA, USA; 2% bovine serum albumin, A9085, Sigma-Aldrich, St. Louis, MO, USA; 0.5% triton X, in PBS) prior to incubating in one of the polyclonal primary antibodies for 48 hr (anti-GPER [1:4,000] a gift from Dr Edward Filardo, anti-ERα [1:10,000] Santa Cruz Biotechnology (Santa Cruz, CA, USA), cat# HC-20 sc-543, and anti-PR [1:20 from ready-to-use pre-diluted stock]

MyBioSource (San Diego, CA, USA), cat# MBS300415). All antibodies were raised in rabbit and directed against the C-terminus of the corresponding human hormone receptor. Tissues were then incubated in biotinylated goat anti-rabbit secondary antibody for 1 hr (1:600; BA-1000; Vector Laboratories, in 5% NGS) and avidin-biotin-peroxidase complex for 1 hr (PK 6200; Vector Laboratories). Target was visualized with a 3,3'-diaminobenzidine (DAB)/peroxidase reaction (SK-4100; Vector Laboratories) for GPER and ER $\alpha$ , and nickel-DAB for PR, on ice. Sections were mounted on glass slides, dried overnight, and counterstained with methyl green (0.5% in 0.1 M Na acetate buffer pH 4.2, MP Biomedicals LLC, Solon, OH, USA), a nucleic acid stain (Kurnick, '50), to visualize the nuclei of all cells before affixing the cover glass with DPX (44581; Fluka, Steinheim, Germany). The GPER antibody, described by Filardo et al., has previously been validated via peptide block in rat and western blot in both rat and monkey (Filardo et al., 2000, 2007; Noel et al., 2009; Hammond et al., 2011; Kenealy et al., 2011a). Prior validation of the ER $\alpha$  antibody was carried out via Western blot in human (Long and Nephew, 2006; Gorosito et al., 2008) and peptide block in monkey (Wang et al., 2010). Although we could not perform preadsorption controls or Western blots of the PR antibody (the vendor does not disclose the antigen sequence, and the antibody is not compatible with western blots), this antibody was previously utilized in the California mouse, *Peromyscus californicus* (Fuxjager et al., 2010), and that study showed nuclear labeling very similar to that found in our macaque hypothalamic tissues. Further, the PR antibody cellular localization (nuclear) and distribution in the nervous system herein matches that of other PR antibodies as published in monkey and rats (Bethea and Fahrenbach, '92; Bethea et al., '96; Quadros and Wagner, 2008; Quadros et al., 2008; Furuta et al., 2010). All IHC runs contained negative-control sections that excluded the primary antibody and had no immunoreactivity.

### Stereological Analysis

StereoInvestigator software (MicroBrightField Bioscience, Williston, VT, USA) and an Olympus BX-61 microscope were used for quantification. The optical fractionator method was used (Gundersen and Jensen, '87; West et al., '91; Glaser and Wilson, '98), with parameters appropriate to achieve coefficients of error (CEs) no greater than 0.12 (Gundersen  $m = 1$ ). Abbreviations and terminology used throughout were taken from a combination of three atlases and modified to reflect most current usage (Bleier, '84; Paxinos et al., '90, Brainmaps.org). Counter-stained cells were used to identify the borders of the ARC and PERI according to neuroanatomical landmarks found in two atlases of the rhesus macaque brain (Bleier, '84; Paxinos et al., '90). The boundaries of the ARC were the median eminence (ventral), 3rd ventricle (medial), ventromedial hypothalamus and medial preoptic area (lateral and dorsal), and the anterior hypothalamic area and paraventricular nucleus PVN; (dorsal). The PERI was caudal to the ventral diagonal band; medial to the medial preoptic nucleus and anterior hypothalamic area; lateral to the 3rd ventricle; and ventral to the PVN. Note that our PERI area is a composite from the atlases and is slightly more extensive based on methyl green density. Immunoreactive (IR) cells were counted at the following magnification: GPER 40 $\times$ , ER $\alpha$  100 $\times$ , PR 100 $\times$ , and methyl green labeled cells at 40 $\times$ . Cell density (population estimate of IR cells/volume sampled  $\mu\text{m}^3$ ) is reported, rather than total population, due to an uneven number of sections per region between animals due to tissue damage/loss. The percentage of IR cells was further calculated (population estimate

of IR cells/population estimate of all cells), in the respective region, for each individual. Additionally, we used the Nucleator method in conjunction with optical fractionator to measure the cross-sectional area of GPER cell bodies (six rays, centered in the middle of nucleus at the widest part of each cell) (Gundersen et al., '88; Korbo and Andersen, '95). The percent of cells that fell within size bins was found for each animal and the average of the group is presented. Animals were excluded only if there were fewer than three sections containing the corresponding region. All slides were labeled with a random code and were quantified blind to group.

### Statistical Analysis

All analysis was done using R statistical packages (Fox and Weisberg, 2011; R Development Core Team, 2012). Due to small sample sizes, high intra-group variability, and non-normal data distribution (analysis as determined by the Shapiro–Wilks normality and Levene's equality of variance tests), we used non-parametric tests for analysis. Kruskal–Wallis ( $P$ ) was used to determine significant main effects [ $P \leq 0.05$ ], followed by a post-hoc test using pairwise Wilcoxon (W) with a Benjamini and Hochberg false discovery rate correction for multiple comparisons (Wilcoxon, '45; Kruskal and Wallis, '52; Shapiro and Wilk, '65; Hochberg and Benjamini, '90; Benjamini and Hochberg, '95). Correlations between all variables were calculated using the Pearson coefficient, also followed by a Benjamini and Hochberg correction. Outliers were determined with the Grubbs-test, using a cut off of  $2.5 \times$  the standard deviation from mean of all animals, for each receptor and within each region, regardless of group. Only significant [ $P \leq 0.05$ ] and non-significant trends [ $0.05 < P \leq 0.10$ ] are reported here. We opted to present trends because of the low power and the variability of monkeys. Table S2 lists the  $P$ -value results of all statistical tests.

## RESULTS

Both ER $\alpha$  and PR were densely expressed in both the ARC and PERI of young and aged female rhesus macaques, while GPER was less dense. Examples of the boundaries of the ARC and PERI, and immunolabeling of each receptor in the two regions of a representative monkey, are provided in Figure 1.

### GPER

GPER immunoreactivity was observed on cell processes and in perikarya (Fig. 2) and was widely dispersed throughout the hypothalamus and surrounding regions. It is noteworthy that cellular labeling was relatively light in the ARC and PERI (Figs. 1 and 2), as well as the PVN (another hypothalamic region) compared to other non-hypothalamic regions with darker labeling, shown for basal nucleus of Meyert (BNM), bed nucleus of the stria terminalis (BNST), and the substantia innominata (SI; Fig. 2). The qualitative distribution and density of cells is shown in Table 1 and high-resolution images are available in Figure S1. Stereological counting of GPER-IR cells in the PERI demonstrated significant main effect of age, with aged animals having a greater density ( $P = 0.01$ ) and a trend for a higher percentage of GPER ( $P = 0.06$ ) regardless of treatment (Fig. 3). Post-hoc analysis revealed that AE monkeys had significantly higher density and % GPER-IR cells than YE monkeys ( $W = 0.01$ ,  $W = 0.03$ , respectively). There was also a trend of AE animals having a greater

density of GPER-IR than YV ( $W = 0.10$ ). In the ARC, there was a trend for an age effect, with older animals having greater GPER-IR density than young ( $P = 0.07$ ).

During the analysis of GPER we noticed that many of the young animals had very small GPER-IR cells (defined as  $<150 \mu\text{m}^2$ ), while aged monkeys had fewer such cells. We quantified cell cross-sectional area using the Nucleator tool in StereoInvestigator. Figure 4 shows the distribution of GPER size, divided into  $50 \mu\text{m}^2$  bins, for both regions. In the ARC, aged animals tended to have fewer cells between  $100$  and  $149 \mu\text{m}^2$ , with  $YE > AE$  and  $YE > AV$  ( $P = 0.07$ ,  $W = 0.08$ ,  $W = 0.09$ , respectively) and aged monkeys tended to have more cells between  $200$  and  $249 \mu\text{m}^2$  than the young, regardless of treatment ( $P = 0.09$ ). In the PERI, young animals tended to have more cells between  $50$  and  $99 \mu\text{m}^2$  than aged ( $P = 0.07$ ), post-hoc analysis showed that this was driven by YE having a greater percentage than AE or AV ( $P = 0.07$ ,  $P = 0.07$ , respectively). Additionally, within cells between  $150$ – $199 \mu\text{m}^2$ , there was a trend for an effect of treatment and group ( $P = 0.09$  and  $P = 0.06$ , respectively). This was driven by YE having fewer cells of this size than YV, AV, or AE ( $W = 0.02$ ,  $W = 0.03$ ,  $W = 0.07$ , respectively). Finally, in the PERI, it was noteworthy that the YV group had no cells between  $350$  and  $400 \mu\text{m}^2$ , and only 1 cell over  $400 \mu\text{m}^2$ .

### ER $\alpha$

ER $\alpha$  immunoreactivity was predominantly detected in cell nuclei, but some cytoplasmic and/or membrane labeling was observed in the ARC and PERI (Fig. 1). In the ARC, there was a trend for a treatment effect ( $P = 0.08$ ) driven by YE having greater ER $\alpha$ -IR cell density than YV ( $W = 0.05$ ; Fig. 5). No significant effects of aging, treatment, or interactions, were found for the density or percent of ER $\alpha$ -IR cells in the PERI (Fig. 5).

### PR

PR was almost exclusively nuclear in cellular localization (Fig. 1). There was a trend for a treatment effect in the PERI with YE having a higher percentage of PR-IR cells than AV ( $P = 0.10$ ,  $W = 0.08$ ), but no effects on the density (Fig. 6). In the ARC, no effects of age, treatment or interaction were found in the density or percent of PR-IR.

### Total Cell Density in ARC and PERI

There were no significant effects of aging, treatment, or interaction in the total number of cells in either the ARC or PERI (see Table S2 for statistical results).

### Correlation Network

Figure 7 depicts the relationship between the percent of GPER, ER $\alpha$  and PR-IR cells, percent large GPER ( $>150 \mu\text{m}^2$ ), body weight, age and number of live births (see Supplemental Table S1). Supplemental Table S2 contains all of the Pearson's correlation coefficients ( $r$ ) and corresponding significance levels ( $P$ ). We excluded cell density and total cells # because they were highly correlated with percent immunoreactive cells and inclusion of all three measures occluded the relationships with the demographic data. Only correlations with  $P \leq 0.10$  were included. The lines indicate the significance level (shade) and correlation coefficient (thickness).

## DISCUSSION

This study investigated the effects of aging, E<sub>2</sub> treatment, and their interactions on the expression of hormone receptors in two hypothalamic regions of female rhesus macaques. Our results extend previous work in several ways. First, the evolutionary conservation of the neurobiology of reproduction, especially similarities in menstrual cycles and hypothalamic circuitry between monkeys and humans, makes macaques an important translational model. Second, the experimental model of long-term, cyclic E<sub>2</sub> given over at least a 2-year period, more closely mimics the therapeutic estrogen treatments used in many perimenopausal women. Third, we focused on ARC and PERI, because of their role in hormone feedback and other neuroendocrine functions (Bethea et al., '96; Skinner et al., '98; Blurton-Jones et al., '99; Mills et al., 2002; Petersen et al., 2003; Rapp et al., 2003; Tsai et al., 2004; Michael et al., 2005). To our knowledge, studies assessing the expression of steroid hormone receptors in a model of aging and long-term E<sub>2</sub> treatment have not been performed in these regions in monkeys. Finally, research on the neurobiological expression of GPER is a relatively new area, with limited data on its expression in the adult and aging hypothalamus. Overall, our results were surprising in that E<sub>2</sub> and aging had limited effects on the numbers of ER $\alpha$  or PR-IR cells in the ARC or PERI of female macaques. By contrast, the GPER system in the PERI, and to a lesser extent the ARC, had higher expression in aging monkeys, as well as an interaction of age and E<sub>2</sub>. Additionally, we identified differences in populations of GPER neurons, with aged animals having a larger proportion of large GPER-IR cells, and fewer smaller cells, than young animals. Finally, we are the first to describe the distribution of GPER throughout the rhesus macaque hypothalamus and adjacent areas. An interpretation of these data is provided below.

### GPER

GPER is a membrane ER that mediates rapid actions of E<sub>2</sub> (Filardo et al., 2002; Noel et al., 2009; Kenealy et al., 2011a,b). GPER is becoming increasingly well-established as involved in metabolic, cardiovascular, and immune physiology (Wang et al., 2008; Prossnitz and Barton, 2011; Sharma and Prossnitz, 2011; Filardo and Thomas, 2012). While expressed in the hypothalamus of monkeys, rats, mice and hamsters (Brailoiu et al., 2007; Canonaco et al., 2008; Hazell et al., 2009), to our knowledge a functional role for GPER in HPG regulation has not yet been identified. In fact, the GPER knockout mouse, to our knowledge, has no reproductive phenotype (Brailoiu et al., 2007; Prossnitz and Barton, 2011). Nevertheless, in monkeys, GPER is expressed in cultured fetal and adult GnRH neurons and mediates rapid GnRH release (Noel et al., 2009; Kenealy et al., 2011a,b). This is consistent with the possibility that GPER plays a role in the E<sub>2</sub>-mediated regulation of the HPG axis. While expressed in hypothalamus (Brailoiu et al., 2007; Canonaco et al., 2008; Hazell et al., 2009; Noel et al., 2009), to our knowledge, quantitative measures of GPER hypothalamic expression and its regulation by estradiol and aging have not been reported in any species prior to our study. Of the three receptors studied herein, GPER was the only one to have robust changes in its expression and morphology with aging and E<sub>2</sub> treatment in both nuclei examined, though we cannot speculate as to the physiological role these changes play. Nevertheless, two observations were potentially most important. First, GPER-IR cell density in the PERI was highest in the AE monkeys. Second, we discovered that young monkeys



have fewer large and more small GPER cells than aged monkeys, raising the possibility that GPER cells undergo hypertrophy with age in the two brain regions examined. It is unknown whether the larger GPER cells represent a novel population of neurons in aging monkeys, or if a subpopulation of GPER neurons undergoes hypertrophy with aging. We favor the latter explanation in light of reports that other neuronal subpopulations in the medial and caudal ARC of humans undergo age-related hypertrophy (Rance et al., '90; Abel and Rance, '99; Rometo and Rance, 2008; Rance, 2009). In our current study, the large GPER cells were predominantly (87%) found in the same area of the ARC. Some of these same neurons that undergo hypertrophy are integral to the regulation of the HPG axis, including those that express kisspeptin, prodynorphin, and neurokinin B. Additionally, to our knowledge, there are no reports of age-related hypertrophy in the PERI region in any animal. Future work should investigate whether these cells also express GPER.

### ER $\alpha$

We chose to examine ER $\alpha$  due to its critical role in reproduction, as demonstrated by the infertility of ER $\alpha$ -knockout mice (Lubahn et al., '93). The only significant finding was that YE had greater ER $\alpha$  density than the YV group in the ARC, an effect not found in the aging monkeys. Previous work in rodents has been mixed, with evidence that E<sub>2</sub> treatment can increase, a decrease or have no effect on ER $\alpha$  mRNA or protein, with differences potentially attributable to species, hypothalamic region, and the nature of E<sub>2</sub> treatment (Lauber et al., '91; Shughrue et al., '92; Blaustein, '93; Olster, '98; Chakraborty et al., 2003). An age-related increase of ER $\alpha$ -IR cell numbers in rats was found in the anteroventral periventricular (AVPV), but not the MPN or ARC, with no effect of short-term E<sub>2</sub> treatment observed in any age group (Chakraborty et al., 2003). Interactions of E<sub>2</sub> and age have also been reported in these and other studies (Funabashi et al., 2000; Chakraborty et al., 2003). For example, Funabashi et al. (2000) found that in young rats, 4 days of E<sub>2</sub> had no effect on ER $\alpha$  mRNA in the preoptic area (POA) and reduced expression in the ARC and ventromedial hypothalamus (VMH). By contrast, middle-aged animals had decreased ER $\alpha$  mRNA with E<sub>2</sub> in the POA only, and old animals showed no effect of E<sub>2</sub> treatment (Funabashi et al., 2000). Aged rats had a decline in ER $\alpha$  mRNA in the periventricular preoptic nuclei, but not in the medial preoptic nucleus or VMN. Differences in results are likely due to species as well as duration of E<sub>2</sub> treatments.

There are limited data on changes to ER $\alpha$  with aging or E<sub>2</sub> in nonhuman primates. In young adult monkeys, Bethea et al. showed no effect of E<sub>2</sub> on ER $\alpha$  mRNA or protein in the ARC, PVN, VMN or pituitary (Bethea et al., '96). In companion studies performed on prefrontal cortex and hippocampus of the same monkeys used in our current study, no changes in ER $\alpha$ -IR cells were found (Adams et al., 2002; Wang et al., 2010). It is therefore not entirely surprising that we found few differences in ER $\alpha$ -IR cell density, and no change in the percentage of these cells in the PERI and ARC, with age or E<sub>2</sub> treatment.

We were unable to perform immunohistochemistry on ER $\beta$  in this study due to the absence of a reliable antibody to detect this protein in perfused brains (Snyder et al., 2010). However, there are data showing that ER $\beta$  is co-expressed in GnRH neurons, and mediates rapid GnRH responses to estrogen treatment in rats (Hrabovszky et al., 2001; Abrahám et

al., 2003). In addition, ER $\beta$  mRNA is expressed in several hypothalamic regions, including paraventricular nucleus, preoptic area, and ventromedial nucleus, in the rhesus macaque brain (Pau et al., '98; Gundlach et al., 2000). Therefore, this is an important area for future studies when a validated ER $\beta$  antibody becomes available.

## PR

The hypothalamic expression of PR, a member of the nuclear hormone receptor family, is integral to normal reproduction. Female PR knockout mice have substantial impairments, including deficits in sexual behavior that indicate that PR in the brain is necessary for normal reproductive functionality (Lydon et al., '96). Although E<sub>2</sub>-mediated up-regulation of PR is well documented, it is age, sex, brain region and species dependent (Blaustein and Turcotte, '89; Bethea and Fahrenbach, '92; Olster, '98; Mills et al., 2002; Quadros and Wagner, 2008; Rometo and Rance, 2008). Here, we saw no significant effects of age or long-term cyclic E<sub>2</sub>, though there was a trend for higher % PR cells in E<sub>2</sub> compared to vehicle monkeys of both ages. The small magnitude of these results may be explained by the low power of this study. In fact, there is considerable variation in expression of PR (and other receptors) across individuals. Considering the variability in life history in monkeys up to 25 years of age (see Supplemental Table S1), this is to be expected, and is translationally relevant to humans.

The literature on PR expression in the hypothalamus of rats is predominantly based on short-term E<sub>2</sub> treatment regimens. Similar to what we observed, other groups showed that young monkeys responded to short-term (28 days) constant E<sub>2</sub> treatment with increases in PR-IR cell numbers and mRNA in some (VMN and ARC), but not all (PVN) hypothalamic regions (Bethea and Fahrenbach, '92; Bethea et al., '96). Another study showed that in the hypothalamus of rats, there is a disconnect between mRNA and protein induction by short-term (4 day) E<sub>2</sub> treatment: Furuta et al. (2010) demonstrated that the increase in PR-IR is attenuated with age, while Funabashi et al. (2000) showed that aged rats maintained a robust PR mRNA response to E<sub>2</sub>. Similar to our results in the ARC, young female rats showed no increase in PR-IR in their VMN after an acute injection of E<sub>2</sub>, while the older females responded with increased PR-immunoreactivity (Quadros et al., 2008). By contrast to our results, there was also a consistent PR induction in the medial preoptic nuclei of rats at both ages (Quadros et al., 2008). Taken together, there is a complex relationship among age, duration of E<sub>2</sub> treatment and PR expression, in many species. Ongoing work in our laboratory utilizing different E<sub>2</sub> regimens and comparing chronic to cyclic E<sub>2</sub> may shed further light on this question. Regardless of future results, current data reported here suggest little change in ARC and PERI PR-IR cells with age and E<sub>2</sub> treatment in our monkey model.

## Correlation Networks

Analyses of relationships among the different variables revealed that GPER immunoreactivity (both % and density; data shown in Figure 7 are for % GPER) is a hub in both ARC and PERI. In support of our cell size data, in the PERI age was positively correlated with percent of cells >150  $\mu\text{m}^2$ . Other interesting points are that ER $\alpha$  and PR were highly correlated, both with each other as well as with GPER. In both regions, reproductive parity was highly correlated with expression of all three receptors in the PERI,

but only with GPER in ARC. Though interesting, correlation results must be interpreted carefully as they do not necessarily indicate causation.

## IMPLICATIONS AND CONCLUSIONS

Our study demonstrates that the aged primate hypothalamus retains the ability to express steroid hormone receptors at levels comparable to young adults. There were also few effects of long-term E<sub>2</sub> on ER $\alpha$  and PR cell % or density. Additionally, there were no changes in the total number of cells in either region, which is consistent with findings in other populations of macaques (Roberts et al., 2012). Differences between our model and published data using shorter-term E<sub>2</sub> treatment suggest that changes previously observed in aged animals in the expression of ER $\alpha$  caused by HRT may be relatively transient, and that the nonhuman primate brain eventually returns to the pre-treatment baseline. Novel data on GPER indicate that there are differences between YE and AE monkeys, with the AE group having the highest numbers of GPER-IR cells in the PERI. It is not known, however, how density of populations of hypothalamic cells may play out as functional changes in responsiveness to steroid hormones. Furthermore, our observation of age-related GPER-IR cell hypertrophy is consistent with other hypertrophic neuronal populations in humans and monkeys including kisspeptin and neurokinin B (Ule et al., '83; Rance et al., '90; Rometo and Rance, 2008; Rance, 2009). In nonhuman primates, these data suggest that, following long-term cyclic E<sub>2</sub>, ER $\alpha$  and PR in the PERI and ARC probably do not contribute significantly to an age-related decline in hormone sensitivity. Additionally, GPER may play a significant role in reproductive aging, either as a contributing or compensatory factor, something that requires future experimentation to resolve its physiological role.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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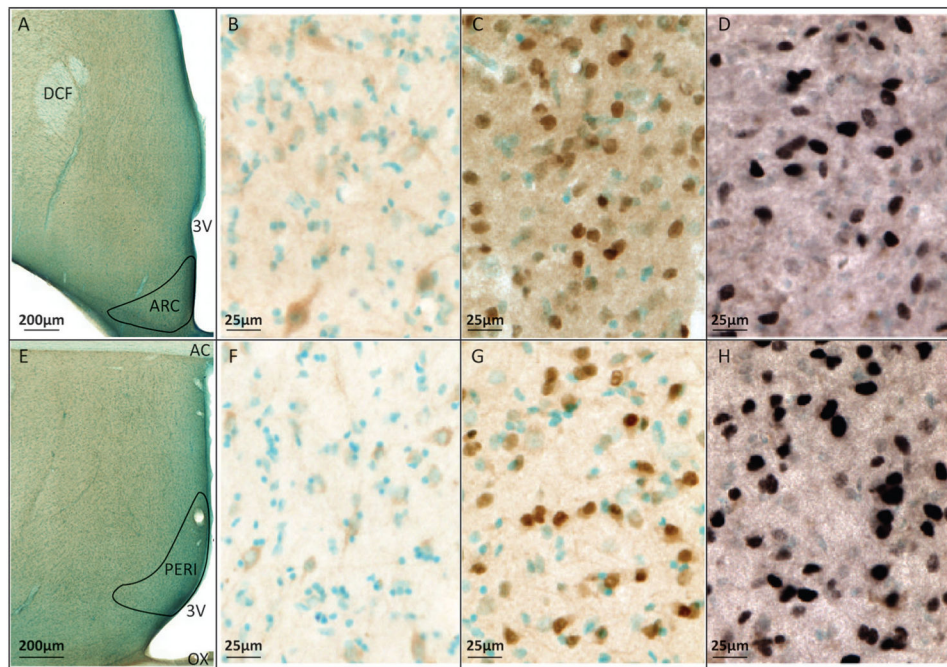
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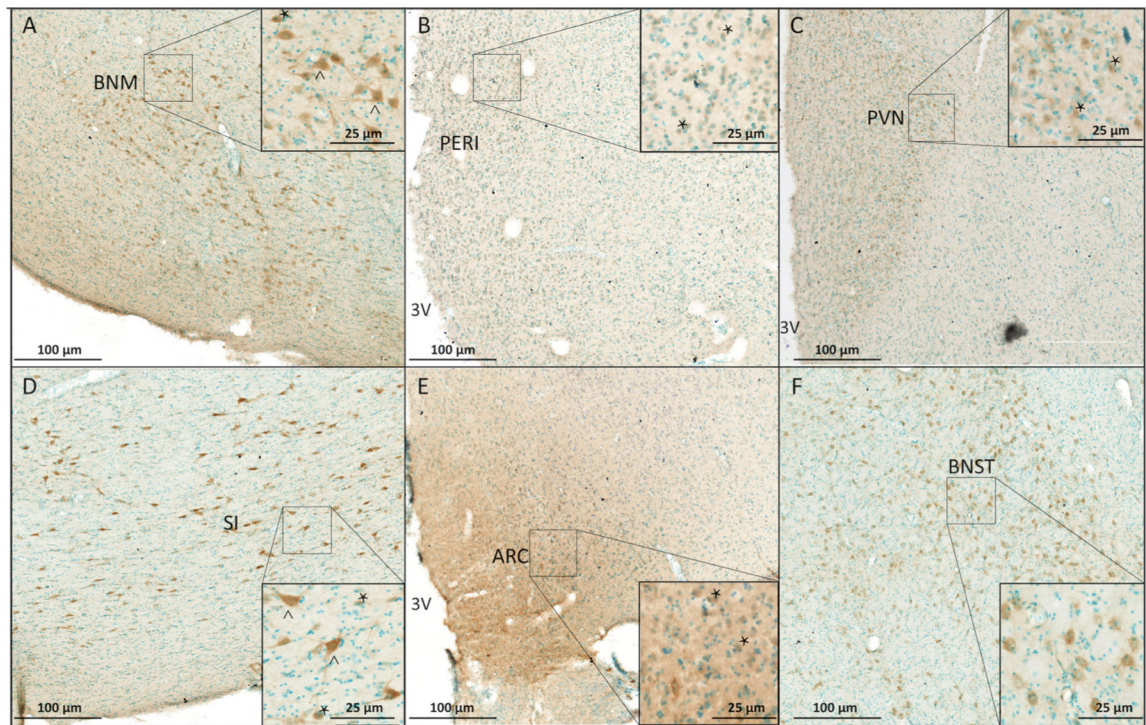


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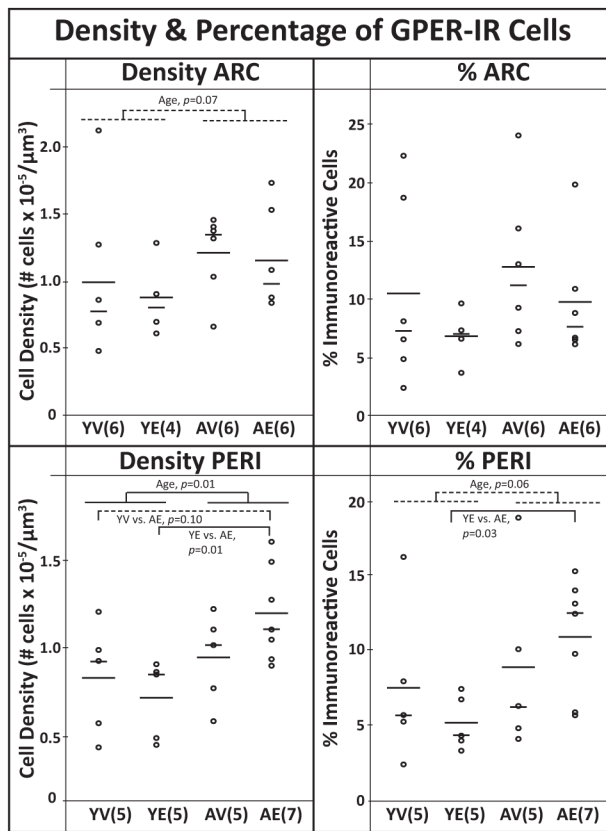
**Figure 1.**

Micrographs showing expression of GPER, ER $\alpha$  and PR in the ARC (top) and PERI (bottom). (A,E) Low magnification of the ARC and PERI regions used for stereologic analysis. Scale bars = 200  $\mu$ m. Boundaries were determined using distribution of counterstained cells and the anatomical landmarks: dorsal column of the fornix (DCF), third ventricle (3V), anterior commissure (AC), and optic chiasm (OX). The remaining representative micrographs were obtained from consecutive sections of monkey #18261 (AV group). (B,F) GPER; (C,G) ER $\alpha$ ; (D,H) PR, are shown in the ARC and PERI, respectively. Counterstained nuclei are blue-green. Scale bars = 25  $\mu$ m (B–D, F–H). Images were modified in Photoshop with slight contrast adjustments to allow for clearer visualization. However, because stereologic counting was done on the microscope using the original slides, photographic adjustments had no influence on the actual quantification of data.

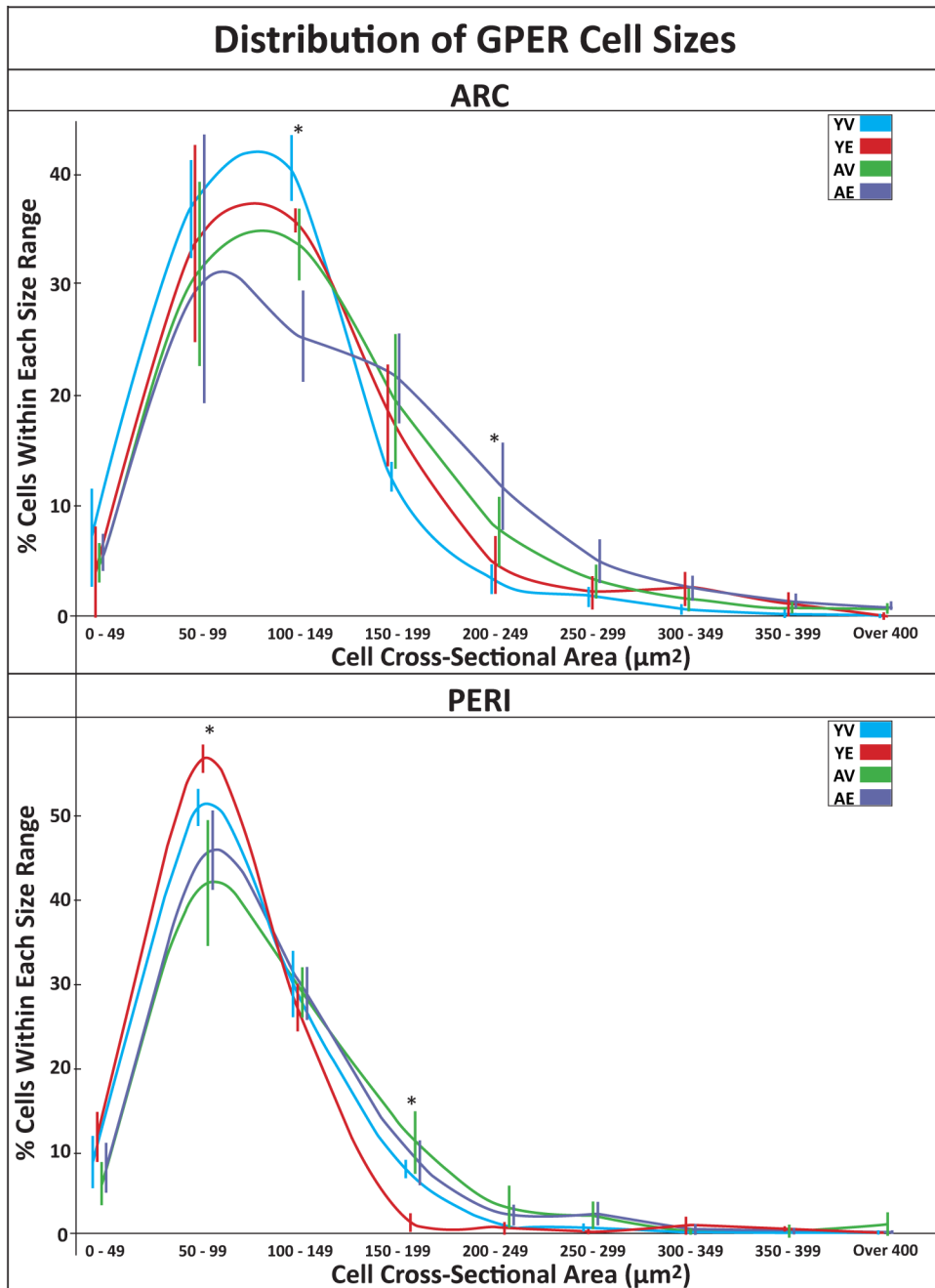


**Figure 2.**

Representative micrographs of distribution and cellular morphologies of GPER-IR cells in the hypothalamus and surrounding areas. The distribution was similar across all monkeys, and GPER immunostained cells are shown from two representative monkeys (#28603 and #29136). Micrographs are shown for: (A) basal nucleus of Meynert (BNM); (B) periventricular region (PERI); (C) paraventricular nucleus (PVN); (D) substantia innominata (SI); (E) arcuate nucleus (ARC) and (F) bed nucleus of the stria terminalis (BNST). Some representative large cells ( $>150 \mu\text{m}^2$ ) are indicated by ^ and small cells ( $<150 \mu\text{m}^2$ ) by \*. Counterstained cells have blue nuclei. Scale bars =  $100 \mu\text{m}$  for low magnification images and  $25 \mu\text{m}$  for the insets.

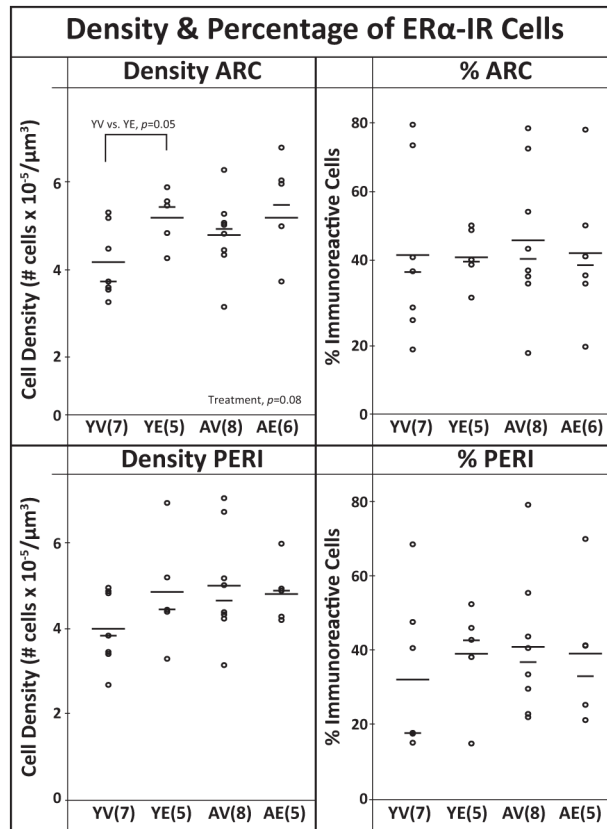


**Figure 3.** The density and percentage of immunoreactive GPER cells in the ARC and PERI. Each graph includes the individual data points (circles), mean (long horizontal line), and median (short horizontal line). In the PERI, aged animals had significantly higher GPER density than young. Post hoc analyses showed AE monkeys > YE and a trend of AE > YV. There was also a trend ( $P < 0.1$ ) of aged animals having a greater % immunolabeled cells than young (shown by the dotted lines), driven by AE > YE. In the ARC there was a trend for more GPER cells in aged compared to young animals, regardless of treatment. Sample sizes are shown in parentheses.

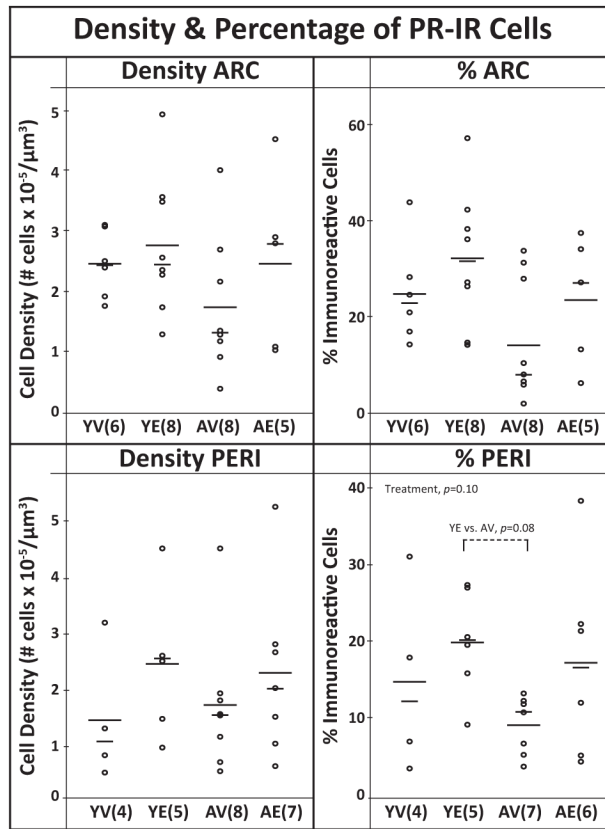


**Figure 4.** Size of GPER cell bodies in ARC and PERI. The size of GPER cells was determined using the StereoInvestigator Nucleator feature. Data shown are for the percentage of cells that fell within  $50 \mu\text{m}^2$  size bins (mean  $\pm$ SEM). The lines and corresponding error bars are color coded with YV in blue, YE in red, AV in green and AE in purple. The error bars are offset to allow better visualization. In the ARC, there was a trend of young monkeys having more small cells between 100 and  $149 \mu\text{m}^2$  than the aged animals ( $P = 0.07$ ), with YV < AE ( $P = 0.09$ ), and YE < AE ( $P = 0.08$ ). There was also a trend of aged monkeys having more large

cells between 200 and 250  $\mu\text{m}^2$ . In the PERI, there was a trend of young monkeys having more small cells between 50 and 99  $\mu\text{m}^2$  than the aged animals ( $P = 0.07$ ), with YV < AE ( $P = 0.07$ ), and YE < AE ( $P = 0.07$ ). There was also a trend of vehicle monkeys having more large cells between 200 and 250  $\mu\text{m}^2$  than treated animals, due to YE < YV ( $P = 0.02$ ), YE < AV ( $P = 0.03$ ), and YE < AE ( $P = 0.07$ ). YV monkeys had no cells between 350 and 400  $\mu\text{m}^2$ , and only 1 cell over 400  $\mu\text{m}^2$  in the PERI. \* $P < 0.10$ .

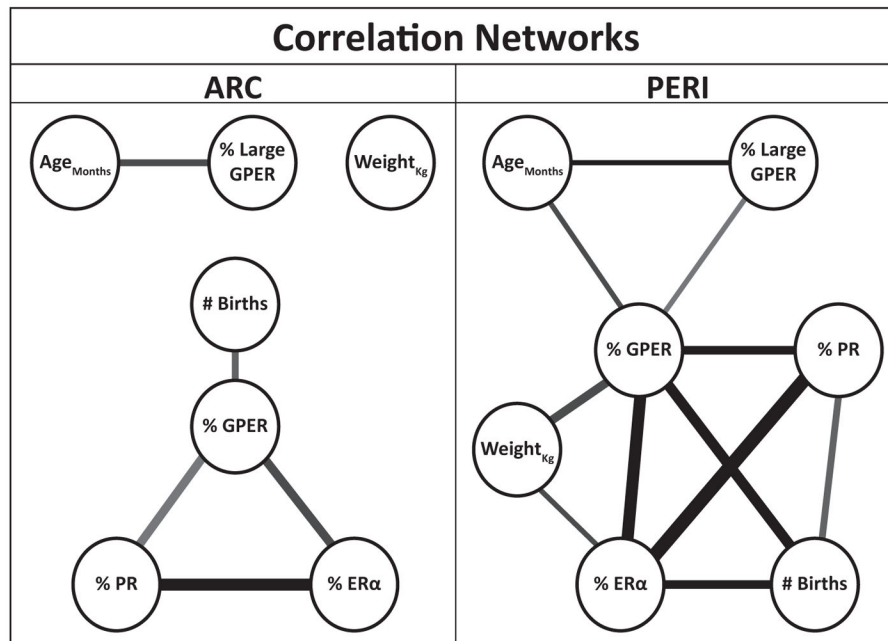


**Figure 5.** The density and percentage of immunoreactive ER $\alpha$  cells in the ARC and PERI. Each graph includes the individual data points (circles), mean (long horizontal line), and median (short horizontal line). In the ARC, there was a trend of higher density in E<sub>2</sub> treated animals compared to vehicle ( $P = 0.08$ ), driven by YE > YV ( $P = 0.05$ ). The percent of ER $\alpha$ -IR cells were not affected by age or treatment. Sample sizes are shown in parentheses.



**Figure 6.** The density and percentage of immunoreactive PR cells in the ARC and PERI. Each graph includes the individual data points (circles), mean (long horizontal line), and median (short horizontal line). The density of PR-IR cells was not affected by age or treatment. In the PERI there was a trend for a treatment effect on % cells ( $P = 0.10$ ), with  $E_2 >$  vehicle. Sample sizes are shown in parentheses.





**Figure 7.**

Correlation network of animal characteristics and % immunoreactive cells. Thickness and shade of lines indicate the strength of correlation and significance level, respectively. All resulting  $r$  and  $P$  values for networks are available in Supplemental Table S2. Cell density, % and total cells # were highly correlated therefore we included only % IR cells for simplicity sake. In both regions, GPER cells formed a hub with the nuclear receptors, # births, and (in the PERI) other characteristics of the animals.

**Table 1**

Distribution and characterization of GPER immunoreactivity throughout the hypothalamus and surrounding regions.

Abbreviation	Nucleus	Density of GPER	Type of label (axons, lg/sm cell)
AcN	Nucleus of anterior commissure	+++	Small to large cell bodies, some processes
AHA	Anterior hypothalamic area	--/+	Processes with some small cell bodies
ARC	Arcuate nucleus	+++	Processes with small to large cell bodies
BNM	Basal nucleus of Meynert	++	Large cell bodies and processes
BNST	Bed nucleus of stria terminalis	-/+	Small to large cell bodies and some processes
Ca	Caudate nucleus	-/+	Small and medium cell bodies
CP	Cerebral peduncle	--	Few large cell bodies
DA	Dorsal hypothalamic area	+	Medium to very large cell bodies
DBh	Nucleus of diagonal band of Broca, horizontal portion	++	Small to large cell bodies and processes
DBv	Nucleus of diagonal band of Broca, vertical portion	-	Small cell bodies
DP	dorsal premammillary nucleus	-/+	Very small to large cell bodies
FF	Fields of Forel	--	Some medium cell bodies
Gp	Globus pallidus	-/+	Large cell bodies and processes
HAA	Anterior hypothalamic area	+++	Very small, small, and medium cell bodies
HDM	Dorsomedial nucleus of hypothalamus	-/+	Some small and medium cell bodies
HLA	Lateral hypothalamic area	-	Small, medium, and large cell bodies
HLmc	Magnocellular nucleus of lateral hypothalamus	+++	Medium to very large cell bodies
IC	Internal capsule	--	Large cell bodies
LSv	Lateral septal nucleus, ventral portion	++	Very small cell bodies
ME	Median eminence	++	Axons, some large cell bodies
MPN	Medial preoptic nucleus	++	Small and medium cell bodies
MPO	Median preoptic nucleus	-	Small and medium cell bodies
MS	Medial septal nucleus	+	Small and medium cell bodies
OLT	Olfactory tubercle	+++	Small cell bodies and processes
OX	Optic chiasm	-	Processes with few large cell bodies
PA	Posterior hypothalamic area	-/+	Small to large cell bodies and processes
PERI	Periventricular region	+++	Very small to medium cell bodies and processes
PVN	Paraventricular nucleus	++	Very small to large cell bodies and processes
Pf	Perifornical nucleus	+++	Small to very large cell bodies with some processes
PM	Premammillary nucleus	+++	Small cell bodies and processes
POA	Preoptic area	-/+	Small and medium cell bodies
SCN	Suprachiasmatic nucleus	+++	Very small and small cell bodies and processes
SI	Substantia innominata	+++	Medium to very large cell bodies and processes
SM	Stria medullaris of thalamus	+++	Very small and small cell bodies and processes
SON	Supraoptic nucleus	+++	Medium to large cell bodies and processes
STr	Stria terminalis	+	Small cell bodies and processes
Sv	Subventricular nucleus	++	Medium cell bodies with processes

Abbreviation	Nucleus	Density of GPER	Type of label (axons, lg/sm cell)
TCA	Area of tuber cinereum	-/+	Processes with some small and medium cell bodies
Th	Thalamus	+ /++	Medium to large cell bodies and processes
TM	Tubero mammillary area	-	Processes with small cell bodies
TU	Lateral tuberal nucleus	+	Very small to large cell bodies and processes
VMH	Ventromedial nucleus of hypothalamus	+	Very small to medium cell bodies
VPa	Ventral pallidum	-/+	Medium and large cell bodies, some processes
ZI	Zona incerta	-/+	Medium cell bodies
3V	Third ventricle	∅	None
AC	Anterior commissure	∅	None
DCF	Descending columns of the fornix	∅	None
LV; OT	Lateral ventricle; optic tract	∅	None; None

∅, none; —, very sparse to none; -, sparse; +, moderate; ++, dense; +++, very dense.

GPER distribution and cellular morphologies in the hypothalamus and surrounding areas. Table provides a list and qualitative description of GPER immunolabeling in specific regions, and a list of abbreviations.

Abbreviations and terminology were taken from a combination of 3 atlases and modified to reflect most current usage (Bleier, '84; Paxinos et al., '90, Brainmaps.org).