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Reproductive Aging & Long-Term Hormone Replacement Therapy in the Rhesus Macaque

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Reproductive Aging & Long-Term Hormone Replacement Therapy in the Rhesus Macaque

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2014

Dedication

I dedicate this dissertation to my husband, Larry Lindsey

and to my graduate cohort:

Bailey Kermath, Colleen Cappellini, Miriam Meister, Leslie Ramsey, Lindsay Dickey

& Sachin Vaidya.

We all got into this together and you each helped me in your own way.

Acknowledgements

I must first thank my mentor, Dr. Andrea Gore. She has always encouraged me to go the extra mile while giving the freedom to choose my own path. I have learned so much from her, not only about how to be a good scientist, writer and manager, but also how to be a good mentor as well. I feel very privileged to have had the opportunity to work with her.

All of the members of my lab have been a great source of support, inspiration and comradery. Dr. Weiling Yin's rodent work inspired much of this dissertation and she has been very generous with her time and expertise. I thank my assistants and collaborators who worked with me at UT Austin: Dr. Fay Guarraci, Tyler Merceron, Long Nguyen, Christina Tran, Sateria Lozano, Ji Eun Kim and Syed Zafar. I appreciate all of the time and energy you contributed to this body of work.

I am grateful to my collaborators in Dr. John Morrison's lab at Mount Sinai School of Medicine in NYC. Bill Jansen and Rishi Puri have always been quick to answer my questions and to help when requested. I appreciate the time and effort the staff at the California National Primate Research Center at the University of California at Davis put into rearing, caring for and carrying out the hormone treatments on the animals used in this research.

Dr. Dwight Romanovicz, Julie Hayes and John Mendenhall have all taught me a lot about microscopy and their patience and generosity is greatly appreciated.

Finally, I thank my committee members: Dr. Hans Hofmann, Dr. Juan Dominguez, Dr. Mark Hayward and Dr. Peter Thomas, for their time, support, suggestions and enthusiasm. I am very lucky to have had them on my dissertation committee and I appreciate all of the feedback they have given me.

Reproductive Aging & Long-Term Hormone Replacement Therapy in the Rhesus Macaque

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The University of Texas at Austin, 2014

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Menopause is a natural transition heralded by the cessation of menstrual cycles and ovulation, and it occurs in all women at an average of about 50 years of age. While not a disease, menopause is often accompanied by symptoms that interfere with the quality of life and these symptoms are due to the relatively abrupt deprivation of E_2 and P_4 experienced during reproductive aging. Reproductive aging consists of changes in the synthesis and release of hormones from the hypothalamus, pituitary and gonad, which make up the HPG axis. Because gonadal hormones play critical roles in many systems throughout the body and brain, not just reproduction, treatment of menopausal symptoms to date largely involves hormone replacement therapy (HRT) with E_2, P_4 or their combination. While not intended to treat other neurobiological symptoms beyond hot flushes, HRT has the potential to exert widespread actions due to the abundance of hormone receptors throughout the nervous system. Thus, a fuller understanding of the neurobiology of menopause is badly needed. Although much of the research into the mechanisms that underlie reproductive aging focuses on ovarian failure and follicular atresia (cell death), there is evidence that there are significant alterations in the function of the neuroendocrine levels - the hypothalamus and pituitary - that also contribute to this process. As the mean

age of the population increases, the number of post-menopausal women continues to grow with broad economic, healthcare and social costs. It is increasingly important to understand the complex mechanisms underlying reproductive aging and the effects of HRT. In this dissertation, I focus on the question of how the female non-human primate hypothalamus changes both with aging and in response to steroid hormone treatments.

Table of Contents

List of Tables

List of Figures

CHAPTER 1: INTRODUCTION TO FEMALE REPRODUCTIVE SENESCENCE

REPRODUCTION & THE HYPOTHALAMIC-PITUITARY-GONADAL (HPG) AXIS

Reproduction is absolutely necessary for the propagation of a species. The essential nature of reproduction is consistent with evidence that most aspects of reproductive physiology are conserved across vertebrates. Most animals retain the ability to reproduce until death, but some mammals experience a cessation of reproductive capacity that occurs before the end of life. Humans are one of the most extreme examples. The timing of the dramatic reduction in reproductive capacity that culminates in menopause occurs about halfway (on average age 50) through the natural maximum lifespan of humans (approximately 100 years). Males of most species retain the ability to reproduce until very late in life, including in humans, although a decline in fertility and decreased levels of gonadal hormones occur in men. For the rest of this section I will focus on reproduction in females, although it is noteworthy to point out that many aspects of HPG physiology are quite similar between the sexes.

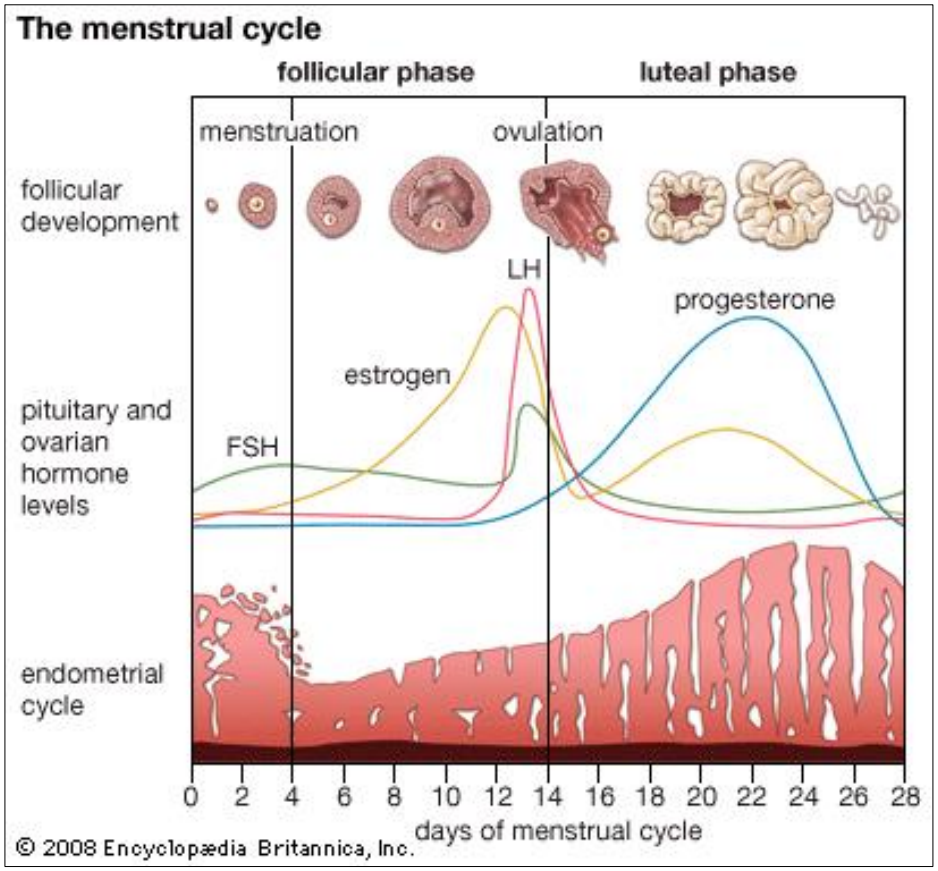
Precisely synchronized hormonal release from the hypothalamus in the brain, the pituitary, and the gonads (ovaries in women and testis in men) is required for normal reproduction (reviewed in (Gore et al., 2014)). The activity of the HPG axis varies throughout the life cycle. During prenatal and early development, the HPG axis is hyperactive, triggering sexual differentiation of the brain and body (Gore, 2008; McCarthy et al., 2008; Schwarz and McCarthy, 2008). After a quiescent period during childhood, the activity of the HPG axis ramps up, triggering the

pubertal transition. The HPG axis maintains high activity throughout adulthood, with fluctuations (in females) across reproductive cycles. Finally, accompanying general somatic aging (Kirkwood and Shanley, 2010), there is a gradual decline in HPG function, known as reproductive aging, ultimately leading to reproductive failure.

Gonadotropin-releasing hormone (GnRH) is a ten-amino acid neuropeptide synthesized in about 1000 cells in the basal hypothalamus. The release of GnRH from these neurons provides the primary driving source onto the rest of the HPG axis. GnRH axons extend to the median eminence (ME) at the base of the hypothalamus, where the neuropeptide is released into the portal capillary system that vascularizes the anterior pituitary (Abel and Rance, 2000; Gore, 2002; Yin and Gore, 2010). In the ovaries, the gonadotropins (LH and FSH) stimulate follicular development (folliculogenesis), ovulation, and steroidogenesis of gonadal steroids. A protein hormone, inhibin B, is produced by the growing follicle and is stimulated by FSH (Welt et al., 2001). The ovarian hormones enter the blood stream to exert actions on target cells that express the steroid hormone and/or inhibin receptors. Reproductive tissues, bone, breast, brain, and many other cells express steroid hormone receptors. In addition, feedback of gonadal steroids onto hormone receptors in the brain and pituitary depresses the activity of GnRH neurons and gonadotropes, respectively, forming a negative feedback loop for most of the reproductive cycle in females. In males, gametogenesis and steroidogenesis are driven by regular pulses of GnRH release, causing pulses of LH/FSH, and ultimately, testosterone. Negative feedback from testicular hormones, especially testosterone, dampens the drive from the hypothalamus to enable the peaks and troughs that characterize pulsatile secretion of these HPG hormones. Females, on the other hand,

have a more complex reproductive cycle that depends on hormonal feedback onto GnRH switching from negative to positive. During negative feedback phases (most of the cycle), E_2 and P_4 down-regulate GnRH and gonadotropin output. Late in the follicular phase, this negative feedback switches to positive, under which increases levels of gonadal estrogens cause a surge in GnRH that triggers ovulation (Gore and Terasawa, 2001; Gore et al., 2004; Terasawa, 2001). After ovulation, negative feedback resumes.

Figure 1.1: Human Menstrual Cycle.



Women have a menstrual cycle that lasts ~28 days and is divided into the follicular phase, luteal phase and menstruation, as depicted in **Figure 1.1**. The early follicular phase is characterized by low levels of E_2 and P_4, and low levels of inhibin that otherwise suppress FSH release. This lack of inhibition from inhibin allows a wave of follicles to begin to develop under the influence of FSH. During this period, pulses of GnRH are low in amplitude, and occur every 1-4 hours, and feedback from ovarian steroids is exclusively negative onto the hypothalamus and pituitary (Bentzen et al., 2013; Gill et al., 2002a; Shaw et al., 2010). As the follicle increases in size, it begins to synthesize increasing amounts of E_2 that is responsible for endometrial proliferation in the uterus. While initially causing negative feedback, once the production of E_2 passes a critical threshold, called the preovulatory peak, this causes the HPG-axis to switch from negative to positive feedback. During this preovulatory period, there is a surge in GnRH release, followed by a peak in LH, called the preovulatory LH surge, that triggers ovulation. When the ovum emerges from the ovary the residual follicle collapses into the corpus luteum, a tissue that produces a postovulatory peak in P_4 and starts the luteal phase of the cycle. P_4 induces a thickening of the uterine endometrium lining and increased vascularization, preparing it for implantation of a fertilized egg. If a conception does not occur, the corpus luteum breaks down, P_4 levels plummet, and the endometrium is shed causing menstruation. Unlike males, who have continual gametogenesis, the pool of oocyte and follicular components of women are created in utero and are not renewed in adulthood. Thus, with aging, as the follicular reserves decline, there are diminishing E_2 levels. With the final ovulation and the lack of developing follicles, E_2 levels stop cycling, and circulating levels are comparable to those pre-pubertally.

GnRH Neurons

Gonadotropin-releasing hormone (GnRH) is a decapeptide that is released from hypophysiotropic neurons in the hypothalamus. GnRH cells are the final output from the hypothalamus to pituitary. This small population of neurons receives a broad array of input from other neurons and glia, enabling the GnRH neurons to compile signals from across the brain into a single pathway that drives pituitary activity and thus the function of the HPG axis as a whole (Gore, 2002; Knobil, 1974; Knobil et al., 1980; Richter and Terasawa, 2001; Sisk and Foster, 2004). The GnRH peptide, as well as its role in reproduction is highly conserved throughout Kingdom Animalia, and the gene and peptide have been well-characterized in mammals, birds, reptiles, fishes, mollusks and insects (Dubois et al., 2002; Gorbman and Sower, 2003; Roch et al., 2011).

GnRH neurons release their neuropeptide in a pulsatile pattern of release, at increments between 30 minutes to several hours depending upon species and reproductive status. This pattern of pulses, with a rapid increase in release followed by an exponential decay over time, is necessary for the maintenance of reproductive tissues/ hormones/fitness in both sexes. In females, a second mode of release, the surge of GnRH, is obligatory for ovulation (Dalkin et al., 1989; Filicori and Crowley, 1984; Gill et al., 2002a; Moenter et al., 1992; Shaw et al., 2010; Terasawa, 1995). Both pulsatile and surge GnRH release are tightly coordinated events that involve complex stimulatory and inhibitory inputs to GnRH cells from neurotransmitters, neurotrophic factors, and peripheral hormones.

The perikarya of neurons that synthesize GnRH are dispersed in the basal hypothalamus, rather than concentrated in a single discrete hypothalamic nucleus. Although there are some species differences in number and distribution, the

neuroanatomy of these neurons is reasonably well conserved in all mammals examined thus far (King and Rubin, 1995). Compared to non-primates, humans and monkeys have slightly more GnRH neurons, which are localized predominantly within the basal hypothalamus. By contrast, in rodents, GnRH perikarya are located in more rostral regions of the hypothalamus, particularly the preoptic area (Abel and Rance, 2000; King and Rubin, 1994; King et al., 1985).

Within the cell bodies of neurons, GnRH is packaged into secretary vesicles and transported, via axonal projections, to the median eminence at the base of the hypothalamus. There, upon stimulation, the secretory vesicles undergo exocytosis to release the neuropeptide into the perivascular space of the portal capillary system that vascularizes the anterior pituitary (King et al., 1982; Rangaraju et al., 1991; Yin and Gore, 2010).

For successful reproduction to occur, animals must respond to their physiological and environmental state, thus GnRH neurons integrate signals from a vast array of neural and hormonal inputs. GnRH perikarya receive heterogeneous inputs from many classes of neurotransmitters, including glutamate, GABA, NPY, catecholamines, among others (Almey et al., 2012; Clarke and Pompolo, 2005; Gore, 2004; Romanò et al., 2008). Because most of these neural classes are steroid-sensitive (e.g. express steroid hormone receptors) they also mediate effects of hormone input to the GnRH system, as discussed later in this chapter.

THE MEDIAN EMINENCE (ME)

GnRH neurons usually have 2 processes, one that extends to the ME, and a second that extends to other parts of the brain. GnRH neurons are referred to as

"hypophysiotropic neurons" because of their function in stimulating the pituitary (hypophysis) through the release of the GnRH peptide in the pericapillary space in the ME. The release of GnRH from many neurons, whose somata are scattered over a relatively large area in the basal hypothalamus, must be tightly coordinated for pulsatile release. Therefore, the convergence of GnRH neuroterminals allows for close proximity of GnRH axons and for the possibility of coordinated release. Several features of the ME facilitate the ability of GnRH neurons to orchestrate release of the peptide, and to receive inputs from local glia and neuroterminals.

At the base of the hypothalamus, the ME the base of the third ventricle, just below the arcuate nucleus of the hypothalamus, and forms a bridge to the anterior pituitary. This is one of the few circumventricular regions of the brain that has a leaky, or fenestrated blood-brain barrier, allowing for communication of blood vessels in the basal hypothalamus with the peripheral circulation. There are few synapses in ME and cellular communication is dominated by diffusion of hormones and neurotransmitters in a manner similar to volume transmission (Agnati et al., 1995). The ME is separated into different layers, or zones, although terminology varies across studies. There is agreement upon a subdivision of the ME into the internal and external zones - (Anthony et al., 1984). Others further subdivide the to the third ventricle, myelinated axons, and the pericapillary region (Ojeda et al., 2008; Yin and Gore, 2010). For my dissertation I primarily refer to the internal and external zones of the ME. The internal zone abuts the third ventricle and contains the cell bodies of tanycytes (specialized glial cells) and GnRH and other releasing-hormone neural processes. The external zone contains the portal capillary bed and has a high density of secretory neuroterminals, microglia and tanycytic endfeet.

Besides reproduction, the hypothalamus also controls metabolism, stress, stress, and growth via hypophysiotropic releasing hormones. Like GnRH in the HPG-axis, thyrotropin-releasing hormone (TRH; metabolism), growth hormone-releasing hormone (GHRH; growth) and corticotropin-releasing factor (CRF; stress), have neural cell bodies in the hypothalamus and send long (unmyelinated) projections to the ME and portal capillary system to stimulate the release of their respective hormones in the anterior pituitary: somatotropes (GH); prolactins (PRL); gonadotropes (LH and FSH); corticotropes (ACTH) and thyrotropes (TSH). Thus, analyses of the morphology and ultrastructure of the ME cuts across all of these neuroendocrine systems.

In addition to neural projections, the ME contains four types of glia that support, nourish, protect and guide neuroterminals containing peptides and neurotransmitters (Baroncini et al., 2007; Ojeda et al., 2008; Prevot, 2002; Prevot et al., 2010; Yin and Gore, 2010). Ependymal cells form the lining of the third ventricle and have cilia that keep the cerebrospinal fluid (CSF) circulating throughout the ventricular system in the brain. Microglia are a type of macrophage that serves as the immune cells of the central nervous system (CNS), engulfing infectious agents that make it past the blood-brain barrier (BBB), and clear dead or dying neurons and glia. Since the ME has a leaky BBB, these glia are prolific in this region. Astrocytes are the 'nurse cells' in the CNS, supporting and nourishing neurons. Additionally, they play a critical role in the glutamate cycle, which is a key regulator of peptide release in the ME. Tanycytes are a specialized subtype of astrocytes, that control neuroterminals' access to the portal capillary system.

Tanycytes appear to be particularly important in the regulation of hypophysiotropic neuroterminals, including GnRH. The tanycytic cell bodies are

located near the wall of the third ventricle and send long projections to line the basal lamina (the membrane that separates tissues from capillary beds). Their 'end feet' help form the fenestrated BBB. With direct access to the cerebrospinal fluid, and thus the rest of the central nervous system, and the portal capillaries, they form a bridge between the two circulatory systems. The tanycytic 'end feet' allow for rapid communicate between the hypothalamus and the pituitary through their interactions with the portal capillaries. Additionally, these cells are very motile, wrapping around neuroterminals to guide them to the capillary, as well as retracting their 'end feet' from the basal lamina, giving access to the terminals to release their hormones (Akmayev and Fidelina, 1981; Baroncini et al., 2007; Langlet et al., 2013; Ojeda et al., 2008; Prevot et al., 2010). They are responsive to peripheral hormones, especially estradiol, and various neurotransmitters that modify the morphologic and transport activity of these cells. These processes are very important in the regulation of GnRH release at the level of the portal capillary (Flament-Durand and Brion, 1985; Prevot, 2002; Zoli et al., 1995).

ESTROGEN AND ESTROGEN RECEPTORS

Estrogens are produced predominantly by the gonads, but also in low levels by the adrenal, and locally in tissues throughout the body and brain. Estradiol is critical for sexual differentiation of the fetal brain, for development of secondary sexual characteristics in females, and the acquisition and retention of adult sexual behavior (McEwen 1983). In addition to its well-established role in reproduction, estrogens are active in many processes in the central nervous system (CNS) as underscored by the widespread expression of estrogen receptors throughout the

brain, including regions that are involved in memory, attention, mood, pain sensitivity, and locomotor activity (reviewed in (Dumitriu et al., 2010; McEwen, 2002; McEwen and Alves, 1999; Morrison et al., 2006). Interest in estrogen's role in learning and memory is growing rapidly now that it is established that E_2 is involved in the formation and breakdown of neuronal spines and synapses and that it affects behavioral tasks of learning and memory. Moreover, there is a hormone-sensitive ebb and flow of synaptogenesis that occurs with the normal rise and fall of E_2 levels throughout the female cycle or in models of ovariectomy plus estrogen replacement (Brinton, 1993; Woolley and McEwen, 1992; Woolley et al., 1990). This mounting evidence for estrogen's importance in neuronal excitability and the dynamic restructuring of synapses raise critical questions about how the depletion of E_2 in post-menopausal women affects the plasticity of the aged brain (Brinton, 1993; Nabekura et al., 1986; Woolley, 2007; Woolley and McEwen, 1992; Woolley et al., 1990).

ESTROGEN RECEPTORS

Initially, estrogen's actions were thought to be exclusively genomic, mediated by the nuclear estrogen receptors ERα and ERβ. Following binding of estradiol to these receptors, dimerization of the receptor-ligand complex, and interactions with co-regulatory factors (co-activators and/or co-repressors), these receptors act as transcription factors by binding to estrogen response elements (ERE) on target genes, thereby modulating gene transcription (Paech et al., 1997). The nuclear ERs are widely distributed throughout the body and brain, with considerable complementarity in their localization, and some overlap. Dense ERα expression is

found in the pituitary, kidney, epididymis, adrenal, ovary, testis, and uterus (Kuiper and Gustafsson, 1997; Kuiper et al., 1998). ERβ is more abundant in the prostate, lung, bladder, ovary, testis, and uterus (Kuiper and Gustafsson, 1997; Kuiper et al., 1998). Both ERs are densely expressed in multiple hypothalamic regions, including that of the monkey (Blurton-Jones et al., 1999; Chakraborty et al., 2003; Shughrue et al., 1992; Simerly et al., 1990). Less is known about ERβ due to a lack of good antibodies in the last decade of research.

Although it has been known for many years that estrogens can have rapid actions when applied to the membrane of hypothalamic cells, the identity and nature of those receptors was not known for many years after that initial discovery (Kelly et al., 1984; Lagrange et al., 1995). Then, several candidates emerged as mediating rapid effects of E_2. One important molecule is GPER, previously called GPR30, a member of the 7-transmembrane G-protein coupled receptor superfamily. In addition, there was increasing evidence that the "nuclear" ERs could be expressed on membranes and mediate rapid actions of E_2 through nongenomic intracellular signaling pathways (Brinton, 2001; Kelly and Levin, 2001; Kelly and Wagner, 1999; Lee and McEwen, 2001; Spary et al., 2009). GPER was isolated by several groups in 1996 (Carmeci et al., 1997; Feng and Gregor, 1997; Kvingedal and Smeland, 1997; Owman et al., 1996; Takada et al., 1997). Other putative membrane ERs such as the STX-sensitive receptor and ER-X are not as well understood, with more research merited in this area (Qiu et al., 2003; Toran-Allerand et al., 2002).

GPER was initially identified for its role in cancer biology, but over the past 15 years its role in cardiovascular, metabolic and immune systems has also been established (Carmeci et al., 1997; Feng and Gregor, 1997; Kvingedal and Smeland, 1997; Owman et al., 1996; Thomas et al., 2005). Because of its ability to bind

estradiol, interest turned to its possible role in reproduction. GPER is expressed in hypothalamus, pituitary, and ovary (Chimento et al., 2014; Rudolf and Kadokawa, 2013). It is densely expressed in the brain, including the hypothalamus (Brailoiu et al., 2007; Hazell et al., 2009; Naugle et al., 2014; Walker et al., 2012). The expression of GPER is not limited to the plasma membrane; unlike many other G protein-coupled receptors, it has been shown to actively bind E_2 in the cytosol and in the endoplasmic reticulum (Brailoiu et al., 2007; Hazell et al., 2009; Prossnitz et al., 2008). Because the GPER knockout mouse does not have an obvious reproductive phenotype (Brailoiu et al., 2007; Meyer et al., 2012; Prossnitz and Barton, 2011)(Brailoiu et al., 2007; Meyer et al., 2012; Prossnitz and Barton, 2011), however, research in the realm of GPER and reproductive biology is relatively limited.

ESTROGEN RECEPTORS AND GNRH

Until about 15 years ago, it was the prevailing dogma that E_2 feedback onto GnRH neurons was indirectly mediated interneurons and glial cells. The reason for this dogma was that GnRH cells do not express most of the nuclear hormone receptors that are required for reproduction, particularly ERα and the progesterone receptor (Shivers et al., 1983; Wintermantel et al., 2006). GnRH neurons do not co-express ERα, although they do co-express ERβ (Abrahám et al., 2003; Herbison and Pape, 2001; Hrabovszky et al., 2000, 2001, 2007; Sharifi et al., 2002; Skinner and Dufourny, 2005; Sullivan et al., 1995; Wintermantel et al., 2006). Although GnRH neurons express ERβ, it is the ERα knockout (KO) mice that are unable to reproduce, while ERβ-KO mice can reproduce, albeit sub-optimally (Herbison and Pape, 2001;

Korach et al., 1996; Levin-Allerhand et al., 2001), leading to the idea that ERα is the dominant nuclear ER mediating E_2 feedback, indirectly, onto the GnRH system.

Thus, research turned to investigating whether feedback actions of E_2 were mediated indirectly by other neurons or glia that co-express the nuclear ERs (Herbison and Pape, 2001). It was discovered that many classes of neurons, with diverse neurotransmitter or neuropeptidergic phenotypes that regulate GnRH release, co-express ERs. These include glutamate, GABA, NPY, and catecholamines (Almey et al., 2012; Clarke and Pompolo, 2005; Franceschini et al., 2006; Herbison, 1998; Leranth et al., 1992; Romanò et al., 2008; Stumpf and Jennes, 1984). The more recent discovery of the neuropeptide kisspeptin, which has high ERα expression, added evidence for indirect E_2 regulation of GnRH release via kisspeptinergic inputs (García-Galiano et al., 2012).

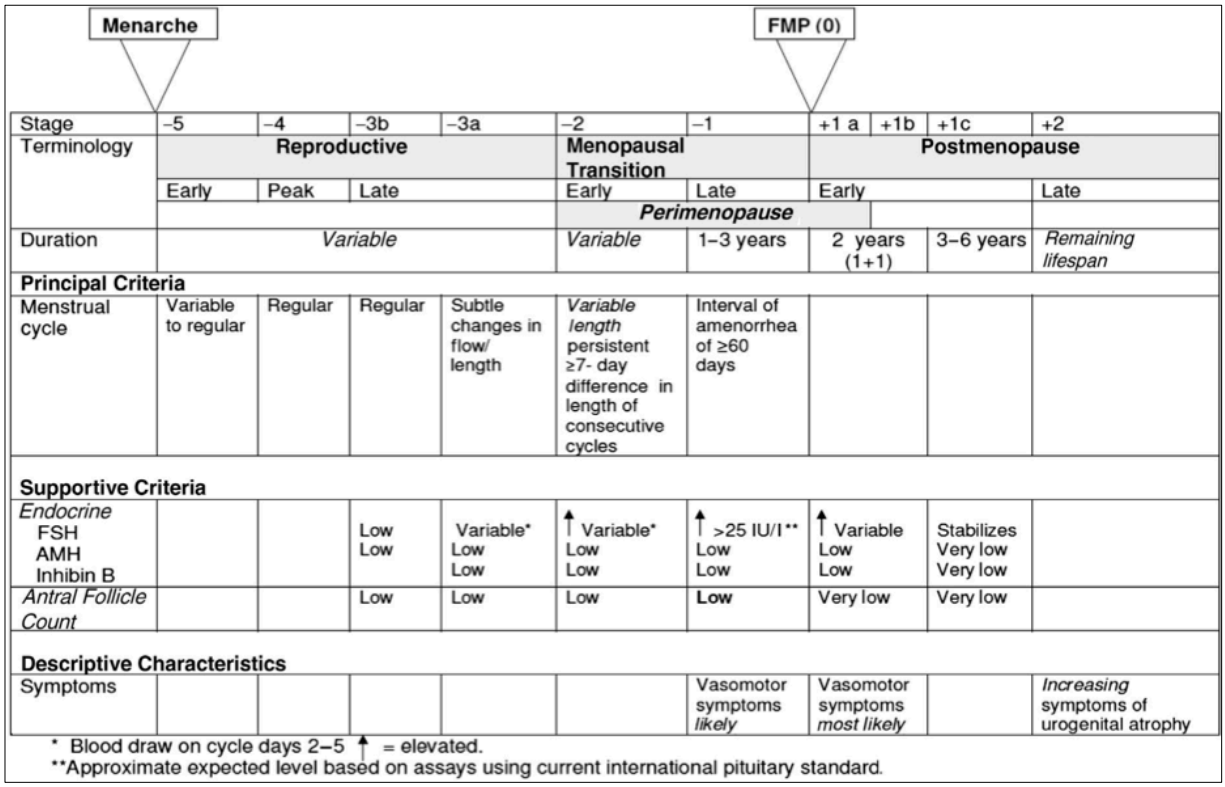
Despite the "dogma" for indirect effects of E_2 on GnRH cells, there is actually a longer history showing that GnRH neurons in hypothalamic slices responded rapidly to application of E_2 with hyperpolarization (Kelly et al., 1984). This early observation, however, was not followed up on until about 10 years ago. Then, a series of studies showed that GnRH cells in vitro respond to E_2 exposure with biphasic [rapid (within a few minutes) and relatively slower (>15 minutes) time scales] intracellular Ca^{2+} oscillations, increased action potential rate, and GnRH peptide release (Abe and Terasawa, 2005; Abe et al., 2008; Abrahám et al., 2003; Chu et al., 2009; Hrabovszky et al., 2007; Jacobi et al., 2007; Kenealy et al., 2011a, 2011b; Noel et al., 2009; Sullivan et al., 1995; Sun et al., 2010; Temple et al., 2004; Zhang et al., 2010). These very important findings indicate that there are multiple mechanisms involved in the direct action of E_2, including directly on GnRH cells.

The reconciliation of this work happened through the increased discovery and understanding of membrane ERs. That this is relevant to GnRH neurons was shown by experiments showing that when ERα and ERβ receptors were blocked, GnRH cells respond rapidly to E_2 with increased action potential rate and intracellular Ca^{2+} oscillations (Abe and Terasawa, 2005; Abrahám et al., 2003; Filardo et al., 2002; Hrabovszky et al., 2007; Jacobi et al., 2007; Kenealy et al., 2011a; Noel et al., 2009; Temple et al., 2003). As already discussed, there are several candidate ERs for these actions, among which, I have focused on GPER in my dissertation. GT1-7 cells, an immortalized GnRH cell line, co-express GPER, and prior to my work there was one report of a monkey GnRH neuron co-expressing GPER (Jacobi et al., 2007; Terasawa et al., 2009). GPER is also expressed on neurons and glia in the vicinity of GnRH perikarya and processes (Almey et al., 2012). Thus, the hypothalamic GPER may mediate both direct and indirect rapid actions of E_2 on the GnRH system.

REPRODUCTIVE AGING IN WOMEN

In women, neuroendocrine and ovarian factors contribute to reproductive aging. Ovarian follicular decline begins around the age of 35 and the rate of follicular atresia increases with age, culminating in the inability to produce viable follicles, a failure to ovulate and subsequently generate a corpus luteum, and a resulting decline in E_2 and P_4 levels (Burger et al., 2002; Gosden and Faddy, 1994; Zapantis and Santoro, 2003). The reproductive lifespan in women is broken down into the reproductive, menopausal transition (peri-menopause), and post-menopause phases, each of which is further subdivided into early and late phases as well as an

Figure 1.2: Definition of the Stages of Reproductive Aging.



Menarche						FMP (0)				
Stage	−5	−4	−3b	−3a	−2	−1	+1 a	+1b	+1c	+2
Terminology	Reproductive				Menopausal Transition		Postmenopause			
	Early	Peak	Late		Early	Late	Early			Late
					Perimenopause					
Duration	Variable				Variable	1–3 years	2 years (1+1)		3–6 years	Remaining lifespan
Principal Criteria										
Menstrual cycle	Variable to regular	Regular	Regular	Subtle changes in flow/ length	*Variable length* persistent ≥7- day difference in length of consecutive cycles	Interval of amenorrhea of ≥60 days				
Supportive Criteria										
Endocrine										
FSH			Low	Variable*	↑ Variable*	↑ >25 IU/l**	↑ Variable		Stabilizes	
AMH			Low	Low	Low	Low	Low		Very low	
Inhibin B			Low	Low	Low	Low	Low		Very low	
Antral Follicle Count			Low	Low	Low	**Low**	Very low		Very low	
Descriptive Characteristics										
Symptoms						Vasomotor symptoms *likely*	Vasomotor symptoms *most likely*			*Increasing* symptoms of urogenital atrophy

* Blood draw on cycle days 2–5 ↑ = elevated.
**Approximate expected level based on assays using current international pituitary standard.

The Stages of Reproductive Aging Workshop +10 (STRAW+10) system for reproductive aging in women. FMP, final menstrual period; FSH, follicle-stimulating hormone; AMH, anti-Mullerian hormone. *Harlow et al., 2012.*

additional peak reproductive phase (as defined in the Stages of Reproductive Aging Workshop +10 (STRAW+10) reproductive staging system; (Harlow et al., 2012); **Figure 1.2**).

The early menopausal transition is characterized by lengthened menstrual cycles, increased FSH, attenuation of the GnRH surge, and a decline in total number of follicles within the ovary and the percentage of growing follicles in each cycle; age-related hormonal changes are shown in **Figure 1.3** (Gougeon, 1998; Wise,

1984). Once a woman experiences amenorrhea of 60 days, she has entered the late menopausal transition phase. This is accompanied by extreme fluctuations in FSH levels, increased E_2 and frequent anovulation. Post-menopause occurs after the final menstrual period, E_2 decreases and FSH rises for about 2 years, after which women no longer produce ovarian E_2 or P_4. These decreased steroid hormone levels result in a decline in negative feedback on the hypothalamus and pituitary, causing GnRH, LH, and FSH levels and pulse amplitude to increase in women and monkeys (Gore, 2002; Gore et al., 2004; Hall, 2004). Elderly women may go through a further transition in which LH pulse amplitude and inter-pulse interval decrease and FSH levels fall, indicating that there is an eventual decline in the central neuroendocrine axis in addition to gonadal dysfunction (Genazzani et al., 1997; Hall et al., 2000).

Although the role that ovarian dysfunction plays in human reproductive senescence is well established, there is a growing body of evidence that suggests that aging of the hypothalamus and pituitary is a key factor in this process. In fact, reproductive failure is driven by the loss of GnRH drive from the hypothalamus in rodents, and their ovaries remain functional through the end of life (Kermath and Gore, 2012). In women, there is evidence of age-related declines in both pituitary and hypothalamic function. An erosion of positive feedback has been found in a subset of women, with only half of peri-menopausal women with E_2 peaks respond with a LH surge (Shaw et al., 2011; Weiss et al., 2004). Additionally, women

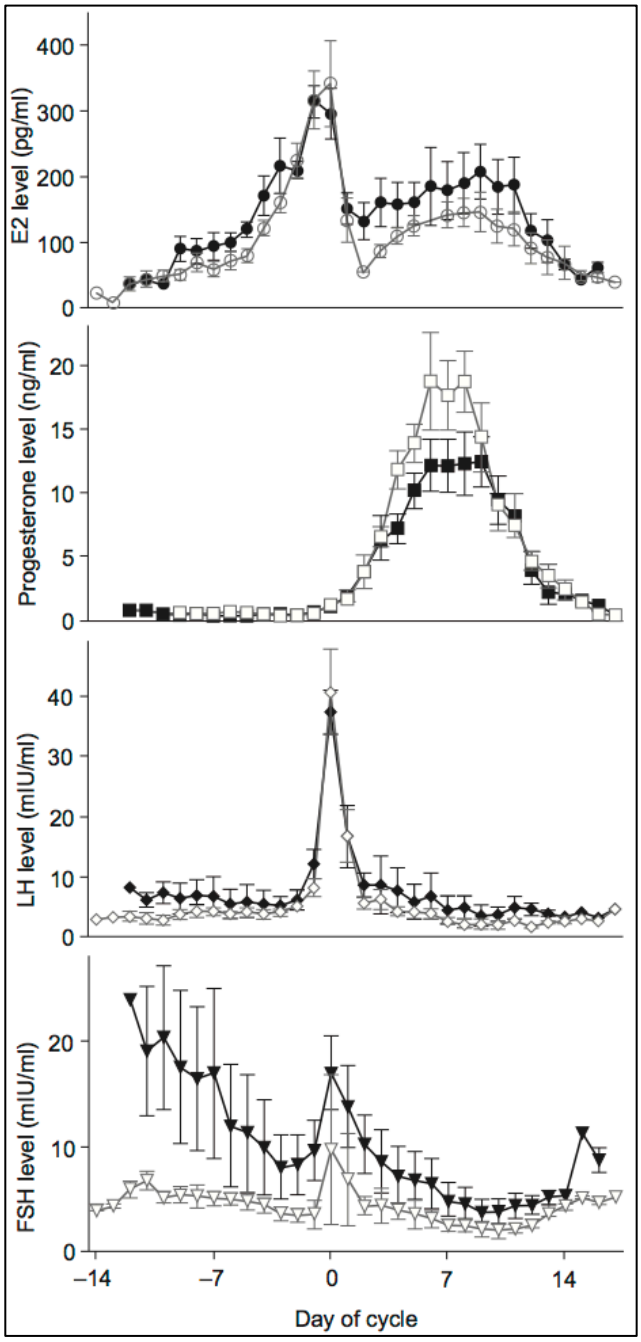


Figure 1.3: Relationship Between Pituitary and Ovarian Hormones Throughout the Menstrual Cycle.

Open symbols represent women between 18-38 and closed symbols represent reproductive women 43-53. *Reprinted with permission from Santoro N et al., 1998.*

between the ages of 50 and 75 have a 30% decrease in LH and FSH levels, in the absence of E_2 negative feedback (Hall, 2004; Santoro et al., 1998). However, other studies have found contrary evidence that older and younger post-menopausal woman have similar levels of GnRH suppression by estrogen and progesterone negative (Gill et al., 2002a, 2002b). Another line of work has identified age-related dysfunction in ability of the hypothalamus to respond to E_2 positive feedback, as shown by dramatic increases in pituitary metabolism that were absent in the hypothalamus, with FDG-PET scans (Ottowitz et al., 2008). Together these studies suggest that there is age-related hypothalamic

dysfunction in addition to gonadal dysfunction, at least in some women, and that this occurs later in life.

ANIMAL MODELS OF REPRODUCTIVE AGING

Nonhuman models of reproductive senescence are critical in understanding the mechanisms that underlie the multitude of physiological processes involved with this transition. With animal models, it is possible to control for variable that are common confounds in humans studies such as diet, social interactions, hormone levels, housing conditions, exposure to exogenous hormones and genetic variation. Additionally, there are strict limitations on experimental manipulations that are acceptable for human subjects. There are a wide variety of methods and animal models available to investigate how hormones and age interact (summarized in **Table 1.1**). In this dissertation, I will focus predominantly on primate research.

Rodents are the most commonly studied mammals because they are easier and less expensive to house, age faster, easier to manipulate than larger animals, allowing for larger sample sizes that increase the statistical power, which is sorely lacking in primate studies. Additionally, genetic manipulations such as transgenic knockouts and GFP proteins are hard to develop in longer-lived mammals. The rodents' HPG axis is highly conserved with other mammals and they experience a process that is similar to menopause in middle age (called estropause) that consists of a transition to acyclicity due to a loss of drive from GnRH neurons (Gore and Terasawa, 2001; LeFevre and McClintock, 1988). They are a great model for investigating the mechanisms that underlie the age-related changes in the central neuroendocrine system, without the confound of ovarian dysfunction, because their

reproductive senescence is driven by hypothalamic dysfunction, as shown by their age-related decreases in GnRH, LH and FSH. Transplantation studies have further demonstrated that ovaries of aged animals functioned normally in young animals (Brann and Mahesh, 2005; Downs and Wise, 2009; Kermath and Gore, 2012; Krohn, 1962; LeFevre and McClintock, 1988). This is a double-edged sword, though, because the fundamental species differences in reproductive aging of these animals limit the translational applicability of these studies to human health.

New world monkeys, such as the common marmoset and tamarins, are much easier to study in laboratory settings due to their small size, short lifespan and quick adaptation to experimental conditions (Tannenbaum et al., 2007; Tardif, 2008). But, like the rodent model, their reproductive systems are considerably different from humans and show little evidence of declining follicular reserves with age, and they do not have menstrual cycles (Bales et al., 2001; Tannenbaum et al., 2007; Tardif, 2008; Tardif and Ziegler, 1992).

Table 1.1: Summary of Reproductive Senescence in Primates and Rodents.

Species	Cycle length	Natural Reproductive Senescence	GnRH / LH	Ovarian Function	References
Human	~28 days	Yes	Initial rise, then fall late in life	Complete failure, with depletion of viable follicles	Hall 2004, Gore 2014
Rhesus Monkey	24-34 days	Yes: very late in life, on a rapid timescale	Increase with age	Decline in number of viable follicles with age	Gilardi 1997, Shideler 2001, Bachevalier 1991, Tigges 1988, Walker 2008, Gore 2004, Kim 2009, Woller 2002, Downs 2006
Baboon	~35 days	Yes: with both peri-menopause and post-menopausal phases	Fluctuating serum gonadotropin	Shortened follicular phases with increasing frequencies of anovulatory cycles prior to the cessation of menses	Martin 2003, Santoro 1996, Bellino 2003, Shideler 1989
Chipanzee	32-35 days	Not complete reproductive cessation, but do have irregular cycles and a decline in conception rate with age	Increase with age	Decline in number of viable follicles with age	Bellino 2002, Graham 1979, Gould 1981
Tamarins	15-31 days	No functional change in reproductive capacity	Similar in young and aged	Irregular cycles with some anovulation, slight decline in viable follicles with age	Tardif 1992,Tardif 2008, Tannenbaum 2007
Rodents	4-5 days	Estropause: irregular cycles to acyclicity at middle age	Surges delayed and attenuated	Maintain viable follicles until late in life	Kermath 2012, Gore 2014

Old world nonhuman primates and humans have very similar reproductive systems that make them a good model for investigating human reproductive senescence (Bellino, 2002; Gilardi et al., 1997; Kaplan, 2004). Macaques are the most commonly studied monkeys because they are fairly easy to raise and house in captive colonies because they are the smallest and have the shortest lifespan (~30 years) of the old world nonhuman primates. They have reproductive cycles that closely parallel those of humans, and they also undergo natural reproductive senescence with 28-day menstrual cycles, similar endocrine, ovarian and neurobiological changes as women (Dierschke, 1985; Graham, 1979; Hodgen et al., 1977). Macaques start showing signs of reproductive aging around 20 years and do not experience complete menopause until ~27 years old, which is later in life than the human menopausal transition and roughly equivalent to women that are 60 and 81 years, respectively (Bachevalier et al., 1991; Gilardi et al., 1997; Maffucci et al., 2008; Peters et al., 1996). Baboons have a menopausal transition that is the most similar to humans than any other primate, but there are significant practical barriers to starting and maintaining captive baboon colonies (VandeBerg and Williams-Blangero, 1997). Surprisingly, our closest evolutionary relative, the chimpanzee, does not have complete reproductive senescence, though they do have reduced reproductive capacity and an increase in gonadotropins with age (Bellino, 2002; Graham, 1979).

AGE-RELATED CHANGES TO THE HYPOTHALAMUS

The necessity of GnRH for normal reproduction underscores the importance of understanding the role it plays in reproductive senescence. There are significant

differences in the impacts of age on GnRH expression and secretion, both within and between species (summarized in **Table 1.2**). The contrary findings within the same species (mostly in rodents) are most likely due to different experimental manipulations and ages that are compared.

Hypothalamic aging drives reproductive aging in rodents, as shown by decreased mean levels, pulse frequency and amplitude of GnRH as well as a delayed and attenuated LH surge (Le et al., 2001; Lloyd et al., 1994; Rubin and Bridges, 1989; Rubin et al., 1994; Scarbrough and Wise, 1990; Wise et al., 1988). In fact, in most animals studied thus far, there are age-related changes in some aspects of GnRH release, including mean basal levels, pulse amplitude and frequency as well as the preovulatory surge. However, these differ by species. Due to the invasive nature of direct measurements of GnRH release, LH is often used as a proxy because its release closely follows that of GnRH, but the glycoprotein free alpha subunit (FAS) has been shown to be a more reliable measure of rapid GnRH release (Hayes et al., 1999).

In humans, the dynamics of GnRH secretion is altered with age, demonstrated by the increase of basal GnRH secretion in post-menopausal vs. pre-menopausal women, in spite of a decrease in pulse frequency, in addition to the increase in response to the removal of E_2 and P_4 negative feedback that occurs with ovarian decline (Gill et al., 2002b; Hall, 2004; Hall et al., 2000; Rossmanith, 1995). In elderly post-menopausal women this process is reversed, with declines in gonadotropin levels, indicating that hypothalamic aging occurs at a slower rate than ovarian aging (Chakravarti et al., 1976; Hall, 2004). Rhesus monkeys have similarly

Table 1.2: Age-Related Changes in GnRH Secretion and Expression in the Hypothalamus.

GnRH Secretion			
Species	**Age**	**GnRH/LH Results**	**Reference**
Human	Peri-menopausal	LH surge is detected in only half of women	Weiss et al., 2004
Human	Early / Late post-menopausal	Pulse frequency, LH and FAS pulse amplitude are lower in late than early post-menopausal women	Hall et al., 2000; Gill et al., 2002
Human	Early / Late post-menopausal	Decrease in LH and FSH with age	Chakravarti 1976, Hall 2004
Human	Pre- / Post-menopausal	Increase in LH and FSH with age	Hall 2004
Rhesus monkey	Young / Old	Mean secretion level increases; pulse amplitude increases; no change in pulse frequency with age	Gore 2004, Woller 2002, Downs & Urbanski 2006
Japanese macaque	Young / Old	LH is higher peri-menopausal than young	Nozaki et al., 1995
Marmoset	Adults	Pulsatile release is similar in intact mid-follicular phase and OVX animals	Tannenbaum 2007
Rat	Young / Middle Aged / Old	Mean secretion level, pulse frequency, amplitude decrease with age	Wise 1988, Scarbrough & Wise 1990
Rat	Young / Middle Aged	Surge is delayed and attenuated with age	Rubin 1994, Lloyd 1994, Wise 1982, Rubin & Bridges 1989, Le 2001

Table 1.2: Continued

GnRH in Hypothalamus			
Species	**Age**	**GnRH/LH Results**	**Reference**
Human	Pre- / Post-menopausal	Increased mRNA with age	Rance & Uswandi 1996
Rhesus monkey	Pre- / Post-menopausal	Increased mRNA with age	Kim 2009
Rhesus monkey	Juvinile (2yr) / Old (22yr)	No change in cell number or morphology	Witkin 1986
Rat	Young / Middle Aged / Old	Increased mRNA with age	Gore 2000, Gore 2000b; Mieda & Sakurai 2011
Rat	Young / Old	More cells in old than young	Merchenthaler el al., 1980
Rat	Young / Old	No change in mRNA with age	Gore et al. 2002
Rat & Mouse	Young / Middle Aged / Old	No cell number difference	Miller & Gore 2002, Hoffman & Finch 1986
Rat	Young / Middle Aged	GnRH-IR, cell number and distribution is similar	Rubin 1984, 1994
Rat	Young / Old	GnRH axon number, area and immunoreactivity decreases with age; GnRH concentration is similar	Bestetti 1991, Hoffman & Sladek 1980
Rat	Young / Middle Aged	Decreased mRNA with age	Miller & Gore 2001, Li 1997
Rat & Mouse	Young / Old	Decrease cell number with age	Funabashi & Kimura 1995, Miller et al., 1990
Rat	Young / Middle Aged	Young have an increase in mRNA proestrous and during surge, but not middle aged	Robin et al. 1997, Gore et al. 2000
Rat	Young / Middle Aged	GnRH immunoreactivity decrease more after LH surge in young	Rubin & King 1995
Rat	Young / Middle Aged	Distribution and morphology is similar	Rubin et al., 1984

altered patterns of GnRH secretion late in life, with increased basal levels and pulse amplitude of GnRH release (Downs and Urbanski, 2006; Gore et al., 2004; Nozaki et al., 1995; Woller, 2002). In further support of these findings, there is a greater amount of GnRH mRNA found in the mediobasal hypothalamus of post-menopausal women and monkeys (Kim et al., 2009; Rance and Uswandi, 1996). The observed increases in GnRH mRNA of the monkey (and most likely humans as well) is due to higher production and not to an increase in the number of cells that produce GnRH (Witkin, 1986).

There have been few studies examining age-related changes in the ME of humans or nonhuman primates, but there are some very intriguing results about the ME of aging rodents. Yin et al found that the ultrastructural organization of the ME changes dramatically with age with a decline in the apposition of glia and GnRH neuroterminals as well as significant morphological changes in tanycytes and disorganization in the pericapillary region (Yin et al., 2009a, 2009b). No age differences in the distribution of GnRH processes or amount of GnRH peptide contained in the ME have been found, as determined by RIA or immunoreactivity (Bestetti et al., 1991; Rubin et al., 1984; Yin et al., 2009b). These results suggest that age-related alterations in the physical relationship between tanycytes and neurosecretory terminals, not GnRH synthesis, may contribute to hypothalamic dysfunction with age, in the rat.

HORMONE REPLACEMENT THERAPY AND WHI

There are a multitude of changes that occur with menopause, besides infertility, that can significantly impair quality of life. Many women experience at least one, but often a combination of the following symptoms: vasomotor hot flashes, sleep disturbances, mood changes such as irritability and depression, urogenital symptoms, increased probability for neurodegenerative disorders, osteoporosis and cardiovascular disease (Benedetti et al., 2001; Nelson, 2008; van der Schouw et al., 1996; van Der Voort et al., 2003; Wich and Carnes, 1995). Thus, an understanding of the mechanisms for reproductive aging, and the causes and consequences of ovarian follicular loss and the consequent depletion of E_2, is extremely important. Furthermore, there is great need for better clinical treatments, when appropriate, for these menopausal symptoms.

The Women's Health Initiative (WHI) was a longitudinal randomized clinical study looking at the most commonly prescribed hormone therapy; conjugated equine estrogens (CEE) taken with or without medoxyprogesterone acetate (MPA; a synthetic progestin). The intention was to characterize the benefits and risks associated with the most commonly prescribed hormone replacement therapy. The study included 27,000 women assigned to one of two groups: CEE and CEE+MPA, both of which had a placebo control. There were some significant benefits found for each treatment, but the health risks were considered so serious that the study was canceled early. The recommendations were that women should only take HRT as the last resort and for the shortest duration possible.

In the last 10 years the results of this study has been challenged due to major flaws in experimental design and analysis. For example: women of all ages were lumped together into a single group, from 50 to 80 years of age. The majority of

these women did not have HRT before the study, often being without hormones for more than 10 years. This population was not at all representative of women who take hormone treatments. Most are in the peri-menopausal stage, closer to 50 years of age, and many have past hormone use. Thus, retrospective analysis of the WHI work subdivided these groups by age and previous hormone status. This reanalysis found that the benefits of HRT are critical for the heath of many women (Gass, 2008; Santen et al., 2010). From this work an estimate of 19,000 – 92,000 women have died unnecessarily due to estrogen avoidance (Panay and Fenton, 2013).

There is also great variability in the types of hormone treatments given to women. Women with a uterus must take E_2 in combination with P_4 to avoid endometrial proliferation leading to cancer. However, there are numerous formulations of these hormones, each with different bioavailability, duration of action, and strength of binding. Estrogens are often sourced from bovine and equine animals, but synthetic estrogens are also used. For the WHI, conjugated equine estrogens (CEE) were used, that are predominantly comprised of estrone sulfate. Estrone has a lower bioavailability and estrogen-receptor activity than estradiol (O'Connell, 1995). Additionally, unopposed estrogens have been shown to have cardiovascular-protective effects in women, while conjugated equine estrogens have none (Williams and Suparto, 2004). Beyond the treatment regimen, length of hormone-free time before HRT, dosage and length of treatment, have an impact on alleviation of symptoms and relative health risks (Canonico et al., 2008; Herrinton and Weiss, 1993; Terauchi et al., 2012).

GOALS OF THIS DISSERTATION:

The goal of this dissertation work was to determine what role the aging hypothalamus plays in the response of female primates to hormone replacement therapy. My hypothesis was that there are age-related changes in hypothalamic networks that regulate reproduction and these changes are influenced by hormone status. To address this, I designed and carried out three experimental studies 1) Quantify the expression of hormone receptors in two hypothalamic regions that are important to female reproduction; 2) Determine the pattern of co-expression of GnRH and GPER; and 3) Describe the morphology of the microenvironment surrounding secretory neuroterminals in the median eminence.

I used ovariectomized adult female rhesus macaques as a model of human menopause. I used monkeys of two age groups to determine age-specific changes in the GnRH regulatory network: young and aged adults that were roughly equivalent to women that are 33 and 75 years old. To establish the effects of long-term hormone treatment, and possible interactions between age and hormones, these animals were given E_2, $E_2 + P_4$ or no hormone, for a period of two years.

Together, these studies will provide insight into the neuroendocrine aging processes of female primates.

CHAPTER 2: G-PROTEIN COUPLED ESTROGEN RECEPTOR, ESTROGEN RECEPTOR A, AND PROGESTERONE RECEPTOR IMMUNOHISTOCHEMISTRY IN THE HYPOTHALAMUS OF AGING FEMALE RHESUS MACAQUES GIVEN LONG-TERM ESTRADIOL TREATMENT

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_2) treatment, and their interactions, on expression of G protein-coupled estrogen receptor (GPER), estrogen receptor α (ERα) and progesterone receptor (PR) immunoreactivity (IR) 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_2 every 3 weeks for 2 years. Immunohistochemistry and stereologic analysis of ERα, 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-IR 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 ERα, E_2 treated monkeys tended to have higher cell density than vehicle monkeys in the ARC. The percent of PR-IR in the PERI tended to be higher in E_2 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_2 treatment has few effects on their expression, although GPER was affected more than ERα or PR. This result is surprising in light of evidence for E_2 regulation of the receptors studied here, and differences may be due to the selected regions, long-term nature of E_2 treatment, among other possibilities.

The text in this section is excerpted, with permission, from Naugle MM, Nguyen LT, Merceron TK, Filardo E, Janssen WGM, Morrison JH, Rapp PR, and Gore AC (2014), Journal of Experimental Zoology Part A: Ecological Genetics and Physiology. Dr. John Morrison, Dr. Peter Rapp, Bill Janssen, were involved in planning the hormone treatments and collecting the tissue specimens. Long Nguyen, Tyler Merceron aided in tissue preparation and stereological quantification. Dr. Ed Filardo provided the GPER antibody and editorial feedback. Dr. Andrea Gore was the primary investigator on this project.

INTRODUCTION

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_2) and progesterone (P_4) (Burger et al., 2002; Trévoux et al., 1986). 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, 1990; 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 vs. benefits of health outcomes are highly controversial (Canonico et al., 2008; Fitzpatrick et al., 2000; Herrinton and Weiss, 1993; Manson et al., 2013; Prentice et al., 2009; Rossouw et al., 2002; Talboom et al., 2008; Terauchi et al., 2012), 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; Maffucci and Gore, 2006; Rapp et al., 2003). 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 (Kermath and Gore, 2012; Maffucci and Gore, 2006). 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 (Downs and Wise, 2009; Gill et al., 2002a, 2002b; Gore et al., 2000a; Gougeon et al., 1994; Richardson et al., 1987; Weiss et al., 2004; Wise, 1984). 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 (Downs and Wise, 2009; Gore et al., 2000b; Hall, 2007; van Look et al., 1977; Rance, 2009; Shaw et al., 2011; Tsai et al., 2004; Wise and Ratner, 1980). 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 a (ERα), and progesterone receptor (PR), among others (Dorling et al., 2003; Glidewell-Kenney et al., 2007; Liu and Yen, 1983; van Look et al., 1977; Petersen et al., 2003; Skinner et al., 1998; Sullivan et al., 1995; Terasawa, 1995; Wilson et al., 2002). An important research gap is whether, and how, hormone feedback on the hypothalamus may change with aging and by which cells these effects are mediated. The mechanism for these changes is also unclear and 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., 1997; 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 (Archer, 2004; Gilardi et al., 1997; Gore et al., 2004; Krey et al., 1975). 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 (Castellano et al., 2010; Downs and Wise, 2009; Hofman, 1997; Mittelman-Smith et al., 2012; Wiegand and Terasawa, 1982). These hypothalamic areas also express high levels of steroid hormone receptors and are important targets of E_2 feedback in the regulation of the HPG axis (Bethea et al., 1996; Blurton-Jones et al., 1999; Michael et al., 2005; Mills et al., 2002; Petersen et al., 2003; Rapp et al., 2003; Skinner et al., 1998; Tsai et al., 2004). To determine whether there are age-related changes in steroid hormone receptors, and altered responses of these receptors to E_2 feedback, we quantified the density and percentage of cells that express GPER, ERα, and PR in the ARC and PERI of young and aged macaques that were ovariectomized (OVX) and given E_2 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 & 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 2.1** shows animal demographics, age distributions, and age at euthanasia, with 21 young adults (mean age 11 ± 4.4 years) and 15 aged adults (mean age 24.9 ± 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.

Table 2.1: Descriptive Data for Each Monkey Used in the Current Study.

Young Vehicle						
Animal ID	Age	Birth Date	Necropsy Weight (kg)	Necropsy Date	Conceptions	Live Births
27006	11.8	05/27/92	11.8	03/31/04	5	5
28392	13.3	05/15/94	7.6	08/21/07	4	2
28816	10.4	04/12/95	9.0	09/28/05	1	0
28973	8.8	05/10/95	8.9	03/31/04	1	1
29357	9.0	03/26/96	10.2	04/13/05	1	1
29431	8.3	04/03/96	9.0	07/14/04	0	0
30691	7.5	03/23/98	8.9	09/28/05	1	1
31998	10.2	01/01/98	5.0	03/04/08	4	4

Young Estradiol						
Animal ID	Age	Birth Date	Necropsy Weight (kg)	Necropsy Date	Conceptions	Live Births
26326	12.8	06/12/91	10.2	04/21/04	6	5
27424	14.6	04/02/93	8.5	11/13/07	8	7
27697	11.8	05/17/93	12.3	04/13/05	5	5
27723	11.1	05/21/93	11.4	07/14/04	4	4
27756	12.1	05/28/93	10.4	07/06/05	5	5
28733	9.2	03/29/95	11.3	06/23/04	2	2
29265	8.3	02/28/96	5.3	06/23/04	2	2
29583	9.2	04/18/96	9.6	07/06/05	2	1
29628	7.8	06/18/96	8.2	04/21/04	2	2
29704	11.8	05/05/96	8.5	03/04/08	4	1
31660	8.4	04/14/99	6.5	09/18/07	2	2
31993	10.1	03/30/98	5.3	05/27/08	5	2
32987	7.0	03/22/01	6.9	04/01/08	2	1

Table 2.1: Continued

Aged Vehicle						
Animal ID	Age	Birth Date	Necropsy Weight (kg)	Necropsy Date	Conceptions	Live Births
17416	25.0	09/14/77	9.9	10/02/02	12	12
17676	24.0	05/09/78	10.1	05/29/02	8	7
20568	23.4	01/09/80	7.8	07/02/03	15	12
28619	24.3	01/01/78	7.1	05/29/02	1	0
29177	27.9	10/01/74	9.5	09/11/02	0	0
30518	23.3	04/01/79	9.3	07/10/02	0	0
31787	28.9	04/25/74	8.8	04/09/03	0	0
31796	28.3	06/28/74	7.7	11/13/02	0	0

Aged Estradiol						
Animal ID	Age	Birth Date	Necropsy Weight (kg)	Necropsy Date	Conceptions	Live Births
17164	23.4	04/17/77	9.7	09/24/00	11	8
18261	23.5	03/06/79	11.1	10/02/02	15	13
20742	20.8	09/12/82	7.6	07/02/03	9	8
28594	25.3	01/01/77	9.2	05/08/02	1	1
28603	25.3	01/01/77	8.4	05/08/02	0	0
29163	28.5	01/01/74	10.7	07/10/02	0	0
30519	24.4	04/01/78	9.6	09/11/02	0	0

Monkeys were ovariectomized approximately 2.5 years prior to euthanasia, with estradiol or vehicle injections beginning ~3 months post-OVX.

Ovariectomy & 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_2C; 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 2.1**) were excluded after the study started due to illnesses, sudden death or incomplete OVX. The efficacy of E_2C injections was previously demonstrated by a rapid rise in circulating E_2 that peaked within 24 hours, at levels comparable with the preovulatory surge in intact female monkeys and similar to women (Hao et al., 2007; Oriowo et al., 1980; Rahimy et al., 1999; Shideler et al., 2003). Treatment duration was 2-3 years with all injections coded and administered in a blind fashion.

Perfusion & Tissue Processing

Twenty-four hours after the last E_2C or vehicle injection, animals were deeply anesthetized and perfused as per (Hao et al., 2003). Fixative was 1% paraformaldehyde (PFA) for 1 minute, then 4% PFA for 12 minute in phosphate buffered saline (PBS) with post-fixation for 6 hrs in 4% PFA. At the time of euthanasia, at 24 hours post-E_2, monkeys were expected to be in the negative feedback phase of steroid feedback onto the HPG axis (Oriowo et al., 1980; Rahimy et al., 1999; Terasawa, 1995). Group assignment was validated at perfusion, resulting in the exclusion of 2 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 3 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, 5 and 7 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 **Figures 2.3, 2.5** and **2.6**; they represent the maximum number of monkeys for each antibody for which a complete series could be collected. Attrition was due primarily due 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 (3 x 10 minutes) were performed prior to all steps, with the exception of the primary antibody. The tissue was treated to quench endogenous peroxidase activity for 20 minutes (30% of 3:1 methanol: 3% H_2O_2 in PBS) and to block non-specific binding for 1 hour (10% normal goat serum (NGS), S-1000, Vector Laboratories, Burlingame, CA, USA; 2% bovine serum albumin, A9085, Sigma-Aldrich, St Louis, MO; 0.5% triton X, in PBS) prior to incubating in one of the

polyclonal primary antibodies for 48 hours (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 hour (1:600; BA-1000; Vector Laboratories, in 5% NGS) and avidin-biotin-peroxidase complex for 1 hour (PK 6200; Vector Laboratories). Target was visualized with a 3,3'-diaminobenzidine (DAB)/peroxidase reaction (SK-4100; Vector Laboratories) for GPER and ERa, and nickel-DAB for PR, on ice. Sections were mounted on glass slides, dried overnight, and counterstained with methyl green (MP Biomedicals LLC, Solon, OH, USA; 0.5% in 0.1M Na acetate buffer pH 4.2), 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., 2007, 2000; Hammond et al., 2011; Kenealy et al., 2011b; Noel et al., 2009). Prior validation of the ERα antibody was carried out via western blot in human (Gorosito et al., 2008; Long and Nephew, 2006) 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 et al., 1992, 1996; Furuta et al., 2010; Quadros and Wagner, 2008; Quadros et al., 2008). 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 (Glaser and Wilson, 1998; Gundersen and Jensen, 1987; West et al., 1991), with parameters appropriate to achieve coefficients of error (CEs) no greater than 0.12 (Gunderson m=1). Abbreviations and terminology used throughout were taken from a combination of 3 atlases and modified to reflect most current usage (Bleier, 1984; Paxinos et al., 1990; Brainmaps.org). Counterstained 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, 1984; Paxinos et al., 1990). 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 (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 paraventricular nucleus. 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 40X, ERα 100X, PR 100X, and methyl green labeled cells at 40X.

Cell density (population estimate of IR cells / volume sampled μm^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 (6 rays, centered in the middle of nucleus at the widest part of each cell) (Gundersen et al., 1988; Korbo and Andersen, 1995). 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 3 sections containing the corresponding region. All slides were labeled with a random code and were quantified blind to group.

Statistical Analysis

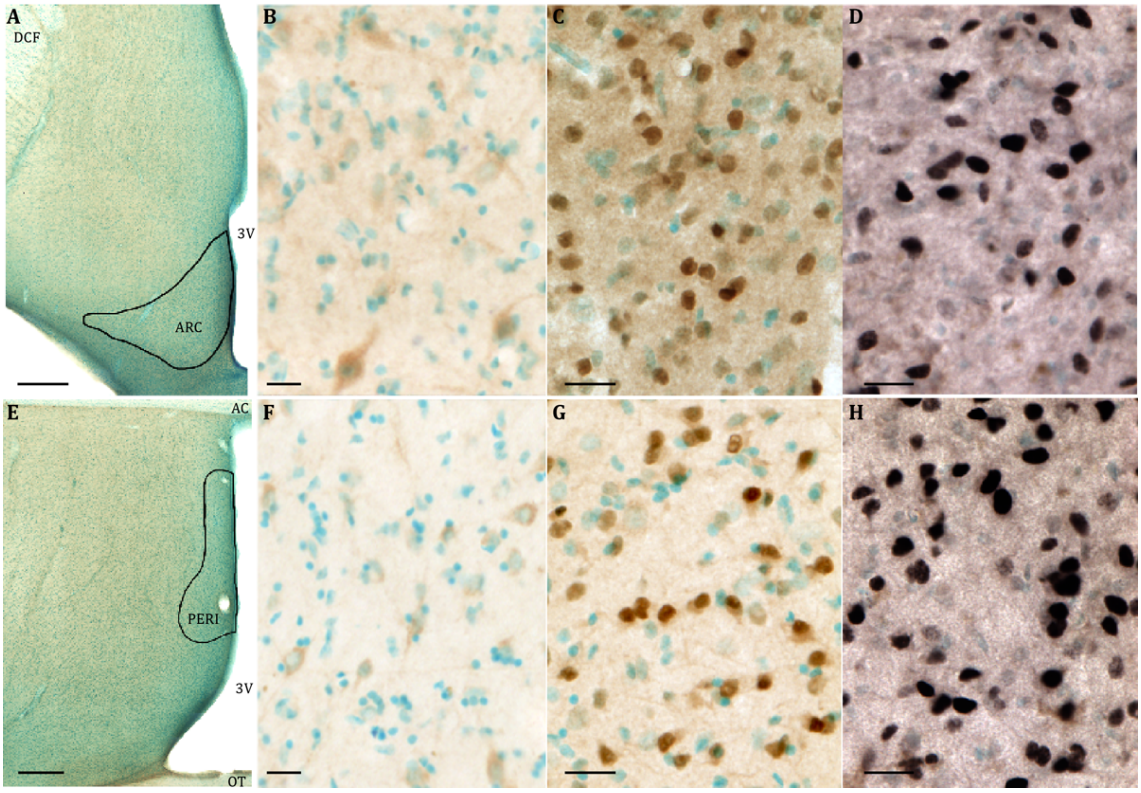
All analysis was done using R statistical packages [R Development Core Team]. Due to small sample sizes, high intra-group variability, and non-normal data distribution (analysis as determined by the Shapiro-Wilks normality and Levene's equality of variance tests), we used non-parametric tests for analysis. 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 (Benjamini et al., 2001; Hochberg and Benjamini, 1990; Kruskal and Wallis, 1952; Wilcoxon, 1945). 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 2.5X 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.

RESULTS

Both ERα 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 2.1**.

Figure 2.1: Photomicrographs Showing Expression of GPER, ERα and PR in the ARC and PERI.

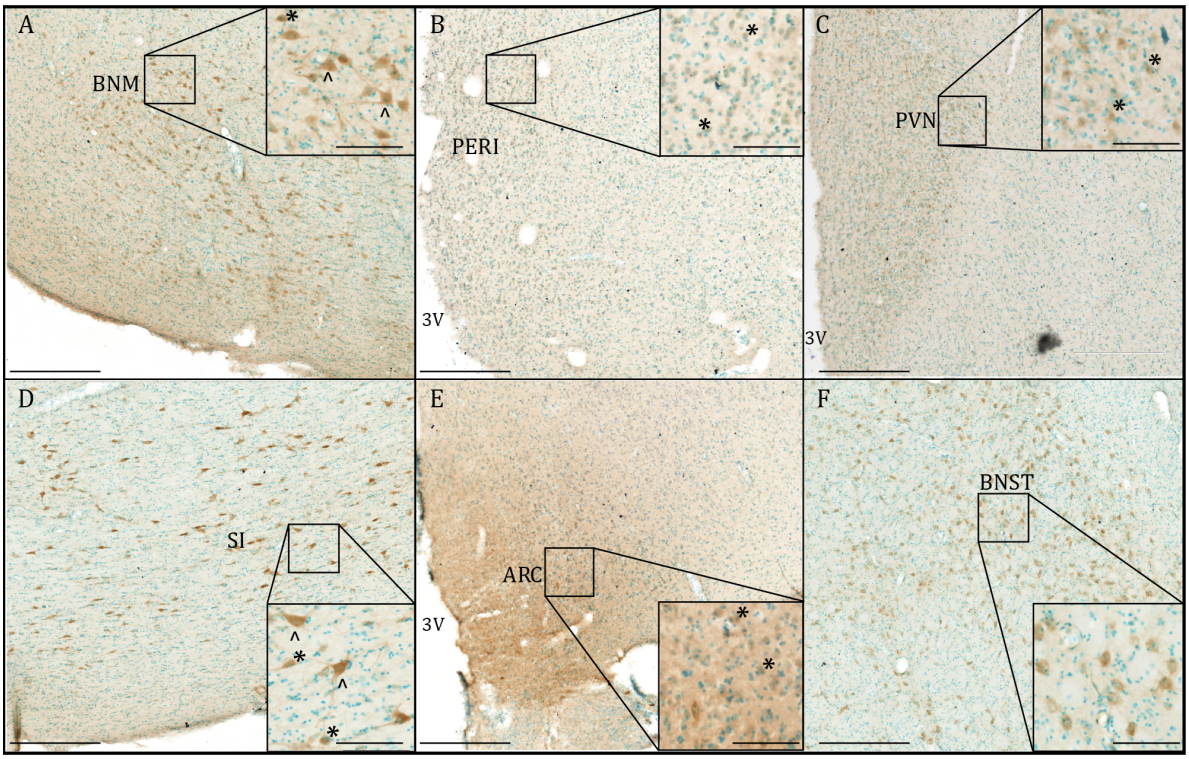


A, E: Low magnification of the ARC and PERI regions used for stereologic analysis. Scale bars = 200 μm. Boundaries were determined using distribution of counterstained cells and the anatomical landmarks: dorsal column of the fornix (DCF), third ventricle (3V), anterior commissure (AC), and optic chiasm (OX). The remaining representative micrographs were obtained from consecutive sections of monkey #18261 (AV group). B, F: GPER; C, G: ERα; D, H: PR, are shown in the ARC and PERI, respectively. Counterstained nuclei are blue-green. Scale bars = 25 μm (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.

GPER

GPER immunoreactivity was observed on cell processes and in perikarya (**Figure 2.2**) and was widely dispersed throughout the hypothalamus and surrounding regions. It is noteworthy that cellular labeling was relatively light in the ARC and PERI (**Figure 2.1** and **2.2**), as well as the paraventricular nucleus (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; **Figure 2.2**). The qualitative distribution and density of cells is shown in **Table 2.2** and high-resolution images are available in Supplemental **Figure 2.1**. 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 (**Figure 2.3**). 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 ($p=0.07$).

Figure 2.2: 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 μm^2) are indicated by ^ and small cells (< 150 μm^2) by *. Counterstained cells have blue nuclei. Scale bars = 100 µm for low magnification images and 25 µm for the insets.

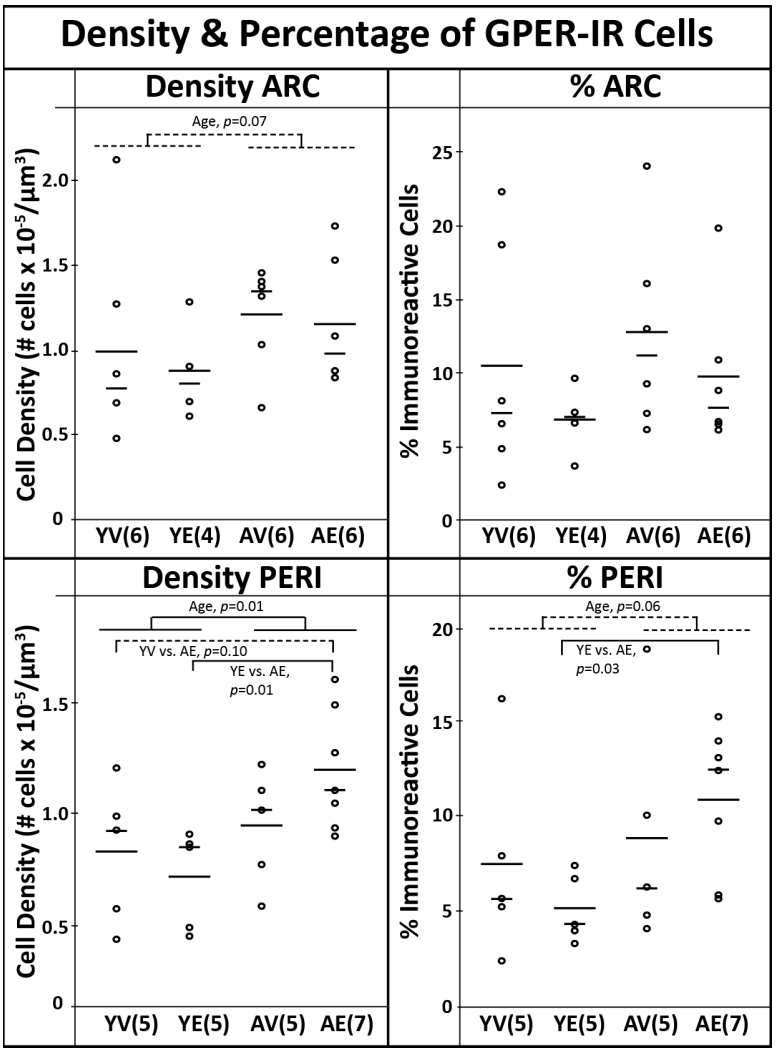


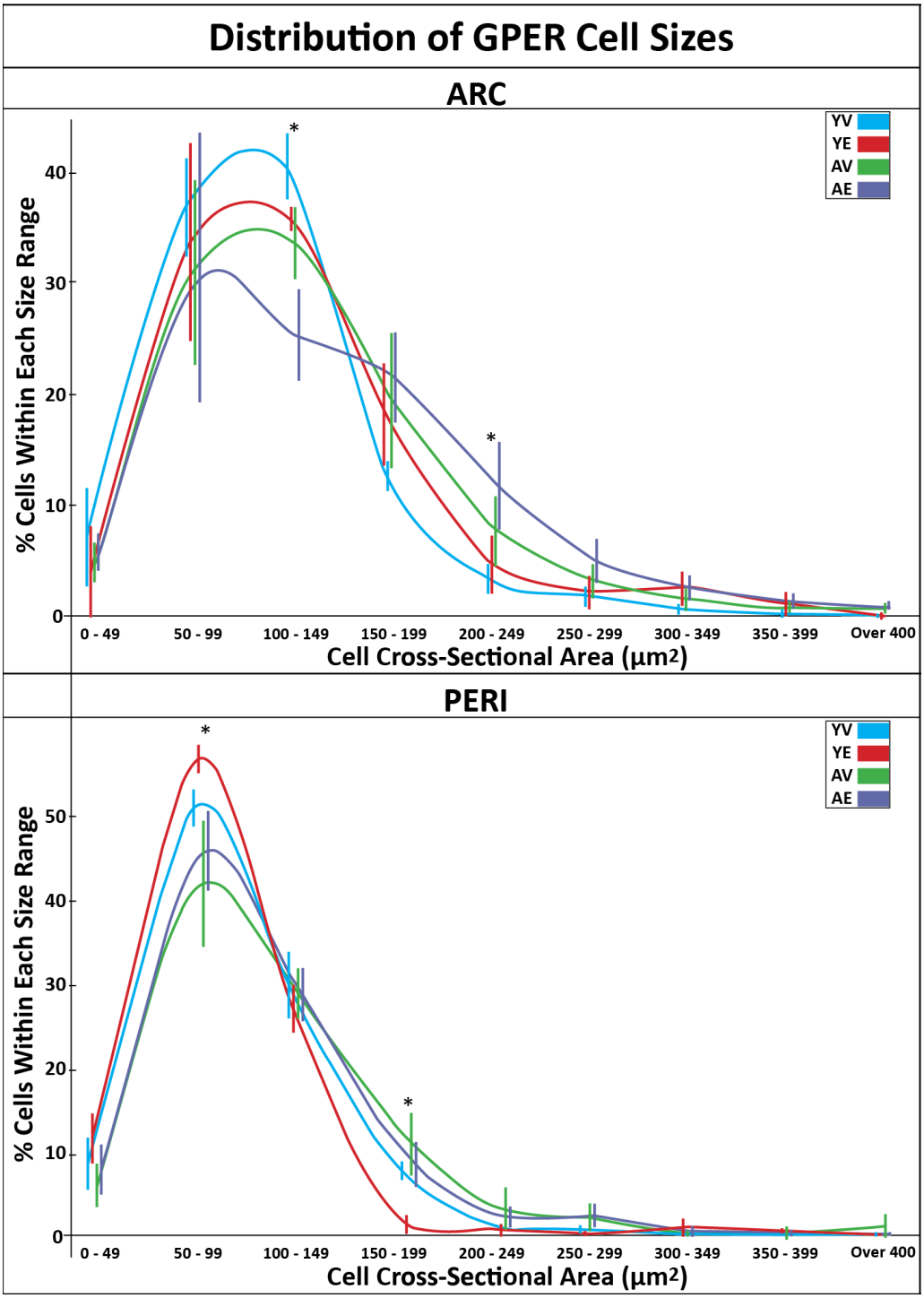
Figure 2.3: The Density and Percentage of Immunoreactive GPER cells in the ARC and PERI.

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.

During the analysis of GPER we noticed that many of the young animals had very small GPER-IR cells (defined as < 150 μm^2), while aged monkeys had fewer such cells. We quantified cell cross-sectional area using the Nucleator tool in StereoInvestigator. **Figure 2.4** shows the distribution of GPER size, divided into 50 μm^2 bins, for both regions. In the ARC, aged animals tended to have fewer cells

between 100-149 μm^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 200-249 μm^2 than the young, regardless of treatment (p=0.09). In the PERI, young animals tended to have more cells between 50-99 μm^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 μm^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 350-400 μm^2, and only 1 cell over 400 μm^2.

Figure 2.4: 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 μm^2 size bins (mean ± 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 100-149 μm^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 200-250 μm^2. In the PERI, there was a trend of young monkeys having more small cells between 50-99 μm^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 200-250 μm^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 350-400 μm^2, and only 1 cell over 400 μm^2 in the PERI. * $p \leq 0.10$.

Table 2.2: GPER Distribution and Cellular Morphologies in the Hypothalamus and Surrounding Areas.

Abreviation	Nucleus	Density of GPER	Type of label
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

Table 2.2: Continued

Abreviation	Nucleus	Density of GPER	Type of label
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

Table 2.2: Continued

Abreviation	Nucleus	Density of GPER	Type of label
SON	supraoptic nucleus	+++	medium to large cell bodies and processes
STr	stria terminalis	+	small vell bodies and processes
Sv	subventricular nucleus	++	medium cell bodies with processes
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 palladum	-/+	medium and large cell bodies, some processes
ZI	zona incerta	-/+	medium cell bodies
3V	third ventricle	~~0~~	none
AC	anterior commissure	~~0~~	none
DCF	descending columns of the fornix	~~0~~	none
LV	lateral ventricle	~~0~~	none
OT	optic tract	~~0~~	none

~~0~~ none; -- very sparse to none; - sparse; + moderate; ++ dense; +++ very dense.

This 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, 1984; Paxinos et al., 1990; Brainmaps.org).

ERα

ERα immunoreactivity was predominantly detected in cell nuclei, but some cytoplasmic and/or membrane labeling was observed in the ARC and PERI (**Figure 2.1**). In the ARC, there was a trend for a treatment effect (p=0.08) driven by YE

having more ERα-IR cells than YV (W=0.05; **Figure 2.5**). No significant effects of aging, treatment, or interactions, were found for the density or percent of ERα-IR cells in the PERI (**Figure 2.5**).

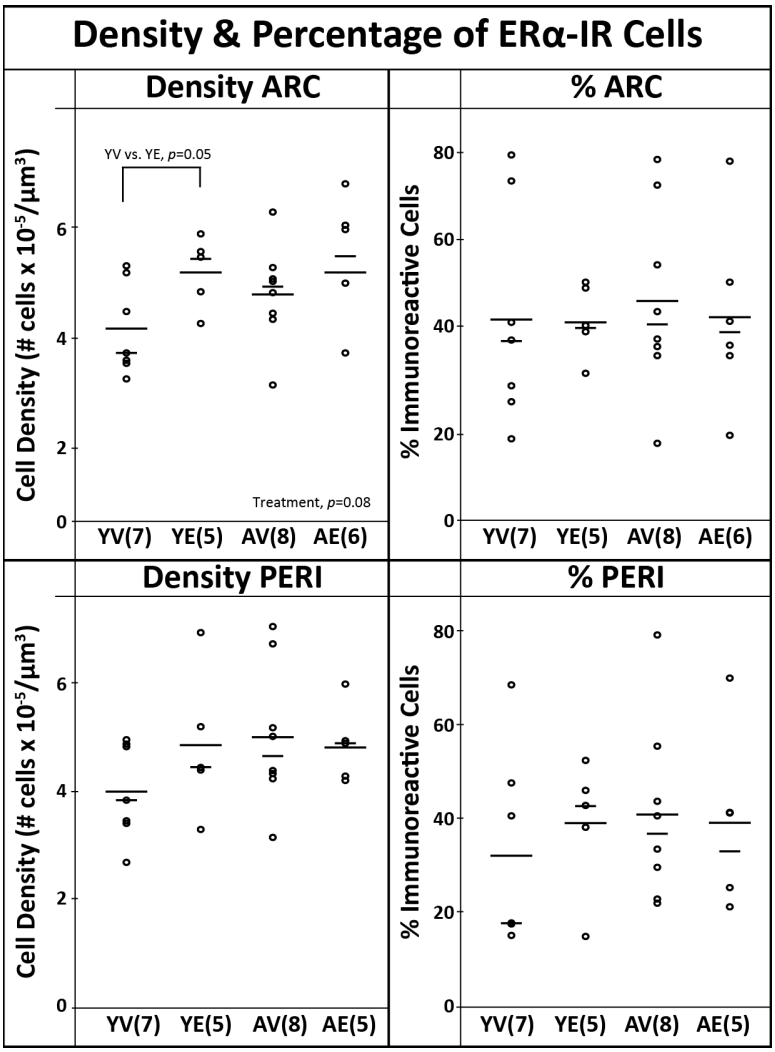


Figure 2.5: The Density and Percentage of Immunoreactive ERα cells in the ARC and PERI.

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_2 treated animals compared to vehicle (p=0.08), driven by YE > YV (p=0.05). The percent of ERα-IR cells were not affected by age or treatment. Sample sizes are shown in parentheses.

PR

PR was almost exclusively nuclear in cellular localization (**Figure 2.1**). 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. In the ARC, no effects of age, treatment or interaction were found in the density or percent of PR-IR (**Figure 2.6**).

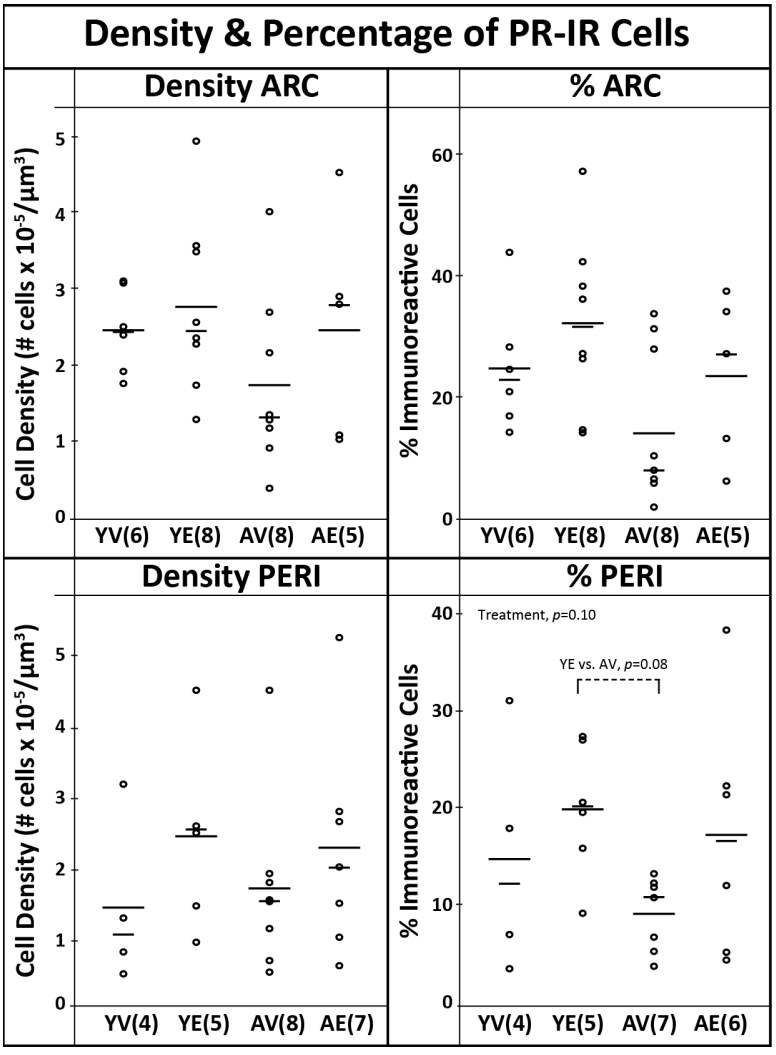


Figure 2.6: The Density and Percentage of Immunoreactive PR Cells in the ARC and PERI.

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 (p=0.10), with E_2 > vehicle. Sample sizes are shown in parentheses.

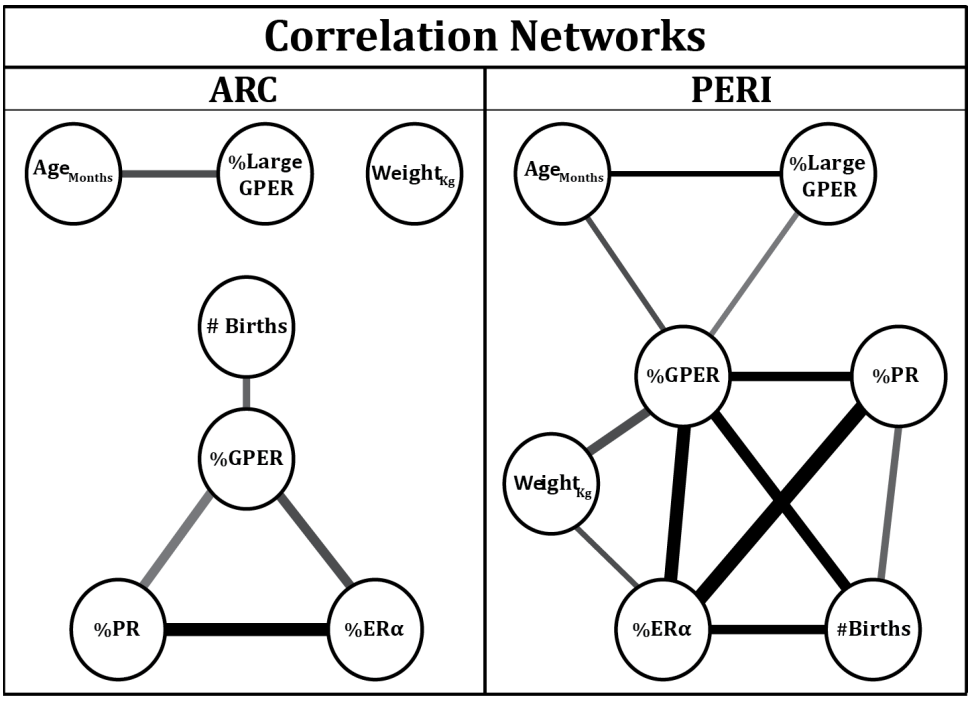
Total Cell Density in ARC & PERI

There were no significant effects of aging, treatment, or interaction in the total number of cells in either the ARC or PERI.

Correlation Network

Figure 2.7 depicts the relationship between the percent of GPER, ERα and PR-IR cells, percent large GPER (>150 μm^2), body weight, age and number of live births (see **Table 2.1**). **Table 2.3** 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).

Figure 2.7: 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 **Table 2.3**. 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 2.3: *P* Values for Correlation Networks.

ARC								
Correlation *p* values	# Births	Weight (Kg)	Age (Months)	% GPER	% ERα	% PR	% Large GPER	**Pearson's *r* values**
# Births	\	0.26	0.29	**0.49**	0.36	0.36	-0.51	# Births
Weight (Kg)	0.23	\	0.02	0.22	0.35	0.20	-0.21	Weight (Kg)
Age (Months)	0.35	0.40	\	0.21	0.17	0.08	***0.47***	Age (Months)
% GPER	**0.04**	0.43	0.34	\	***0.54***	**0.58**	-0.06	% GPER
% ERα	0.13	0.16	0.56	***0.01***	\	***0.71***	-0.24	% ERα
% PR	0.11	0.82	0.18	**0.07**	***0.00***	\	-0.23	% PR
% Large GPER	0.34	0.85	***0.01***	0.54	0.48	0.65	\	% Large GPER

PERI								
Correlation *p* values	# Births	Weight (Kg)	Age (Months)	% GPER	% Erα	% PR	% Large GPER	**Pearson's *r* values**
# Births	\	0.13	0.31	***0.61***	***0.55***	**0.40**	0.39	# Births
Weight (Kg)	0.23	\	0.08	***0.53***	**0.32**	0.19	-0.11	Weight (Kg)
Age (Months)	0.35	0.40	\	***0.33***	0.10	0.20	***0.35***	Age (Months)
% GPER	***0.01***	***0.02***	***0.03***	\	***0.74***	***0.59***	**0.35**	% GPER
% ERα	***0.00***	**0.04**	0.33	***0.00***	\	***0.84***	-0.11	% ERα
% PR	**0.05**	0.52	0.70	***0.01***	***0.00***	\	-0.07	% PR
% Large GPER	0.34	0.58	***0.00***	**0.08**	0.97	0.60	\	% Large GPER

Pearson's r-values (top) are highlighted in grey. All p values < 0.1 are bolded. Italicized p values also passed the Benjamini and Hochberg false discovery rate correction (ARC $p \leq 0.01$, PERI $p \leq 0.03$). Cell density, %, and total cells # were highly correlated therefore we included only % ir-cells for simplicity sake.

DISCUSSION

This study investigated the effects of aging, E_2 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_2 given over at least a two-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., 1996; Blurton-Jones et al., 1999; Michael et al., 2005; Mills et al., 2002; Petersen et al., 2003; Rapp et al., 2003; Skinner et al., 1998; Tsai et al., 2004). To our knowledge, studies assessing the expression of steroid hormone receptors in a model of aging and long-term E_2 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_2 and aging had limited effects on the numbers of ERα 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_2. 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_2 (Filardo et al., 2002; Kenealy and Terasawa, 2011; Kenealy et al., 2011b; Noel et al., 2009). GPER is becoming increasingly well-established as involved in metabolic, cardiovascular and immune physiology (Filardo and Thomas, 2012; Prossnitz and Barton, 2011; Sharma and Prossnitz, 2011; Wang et al., 2008). 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 (Kenealy and Terasawa, 2011; Kenealy et al., 2011b; Noel et al., 2009). This is consistent with the possibility that GPER plays a role in the E_2-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_2 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 numbers in the PERI were 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. 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 (Abel and Rance, 1999; Rance, 2009; Rance et al., 1990; Rometo and Rance, 2008). 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.

ERα

We chose to examine ERα due to its critical role in reproduction, as demonstrated by the infertility of ERα-knockout mice (Lubahn et al., 1993). The only significant finding was that YE had greater ERα 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_2 treatment can increase, a decrease or have no effect on ERα mRNA or protein, with differences potentially attributable to species, hypothalamic region, and the nature of E_2 treatment (Blaustein, 1993; Chakraborty et al., 2003; Lauber et al., 1991; Olster, 1998; Shughrue et al., 1992). An age-related increase of ERα-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_2 treatment observed in any age group (Chakraborty et al., 2003). Interactions of E_2

and age have also been reported in these and other studies (Chakraborty et al., 2003; Funabashi et al., 2000). For example, Funabashi et al. found that in young rats, 4 days of E_2 had no effect on ERα mRNA in the preoptic area (POA) and reduced expression in the ARC and ventromedial hypothalamus (VMH) (Funabashi et al., 2000). By contrast, middle-aged animals had decreased ERα mRNA with E_2 in the POA only, and old animals showed no effect of E_2 treatment (Funabashi et al., 2000). Aged rats had a decline in ERα 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_2 treatments.

There are limited data on changes to ERα with aging or E_2 in nonhuman primates. In young adult monkeys, Bethea et al. showed no effect of E_2 on ERα mRNA or protein in the ARC, paraventricular nuclei (PVN), VMN or pituitary (Bethea et al., 1996). In companion studies performed on prefrontal cortex and hippocampus of the same monkeys used in our current study, no changes in ERα-IR cells were found (Adams et al., 2002; Wang et al., 2010). It is therefore not entirely surprising that we found few differences in ERα-IR cell density, and no change in the percentage of these cells in the PERI and ARC, with age or E_2 treatment.

We were unable to perform immunohistochemistry on ERβ 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 ERβ is co-expressed in GnRH neurons, and mediates rapid GnRH responses to estrogen treatment in rats (Abrahám et al., 2003; Hrabovszky et al., 2001). In addition, ERβ mRNA is expressed in several hypothalamic regions, including paraventricular nucleus, preoptic area, and ventromedial nucleus, in the rhesus macaque brain (Gundlah et al., 2000; Pau et

al., 1998). Therefore, this is an important area for future studies when a validated ERβ 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., 1996). Although E_2-mediated up-regulation of PR is well documented, it is age, sex, brain region and species dependent (Bethea et al., 1992; Blaustein and Turcotte, 1989; Mills et al., 2002; Olster, 1998; Quadros and Wagner, 2008; Rometo and Rance, 2008). Here, we saw no significant effects of age or long-term cyclic E_2, though there was a trend for higher % PR cells in E_2 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 **Table 2.1**), 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_2 treatment regimens. Similar to what we observed, other groups showed that young monkeys responded to short-term (28 days) constant E_2 treatment with increases in PR-IR cell numbers and mRNA in some (VMN and ARC), but not all (PVN) hypothalamic regions (Bethea et al., 1992, 1996). Another study showed that in the hypothalamus of rats, there is a disconnect between mRNA and

protein induction by short-term (4 day) E_2 treatment: Furuta et al. demonstrated that the increase in PR-IR is attenuated with age, while Funabashi et al. showed that aged rats maintained a robust PR mRNA response to E_2 (Funabashi et al., 2000; Furuta et al., 2010). 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_2, while the older females responded with increased PR-immunoreactivity (Quadros and Wagner, 2008). By contrast to our results, there was also a consistent PR induction in the medial preoptic nuclei of rats at both ages (Quadros and Wagner, 2008). Taken together, there is a complex relationship among age, duration of E_2 treatment and PR expression, in many species. Ongoing work in our laboratory utilizing different E_2 regimens and comparing chronic to cyclic E_2 may shed further light on this question. Regardless of future results, current data reported here suggest little change in ARC and PERI PR with age and E_2 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 2.7** 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 μm^2. Other interesting points are that ERα 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 & Conclusions

Our study demonstrates that the aged primate hypothalamus retains the ability to express steroid hormone receptors at comparable levels to young adults. There were also few effects of long-term E_2 on ERα and PR cell 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_2 treatment suggest that changes previously observed in aged animals in the expression of ERα 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 (Rance, 2009; Rance et al., 1990; Rometo and Rance, 2008; Ule et al., 1983). In nonhuman primates, these data suggest that, following long-term cyclic E_2, ERα 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.

ACKNOWLEDGMENTS

Additionally, we are grateful to Dr. Jeffrey Roberts and Heather McKay at the California National Primate Research Center for expert care of the animals. Dr. Weiling Yin and Megan Noel assisted with preparation and sectioning of hypothalamic tissues. Christina Tran, Sateria Lozano, and Syed Zafar were very helpful with data collection and organization. Dr. Hans Hofmann and Dr. Theresa Jones provided important advice on the analysis of correlations and stereological results. This work was supported by NIH Grant P01 AG16765.

CHAPTER 3: GNRH NEURONS OF YOUNG AND AGED FEMALE RHESUS MONKEYS CO-EXPRESS GPER BUT ARE UNAFFECTED BY LONG-TERM HORMONE REPLACEMENT

Abstract: Menopause is caused by changes in the function of the hypothalamic pituitary gonadal (HPG) axis that controls reproduction. Hypophysiotropic gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus orchestrate the activity of this axis and are regulated by hormonal feedback loops. The mechanisms by which GnRH responses to the primary regulatory sex-steroid hormone, estradiol (E_2) are still poorly understood in the context of menopause. Here we used immunofluorescence double labeling to characterize the co-expression of G protein-coupled estrogen receptor (GPER) in GnRH neurons in the hypothalamus of young and aged rhesus macaques. Animals were given long-term hormone treatments (E_2, E_2 + progesterone, or vehicle, for two years) that mimic currently prescribed hormone replacement therapies used for the alleviation of menopausal symptoms in women. We found that about half of GnRH perikarya express GPER, while only about 12% of GnRH processes and terminals in the median eminence (ME) are double labeled. Additionally, many GPER labeled processes were in direct contact with GnRH neurons, often wrapped around the perikarya and processes and in close proximity in the ME. These results extend prior work by showing robust colocalization of GPER in GnRH in a clinically relevant model, and support the possibility that GPER-mediated E_2 regulation of GnRH occurs both in the soma and terminals in nonhuman primates.

INTRODUCTION

Menopause is often accompanied by one or more neurobiological symptoms such as hot flushes, depression, irritability, sleep disruptions, lowered energy levels, memory deficiency, sexual dysfunction, and cognitive decline (Avis et al., 1994, 2009; Hall, 2004; McEwen, 2002). The most common treatment for menopausal symptoms is hormone replacement therapy (HRT), used primarily to improve quality-of-life from vasomotor symptoms, vaginal dryness, and osteoporosis (Cauley et al., 2014; Maki, 2013). While not prescribed for neurobiological symptoms other than hot flushes, HRT undoubtedly has widespread effects on the brain. As the mean

age of the population increases, the number of post-menopausal women continues to grow, with broad economic, healthcare and social costs. Thus, it is increasingly important to understand the complex mechanisms underlying reproductive aging and the effects of HRT on the brain.

Reproduction is controlled by the precise synchrony of hormones released by the hypothalamus, pituitary and gonad, which make up the HPG axis. Gonadotropin-releasing hormone (GnRH) is a neuropeptide that is synthesized by neurons in the mediobasal hypothalamus (MBH) in primates, and released from nerve terminals at capillaries within the median eminence (ME; (Gore, 2002; King and Anthony, 1984; Rangaraju et al., 1991; Richter and Terasawa, 2001; Sisk and Foster, 2004)). GnRH secretion triggers the release of the gonadotropins from the anterior pituitary that stimulates the production of sex steroid hormones, predominantly estradiol (E_2) and progesterone (P_4) in the ovaries. These hormones then feed back onto the hypothalamus and pituitary, ultimately modulating the production and release of GnRH. Reproductive aging is characterized by changes to all three levels of the HPG axis (Gore et al., 2014), with the decline in E_2 and P_4 leading to many of the somatic and neurobiologic functional changes that accompany the menopausal transition. The role of GnRH is less clear; while rodent studies suggest that a loss of GnRH drive is a primary mechanism for reproductive failure, in the human, changes to the hypothalamus may be secondary to pituitary and/or ovarian failure (Gill et al., 2002a, 2002b; Gore et al., 2000b; Weiss et al., 2004).

Our understanding of the feedback regulation of GnRH via steroid hormones has evolved considerably over the past twenty years. Although GnRH cells do not co-express ERα, they do express ERβ, and GnRH cells respond rapidly to E_2 with

increased action potential rate, intracellular Ca^{2+} oscillations, and GnRH peptide release (Abe and Terasawa, 2005; Abe et al., 2008; Abrahám et al., 2003; Hrabovszky et al., 2007; Jacobi et al., 2007; Kenealy et al., 2011a; Noel et al., 2009; Sullivan et al., 1995; Sun et al., 2010; Zhang et al., 2010). At least some of these rapid responses are mediated via the G protein-coupled membrane estrogen receptor (GPER, sometimes called GPR30; (Abe and Terasawa, 2005; Abe et al., 2008; Kenealy et al., 2011a; Noel et al., 2009; Sun et al., 2010)). There is a single report showing co-localization of GPER on one GnRH neuron in the primate brain; other work in this arena was conducted *ex vivo* or electrophysiologically (Chu et al., 2009). Our laboratory recently reported age-related increases in the density of GPER-immunoreactive cells in the arcuate nucleus and periventricular nucleus of female rhesus monkeys and confirmed that GPER-positive cells are found in regions where GnRH neurons are expressed in the monkey (Naugle et al., 2014). To provide new insights into the possible co-expression of GPER in GnRH neurons, we used fluorescent dual-label immunohistochemistry (IHC) and confocal microscopy to quantify the percent of GnRH perikarya that expressed GPER in the mediobasal hypothalamus, and co-expression in GnRH processes in the median eminence, of young and aged monkeys given different clinically relevant hormone replacement treatments.

METHODS

Animals

A total of 39 rhesus monkeys (*Macaca mulatta*) of two age ranges were included in this study: young adult (n = 17; aged 9.9 yr ± 2.3 yr) and aged (n = 22;

aged 22.6 yr ± 1.6 yr). Young animals were all premenopausal and aged animals were all peri- or post-menopausal, as determined by either normal cycling lengths of 24-34 days, or irregular cycles longer than 35 days, respectively (Gilardi et al., 1997). Cycle length was established by daily visual inspection of vaginal bleeding for 2 years before initiation of the study. Additionally, all of the animals in the aged group were over 19 years because prominent endocrine and neurobiological signs of aging do not occur in monkeys before reaching that age (Bachevalier et al., 1991). The animals were housed and experiments carried out at the California National Primate Research Center, as described previously (Hao et al., 2003, 2006, 2007; Hara et al., 2014; Rapp et al., 2003; Wang et al., 2010). Procedures were approved by the Institutional Animal Care and Use Committee at the University of California and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All of the monkeys were selected based on the following criteria: they had no long-term or invasive dietary or pharmacological interventions, and they were all in good physical health with no physical abnormalities or severe arthritis.

Ovariectomy & Hormone Treatment

All animals were ovariectomized (OVX) as previously described (Hao et al., 2003, 2006, 2007; Hara et al., 2014; Rapp et al., 2003; Wang et al., 2010). Briefly, they were sedated with ketamine (10 mg/kg) and atropine (0.4 mg/kg), anesthetized with isoflurane, and their ovaries and Fallopian tubes removed. They were given a 2-3 month recovery period before assignment to one of the three treatment groups, designed to mimic current hormone regimens commonly

prescribed to women. These consisted of the following conditions: vehicle (young n = 7 [YV], aged n = 8 [AV]), continuous estradiol (young n = 5 [YE], aged n = 6 [AE]) and continuous estradiol plus cyclic progesterone (young n = 5 [YEP], aged n = 8 [AEP]). Some animals were excluded from the study due to illness or incomplete OVX as determined by hormone analysis; the n's reported here are the final numbers used in this study. For the hormone groups, two Silastic capsules (3 cm long and 0.46 inner diameter), containing crystalline estradiol (E_2, Sigma) were implanted subcutaneously between the shoulder blades, achieving a mean serum level of 150 pg/ml, and were replaced every three months. The vehicle group received empty capsules on the same schedule, plus an intramuscular injection of 1 ml peanut oil to mimic a fourth treatment group (cyclic estradiol) that we were unable to include in this study due to the lack of aged-matched animals. The E_2 + cyclic P_4 group received 100 mg of micronized progesterone (Catalent Pharm Solutions), mimicking luteal phase P_4 levels, in their daily fruit treat, for 10 out of every 28 days, while the other groups received untreated fruit.

Euthanasia & Tissue Processing

The monkeys were perfused 8-10 days after the last P_4 treatment and the corresponding day for the other groups as previously described (Baxter et al., 2013; Ohm et al., 2012). Briefly, they were deeply anesthetized with ketamine (25 mg/kg) and pentobarbital (20–35 mg/kg) and were perfused transcardially with ice-cold 1% paraformaldehyde, followed by 4% paraformaldehyde in phosphate buffer (PB), for 1 and 12 minutes (at 185 ml/min), respectively. The brain was removed and the hypothalamus was dissected then postfixed for 6 hours in 4% paraformaldehyde

and 0.125% glutaraldehyde in PB and shipped to the University of Texas at Austin in PB for further processing. The mediobasal hypothalamus (MBH) was sectioned at 100 μm using a vibrating microtome (Leica VT1000 S; Leica, Bannockburn, IL). Half of the median eminence (ME), when present, was dissected for use in companion studies and the remaining sections were cryoprotected in a sucrose series (12%, 18%, 30% for 48 hrs each) and stored at -20 °C in 30% glycerol for later use.

Immunohistochemistry

We used dual-label fluorescence immunohistochemistry (IHC) to visualize GnRH and GPER distribution in the MBH. Four to five sections containing the MBH were collected from each animal and allocated into rostral, medial and caudal groups for IHC. There were an uneven number of sections available from each region due to tissue damage and loss from poor perfusions and processing errors. A second IHC run was performed to fill in gaps from animals in the first run in which fewer than 5 GnRH neurons were found. Between each step of the IHC, sections were washed in phosphate buffered saline (PBS; pH 7.4) 3 x 10 minutes. Due to the thickness of the sections, we used a citrate buffer (pH 8.5; preheated to 70 °C; 30 min) for antigen retrieval. Nonspecific binding sites were blocked with a solution of 10% normal goat serum (NGS; S-1000, Vector Laboratories, Burlingame, CA, USA) and 2% bovine serum albumin (BSA; Sigma-Aldrich, USA) for 90 minutes. We incubated sections for 48 hours (at 4°C with constant agitation) in mouse anti-GnRH (HU11b, diluted to 1:1,000; a gift from Dr Henryk Urbanski) and rabbit anti-GPER primary antibody (diluted 1:1,000; a gift from Dr. Edward Filardo), in 2% NGS in PBS. We then incubated the sections simultaneously in the following secondary

antibodies for 2 hours: Alexa Fluor 594 conjugated goat anti-mouse IgG and Alexa 488 anti-rabbit IgG (both diluted 1:400; Life Technologies, Eugene, OR, USA), in a solution of 5% NGS in PBS. The primary antibodies have been thoroughly validated in rodent and primate tissue (Filardo et al., 2007, 2000; Hammond et al., 2011; Kenealy et al., 2011b; Kermath et al., 2013; Miller and Gore, 2002; Noel et al., 2009; Urbanski, 1991). Here, the primary antibodies were omitted in experimental controls and no specific labeling for either fluorophore was found. To reduce lipofuscin autofluorescence, we treated the tissue with 10 mM CuSO4 in 50 mM ammonium acetate for 20 minutes. The sections were then mounted on glass slides, allowed to dry for 10 minutes and coverslipped using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The edges of the coverslip were sealed with clear nail polish. The slides were stored light-protected at 4°C.

Confocal Microscopy

We used a Zeiss LSM 710 microscope running Zen Black software (Carl Zeiss International, version date: 2012) with a Plan-Apochromat 40x/1.4 Oil DIC M27 objective for imaging the MBH of each animal. At the beginning of each imaging session, the laser power, gain and offset were adjusted using the negative control as a reference. For imaging the perikarya and ME, the two fluorophores were scanned sequentially to prevent signal bleed-through and the average of two scans for each line was recorded to decrease background. The GnRH scan was done with an excitation wavelength 561 nm (detection range 566 nm - 689 nm) followed by GPER with an excitation wavelength 458 nm (detection range 492 nm - 598 nm). To map the distribution of GnRH immunoreactive (IR) cell bodies, we imaged the entire

MBH plus some surrounding regions where hypophysiotropic GnRH perikarya are distributed (Goldsmith and Song, 1987; Witkin, 1986). GnRH neurons are 5-10 μm in thickness and to ensure that no GnRH perikarya would be missed, we took a z-stack with a voxel depth of 5 μm and a step size of 9 μm through the whole section. GnRH neurons were detected through the entire thickness of the sections, indicating that there was complete penetration of the antibodies. The location of each GnRH-IR neuron was marked as single (GnRH only) or double (GnRH+, GPER+) on a diagram of the MBH representing the rostral, medial and caudal levels based on four to six overlapping slices (depending on neuron orientation; with voxel depth of 2 μm) through each neuron for GnRH-IR followed by GPER-IR. To determine the relationship between GPER-IR and GnRH neuroterminals in the ME, we took a high-resolution image stack of the internal and external zones of 1-2 ME sections (depending on tissue availability) for each animal.

Analysis & Statistics

All perikarya were analyzed with Fiji software (Schindelin et al., 2012), and MatLab software (version R2013a; MathWorks, Natick, MA) was additionally used for calculating the percent colocalized puncta in the ME. We used 2 methods in concert to categorize the immunoreactivity of the perikarya, both the traditional pseudocolored merge of the 2 channels and a grey-scale analysis to control for any optical illusions due to color perception bias. This was done with an image calculator plug-in in Fiji that created a single gray-scale image that shows the minimum intensity for each pixel of the 2 channels (i.e. if there is no colocalization then the minimum intensity is black). We rated the amount of GPER-IR, from 1-6, in

each GnRH-IR perikarya with both methods, and re-scored a random subset of the images to ensure consistency. The scores from the two categorization methods were compared, resulting in three categories: no GPER-IR, moderate/punctate colocalization, and heavy colocalization.

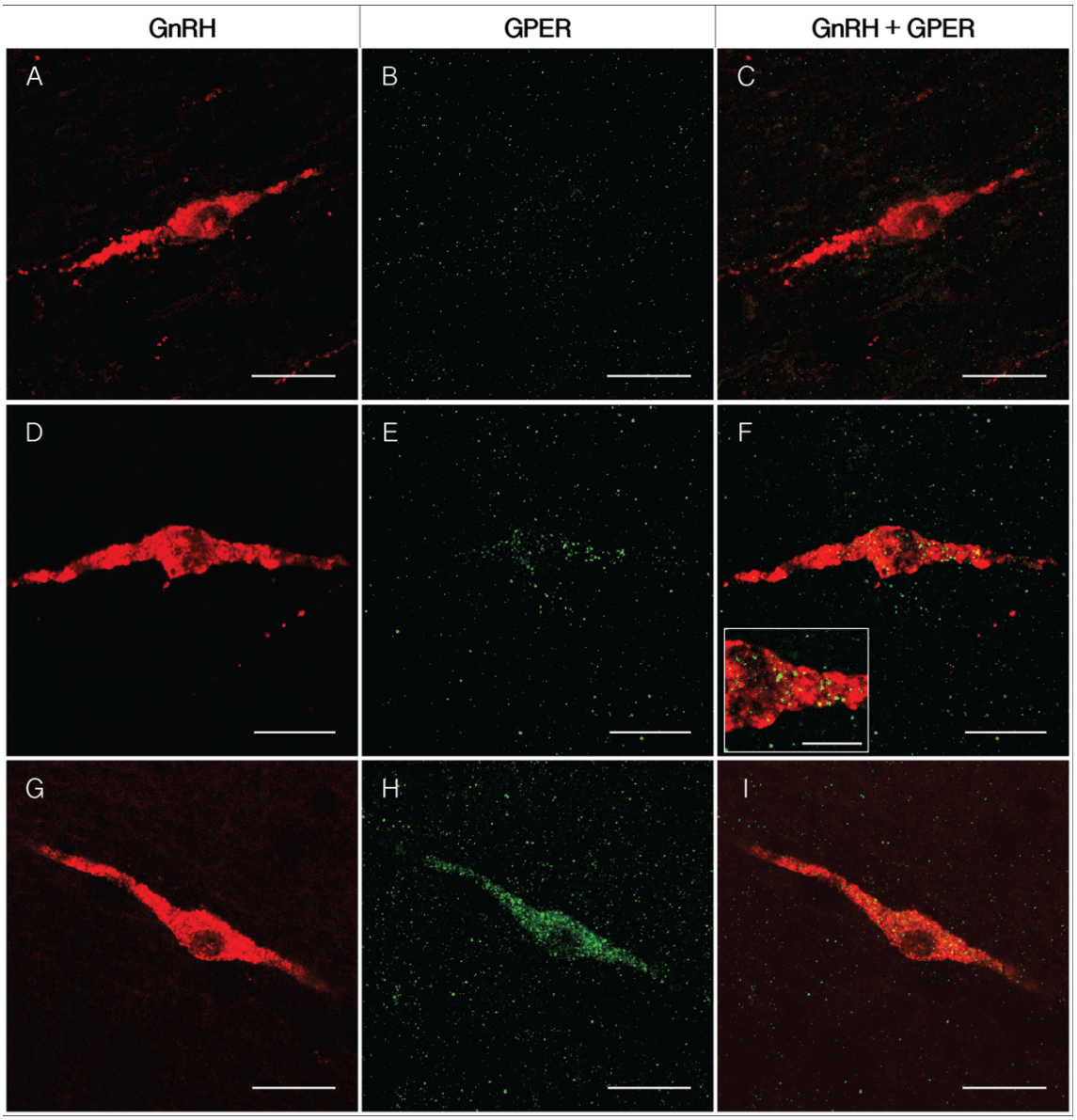
We used R statistical packages (R Development Core Team, 2012) for all statistical analyses. Kruskal-Wallis (p) nonparametric test were used because our dataset was not normally distributed as was determined by the Shapiro-Wilks normality and Levene's equality of variance tests. Our alpha level was set to determine significant main effects ($p \leq 0.05$) or trends ($0.05 < p < 0.10$) followed by a pairwise Wilcoxon post-hoc test to identify specific interactions (Kruskal and Wallis, 1952; Wilcoxon, 1945).

RESULTS

GnRH Perikarya in the MBH Co-express GPER

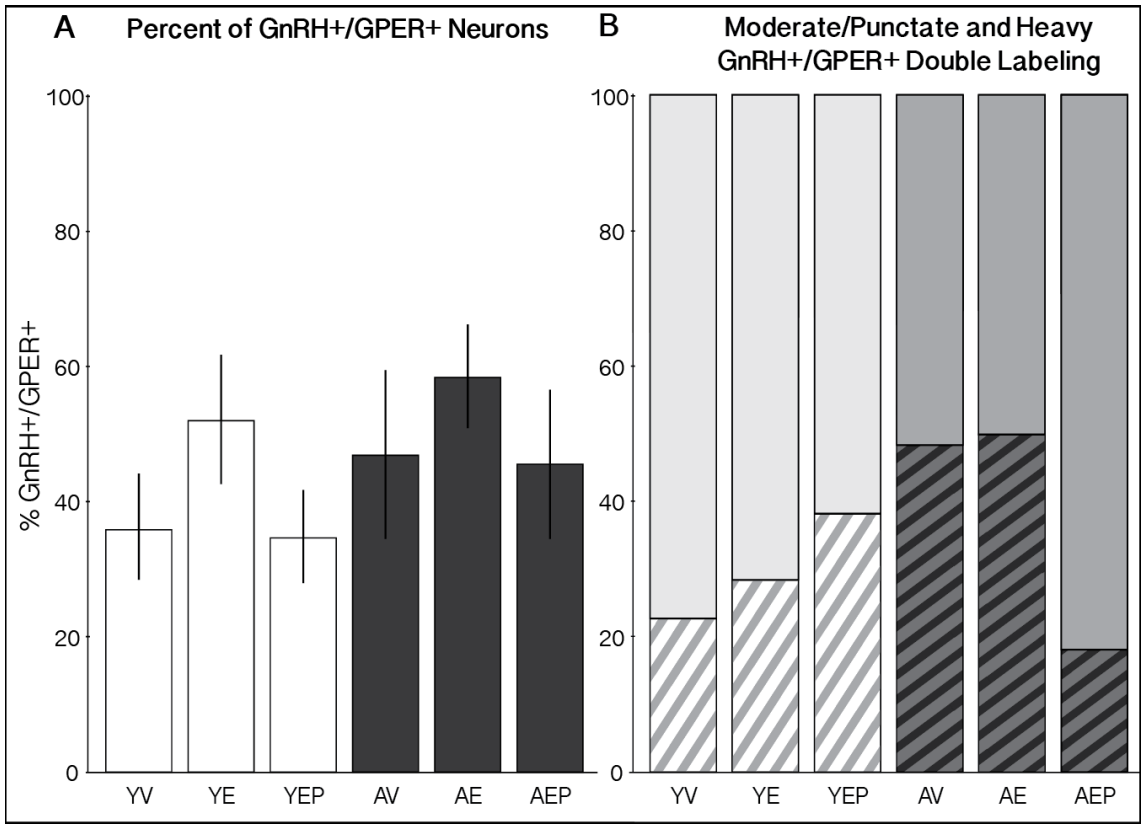
Examples of GnRH perikarya in the MBH that did or did not co-express GPER are shown in **Figure 3.1**. GnRH neurons in the 6 groups of monkeys (YV, YE, YEP, AV, AE, AEP) had the characteristic morphology of bipolar or unipolar perikarya with large nuclei, and were scattered through the basal hypothalamus. No significant main effects of age or hormone treatment were found for the morphology or average number of GnRH neurons per section (data not shown). For double labeling, about half of GnRH-IR neurons expressed GPER, regardless of age or hormone treatment (**Figure 3.2A**). Labeling of GPER in the GnRH neurons was variable, with some GnRH cells showing punctate or moderately intense labeling and others with very heavy labeling throughout the cytoplasm and into the

Figure 3.1: Representative photomicrographs of GPER expression within GnRH neurons.



Photomicrographs of GnRH-IR (left column), GPER-IR (center column) and double labeling (right column). Representative micrographs of the categories of GPER (green) expression within GnRH neurons (red) are shown for unlabeled (A-C), moderate / punctate GPER labeling within GnRH perikarya (D-F), and heavy cytoplasmic double labeling (G-I). The high magnification inset (F) shows punctate GPER-IR. Scale bars = 25 μm (A-I), 10 μm (F inset).

Figure 3.2: Percent of GnRH perikarya that express GPER (GnRH+/GPER+) and the subset of those double-labeled cells that have heavy cytoplasmic label.

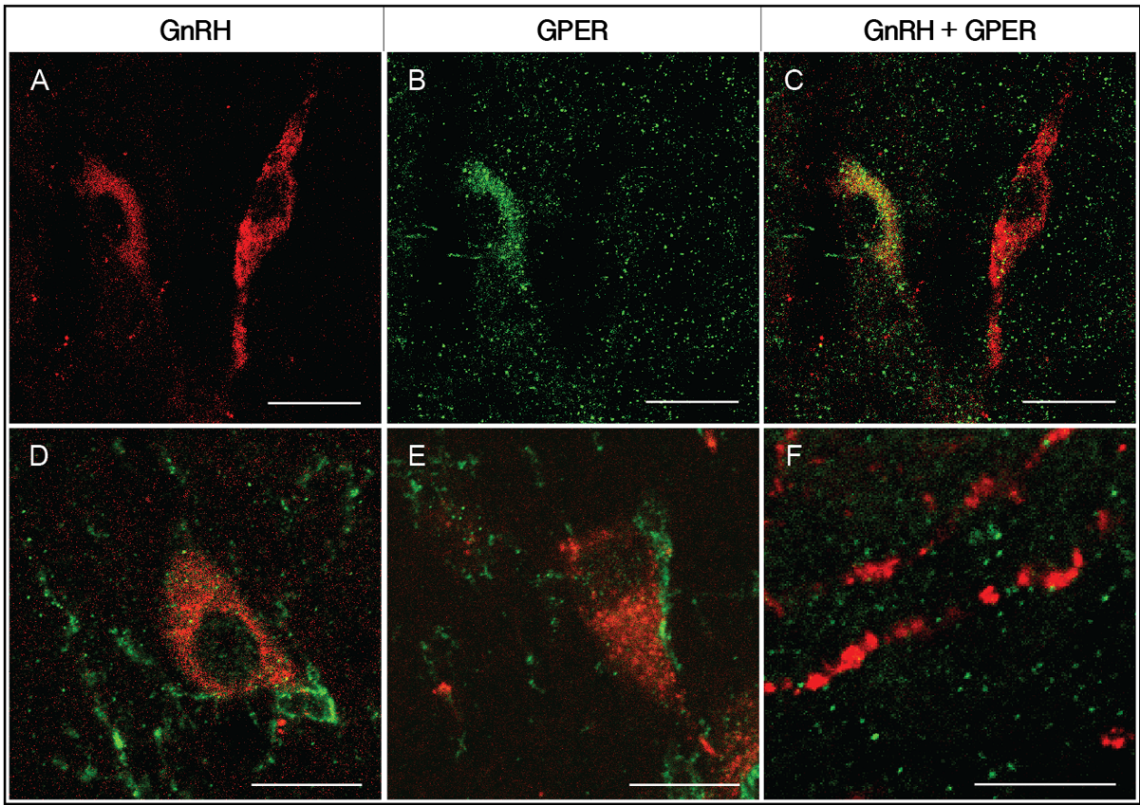


(A) The average percent (± SEM) of GnRH+/GPER+ perikarya throughout the entire MBH for young (white bars) and aged (black bars) monkeys is shown. (B) The percent of GnRH+/GPER+ cells with either moderate/punctate (top, solid) or heavy cytoplasmic (bottom, striped) GPER-IR, for each group. There were no significant effects of age or treatment in the total percent or type of double-label.

varicosities (**Figures 3.1** & **3.3**). Additionally, we observed many GPER-IR processes in close contact with GnRH perikarya and/or proximal processes (**Figure 3.3**). During analysis we noticed great variability in the intensity of GPER immunolabeling in GnRH neurons. Therefore, we further subdivided double-labeled GnRH cells qualitatively into moderate/punctate or heavy GPER co-localization.

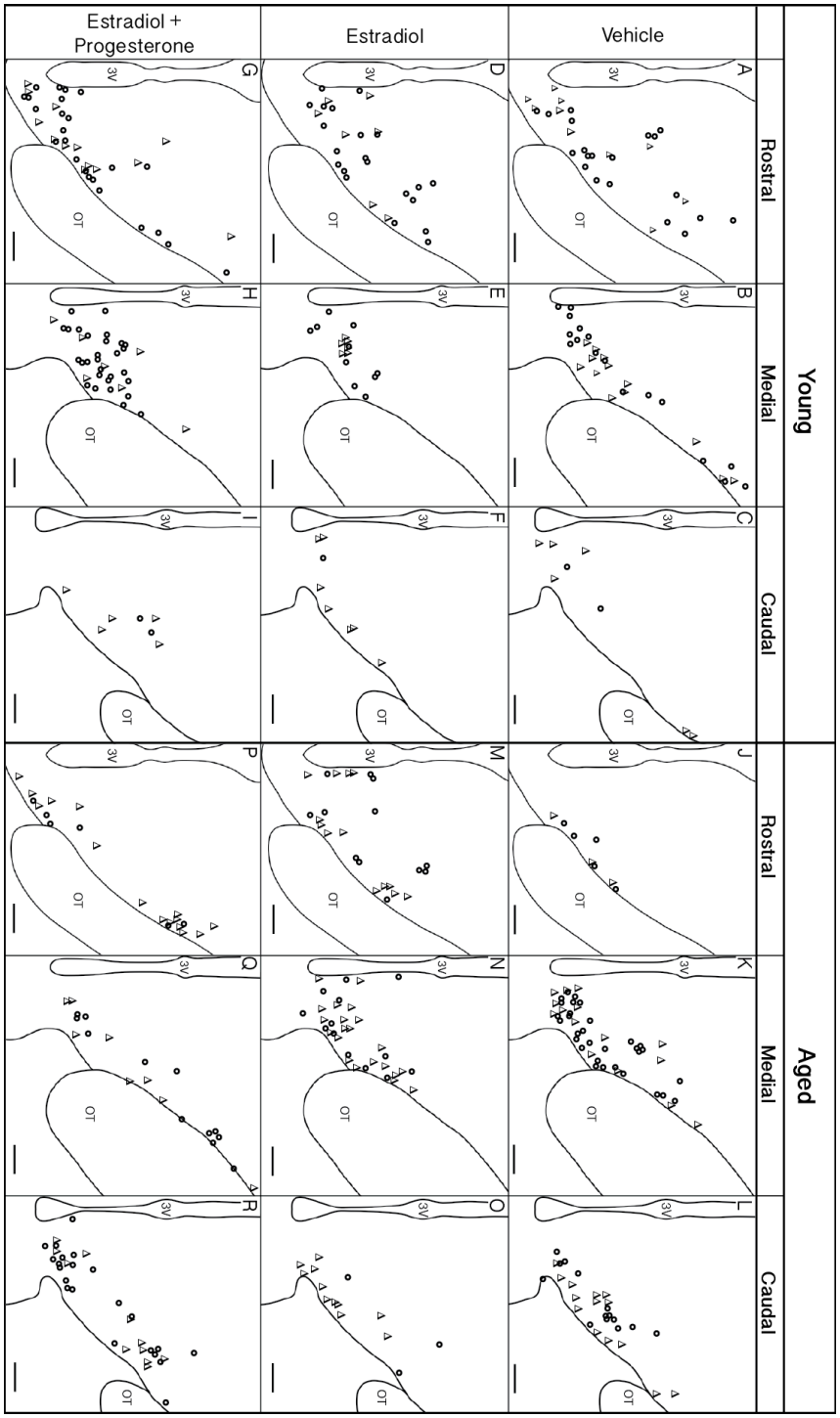
There were no significant effects of age or treatment on the percent or type of double-labeled GnRH+/GPER+ neurons (**Figure 3.2B**).

Figure 3.3: Photomicrographs of double labeling of GnRH perikarya, and the proximity between GPER processes and GnRH neurons.



Panels A-C show two GnRH neurons, one heavily colocalized with GPER (left) next to one that is not labeled with GPER (right). Examples of GnRH perikarya (D & E, red) and processes (F) that are in direct contact with GPER-IR processes (green). Scale bars = 35 µm (A-C), 15 µm (D & E), 10 µm (F).

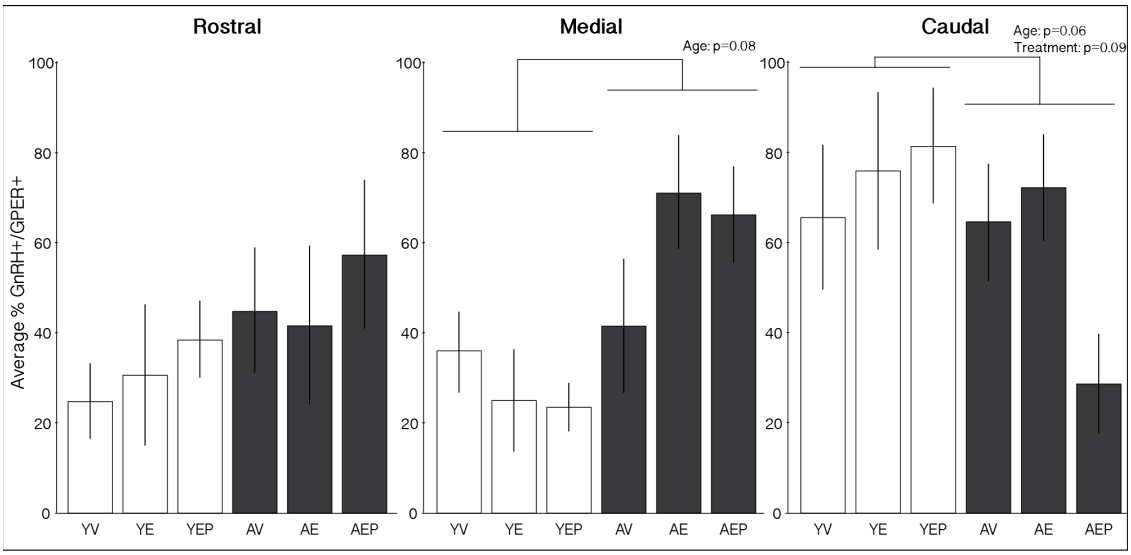
Figure 3.4: Distribution of GnRH neurons in the medial basal hypothalamus.



GnRH neurons were mapped onto a representation of coronal sections of the MBH from rostral (A, D, G – young; J, M, P – aged), medial (B, E, H – young; K, N, Q – aged) and caudal (C, F, I – young; L, O, R – aged) in young (left) and aged (right) adult rhesus monkeys of the three hormone treatment groups. GnRH+/GPER+ double-labeled perikarya are labeled with **Δ** and GnRH+/GPER- with **o**. The third ventricle (3V) and optic tract (OT) are labeled for orientation. Scale bars = 350 mm.

The distribution of GnRH neurons that were single (GnRH+/GPER-) or double (GnRH+/GPER+) labeled was mapped onto representations of the MBH from rostral to caudal, separately for the 6 groups (**Figure 3.4**). The mean percentage of colocalized neurons was also calculated across the three regions (**Figure 3.5**). In the medial MBH, there was a trend for an effect of age (p=0.08), with aged animals

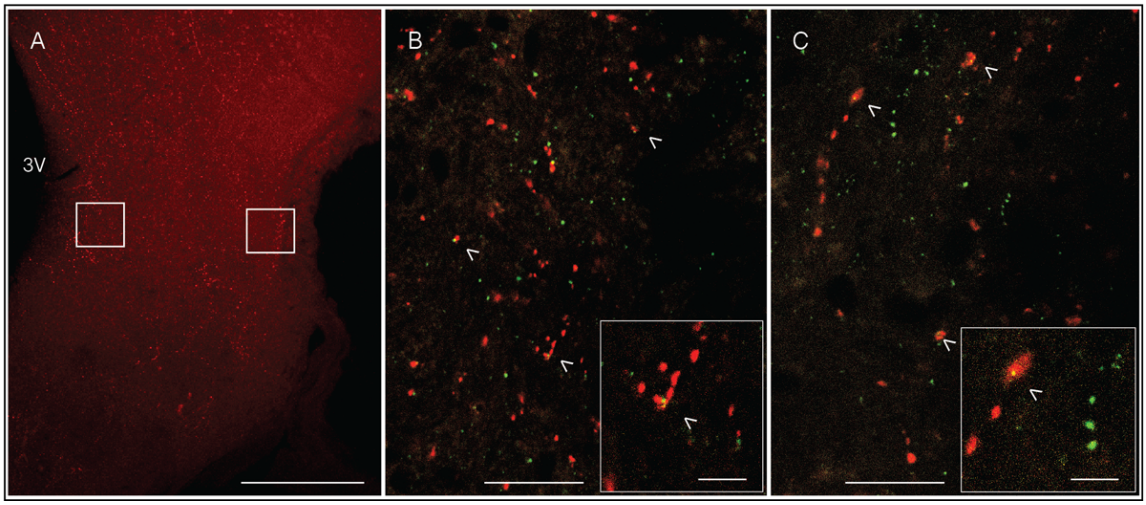
Figure 3.5: Distribution of GnRH+/GPER+ cells in the MBH.



These graphs show the average percent (±SEM) of GnRH+/GPER+ perikarya in the rostral (left), medial (middle) and caudal (right) MBH. There were trends of age effects in the medial and caudal regions (p=0.08 and p=0.06, respectively), as well as a treatment effect in the caudal MBH (p=0.09).

having a higher percentage of GnRH+/GPER+ than young groups. In the caudal MBH there were trends for effects of both age and treatment (p=0.06, p=0.09, respectively).

Figure 3.6: Representative micrographs of GnRH and GPER immunolabeling in the ME.



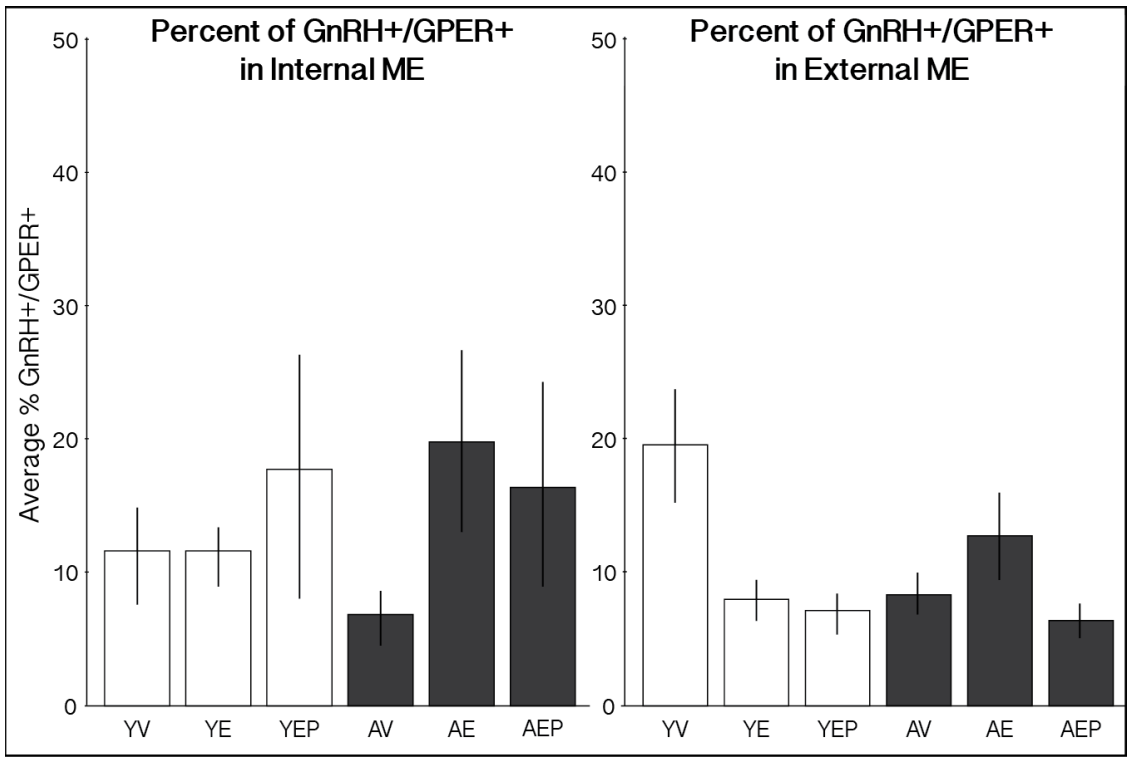
A low magnification image of GnRH-IR in a coronal section through the ME is shown (A) with boxes around the regions of the internal (left) and external (right) zones that were analyzed. The third ventricle (3V) is labeled for orientation. High magnification micrographs of punctate GnRH-IR (red) and GPER-IR (green) are shown in the internal (B) and external (C) zones of the ME. Colocalized puncta (yellow) are indicated with arrowheads. The insets show a high magnification example of colocalization in each region. Scale bars = 350 µm (A), 20 µm (B & C), 5 µm (insets).

GnRH Fibers Have Low Co-expression of GPER in the Median Eminence

Both GnRH and GPER were detectable as punctate labeling in the ME (**Figure 3.6).** Co-localization of GPER in GnRH+ neural puncta was quantified separately in

the internal and external zones of the ME. In both regions there was an average of 12% colocalization of GnRH-IR and GPER-IR puncta. There were no significant effects of age or treatment (**Figure 3.7**).

Figure 3.7: Percent colocalization of GnRH+/GPER+ puncta in the ME.



The mean percent (±SEM) of GnRH puncta that were colocalized with GPER-IR in the internal and external ME are shown for each group. There were no significant effects of age or treatment in either region.

DISCUSSION

The goal of current work was to add to limited knowledge about expression of GPER, a membrane estrogen receptor/binding partner, on hypophysiotropic GnRH neurons. Prior work, mainly conducted *ex vivo*, has largely supported this possibility, but few studies have investigated this relationship in a physiological system. Our current data addressed three novel pieces of information. First, we ascertained whether GnRH cells co-express GPER in adult rhesus monkeys; second, we determined the localization of this co-expression on cell bodies and processes/terminals in the ME; and third, we investigated whether aging and long-term hormone treatments affected co-expression in a clinically-relevant rhesus macaque model of menopause.

GnRH Perikarya Co-express GPER

The expression of GPER in the brain, including the hypothalamus, is quite abundant in primates and rodents (Brailoiu et al., 2007; Canonaco et al., 2008; Hazell et al., 2009; Naugle et al., 2014; Walker et al., 2012). Here, using hypothalami from OVX monkeys of two ages and three hormone treatments, we found that approximately half of the GnRH perikarya in the MBH were GPER-immunoreactive. Although there were some effects of age and hormone treatment, none were robust, suggesting that co-localization of GPER in GnRH neurons of adult female monkeys is a stable part of the GnRH phenotype. Other studies, in general, support co-localization. Cultured embryonic monkey GnRH neurons and GnRH GT1-7 cell lines co-express GPER (Jacobi et al., 2007; Noel et al., 2009). Sun et al. reported that about one-third of GnRH neurons were electrophysiologically activated by the GPER agonist, G1, in mice (Sun et al., 2010). However, conflicting results were found by

Romano et al., who did not detect rapid responses to G1 in any of the seven GnRH neurons examined (Romanò et al., 2008). Some alternate pathways, involving the classical receptors ERα and ERβ, that mediate rapid E_2 activation of GnRH have been found in mice that are not present in primates (Cheong et al., 2014; Chu et al., 2009; Kenealy et al., 2011a; Noel et al., 2009; Romanò et al., 2008; Sun et al., 2010). For example, in mice, Abraham et al. discovered ERβ induced CREB phosphorylation in the GnRH neurons, and Romano et al. found an ERα-dependent pathway involving GABAergic modulation of GnRH Ca^{2+} dynamics (Abrahám et al., 2003; Romanò et al., 2008). Thus, the role that these ERs play in the regulation of GnRH in primates likely differs from rodents and further research is merited to resolve these differences.

The functional role of GPER in GnRH neurons is not known, although we speculate that it may mediate some aspects of hormone feedback and/or synchronization required for pulsatile release. There are still substantial gaps in knowledge about the receptors that mediate positive and negative effects of estrogens on GnRH neurons, making the GPER of potential interest beyond ERα, ERβ and STX-sensitive receptors (Cheong et al., 2014; Christian and Moenter, 2010; Kenealy et al., 2011b). The lack of an obvious reproductive phenotype of the GPER-knockout mice may have hindered research in this area, as the global knockout may have developmental compensation for physiological roles normally mediated by GPER (Kenealy et al., 2011b; Meyer et al., 2012; Prossnitz and Barton, 2011; Zhang et al., 2010).

The Relationship Between GnRH Perikarya & GPER is Highly Heterogeneous

Our analyses revealed that the intensity and pattern of GPER-IR varied between individual GnRH neurons: some cells had exclusively punctate and sparse labeling, while others had GPER-IR distributed densely and evenly throughout the cell. Many of the labeled GnRH perikarya in these monkeys showed GPER labeling in proximal processes and, in a few cases, more distal from the GnRH soma. Unlike many other G protein-coupled receptors, GPER is expressed in multiple intracellular compartments as well as on the cell membrane, in a tissue and species-specific manner, though the physiological role that this plays is currently unknown (Almey et al., 2012; Brailoiu et al., 2007; Cheng et al., 2011; Hazell et al., 2009; Isensee et al., 2009; Naugle et al., 2014; Noel et al., 2009). We cannot say if these differences observed in our study are due to changes in the amount of GPER protein or a change in the translocation or trafficking of GPER to different intracellular compartments.

Some GnRH neurons, while not double-labeled, were found in very close association with GPER-IR varicosities. In many cases, the GPER-IR processes appeared to wrap around GnRH soma, proximal dendrites or axons. Electron microscopy would be needed to verity whether or not these closely apposed cells have synapses or gap junctions, but if that were the case, then GPER+ cells may also mediate effects of E_2 indirectly onto the GnRH neurons they contact. These GPER-IR processes could arise from cells that are either neuronal or glial, as GPER has been found in both types of cells in the regions near GnRH perikarya (Almey et al., 2012). Together, these results suggest that GPER likely mediates direct and indirect E_2 regulation of GnRH neural activity at or near the soma.

We found some regional specificity in the rostral to caudal distribution of GnRH+/GPER+ neurons. In young monkeys, more GnRH neurons were detected in

the rostral and medial MBH, while in aged animals they were located more mediocaudally. Due to limitations in the numbers of animals and tissues we do not want to overinterpret these data. Nevertheless, consistent with our results, age-related changes in the distribution of GnRH cells in rodents have been reported (Miller and Gore, 2002; Rubin and King, 1994). Rubin et al. found that there was an increase GnRH neurons in the dorsomedial portion of the preoptic area of young rats preceding the LH surge and this was not observed in middle-aged animals (Rubin and King, 1994). Additionally, Miller et al. found that there were fewer GnRH neurons in the medial septum of middle-aged irregular and acyclic rats in comparison to young or regularly cycling rats (Miller and Gore, 2002). Together this suggests that the distribution of GnRH perikarya is responsive to the hormonal milieu, and age-related alterations in the dynamics of this system may be linked to reproductive senescence in rodents. While the differences in distribution that we measured did not reach significance, these results are suggestive of the possibility that monkeys also undergo age-related reorganization of GnRH perikarya. We did not find any obvious change in GnRH cell number, consistent with observations in monkeys, mice, and rats (Hoffman and Finch, 1986; Miller and Gore, 2002; Rubin et al., 1984; Witkin, 1986). Although a few others have found some age-related changes in number of GnRH neurons in aged rats and mice, these were relatively small effects (Funabashi and Kimura, 1995; Rubin and King, 1994). Thus, the literature as a whole suggests small to no changes in GnRH perikarya numbers with aging, but possible distributional changes.

GnRH Terminals in the ME Have Low Co-expression of GPER

The external zone of the ME contains the portal capillary bed and has a high density of secretory neuroterminals and tanycytic endfeet, whereas the internal zone of the ME contains GnRH processes, and the cell bodies of tanycytes, specialized glial cells that form a selective barrier to the portal capillaries (King and Rubin, 1995; Rodríguez et al., 2010; Yin and Gore, 2010). The ME region undergoes dramatic cytoarchitectural and phenotypic changes with reproductive aging and hormone treatment in rats, especially around the terminals and capillaries, speculated to affect the release of GnRH (King and Letourneau, 1994; King and Rubin, 1995; Ojeda et al., 2000, 2008; Rodríguez et al., 2010; Yin and Gore, 2010; Yin et al., 2009a, 2009b). In fact, changes to GnRH release measured in the ME have been reported in rodents, in which release is decreased, and in monkeys, in which GnRH pulses (especially amplitude) was increased (Gore et al., 2004; Rubin and Bridges, 1989). Species differences are attributable to feedback regulatory differences with aging, underscoring the importance of conducting this work in a non-human primate model.

In the current study, we found colocalization of GPER and GnRH in the internal and external zones of the ME, with no age or treatment effects. There was a much lower percent of colocalization in the ME (~12%), in comparison to the perikarya of GnRH neurons (~50%). There are several interpretations for these cell compartment differences that are not mutually exclusive. GnRH perikarya may transport only a small percentage of synthesized GPER along the axon to the terminals and processes in the ME. Alternatively or in addition, the detectability of GPER in the ME may be below the threshold of sensitivity of confocal microscopy, something that would require electron microscopy to resolve.

A recent study conducted by our laboratory in a model of natural reproductive aging in the rat showed that GPER mRNA is detectable in the microdissected ME (inclusive of both internal and external zones), and that its expression was positively correlated with the genes for ERa, TGFa, prodynorphin, the vitamin D receptor, and the progesterone receptor, and was negatively correlated with the GABA-B2 receptor (Kermath et al., 2014). While follow up work is needed to further study any functional relationships, these data suggest some interesting molecular candidates for a regulatory network of GnRH in the ME, among which is GPER.

Summary & Limitations

In this study conducted in young and aged OVX rhesus macaques, about half of GnRH perikarya express GPER, whereas a smaller percentage of terminals (12%) had co-expression in the ME. GnRH cell bodies and processes were often in close apposition or proximity to GPER-immunoreactive elements. These data add new information about the robust presence of GPER in and around GnRH cells and suggest a viable site of direct action of estrogens on the GnRH system. However, aging and hormone treatments had limited effects on the co-expression of GnRH and GPER in the rhesus monkey model of menopause. This latter result underscores the stability of GnRH neurons across adult development and into aging. As is often the case for studies of non-human primates, we were constrained by the number of animals that were available for this experiment, and the sometimes-poor quality of tissues. Thus the statistical power of this study was limited and data must be interpreted accordingly.

ACKNOWLEDGMENTS

We are grateful to Dr. Jeffrey Roberts and Mary Roberts at the California National Primate Research Center UC-Davis Primate Center for expert care of the animals. Dr. John H. Morrison and William Janssen are acknowledged for monkey work and perfusions. Dr. Weiling Yin and Megan Noel assisted with preparation and sectioning of hypothalamic tissues. Julie Hayes' expert advice with the confocal microscope was essential for the success of this project. Grant Support: NIH PO1 AG16765.

CHAPTER 4: AGE-RELATED CHANGES IN THE ULTRASTRUCTURAL MORPHOLOGY OF THE MEDIAN EMINENCE OF FEMALE RHESUS MACAQUES ARE AFFECTED BY LONG-TERM HORMONE TREATMENT

Abstract: The median eminence (ME) is the common pathway by which all hypothalamic releasing hormones, including the gonadotropin-releasing hormone (GnRH) peptide that controls reproduction, exit the central nervous system. Dynamic interactions between neurosecretory terminals, specialized glial cells and the portal capillary system are necessary for proper homeostasis and reproductive function. Age-related disorganization of this region's ultrastructure has been seen in rodents and may contribute to neuroendocrine dysfunction seen in that species. In this study, we used transmission scanning electron microscopy (tSEM) to examine the ultrastructure of the portal capillary zone in the ME of young and old female rhesus macaques. Because this region is highly sensitive to steroid hormones, we used ovariectomized animals that had been given vehicle, estradiol, or estradiol + progesterone for 2 years, to determine whether there are age-related changes in the response of this region to these hormones. We found that there is little change in the overall organization of the ME, but there is some hypertrophy of neural and glial components near the capillary in aged animals, and that estradiol treatment mitigates or reverses this hypertrophy. Estradiol also decreased the average size of mitochondria in aged, but not young, neuroterminals and glia. This study shows that there are species differences in the structure of the aging ME and that estradiol's effects in monkeys are age-specific.

INTRODUCTION

Menopause is a natural transition occurring in all women at midlife, and is heralded by a decline in estradiol (E_2) and progesterone (P_4) production, and the cessation of menstrual cycles. The menopausal transition is often accompanied by neurobiological symptoms such as vasomotor symptoms, sleep and mood disruptions, and general cognitive decline (Avis et al., 1994; Hall, 2004; Harlow et al., 2012; Mansfield and Voda, 1997; McEwen, 2002). These and other symptoms are predominantly due to the abrupt deprivation of ovarian E_2 and P_4, and may have a

dramatic impact on quality of life. Hormone replacement therapy (HRT), with E_2, P_4 or their combination, is a common treatment used primarily to improve vasomotor symptoms, vaginal dryness, and osteoporosis, though these hormones have wide-ranging actions across the body and brain that are not fully understood (Cauley et al., 2014; Maki, 2013).

Reproduction is controlled by the coordinated action of the hypothalamus, pituitary and gonads, collectively known as the hypothalamic-pituitary-gonadal (HPG) axis (Gore et al., 2014; Hall, 2012; Wise et al., 2002). GnRH stimulates the release of LH and FSH from the pituitary, which in turn trigger the secretion of E_2 and from the ovary. The ovarian hormones feedback onto the hypothalamus and pituitary, modulating their activity. Reproductive aging alters the synchrony of hormonal release from all three levels of HPG hormones (Gore et al., 2014). The majority of research thus far has focused predominantly on the age-related decline of gonadal function, but there is a growing body of evidence that suggests that the hypothalamus and pituitary play a significant role in reproductive senescence, and may even drive this transition (Gill et al., 2002a, 2002b; Gore et al., 2000a; Weiss et al., 2004).

Within the primate hypothalamus, gonadotropin-releasing hormone (GnRH) is a neuropeptide synthesized in a small group neurons scattered through the basal hypothalamus (Rance and Uswandi, 1996). GnRH neurons integrate internal (e.g. other hormones, nutrition), external (e.g. social environment, stress), and developmental life stage (infancy, puberty, adulthood, senescence) cues to appropriately coordinate reproductive function with the organism's state (Gore, 2002; Gore et al., 2004; Knobil, 1974; Knobil et al., 1980; Richter and Terasawa, 2001; Sisk and Foster, 2004). Processes from these neurons carry large dense-core

vesicles (LDCV), containing GnRH, from the hypothalamus to neuroterminals in the median eminence (ME) at the base of the brain. The ME is a circumventricular structure that allows communication between the brain's ventricular system, neurons, glia, and the portal vasculature (Langlet et al., 2013; Prevot et al., 2010; Rodríguez et al., 2005, 2010). Theses hypophysiotropic neuroterminals release their contents into the extracellular space that is adjoining the portal capillary bed. This capillary system vascularizes the anterior pituitary, and release of neuropeptides at this site is essential for direct and rapid communication between the hypothalamus and the pituitary (King et al., 1982; Rangaraju et al., 1991; Rodríguez et al., 2005; Yin and Gore, 2010).

Like GnRH the neuroendocrine hormones that control metabolism, stress, lactation, and growth, are also packed into large dense core vesicles (LDCV) in the basal hypothalamus and transported to neuroterminals in the pericapillary region of the ME for release (Durrant and Plant, 1999; Prevot, 2002; de Seranno et al., 2010; Yin et al., 2009a, 2009b). Tanycytes, a specialized type of astrocyte, are particularly important to the regulation of the endocrine system because their motile processes wrap around the hypophysiotropic neuroterminals and guide them to the capillary and also tanycytic 'endfeet' form part of the blood-brain-barrier, therefore they act as the gatekeeper for hypothalamic hormones (Akmayev and Fidelina, 1981; Baroncini et al., 2007; Flament-Durand and Brion, 1985; Langlet et al., 2013; Ojeda et al., 2008; Prevot, 2002; Prevot et al., 2010; Zoli et al., 1995). Estrogen is important for the normal function of the ME: 1) E_2 stimulates the withdrawal of tanycytes from the capillary to allow neuropeptides to enter the blood stream, and 2) the majority of E_2-stimulated release of GnRH is mediated by glial-neuronal communication, though not all, as shown in Chapter 3 (Micevych et al., 2010; Prevot et al., 2010; de

Seranno et al., 2010). Mitochondria are also important for the normal function of the ME, because the constant reorganization of this region is an energetically intensive process and requires high densities of mitochondria to support this dynamic system. Additionally, mitochondria are required for steroidogenesis and local synthesis of E_2 is involved in the coordination of GnRH release in the ME (Kenealy et al., 2013; Terasawa and Kenealy, 2012). Therefore, the functional relationship between neuroterminals, glia and mitochondria in the ME are critical for the entire neuroendocrine system.

Given the pivotal role that the ME plays in endocrine function, there have been surprisingly few studies examining the effect of age on this region. Our lab has shown striking changes in the morphological organization and cytoarchitecture of the ME of aging rats (Yin et al., 2009a, 2009b). Given the known differences in reproductive senescence between rats and nonhuman primates, such as the age-related decline in GnRH release in rodent in comparison to increased secretion observed in aged humans and primates, this work needs to be extended (Gore et al., 2004; Rubin and Bridges, 1989; Scarbrough and Wise, 1990; Wise et al., 1988). Moneys also allow the use of clinically relevant long-term hormone replacement, compared to just 3 days of estradiol treatment in rodent studies (Naugle et al., 2014; Yin et al., 2009b). Therefore, in this study, we used electron microscopy to examine the ultrastructure and cytoarchitecture of the ME, and the relationship between glia and neuroterminals, in young and aged female rhesus macaques that received long-term HRT, to allow us to determine the effects of age, hormones or their interactions in this critical region of the brain.

METHODS

Animals

Female monkeys (*Macaca mulatta*) that were either young premenopausal adults, or aged peri- or post-menopausal adults were used in this study. Reproductive status was determined by daily visual inspection of vaginal bleeding and only animals under 19 years old, with normal cycle lengths between 24 to 34 days were included in the pre-menomausal group (Gilardi et al 1997; Bachevalier et al., 1991). Housing and experimental conditions at the California National Primate Research Center were described previously (Hao et al., 2003, 2006, 2007; Hara et al., 2014; Rapp et al., 2003; Wang et al., 2010). All procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California.

Ovariectomy & Hormone Treatment

The monkeys were ovariectomized (OVX) as described previously (Hao et al., 2003, 2006, 2007; Hara et al., 2014; Rapp et al., 2003; Wang et al., 2010) using isoflurane anesthesia and atropine (0.4 mg/kg) and ketamine (10 mg/kg) sedatives. After 2-3 months of recovery, the animals were assigned to one of three groups that received either estradiol (YE & AE), estradiol plus progesterone (YEP & AEP) or a blank vehicle (YV & AV) treatment. To achieve a mean serum level of 150 pg/ml, Silastic capsules (0.46 inner diameter and 3 cm long) containing either crystalline estradiol (E_2, Sigma) or empty (for vehicle group) were implanted between the shoulder blades and replaced every three months. To mimic luteal phase P_4 levels,

the YEP and AEP groups received micronized progesterone (100 mg; Catalent Pharm Solutions) in their daily fruit treat for 10 consecutive days within the month. Six to eight animals were originally included in these groups, but there was attrition due to illness, sudden death and incomplete OVX, determined by hormone analysis at the time of euthanasia. Additionally, due to the fragility of the tissue and complicated preparation process, tissue loss resulted in the final group numbers: YV n=4, YE n=5, YEP n=4, AV n=4, AE n=4, AEP n=4. As described previously, the monkeys were euthanized 8-10 days after the last P_4 treatment or the corresponding day for no P groups, after an average of two years of hormone treatment (Baxter et al., 2013; Ohm et al., 2012).

Euthanasia & Tissue Preparation

The animals were deeply anesthetized with pentobarbital (20–35 mg/kg) and ketamine (25 mg/kg) and were transcardially perfused with ice-cold paraformaldehyde (PFA) in phosphate buffer (PB), using 1% PFA for 1 minute and increasing the concentration to 4% PFA for 12 minutes, with the flow rate set to 185 ml/min. The brain was removed to keep the fragile median eminence intact, and the hypothalamus was post-fixed in 0.125% gluteraldehyde and 4% PFA for 6 hours, then shipped, on ice, to the University of Texas at Austin in PB.

Using a vibrating microtome (Leica VT1000 S; Leica, Bannockburn, IL), we collected 10-25 coronal sections at 100 μm, through the entire median eminence region, when present. Sections were cryoprotected via immersion in a series of increasing concentrations of glycerol in 1M PB: 10% 1 hour, 20% 1 hour, and 30% 12 hours, all with constant agitation. Eight of these sections, in a rostral to caudal

series, were selected for electron microscopy (EM) analysis. The dorsal-most portion of the ME was dissected and embedded in a Lowicryl resin block using cryofixation system (Leica CPC, Vienna) with an automatic freeze-substitution unit (AFS; Leica AFS, Vienna) that preserved the antigenicity of the proteins for post-embedding immunohistochemistry, as previously described (Hao et al., 2003; Hara et al., 2011, 2012, 2014; Wang et al., 2010; Yin et al., 2007, 2009a, 2009b; Adams et al., 2001, 2002; Yildirim et al., 2008) and modified from (van Lookeren Campagne et al., 1991; Hjelle et al., 1994; Chaudhry et al., 1995). Briefly, the 100 μm blocks were mounted on the head of pins and rapidly plunged into liquid propane that was cooled to -190°C. The specimens were rapidly transferred to the AFS apparatus that was pre-cooled to -90°C for Lowicryl freeze substitution. The tissue was immersed in 1.5% uranyl acetate in anhydrous methanol for 24 hours, the temperature was increased by 4°C every hour to -45°C, then rinsed in anhydrous methanol and infiltrated with Lowicryl resin HM20 (Electron Microscopy Sciences, Ft. Washington, PA) with a four-step series of increasing concentration of resin to methanol (doubled each time; 1 hour each) and then in 100% Lowicryl for 12 hours. The resin was polymerized by ultraviolet light (360 nm) for 48 hours at -45°C, then 24 hours at room temperature and the resulting blocks were prepared for ultrathin sectioning.

We collected sections for EM imaging using an ultramicrotome (Leica EM UC6, Vienna), positioned on a floating table and surrounded by a Plexiglas enclosure. The block face was trimmed to expose the ME sample with a glass knife and we collected 400 nm semi-thin sections for identification of the boundary between the portal capillary bed and the dorsolateral portion of the ME block. The semithins were mounted on glass slides, dried on a hot-plate for 10 minutes at

100°C, stained with 2% toluidine blue and examined with a bench-top transmitted light microscope (Leica DMLB upright microscope, Germany). After determining where to start cutting ultrathin sections, five to ten 70 nm consecutive sections were cut with a 45^{o} ultra diamond knife (Diatome, Hatfield, PA), collected on separate formvar-coated gold slot grids (Electron Microscopy Sciences, Fort Washington, PA).

Microscopy

The images of the ultrathin sections were captured using transmission-mode SEM (tSEM), a newly developed system that consists of a field-emission scanning electron microscope (Zeiss supra 40VP FE-SEM) fitted with a retractable transmitted electron detector, a 12-grid specimen holder and integrated with the Zeiss ATLAS™ (Kuwajima et al., 2013a, 2013b). Unlike conventional transmission electron microscopes (TEM) this system uses low electron doses that reduce physical and optical distortion and leaves the specimen intact for analysis. Additionally, the 12-grid specimen holder and semi-automated ATLAS™ system, dramatically reduced the time and labor involved in image collection. We took multiple large field, high resolution images along the basal lamina, the thick membrane that separates neural tissue from the portal capillary bed of each animal (field-size = 25,000 x 25,000 pixels @ 2 nm^2 pixel size), which allowed us to collect all of our sample sites from an individual monkey in a single imaging session. We then randomly selected 9-10 sites abutting capillaries on the distal edge of the ME (10,000 x 10,000 nm) for analysis. Within each of these 9-10 sites, we chose a 5,000 x 5,000 nm field that contained at least one neuroterminal with LDCVs for further

analysis. All sampling sites were separated by a minimum of 5,000 nm to prevent re-sampling the same structures.

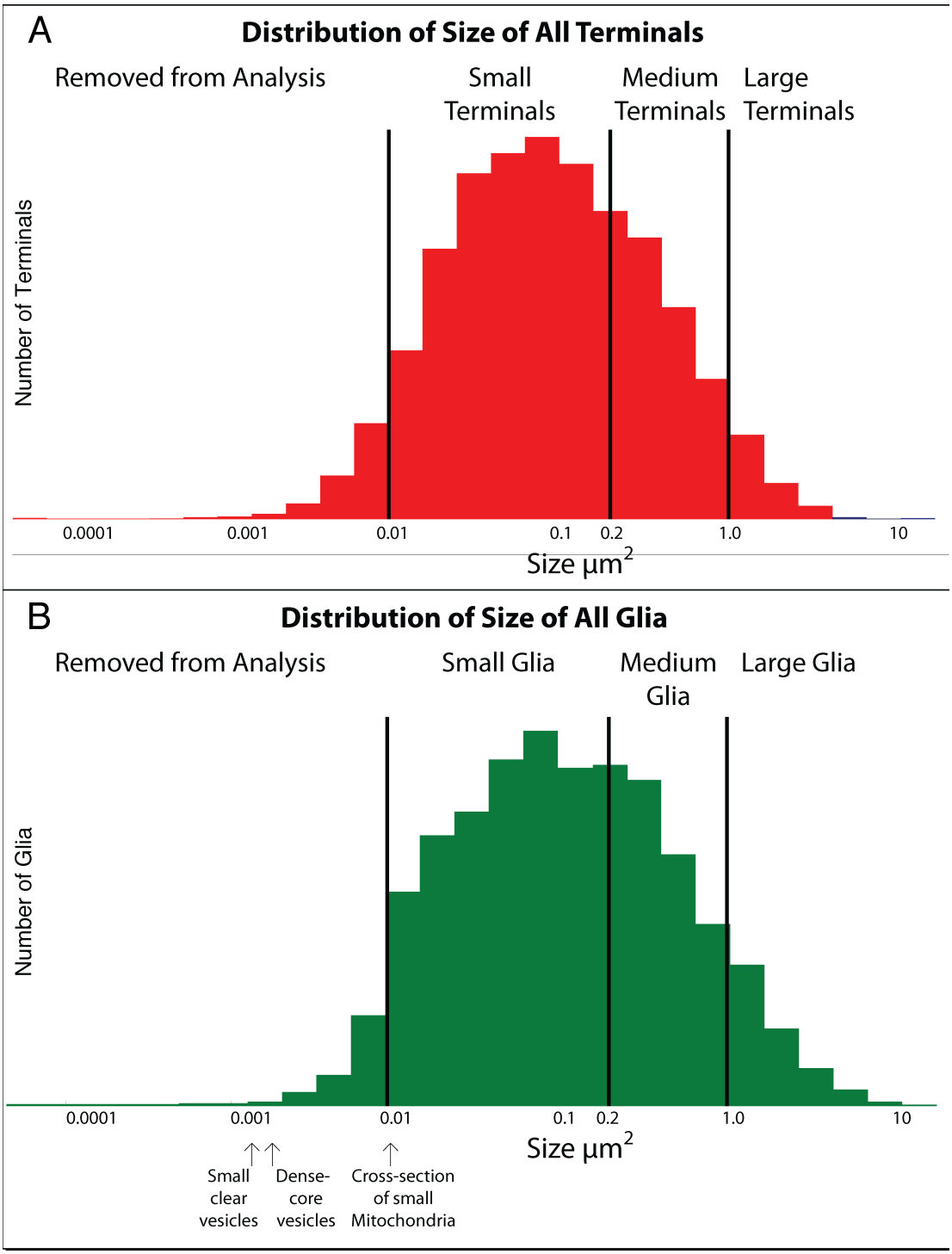
Image & Statistical Analysis

We used TrakEM2, a plugin for Fiji, freely available NIH-sponsored image analysis software, for manual image segmentation (Schindelin et al., 2012). The membrane boundaries of all identifiable neuroterminals, glia, mitochondria and basal lamina were traced and filled in with a color representing the class of the object (red, green, blue, and magenta, respectively). Neuroterminals were distinguished from glia by the presence of large dense-core vesicles (LDCV), small clear vesicles and/or neurofillaments and mitochondria were identified by their cristae (Peters et al., 1991). We separated the terminals into three categories based on the size, the smallest contains only cross-sections of neural processes, the medium contains a mixture of neural processes sectioned at an oblique angle and small neuroterminals, and the largest contains only neuroterminals. The same size categorization was applied to glia and there was no size categorization for mitochondria as shown in **Figure 4.1**.

To determine area, density and class of the segmented components, a second set of images were produced, identical to the first, but with the mitochondria removed. A custom Matlab program (R2013b, Mathworks) was designed to analyze the differences between the image pairs (included in supplement). It operates as follows: For each image pair, a binary (black or white) image, or mask, is created for each type of region by color comparison against the first image in the pair. This mask is composed of several connected components, each representing a single

cross section. We use connected component analysis to extract information about the number of components and the number of pixels that are contained in each. Similarly, we find a mask for mitochondria in the second image by searching for blue pixels. By taking the pixel-wise AND-operation between the mask corresponding to terminals or glia and the one corresponding to mitochondria, we are able to determine the cell type to which each mitochondria corresponds, as well as the size and count for these organelles. The software compiles the information in these images into object count, average object size, and the fraction of non-capillary pixels occupied by terminals, glia, and mitochondria and also categorizes each mitochondria as belonging to a terminal or glial.

Figure 4.1: Distribution of the Sizes of all Neuroterminals and Glia.



This figure shows the distribution of the size (μm^2) of all terminals (A) and glia (B) in the groups combined. Any labeled objects that had a smaller area than the average cross section of small mitochondria were removed from analysis. The neuroterminals and glia were separated into 3 size categories; 1) the small group is entirely composed of cellular processes and axons, 2) the medium consists of a mix of processes sectioned longitudinally and smaller neuroterminals and glial components, 3) the large category is entirely composed of neuroterminals and glial. The mean size of small vesicles, large dense-core vesicles and cross section of small mitochondria are labeled to provide a sense of scale.

We used R for all statistical analysis (R Development Core Team, 2012). Our dataset failed the assumptions required for ANOVA, as determined by Levene's equality of variance and the Shapiro-Wilks normality tests, therefore we used the Kruskal-Wallis non-parametric test to determine significant main effects of age or treatment [$p \leq 0.05$], followed by the pairwise Wilcoxon rank sum post-hoc test to identify specific interactions (Kruskal and Wallis, 1952; Wilcoxon, 1945).

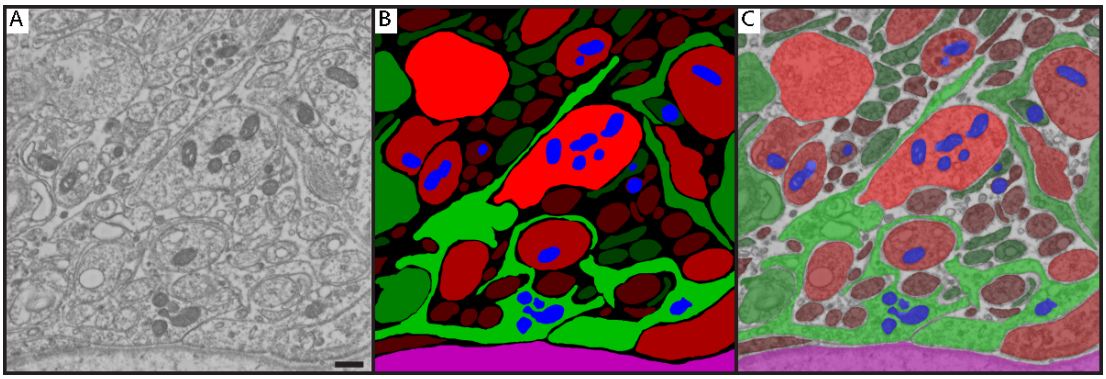
RESULTS

Ultrastructure of ME

The pericapillary region of the ME contained many neuroterminals that contained LDCV as well as small clear vesicles and mitochondria, and these terminals were in close apposition with glia (**Figures 4.2, 4.3** & **4.4**). The basal lamina is high convoluted in both young and aged adults due to the intrusion of the capillaries deeply into the ME, not segregated to the periphery as seen in rodents (Ojeda et al., 2008; Yin et al., 2009a, 2009b). Additionally, the linear organization of tanycytes projections from the third ventricle to the capillary bed seen in rodents is

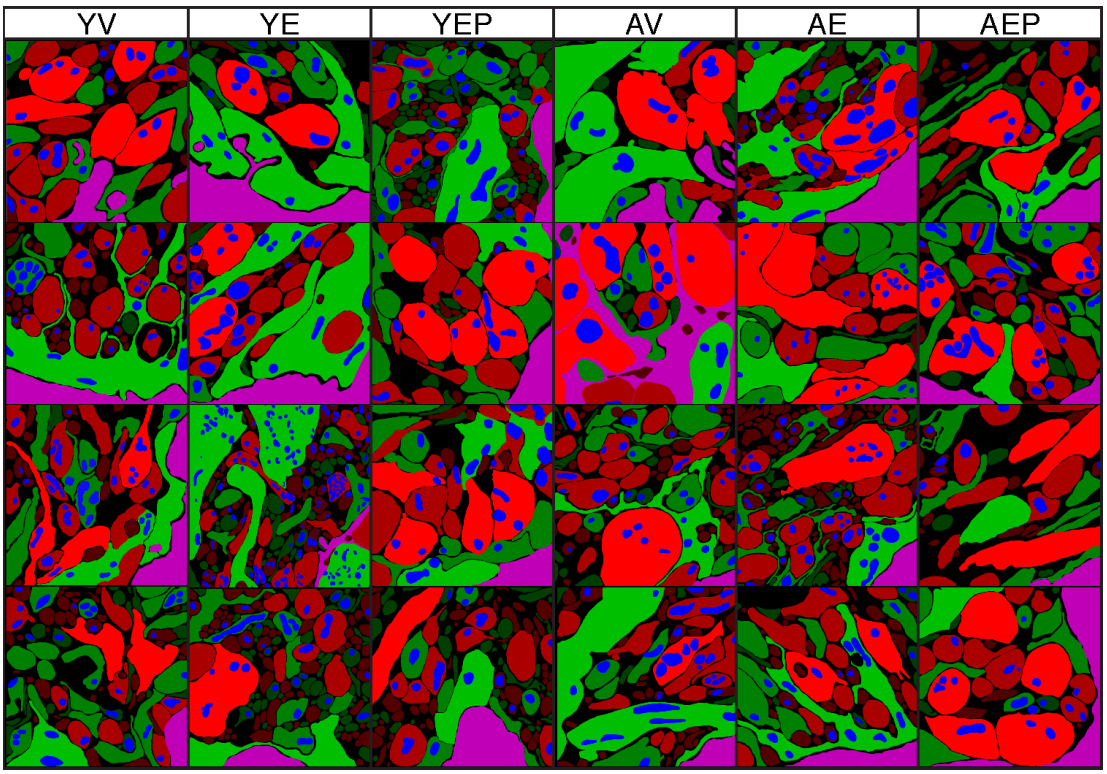
not obvious in adult monkeys of either age (Ojeda et al., 2008; Yin et al., 2009a, 2009b).

Figure 4.2: Image Segmentation.



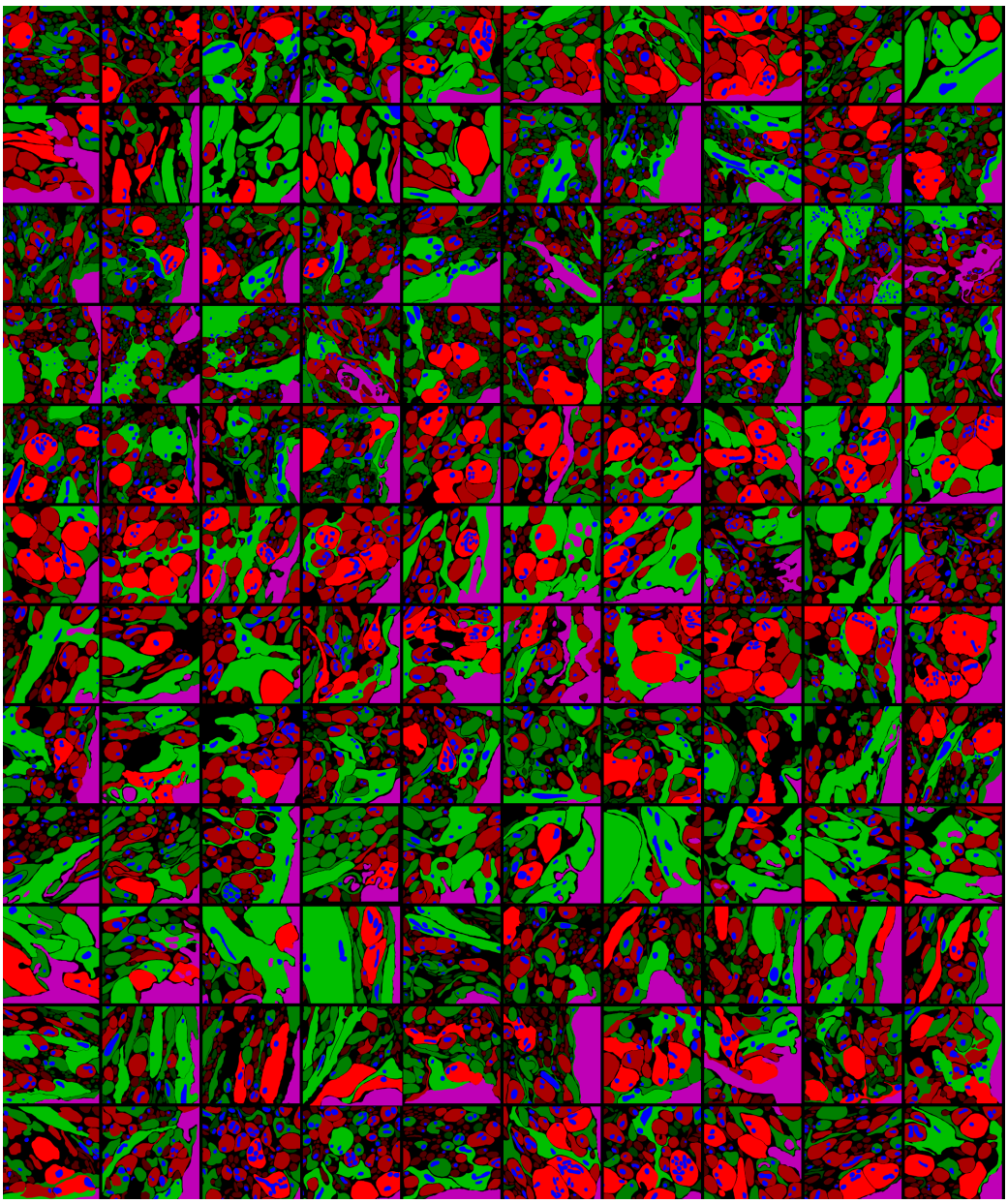
This figure shows an example of a raw image (A) and the final segmented image (C) and an overlay of the two (B). The red areas are neuroterminals, the green are glia, blue are mitochondria and magenta is the capillary bed. The relative intensity of the color corresponds to the size class that each object is assigned to. Scale bar = 400 nm.

Figure 4.3: Representative Micrographs of Ultrastructural Organization of the ME.



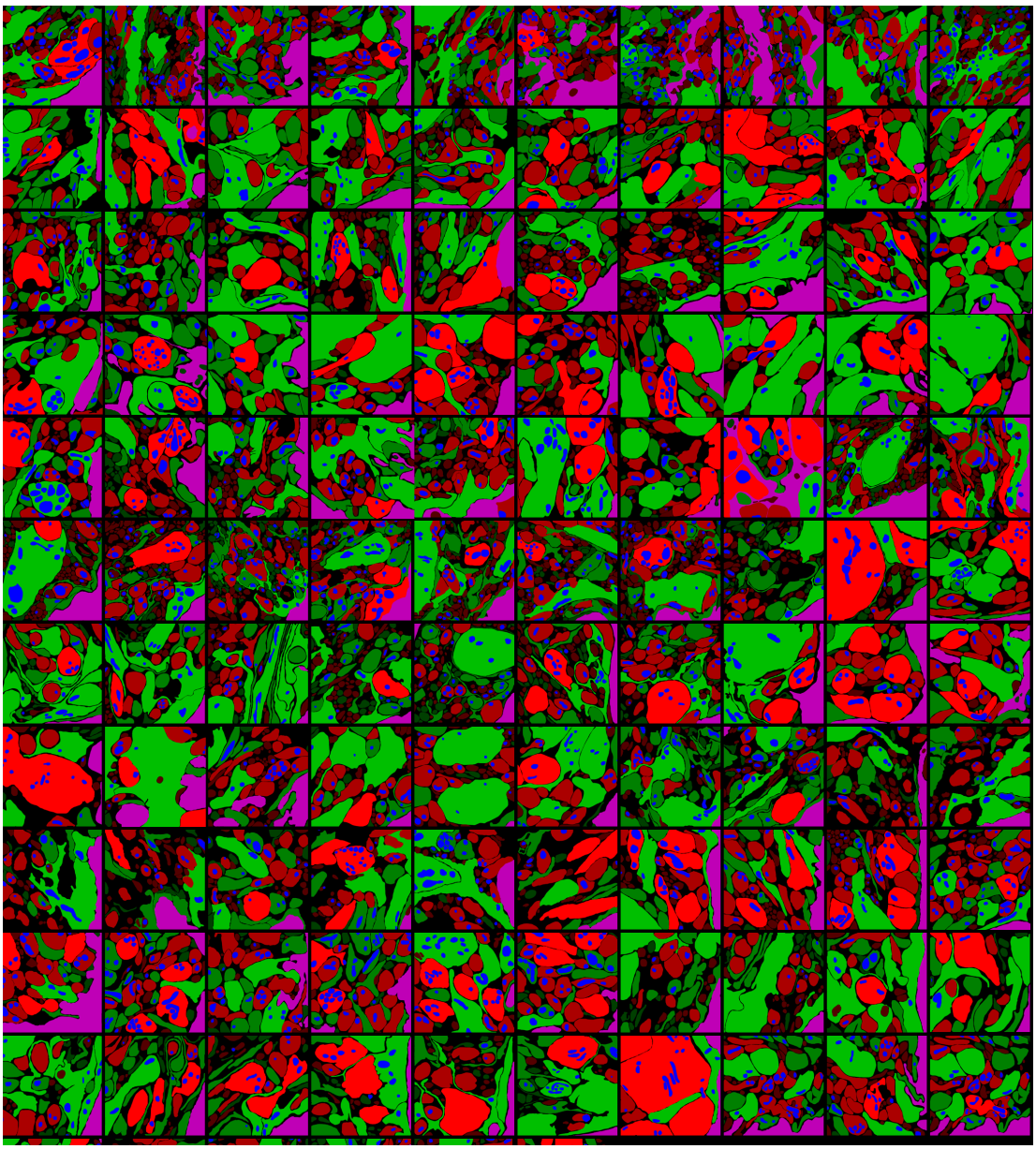
This figure shows a fully segmented representative micrograph for each individual for each group. Young animals are in columns 1-3 and aged animals are in columns 4-6. The red areas are neuroterminals, the green are glia, blue are mitochondria and magenta is the capillary bed. The relative intensity of the color corresponds to the size class that each object is assigned to. Scale bar = 400 nm.

Figure 4.4a: Final Segmented Micrographs from the Young Animals.



This figure shows most of the segmented images for the young animals. The red areas are neuroterminals, the green are glia, blue are mitochondria and magenta is the capillary bed. The relative intensity of the color corresponds to the size class that each object is assigned to. Scale bar = 400 nm.

Figure 4.4b: Final Segmented Micrographs from the Aged Animals.



This figure shows the majority of the segmented images for the aged animals. The red areas are neuroterminals, the green are glia, blue are mitochondria and magenta is the capillary bed. The relative intensity of the color corresponds to the size class that each object is assigned to. Scale bar = 400 nm.

Age & Hormone Treatment Affect Neuroterminal Size & Tissue Fraction

Size: The size of large terminals in aged animals was significantly greater than the young [p<0.00] and there was an interaction of age and treatment [p<0.00, p=0.02] such that hormone treatment dampened this age-related increase (**Figure 4.5**). There were no age, treatment or interaction effects on the size of all terminals, medium terminals or small terminals. Significant results are summarized in **Table 4.1**. The distribution pattern of neuroterminal size also qualitatively indicates group differences, and is shown in **Figure 4.6**.

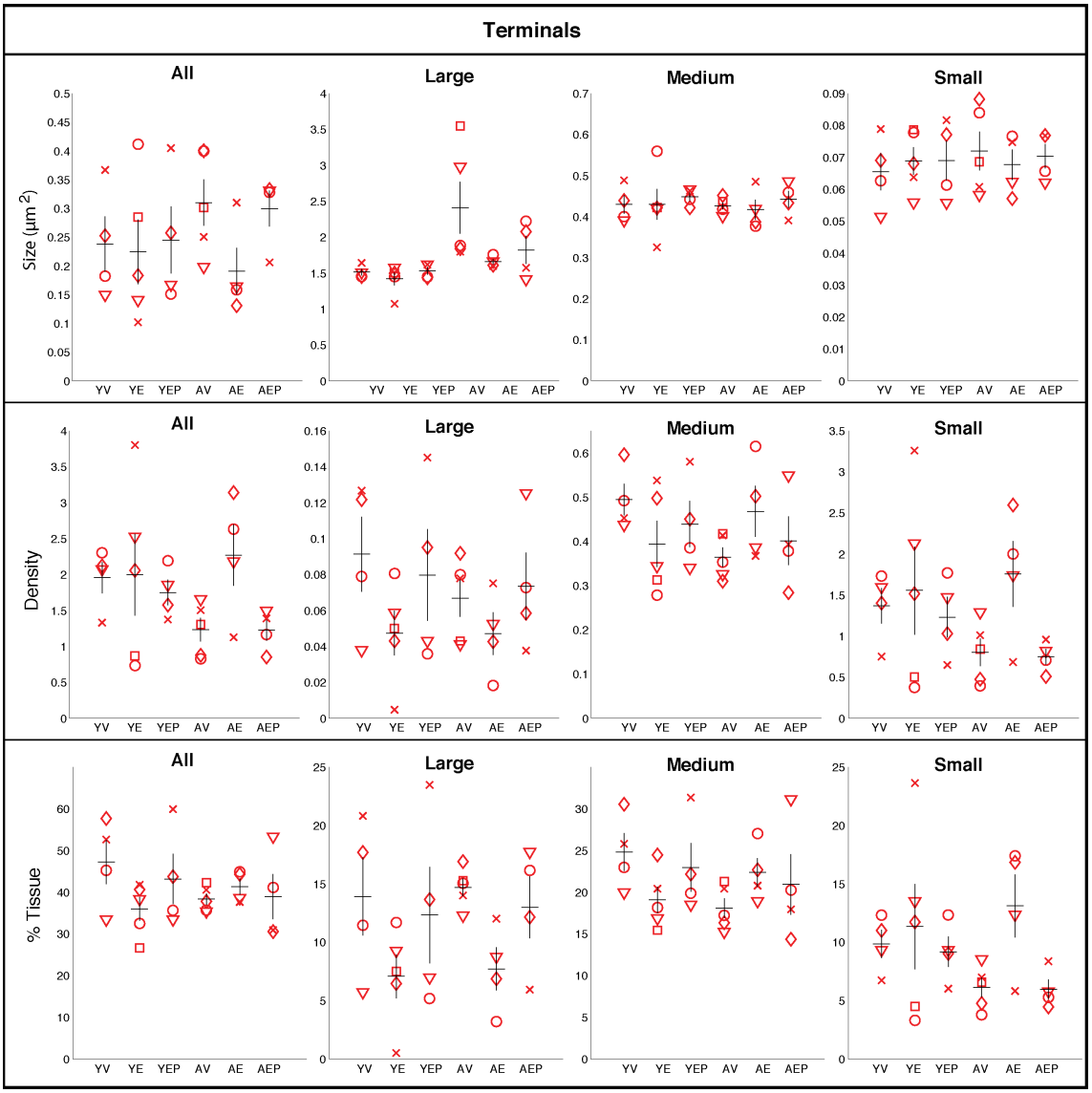
Density: There were no age, treatment or interaction effects on the number of terminals of any size category, as shown in **Figure 4.5**.

Tissue Fraction: There was a significant effect of treatment [p=0.02] on the percent of tissue occupied by large neuroterminals, with E_2 treatment decreasing the tissue fraction in both age groups (**Figure 4.5** & **Table 4.1**).

Age & Hormone Interactions Affect Glia Size, Density & Tissue Fraction

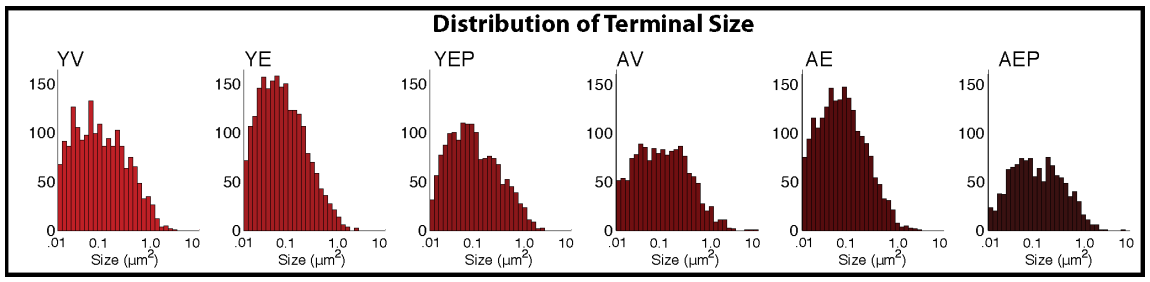
Size: There was a significant increase in the overall size of glia in aged animals [p=0.04] as shown in **Figure 4.7**. There were no significant age, treatment or interaction effects on the size of the large, medium or small glia. The distribution pattern of glia size also qualitatively indicates group differences, as shown in **Figure 4.8**.

Figure 4.5: Neuroterminal Size and Number and Area Fraction.



This figure shows the mean (± SEM) terminal size (1st column), number (2nd column) and tissue fraction (3rd column) for all terminals (top row), large terminals (2nd row), medium terminals (3rd row) and small terminals (last row) in each group. Each individual is represented by a symbol that is consistent throughout all figures. There were significant effects of age and group on the average size of the large terminals ($p<0.00$, $p=0.02$, respectively) and of treatment on the large neuroterminal fraction ($p=0.02$). **Table 4.1** provides the details of the statistical results.

Figure 4.6: Distribution of Size of Neuroterminals by Group.

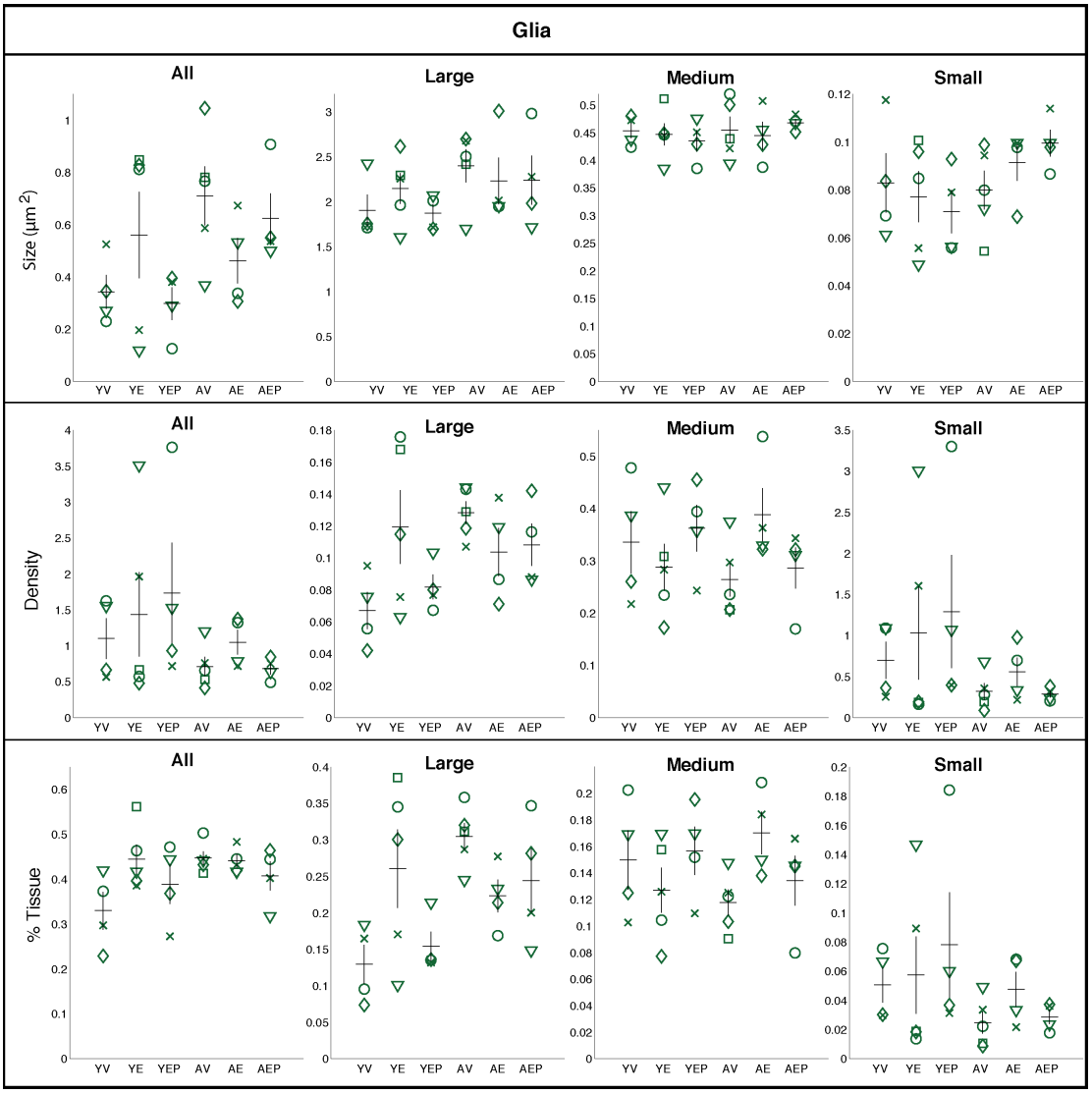


This figure shows the distribution of the area of all terminals for each group. The number of terminals is on the y-axis and the size (μm^2) on the x-axis.

Table 4.1: Statistically significant results.

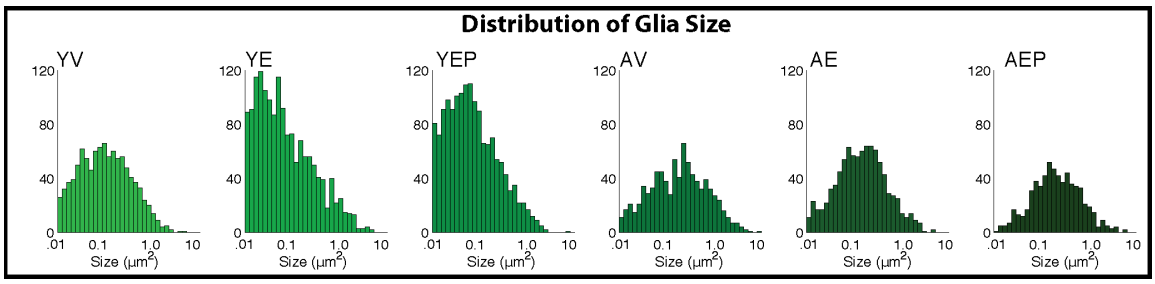
Significant statistical results for terminal size and tissue fraction			
	Average Size of Large Terminals	Large Terminal Tissue Fraction	
Main Effects	Age p < 0.00 Group p=0.02	Treatment p=0.02	
Interactions	AV > YV p=0.02 AV > AE p=0.02 AE > YE p=0.02		
Significant statistical results for glial size, density and tissue fraction			
	Average Size of Glia	Density of Large Glia	Large Glia Tissue Fraction
Main Effects	Age p=0.04	Age p=0.03	Age p=0.03 Group p=0.03
Interactions			AV > YV p=0.02 AV > AE p=0.04
Significant statistical results for mitochondria size			
	Size of All Mitochondria	Size of Mitochondria in Terminals	Size of Mitochondria in Glia
Main Effects	Treatment p=0.04 Group p=0.07	Treatment p=0.01 Group p=0.06	Treatment p=0.04 Group p=0.05
Interactions	AE<AV p=0.02 AE<AEP p=0.03	AE< AV p=0.02	AE < AV p=0.02 AE < AEP p=0.03 AV > YV p=0.07

Figure 4.7: Glia Size and Density and Percent of Tissue Occupied by Glia.



This figure shows the mean (± SEM) glia size (1st row), number (2nd row) and tissue fraction (3rd row) for all glia (1st column), large glia (2nd column), medium glia (3rd column) and small glia (4th column) in each group. Each individual is represented by a symbol that is consistent throughout all figures. There was a significant effect of age on the average size of all glia (p=0.04), on the number of large glia (p=0.03) and on the large glia fraction (p=0.03). There was also a group effect on the fraction of tissue containing large glia (p=0.03). **Table 4.1** provides the details of the statistical results.

Figure 4.8: Distribution of Size of Glia by Group.



This figure shows the pattern of size distribution of all glia for each group. The number of glia is on the y-axis and the size (μm^2) on the x-axis.

Density: The density of large glia in aged animals was significantly greater than the young [p=0.03] and there was no effect of age or treatment on the density of all, medium or small glia (**Figure 4.7**).

Tissue Fraction: There were significant age and interaction effects on the percent of tissue containing large glia [p=0.03 for both effects]. Post hoc analysis revealed that this was due to an age-related increase in the large glia tissue fraction of the vehicle animals, and E_2 treatment reversed this effect (**Figure 4.7** & **Table 4.1**).

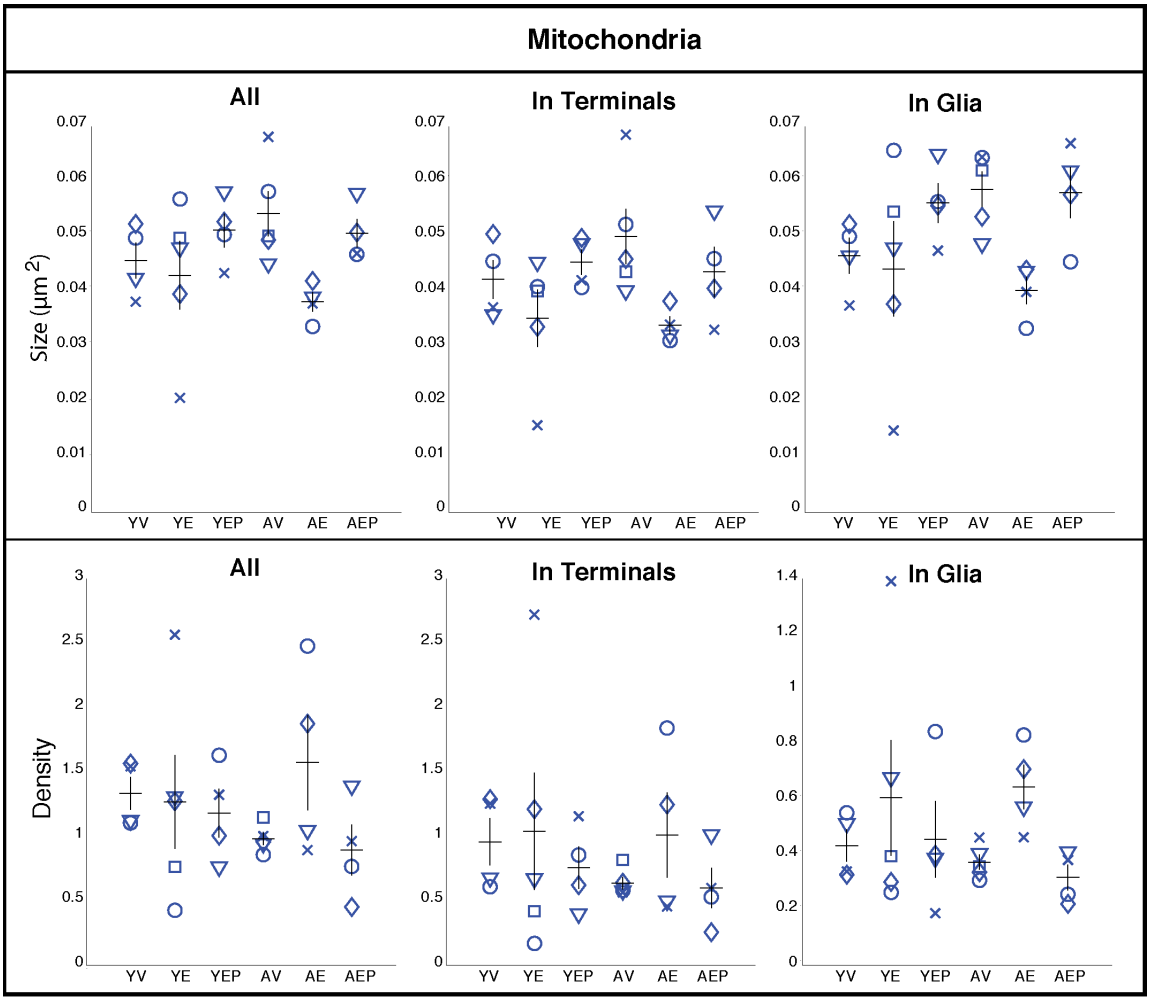
Hormone Treatments Affect Mitochondria Size in Terminals & Glia

Size: There were significant treatment effects on the size of all mitochondria, and within terminals and glia separately. This effect is driven by an age x treatment interaction in the glia, with E_2 reducing size only in aged animals. There was also a trend of an age-related increase in glial mitochondria size in the vehicle treated groups (details in **Table 4.1** & **Figure 4.9**). The distribution of size of all

mitochondria, and within terminals and glia separately indicates qualitative group differences as shown in **Figure 4.10**.

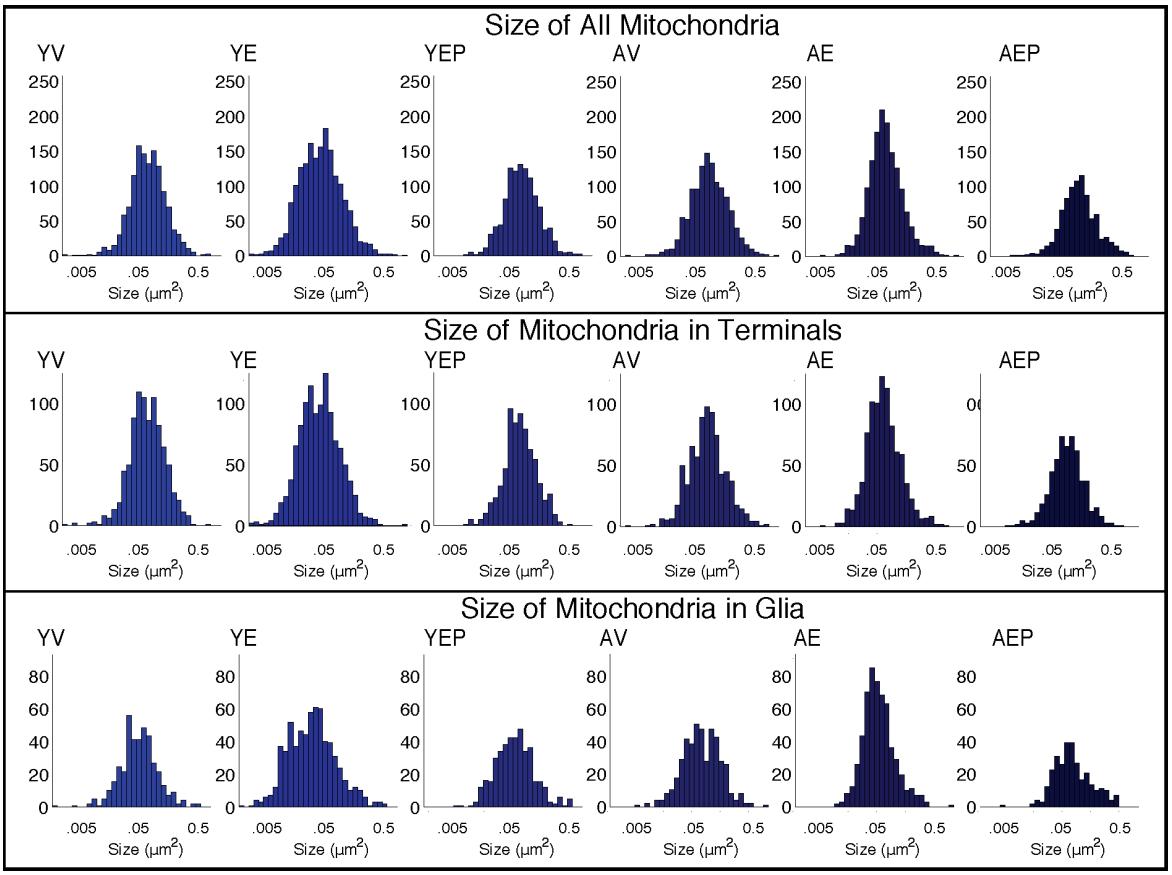
Density: There were no effects of age, treatment or interactions on the number of mitochondria in glia, terminals, or overall (**Figure 4.9**).

Figure 4.9: Size and Density of Mitochondria in Neuroterminals, Glia and Total in ME.



This figure shows the mean (± SEM) percent of mitochondria size (top row) and density (bottom row) for each group. Each individual is represented by a symbol that is consistent throughout all figures. There were significant effects of treatment on the size of all mitochondria and of those in the terminals and glia (p=0.04, p=0.01, p=0.04, respectively). There was a significant group effect in the size of mitochondria in glia, and group trends in all mitochondria and those found in terminals (p=0.05, p=0.06, p=0.07). **Table 4.1** provides the details of the statistical results.

Figure 4.10: Distribution of the Size of All Mitochondria, Mitochondria in Terminals and Mitochondria in Glia.



This figure shows the distribution of mitochondria size in the ME (top row), and the subset of mitochondria found in terminals (middle row) and in glia (bottom row) for each group. The number of terminals is on the y-axis and the size (µm^2) on the x-axis.

Extracellular Space is Not Significantly Affected by Age or Treatment

There were no significant effects of age, treatment or interactions on the percent of extracellular space, although there was a small age effect, with a higher percent of empty space in young than aged animals (p=0.09) as shown in **Figure 4.11**.

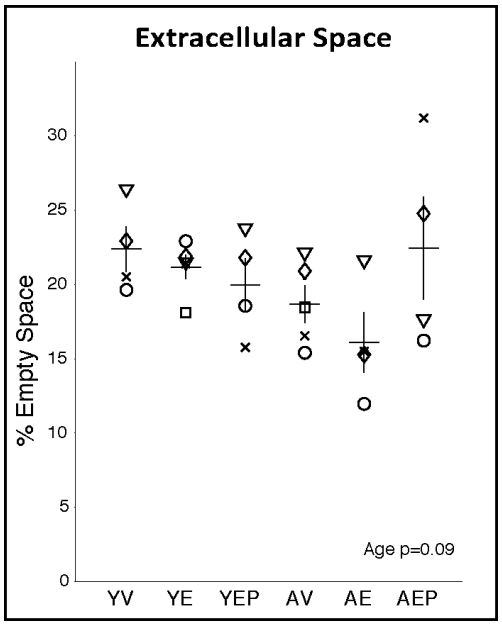


Figure 4.11: Percent of extracellular space.

This figure shows the mean (± SEM) percent of extracellular space in each group. The aged animals had slightly less empty space than the young animals (p=0.09). The symbols each represent an individual and are consistent throughout all figures.

DISCUSSION

To our knowledge, this is the first study to use electron microscopy to characterize the effects of age and hormone treatment on the ultrastructure of the pericapillary region of the median eminence of female rhesus monkeys as a model of menopause. Because the morphological plasticity of the ME is critical for normal functioning of the endocrine system, we focused on the relationship between secretory neuroterminals and glia, as reviewed in (Prevot et al., 2010; Yin and Gore, 2010). We found that there are significant effects of age, hormone and/or interactions between the two on all of these components. Overall, there seems to be age-related hypertrophy in glia and large neuroterminals, as well as increased density in and a corresponding increase in the fraction of tissue occupied by glia. When effects were seen, hormone treatment tended to dampen the age-related changes in terminal and glial ultrastructure. There was one exception; E_2 treatment had identical actions in both age groups, suppressing the tissue fraction containing large neuroterminals. The size of mitochondria was differentially affected by E_2 treatments in the two age groups, resulting in smaller mitochondria in the AE groups. Together these results suggest

that the ultrastructure of the ME changes with age in primates and HRT can mitigate some of these changes.

Ultrastructure of ME

The ME is generally organized into internal and external zones: the internal zone surrounds the base of the third ventricle and the cell body of tanycytes, a specialized type of astrocyte, while the external zone contains a high density of glial support cells such as astrocytes, microglia and tanycyte projections (Anthony et al., 1984; Ojeda et al., 2008; Yin and Gore, 2010). GnRH and other releasing hormones are synthesized and packed into large dense core vesicles (LDCV) in the basal hypothalamus and transported to neuroterminals in the pericapillary region of the ME for release (Durrant and Plant, 1999; Prevot, 2002; de Seranno et al., 2010; Yin et al., 2009a, 2009b). Tanycytes, are particularly important to the regulation of the endocrine system because their 'endfeet' are part of the blood brain barrier and act as the gatekeeper to the portal capillary system failure of tanycyte function would severely limit vesicle release (Flament-Durand and Brion, 1985; Prevot, 2002; Zoli et al., 1995). Therefore, we sampled and analyzed areas that with one or more neuroterminals possessing LDCV, often containing small clear vesicles and mitochondria and in close apposition to glia (Baroncini et al., 2007; Durrant and Plant, 1999; Prevot et al., 1998; Witkin and Romero, 1995; Yin et al., 2009a, 2009b). We found that the general organization of the rhesus ME is qualitatively different from the rodent in both young and aged adults. For example: the basal lamina is very convoluted in both young and aged adults, whereas similar convolution is a marker of hypothalamic aging in rats, although it is also increased during proestrus

(Prevot et al., 1999; Yin et al., 2009a, 2009b). Additionally, the linear organization of tanycytes projections from the third ventricle to the capillary bed seen in rodents is not obvious in monkeys of either age (Ojeda et al., 2008; Yin et al., 2009a, 2009b).

Age & Hormone Treatment Affect Neuroterminal Size & Tissue Fraction

We observed a significant increase in the size of large neuroterminals near the capillary of our aged animals. In monkeys and humans, GnRH release increases with age, and the increased terminal size may be to accommodate larger numbers of LDCV that are waiting to be released (Gill et al., 2002b; Gore et al., 2004; Park et al., 2002). Age-related increases in GnRH peptide content in the pericapillary region have not been described in the monkey, it is generally thought that this does not change with age in rodents (Bestetti et al., 1991; Rubin et al., 1984; Yin et al., 2009b). Although conflicting results were found by our lab in aging rodents; Kermath et al found that there was an increase in GnRH immunoreactivity in the external zone of the ME in naturally aging rats, while Yin et al found no changes in ovariectomized aging rats (Kermath et al., 2013; Yin et al., 2009b). The question of increased peptide content in large terminals could be answered in future studies by quantifying the LDCV present in these large terminals.

The size of large neuroterminals in AV animals was much greater than in the AE or AEP group, which may indicate that the absence of hormones is disrupting release mechanisms and/or the motility of the terminals, or that hormone treatment partially reversed effects of aging. The variability in the size of the large terminals is remarkably similar for all of the young group as well as the AE group, and the highest in the AV group. This may mean that E_2 has a 'normalizing' effect of the size

of neuroterminals. When comparing the patterns in the distribution of terminal sizes in the different groups, we notice that the V and EP groups have similar distribution shapes in both ages and E_2 groups are more similar to each other than to the age-matched groups (**Figure 4.6**). This may mean that the effects of E_2 are stronger than those of age on terminal size.

Since this is one of the first studies to investigate age or hormone-related changes in the ultrastructure of the primate ME, there are very few studies that we can directly compare our results to. There is one group that found that the number and size of GnRH neuroterminals near the basal lamina changes dramatically throughout the estrous cycle of adult rats, but there was no change in the overall number of neuroterminals, regardless of peptide content (Prevot et al., 1998, 1999). Yin et al found that young rats had significantly higher density of GnRH neuroterminals with no effects of treatment and that the 3D morphology of aged terminals changes dramatically with age (Yin et al., 2009b). Future analyses of GnRH terminal morphology will enable more direct comparisons between this rodent work and the monkey.

Age & Hormone Interactions Affect Glia Size, Density & Tissue Fraction

Although it is well established morphological interactions between glia and neuroterminals are very dynamic in the pericapillary region of the ME, to our knowledge, there have been no studies that quantify age-related changes in the morphology of these critical support cells (Baroncini et al., 2007; Ojeda et al., 2008; Prevot et al., 1998, 1999; Yin et al., 2009a, 2009b). We found that there was a significant age-related increase in the size of glia, regardless of size class, and an

associated age-related decline in the density of large glia. E_2 treatment reversed the age-related increase in the glial tissue fraction. Finally, there were significant age and interaction effects on the percent of tissue occupied by large glia, with an age-related increase in the vehicle animals that was reversed with E_2 treatment.

The majority of effects on glia were found in the AV group, indicating that hormone treatment may prevent some age-related changes in glia morphology. It is well established that glia, including tanycytes, express a variety of estrogen receptors and cultured rodent tanycytes have been shown to rapidly retract their processes when exposed to E_2, a mechanism that may contribute to the E_2-induced GnRH surge (Almey et al., 2012; Fuente-Martin et al., 2013; Micevych et al., 2010; de Seranno et al., 2010). Therefore, the increased size and percent of tissue occupied by glia observed in the AV groups may be due to the combination of aging and a lack of the E_2-triggered withdrawal signal, or a compensatory mechanism for the absence of E_2.

Hormone Treatments Affect Mitochondria Size in Terminals & Glia

To our knowledge, this is the first study to demonstrate a change in the size of mitochondria with E_2 treatment in the ME. Mitochondria are the organelles that produce all energy needed for cellular function. In additional to their critical role in the synthesis of E_2 and other steroid hormones, hypothalamic mitochondria express ERs and therefore are directly responsive to E_2 (Alvarez-Delgado et al., 2010; Baulieu and Robel, 1990; Woolley, 2007). The size and number of mitochondria correlate to the local energy demands and degenerating mitochondria swell before decomposing (Fiala et al., 2007). Therefore increase in mitochondrial size or

numbers would correlate with increased cellular metabolism and or dysfunction. We were surprised to see significant treatment effects on the size of all mitochondria, and within terminals and glia separately. There was a trend of an age-related increase mitochondria size in the glia of vehicle treated groups, suggesting that the glia in AV animals have a higher energy demand, the mitochondria within these cells are degenerating, or there is a higher demand for locally synthesized hormones. There was a robust decrease in the size of mitochondria in the AE group, in glia and neuroterminals, indicating that E_2 reduces the need for mitochondria in this region.

In the same monkeys, Hara et al found that there were no changes in the density or size of mitochondria in the hippocampus suggesting site specific effects of hormone (Hara et al., 2014). In contrast to our findings, previous work has shown no age- or hormone-related alterations in mitochondria size in rodent GnRH terminals or perikarya (Witkin and Romero, 1995; Yin et al., 2009b). It is interesting to note that, while most individuals had similar sized mitochondria in the terminals and glia, some had large differences in these two cell types as shown in **Figure 4.9**. Likewise, the majority of animals had an inverse relationship between the size and density of mitochondria, but a few had either a few small, or many large mitochondria, indicating individual differences in mitochondria efficiency.

Extracellular Space is Not Significantly Affected by Age or Treatment

A unique feature of the ME is a relatively large extracellular space where neurotransmitters, neurotrophic factors, and hormones can gain access to blood vessels, act upon receptors, and be taken up by cells in this region, similar to what

has been reported for volume transmission in the brain (Agnati et al., 1995). In the rodent, the percent of extracellular space is not significantly affected by age or hormone treatment, but appears to increase with age (Yin et al., 2009b). In contrast with rodents, there was a trend of decreased extracellular space in our aged animals. Indicating that this region is becoming more tightly packed with age.

Implications and Future Directions

Because this region is the common pathway for all hypophysiotropic-releasing hormones, these morphological changes could have wide-ranging effects on all aspects of endocrine function in in addition to reproduction. Investigation into the role of neuroendocrine aging in reproductive senescence has focused predominantly on cell bodies in the hypothalamus, and the majority of that work was conducted in rodent models. Therefore there is a critical gap in knowledge about the contribution of age-related changes in the ME to reproductive senescence in primates

ACKNOWLEDGEMENTS

We are grateful to Dr. Jeffrey Roberts and Mary Roberts at the California National Primate Research Center UC-Davis Primate Center for expert care of the animals. Dr. John H. Morrison and William Janssen are acknowledged for monkey work and perfusions. Dr. Weiling Yin, Dr. Di Wu and Megan Noel assisted with preparation and sectioning of hypothalamic tissues. Dwight Romanovicz and John Mendenhall provided expert advice with electron microscopy use. Larry Lindsey wrote the Matlab image analysis code and helped create the graphs and figures. This work was funded by NIH grant support: PO1 AG16765.

CHAPTER 5: MAIN DISCUSSION

This study was designed to fill in gaps in knowledge about the actions of different hormone replacement therapy (HRT) regimens in the brain of female primates undergoing reproductive senescence, in the context of human menopause. In the middle of a woman's expected lifespan, she starts to lose estrogen (E2) and progesterone (P4), with broad impacts on virtually every system in the body. There is great controversy about the relative benefits and risks of preventing this hormone loss with exogenous hormones, based on a number of studies with conflicting results (Gass, 2008; Kim et al., 2007; Lobo, 2013; Panay and Fenton, 2013; Yoon et al., 2003). One component underlying this controversy is the lack of knowledge about the effects of these hormones on women's brains, both endogenous and exogenous. Another is the applicability of research findings to perimenopausal, menopausal and postmenopausal women, populations that may differ in age by up to 50 years and have different basal physiologies. In an attempt to fill in some of the gaps in knowledge about this topic, I chose to investigate the structure of the neural circuitry that controls reproduction and set out to determine the effects of aging and a variety of HRT regimens on this system.

For proper reproductive function, the hypothalamic-pituitary-gonadal (HPG) axis depends on a delicate balance between and precise timing of hormonal release from the hypothalamus pituitary and ovary. Any physical changes in the systems that modulate and/or support the synthesis and/or release of GnRH may alter the flow of reproductive processes, ultimately leading to reproductive senescence. The aim of this dissertation was to answer the question: What role does age play in the

hypothalamic response of female primates to hormone replacement therapy? My approach to answer this question was three-fold: 1) analyze expression of hormone receptors in hypothalamic regions that provide indirect hormonal feedback to GnRH neurons; 2) investigate direct routes of hormonal regulation of GnRH neurons, particularly through the membrane estrogen receptor, GPER; and 3) examine the structures in the median eminence that influence the release of GnRH. I chose to use adult ovariectomized female rhesus macaques, of two ages, as a model of aging menopausal women, due to the similarity of their reproductive system and natural menopause. This section will synthesize the empirical findings from my research and frame it in the context of the wider literature and translation to human health.

INDIRECT HORMONE REGULATION OF HPG-AXIS VIA HYPOTHALAMIC INPUTS

I first sought to establish whether there are age-related changes in the ability or capacity to respond to estrogens, as described in Chapter 2: "G-protein coupled estrogen receptor, estrogen receptor α, and progesterone receptor immunohistochemistry in the hypothalamus of aging female rhesus macaques given long-term estradiol treatment." I examined hormone receptor expression as a proxy for measuring the hypothalamic capacity to respond to its respective ligand. This project sought to answer the broad question: What is the contribution of hypothalamic hormone receptor expression to reproductive senescence in primates? My approach to answering this question was to use immunohistochemistry and light microscopy to quantify the number of cells that express one of three hormone receptors that are responsive to E2: GPER, ERα, PR in two hypothalamic regions that are required for normal reproduction. This led to the

development of a more targeted question: How does cyclic estrogen treatment affect the expression of GPER, ERα, PR in hypothalamic regions that mediate E2 and P4 feedback, and are these effects equivalent in different ages of adult monkeys? Through this research, I discovered that E2 treatment caused an increase in the number of cells that expressed estrogen receptors (ERs), particularly the density of GPER in the periventricular area (PERI) in both ages, and of ERα in the arcuate nucleus (ARC) of the young monkeys only. The effects of age were to 1) trigger hypertrophy in GPER cell size and density of GPER cells in the ARC and PERI, regardless of treatment; 2) enhance E2 induction of GPER in the PERI; and 3) prevent the E2 induction of ERα in the ARC.

Due to the high resource demand of non-human primate models, there are few studies available for direct comparison. Many studies have found that hormone receptor expression (mRNA and protein in cells as well as number of cells) is altered by age and/or treatment, but the direction of that change is not consistent and seems to depend on age, species, tissue, brain/hypothalamic region, and HRT timing and regimen used (Bethea et al., 1992; Blaustein, 1993; Blaustein and Turcotte, 1989; Chakraborty et al., 2003; Lauber et al., 1991; Mills et al., 2002; Olster, 1998; Quadros and Wagner, 2008; Shughrue et al., 1992). In concordance with my results, some have found a modest increase or no response of PR and ERα to HRT in the ages examined (Adams et al., 2002; Bethea et al., 1996; Wang et al., 2010). A major contributor to the inconsistencies is likely the duration and regimen of HRT used and the variety in age and species examined, with short-term hormone treatment in rodents as the norm. I found that long-term effects are much more modest in monkeys, suggesting that the hypothalamus can adjust to accommodate HRT and return to the pre-treatment baseline, or that there are species differences.

Together these results suggest that age changes the way that the primates' brain responds to variations in hormonal milieu, and perhaps the enhanced increase of GPER in aged animals is to compensate for a decline in the ERα response to E2. If these results were viewed in light of human health, they would suggest that long and short-term E2 treatments should be investigated separately, and that aged women will respond to treatment, though their response may be mediated by differing pathways than younger women, contributing to the variable efficacy of such treatments.

DIRECT HORMONAL REGULATION OF THE MASTER COORDINATOR OF HPG-AXIS ACTIVITY

I sought to establish the existence of a direct route for E2 to regulate GnRH activity via GPER in adult primates and, if so, what effect age and HRT have on this pathway. This is described in Chapter 3: "GnRH neurons of young and aged female rhesus monkeys co-express GPER but are unaffected by long-term hormone replacement." I chose to focus on the protein expression of GPER in GnRH neurons as a proxy for measuring direct action of E2 on GnRH neurons, as the neurons fail to express ERα. This project sought to answer the broad question: Is direct estrogenic regulation of GnRH affected by age or hormone status? My approach to answering this question was to use fluorescence immunohistochemistry and confocal microscopy to identify and quantify co-expression of the most newly characterized ER, GPER in GnRH neurons. This led to the development of the following questions: 1) Does GPER play a role in estrogenic regulation of GnRH neurons? 2) Does this co-expression fluctuate depending on the region of the GnRH cell, i.e., the perikarya and the nerve process/terminal? And 3) Do age and/or hormones change this co-

expression? Through this research, I identified a direct path for E2 regulation of GnRH in adult primates and discovered that it is predominantly localized in the perikarya of half of GnRH neurons. However, this was relatively unaffected by age or hormone treatment.

This is the first study to show robust expression of GPER in GnRH neurons in adult primates. Due to the logistical complications of the aging monkey model and the relatively recent identification of GPER, there are few studies that we can directly compare to. There is one study, in mice, that found no electrophysiological evidence for GPER-mediated E2 action on GnRH activity (Romanò et al., 2008). However, other rodent and primate studies have found such evidence, suggesting that the protein expression we found is functionally relevant (Kenealy et al., 2011a, 2011b; Noel et al., 2009; Sun et al., 2010).

Together these results suggest that female primates, and potentially rodents, have a novel pathway for E2 regulation onto the main driver of reproduction and that this is unaffected by age or hormones. Perhaps the constant expression of GPER in GnRH neurons partially explains the resilience that the primate hypothalamus shows in aging. If these results were viewed in light of human health, they would suggest that any treatments developed to block GPER action [for example: to treat breast cancer] should be aware of the potential role that this receptor plays in regulating the HPG axis via GnRH.

ULTRASTRUCTURAL REGULATION OF NEUROENDOCRINE HORMONE RELEASE

For my third approach, I aimed to establish whether there are age-related changes in the ultrastructure that controls the secretion of neuroendocrine

releasing-hormones from the ME, as described in chapter 4: "Age-related changes in the ultrastructural morphology of the median eminence of female rhesus macaques are affected by long-term hormone treatment." I examined the morphology surrounding neuroterminals at the site of release into the portal capillary system as a proxy for measuring the capacity to secrete neuropeptides. This project sought to answer the broad question: "Do age-related ultrastructural changes in the ME inhibit endocrine signaling?" My approach to answering this question was to use electron microscopy to quantify morphological changes in cellular components surrounding neuroterminals apposed to the capillary bed. Through this research, I discovered that: 1) age was associated with hypertrophy of neuroterminals and glia; 2) E2 treatment reversed these effects of age, as well as decreased the size of mitochondria; and 3) E2 + P4 treatment had a similar, though much less robust, effect on terminals and glia as did E2 alone, and no effect on mitochondria.

Due to the extremely labor intensive nature of electron microscopy experiments in conjunction with logistical constraints of this model, there are very few studies to which we can directly compare our results. The only two other studies that examined the ultrastructure of the ME in the context of aging and HRT were conducted in rats and found similar, though more dramatic age-related effects (Yin et al., 2009a, 2009b). That study also found little to no evidence for effects of E2 treatment, but it is noteworthy that the treatment was only 3 days, compared to the 2-year HRT regime in the monkeys. Even with the differences in lifespan, the HRT treatment in rats is relatively far shorter. In contrast to this rodent work, human and monkey research has shown that there are some age-related changes in the pattern of hormonal release from the hypothalamus but, overall the hypothalamic capacity to respond to hormones remains intact (Gill et al., 2002a, 2002b; Gore et al.,

2004; Hall et al., 2000). Together these studies suggest a species difference in the response of the aging brain to hormones and that the neuroendocrine aspect of the HPG axis is more robust in primates than rodents (Gore, 2007; Gore et al., 2014; Kermath and Gore, 2012; Morrison et al., 2006; Yin and Gore, 2006, 2010). To translate this work to human health, the effects of E2 HRT, and, to a lesser extent, E2+P4 HRT, may protect against age-related hypothalamic dysfunction.

SYNTHESIS OF RESULTS

My results suggest several novel mechanisms that are consistent with, and may My results suggest several novel mechanisms that are consistent with, and may play a role in humans reproductive aging. GnRH release increases with aging in postmenopausal women, but with altered patterns of release, and the negative feedback effects of E2 and P4 are maintained, while positive feedback is not (Gill et al., 2002a, 2002b; Gore et al., 2004; Hall et al., 2000; Shaw 2011, Park 2002, Weiss 2004). To frame my results in the context of women's health: The decline in the E2 induction of ERα in the ARC may indicate a decrease E2 negative feedback tone onto GnRH. If so, then the increase in GPER expression and hypertrophy of GPER cells in ARC and PERI that could be the mechanism by which the hypothalamus compensates for the reduction in negative feedback. Indeed, the persistent expression of GPER in GnRH neurons may provide an alternate, or collaborative path for E2 negative feedback, regardless the age. Another possibility consistent with this research is that hypothalamic negative feedback is considerably altered with aging and the age-related changes we observed in the morphology and

differential response to E2 in the ME may be the compensatory mechanism that retains negative feedback in primates.

Age-related changes in the pathways that mediate indirect and direct hormonal regulation of hypothalamic function in primates are much less dramatic than what is found in rodents. My results and much of the literature suggests that there are fundamental differences in the regulation of reproduction in rodents and primates. I have found that many of the central assumptions that inform our understanding of primate reproduction are based on the fertility of rodent knockout (KO) models, such as the hierarchy of importance imposed on ERα, ERβ and GPER. Perhaps GPER plays a much more significant role in the E2 feedback regulation of the primate HPG-axis than would be suggested by GPER-KO mice, and the same could be said for ERβ as well. I call into question the direct applicability of KO models to primate reproduction and suggest that any assumptions based on these models be closely examined to ensure that they are not unduly biasing the interpretation of results.

IMPLICATIONS & RECOMMENDATIONS FOR FUTURE RESEARCH

The implications of the empirical findings of this study are that there are protective effects of E2 on the neuroendocrine system that are in agreement with E2's neuroprotective functions throughout the brain. Such results should be taken into consideration when making recommendations for its use in menopausal women, especially when considering actions on the brain (Dumitriu et al., 2010; McEwen, 2002). In addition, this work has greatly added to the existing pool of knowledge about GPER in the primate brain and shown that it may be a much more

significant player in the primate brain than would be expected from rodent literature. Finally, we have identified the ME as a target of hormonal feedback regulation, with potentially great importance to the aging endocrine system.

Due to the nature of postmortem studies, our conclusions were only speculative as to the functional relevance of our results. Three examples of future research that could clarify the results and address the functional relevance of my dissertation work are described below. A companion experiment, using postembedding immunogold labeling to identify which subset of neuroterminals contain GnRH in the pericapillary region of the ME, would strengthen the applicability of our findings directly to the HPG axis. Additional experiments could identify the other releasing hormones to provide insight into differential effects of age and HRT onto the endocrine system as a whole. One study that could ascertain the functional significance of GPER in E2 regulation of primate GnRH in the context of aging, would be to compare the effect of E2 on GnRH secretion (or LH as a proxy) in young and aged monkeys when given G2, the GPER antagonist, either intravenously, or directly into the hypothalamus via cannula or micro-dialysis. However, the difficulty and expense of such a project must be acknowledged. Finally, I suggest that the role of HRT timing be investigated further. Studies that compare the efficacy of HRT on animals that received varying durations of HRT as well as differing durations of hormone depletion would greatly inform the treatment options for women at varying time points post-menopause.

Despite the often-reported apparent harm of HRT in menopausal women, something that has been challenged in recent years, our results suggest that the effects of estrogen on the aging hypothalamus are few and predominantly protective in nature. This study adds to the growing body of research suggesting that the

benefits of E2 hormone therapy should be revisited, and that any policy recommendations concerning HRT should reflect the potentially varied and more nuanced effects of different types of hormones and regimens available. While the focus of this work was on the reproductive axis of females, the results have broader implications for age-related changes throughout the endocrine system, regardless of sex.

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