Cognition and Steroidogenesis in the Rhesus Macaque

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A DISSERTATION

Presented to the Department of Behavioral Neuroscience and the
Oregon Health & Science University School of Medicine
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
November 2013

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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Acknowledgments

It is hard to see only one name on the title page of this dissertation. None of the work I have completed while at Oregon Health & Science University was performed in a vacuum, and nothing would have been achieved without the generous support and collaboration of my colleagues, mentors, and friends during this time.

First and foremost, Dr. Henryk Urbanski has been instrumental in guiding me through the trials and tribulations of primate research, graciously providing me with countless leads on new directions for my work to take. He has been willing to listen to all of my crazy ideas and proposals over the years, some of which even worked out once in a while. Dr. Steve Kohama has become my first resource for answering seemingly out-of-nowhere questions, and has provided me with staggering amounts of expertise in nearly every laboratory technique I have attempted.

I sincerely thank the members of my dissertation committee, Dr. Urbanski, Dr. Kohama, Dr. Kathy Grant, and Dr. Cynthia Bethea, for their willingness to guide me to this point over the last few years. Their understanding of the difficulties of research has wisely talked me down from my over-exuberance for answering every little question, and they have given me wonderfully useful perspectives for the importance of my work.

The behavioral work I have chronicled here could not have been completed without the laboratory of Dr. Martha Neuringer, and I especially owe a genuine thanks to Laurie Renner for the miraculous level of data organization that made my analyses infinitely less time-consuming. Collection of cognitive data was not possible without the help of Alison Weiss, Jamie Garten, and Noelle Landauer. Perhaps the most important members of Dr. Neuringer's lab, however,

have been the monkeys. These animals have been remarkable not only as research subjects, but as fellow beings. Their sacrifice is greatly appreciated.

For his instructional and supportive contributions to this dissertation, I thank graphic design extraordinaire Jeff Hayes. Even in the final hours of panic in completing this work, he was able to patiently teach me how to make a figure look decent, simple, and communicative.

The faculty and administrative staff at both OHSU and ONPRC have been instrumental in both support and working out all the little details to make my experience here as smooth as possible. Dr. Suzanne Mitchell has been immensely helpful in her role as Program Director. Kris Thomason and Nicole Ernst in the Department of Behavioral Neuroscience, and Louise Sacha and Travis Dewey at the ONPRC have always miraculously known the answer to any question posed to them, and have always gone out of their ways to solve any administrative issues I have encountered.

Upon moving across the country, thousands of miles from any family, I made myself a new one. Anna and Lily have been at my side through every step of graduate school, from the first day to the last. While they may have no idea what happens when I walk out the door every morning, they know that when I come home every night all I need is a cat or two on my lap to make it a good day.

The work presented in this dissertation covers the learning and preparation I have completed over the last six years in the Department of Behavioral Neuroscience. I have devoted over one hundred and sixty pages to this time in my life, and while this has been a truly formative process, I find it difficult to devote a scant page or two to acknowledge the learning, experiences, and support that initially brought me to graduate school. I could have attended any graduate school, studied any biological system, succeeded under any mentor, but I never would

have developed the passion for science and learning that brought me here without the help of many people over the years. For cultivating my love of puzzles from before I could even read, I thank my family. For showing me that science, instead of being a rule that exists in text books, is just a series of puzzles picked apart by real people living real lives, I thank my high school Biology teacher Miss Jenny Wolf. She showed me that there was no reason I could not be one of those solvers of puzzles, instead of just an observer, and simply by taking a sincere interest in my future was able to set me on the trajectory to follow a passion I didn't even know I had. And finally, for showing me that the brain is the ultimate of all puzzles, I thank my undergraduate professional mentor, Dr. Michael Baum, and my personal mentor, Dr. Daniel Wesson. They were the first to convince me that someday I too could have those three sacred letters after my name, and I know they would be proud to know that I am almost there.

This work was supported by the following grants from the National Institutes of Health:

AG-023477	HD-007133	OD-011092
AG-029612	HD-029186	RR-000163
AG-036670	HD-018185	

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List of Abbreviations

This list covers common abbreviations used in multiple chapters. Abbreviations used in single chapters are defined upon first use.

Note: According to convention, gene names are italicized and capitalized when referring to the gene or mRNA and appear in capitalized regular type when referring specifically to the protein encoded by the gene.

17BHSD – 17β-hydroxysteroid dehydrogenase

17BHSD5 – 17β-hydroxysteroid dehydrogenase type 5; functionally an aldo-keto reductase enzyme, known as AKR1C3

3BHSD – 3β-hydroxysteroid dehydrogenase

3BHSD2 – 3β-hydroxysteroid dehydrogenase type 2

ACTH – Adrenocorticotropin-releasing hormone

AD – Alzheimer's disease

AMH – Anti-Müllerian hormone

ARO – Aromatase

CRH – Corticotropin-releasing hormone

CYP17A1 – Cytochrome p450 17a1; encodes enzyme with 17α-hydroxylase and 17,20-lyase activities

DCM – Division of Comparative Medicine

DHEA – Dehydroepiandrosterone

DHEA/S – DHEA and DHEAS, collectively

DHEAS – Dehydroepiandrosterone sulfate

DHT – Dihydrotestosterone

DMS – Delayed match-to-sample

DNMS – Delayed non-match-to-sample

E – Estrogen

E_2 – Estradiol

EDTA – Ethylenediaminetetraacetic acid

FSH – Follicle-stimulating hormone

GnRH – Gonadotropin-releasing hormone

HPA – Hypothalamic-pituitary-adrenal

HPC – Hippocampus

HPG – Hypothalamic-pituitary-gonadal

HRT – Hormone replacement therapy

HT – Hormone therapy

LH – Luteinizing hormone

MCI – Mild cognitive impairment

NRC – National Research Council

OHSU – Oregon Health & Sciences University

ONPRC – Oregon National Primate Research Center

OVX – Ovariectomized

P_4 – Progesterone

P450scc – Cholesterol side-chain cleavage enzyme

PFC – Prefrontal cortex

qRT-PCR – Real-time PCR; quantitative reverse transcriptase polymerase chain reaction

RT-PCR – Reverse transcriptase polymerase chain reaction

T – Testosterone

VDR – Variable delayed response

ZF – Zona fascicularis

ZR – Zona reticularis

Abstract

Recent changes in demographics resulting in a drastically increased aging population have sparked significant interest in research combating deficits and diseases occurring in late life. While the treatment of age-related diseases is of utmost importance, improving quality of life by maintaining normal healthy aging shows promise in preventing the development of chronic disease. For example, MCI is an early risk factor for AD, and thus addressing sub-pathological deficits in memory may prevent or delay the onset of dementia.

Gonadal and adrenal steroids play major roles in many cognitive processes, including the formation and maintenance of memory, and these hormones decline markedly with age in humans. The gonadal steroids E_2 and T improve cognitive performance in both rodent models as well as in human studies of memory; however, recent research in the effects of hormone therapy in advanced age raise questions as to the safety and efficacy of these treatments. The adrenal hormone DHEA, however, shows promise in rodent models of age-related cognitive decline, and endogenous levels of this hormone are associated with preserved cognitive performance in the elderly. As a result, treatment with exogenous DHEA may serve as a safer form of HT with the potential to slow cognitive decline. Despite this, trials of DHEA supplementation in the aged find little or no impact on memory.

This dissertation seeks to explore this dissociation between the effects of DHEA on rodent and human cognitive aging using the rhesus macaque (*Macaca mulatta*), a large diurnal primate with gonadal and adrenal endocrine systems similar to those of the human. Using hormone supplementation paradigms including physiological E_2, P_4, DHEA, and T, I have investigated effects of E_2 and DHEA treatment on memory performance in postmenopausal rhesus macaques, as well as effects of hormone supplementation paradigms on peripheral and

central hormone pathways. Additionally, investigation of the intracrine conversion of DHEA to E_2 has shown the potential for this metabolism within the brain, suggesting DHEA administration could possibly improve cognition via local synthesis of E_2 in cognitive areas such as the HPC and PFC. The enzymes responsible for this conversion, however, decline significantly with age. Thus, DHEA supplementation in the elderly may be ineffective due to a decreased ability to synthesize E_2 within the brain itself.

In addition to the interactions of gonadal and adrenal hormones within the brain itself, I show a significant sex-dependent interaction of the two endocrine systems in the periphery. Human and macaque research has previously shown a compensatory interaction between peripheral DHEA and E_2 in females, an effect which I have found also occurs in steroidogenesis in the central nervous system. Moreover, a novel finding in this research is a positive interaction between T and DHEA in male rhesus macaques. Additional data support a mechanism by which adrenal and gonadal systems interact to maintain the hormonal milieu in youth, how and why these mechanisms differ between males and females, and suggest a hormone supplementation paradigm that seeks to restore hormone balance in aged male rhesus macaques.

In summary, the classical approach of hormone therapy for the aged in which a single hormone that is deemed "deficient" is replaced may not be the most physiologically relevant approach to recreate a youthful endocrine composition. Due to the significant compensatory and facilitatory interactions between the adrenal and gonadal axes, a multi-level approach is needed to fully address the impact of the changing hormone environment on cognitive function in the aged.

Introduction

Portions of this section have been published previously in:

Sorwell KG, Urbanski HF (2010) Dehydroepiandrosterone and age-related cognitive decline. Age (Dordr.). *32:61.*

Sorwell KG, Urbanski HF (2013) Causes and consequences of age-related steroid hormone changes: insights gained from nonhuman primates. J Neuroendocrinol. *doi: 10.1111/jne.12064.*

Advances in the treatment and prevention of life-threatening diseases in the Western world have resulted in a dramatic demographic shift, with greater proportions of individuals living longer. The US Census Bureau, for example, projects that the percentage of people over the age of 65 will increase from 13% in 2010 to over 20% in 2050. Additionally the proportion of "oldest-old" (those surviving beyond the age of 85) will more than double, from 1.9% in 2010 to 4.3% in 2050 (Figure I.1). To increase the quality of life for individuals in these demographic ranges, it is essential to determine factors that will result in the maintenance of healthy aging.

Many physiological processes decline with age, including metabolism (Barzilai et al., 2012), cardiovascular health (Lakatta, 2002), bone density (Mirza and Prestwood, 2004), and cognition (Brayne et al., 1995). Concurrently, aging is associated with the deterioration of select endocrine systems, particularly the HPG axis and the HPA axis. The interaction between the endocrine system and other physiological processes has led to the development of HTs to prevent

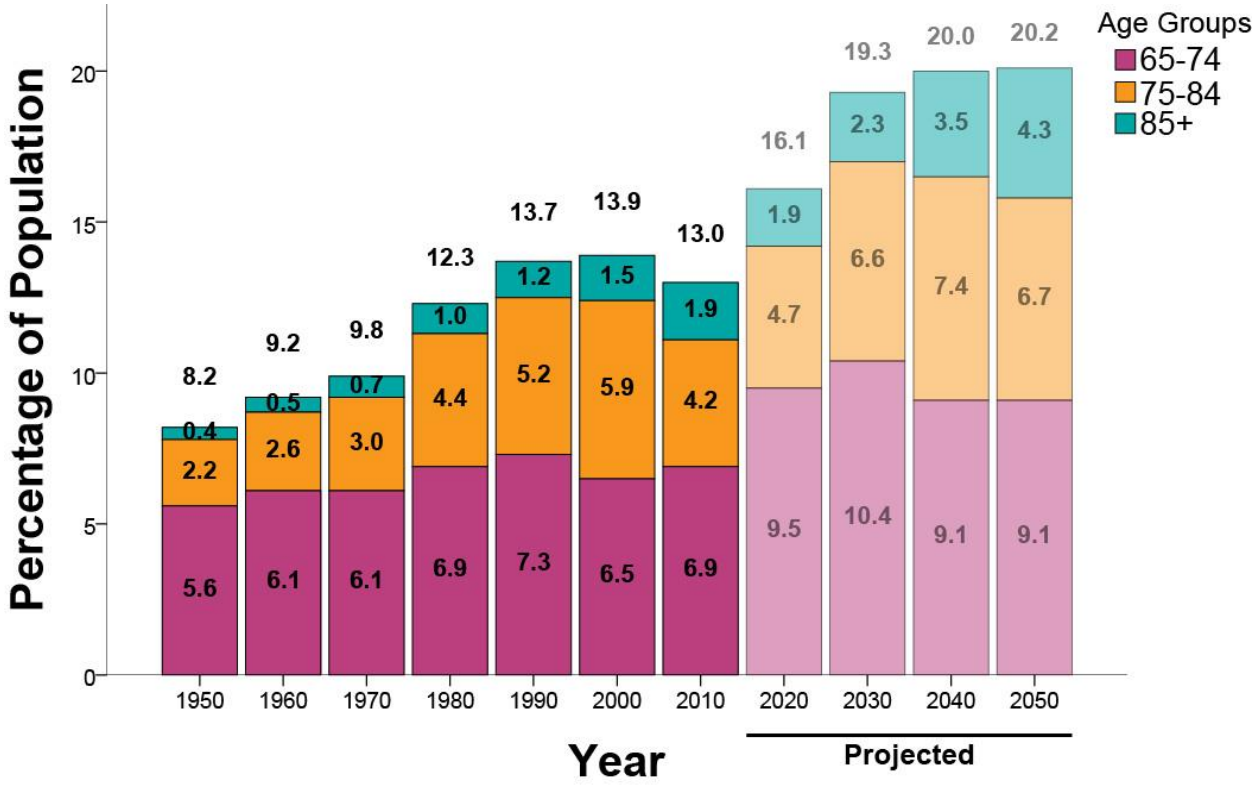


Figure I.1. Aging demographics in the United States from 1950 projected to 2050. Age distribution data from the United States Census Bureau shows a dramatic increase in the elderly population between 1950 and 2010, particularly among those aged 75 years and above. While over the last 30 years the proportion of individuals in this age range has remained relatively steady, these numbers are projected to nearly double in the next three decades. (Hobbs and Stoops, 2002; US Census Bureau, 2011).

or slow physiological decline. This thesis will explore select aspects of these age-related changes, particularly, the interactions of endocrine systems in a rhesus macaque (*Macaca mulatta*) model of aging, as well as the impacts of these systems on cognition and expression of intracrine genes in the brain.

1. Age-related cognitive decline

Human aging, particularly past the fifth decade of life, is accompanied by a number of physiological changes, one of the most widely studied being cognition. Aging is associated with gradual-onset deficits in attention (Commodari and Guarnera, 2008), spatial memory, verbal

memory, working memory (Moffat et al., 2006; Lithfous et al., 2013; Kumar and Priyadarshi, 2013), and executive function (MacPherson et al., 2002). The development of these deficits to a greater extent than is normal for age, but does not meet criteria for dementia, is termed "mild cognitive impairment," (Peterson et al., 1999) and is one of the strongest predictors of more serious forms of dementia, particularly AD (Almkvist et al., 1998; Peterson, 2004; Levey et al., 2006; Maioli et al., 2007). Additionally, cognitive deficits can have grave effects on other aspects of daily living, such as isolation, which may then lead to depression and a worsening of cognitive symptoms (Caccioppo and Hawkley, 2009; James et al., 2011). Cognitive decline is also associated with frailty, with increased risk of falls, fractures, and other accidents (Aueyeung et al., 2011; Robertson et al., 2013). Caregivers of these individuals also face extreme hardships, both psychologically and financially (Lu and Haase, 2009; Trivedi et al., 2013; Hayashi et al., 2013; Seeher et al., 2013). Thus it is of grave importance to develop strategies to intervene in cognitive aging to prevent more devastating disease and to investigate treatments that will slow or possibly prevent cognitive decline in late life.

While nearly the entire brain is involved in some area of cognition, two major regions have become classical targets for cognitive research, particularly in models of aging: the HPC and the PFC. The HPC, a region of the medial temporal lobe that is classically considered to be part of the limbic system, is involved in memory consolidation as well as contextual and relational memory (such as spatial concepts). In rodents, this memory domain is most commonly tested with procedures such as the Morris water maze, in which the animal must remember the location of a hidden platform in a pool of water, or the Barnes maze, in which the animal must remember the location of an escape hole in an open field. In rhesus macaques, the testing of hippocampal memory is often limited to confined tasks, such as a delayed-match-to-sample

(DMS) or delayed-non-match-to-sample (DNMS) task (Zola-Morgan et al., 1994), though a free-moving task similar to the Morris water maze and Barnes maze has been recently developed to test spatial memory in a more ethological manner (Rapp et al., 1997; Haley et al. 2009). In humans, this aspect of memory is often tested with associative memory tasks (Hannula and Ranganath 2008), context recall tasks (Hannula et al., 2013), mental rotation tasks (Inagaki et al., 2002), or computer-based spatial navigation tasks (Piper et al. 2011), all of which are greatly influenced by subjects' ages (Inagaki et al., 2005; Piper et la., 2011; Silver et al., 2012).

The PFC is involved in working memory, or the short-term storage and manipulation of information. This can include cognitive domains such as attention or using trial-specific cues to solve a problem. Prefrontal cortical memory can be tested in rodents using tasks such as T-maze alternation or delayed-response tasks (Evans-Martin et al., 2000; Deacon and Rawlins, 2006), in which the animal must remember earlier aspects of the trial to earn a reward. In the monkey, this domain can be tested using delayed-response tasks, DMS or DNMS, or the Wisconsin Card Sorting tasks (Moore et al., 2005; Hara et al., 2012), in which the animal must adapt to changing rules in a card matching task to earn a reward. In humans, working memory can be tested using the Wisconsin Card Sorting Test, reversal tasks, or trials that involve distractions or non-essential cues. In rodents, monkeys, and humans, aging is associated with a decline in working memory performance as assessed with these and other tasks (Ramos et al., 1993; Beas et al., 2013; Bizon et al., 2012; Samson and Barnes, 2013)

2. The HPG axis during aging

Within the HPG axis, GnRH serves as the primary neuroendocrine link between the brain and the anterior pituitary gland, stimulating the secretion of LH and FSH (Figure I.2). Interestingly, rhesus macaques and humans are one of the few mammalian species in which two distinct molecular forms of GnRH have been identified (GnRH-I and GnRH-II), suggesting that different subpopulations of GnRH neurons contribute differentially to the regulation of reproductive function in primates (Densmore and Urbanski, 2004; Urbanski, 2012). The two pituitary gonadotropins in turn stimulate gametogenesis and sex-steroid hormone production within the ovary (E_2 and P_4) and testis (T). The coordinated release of these reproductive hormones is essential for the onset of puberty and for the subsequent maintenance of fertility in adults, as well as for the development and maintenance of secondary sexual characteristics and other physiological functions.

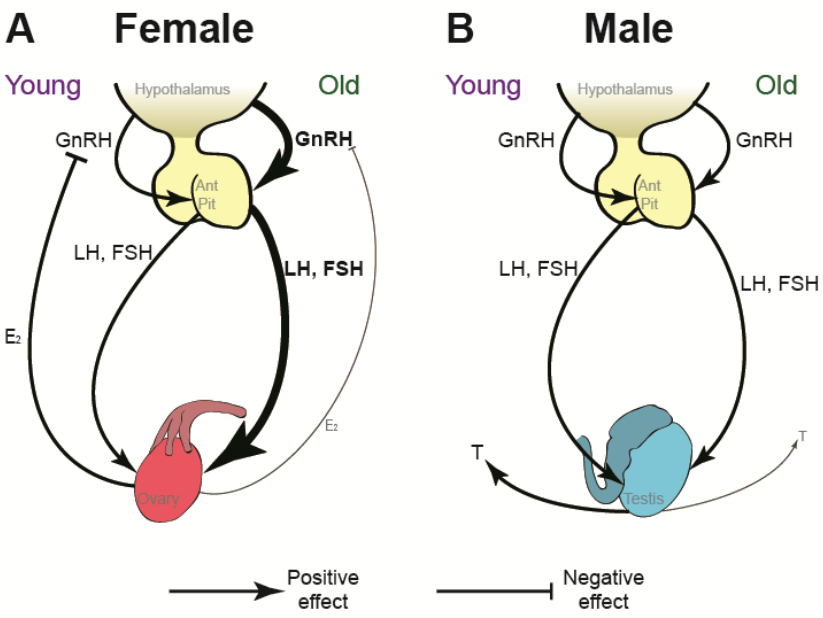


Figure I.2. Female and male hypothalamic-pituitary-gonadal axes. In the female HPG axis (A), the hypothalamus sends GnRH to the anterior pituitary, inducing the release of LH and FSH. These gonadotropins then act on the ovary to release E_2 into the bloodstream, which generally exerts negative feedback on GnRH. In old age, diminished ovarian reserve leads to a lesser release of E_2 from the ovary, resulting in a loss of inhibitory tone and increased circulating LH and FSH. In the male HPG axis (B), LH and FSH induce the secretion of T from the testis. While the mechanism underlying reduced circulating T in old age is unclear, it is believed to be due to a decreased responsiveness of the testis to LH (Liu et al., 2005; Takahashi et al., 2007). Ant Pit, Anterior Pituitary.

2.1 Female reproductive endocrinology during aging

The similarity between the HPG axis of women and adult female rhesus macaques is emphasized by similar hormonal changes that occur across the menstrual cycle, and the subsequent precipitous loss of sex-steroid output after menopause (Downs and Urbanski, 2006; Hall, 2007), which is thought to be triggered by the loss of ovarian follicles (Richardson et al., 1987; Richardson and Nelson, 1990), as well as a reduced responsiveness of the hypothalamus to GnRH and E feedback (Weiss et al., 2004; Shaw et al., 2009; Zhang et al., 2011). This decline in sex-steroid levels is associated with a decrease in the output of other ovarian hormones, such as anti-Müllerian hormone and inhibin B, and a concomitant increase in circulating LH and FSH levels due to the loss of negative feedback to the hypothalamus and pituitary gland (Downs and Urbanski, 2006; Ramezani et al., 2013) (Figure I.2A). This age-related change within the primate HPG axis differs greatly from that observed in rodents, in which the first sign of reproductive senescence appears to be an attenuation of GnRH signaling (Lloyd et al., 1994; Wise et al., 2002), resulting in a dampened and delayed preovulatory LH surge (Wise 1982; Downs and Wise 2009).

2.2 Male reproductive endocrinology during aging

Although male primates show an age-related decline in circulating T levels, this is generally very gradual and less extreme than the precipitous decrease of E_2 observed in females around the time of menopause (Schlatt et al., 2008; Sitzmann et al., 2013). What makes detection of age-related changes in T output particularly difficult to study, however, is its episodic pattern of release, which is driven by pulsatile secretion of GnRH and LH every few hours. In addition, circulating T levels are characterized by a distinctive 24-hour release pattern, which means that

single measurements of T in the circulation are unreliable indicators of overall T output; thus, accurate changes in peak T levels are difficult to determine in aged populations. Despite this difficulty, however, large-scale studies have identified a slow and gradual decline in circulating T in aged men (Harman et al., 2001; Feldman et al., 2002; Schlatt et al., 2008), as well as a dampening of the diurnal rhythm of T (Bremner et al., 1983), possibly due to a reduced responsiveness of the testis to LH stimulation (Liu et al., 2005; Takahashi et al., 2007) (Figure I.2B).

3. The HPA axis during aging

Within the HPA axis, corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. In turn, ACTH signals the adrenal cortex to release cortisol from the ZF and DHEA from the ZR. Under normal circumstances, cortisol exerts negative feedback on both the hypothalamus and pituitary to reduce secretion of CRH and ACTH, respectively (Figure I.3). While acute increases in cortisol can be adaptive in times of stress, prolonged increases can result in hippocampal excitotoxicity (Moghaddam et al., 1994; Goodman et al., 1996) and oxidative damage (Schubert et al., 2008; Sato et al., 2010). In addition, high levels of cortisol can decrease hippocampal volume and interfere with the structural changes necessary for learning and memory (Tata et al., 2006; Conrad 2008; Schloesser et al., 2009; Tata and Anderson, 2010). As the hippocampus, itself, responds to glucocorticoids by exerting additional negative feedback on the hypothalamus, hippocampal damage and the resulting glucocorticoid insensitivity results in a disruption of HPA axis activity and further elevations in cortisol (Tata et al., 2006).

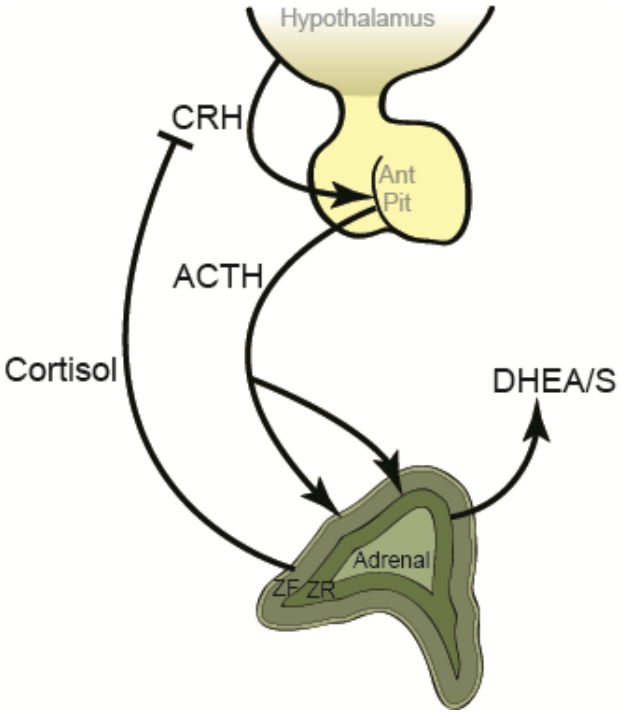


Figure I.3. Hypothalamic-pituitary-adrenal axis. In the human and nonhuman primate HPA axis, CRH from the hypothalamus induces the secretion of ACTH from the anterior pituitary. ACTH in turn activates the ZF of the adrenal cortex to secrete cortisol and the ZR to secrete DHEA/S. Cortisol then exerts negative feedback on CRH to dampen the cycle. Ant Pit, Anterior pituitary.

DHEA, meanwhile, can act as a "functional antagonist" of cortisol (McEwen, 2002; Ferrari and Magri, 2008), in part by promoting neuronal and glial survival (Roberts et al., 1987; Bolog et al., 1987). An increase in circulating cortisol with advanced age has been observed in both humans (Dodt et al., 1994; Deuschle et al., 1997) and nonhuman primates (Downs et al., 2008), with a concurrent marked decline in DHEAS (the sulfated form of DHEA) throughout adulthood (Labrie et al., 1997; Downs et al., 2008; Urbanski et al., 2013), most likely due to degradation of the ZR (Dharia et al., 2005). This resulting increase in the cortisol:DHEA ratio may have drastic implications for many physiological processes, including learning and memory (McEwen, 2002; Ferrari and Magri, 2008), a view that is supported by the finding that higher cortisol:DHEA ratios are associated with greater cognitive impairment (Kalmijn et al, 1998; Van Niekerk et al., 2001).

4. Hormone synthesis pathways

While the steroid hormones of the HPA and HPG axes have been described above distinctly, the synthesis of these hormones are in fact closely tied together. As shown in Figure I.4, the synthesis of E_2 from cholesterol includes both DHEA and T as intermediaries. This suggests that the products of steroidogenic organs are dictated by the presence and absence of the key steroidogenic enzymes expressed. For example, the ZR of the adrenal gland expresses high levels of cytochrome p450scc (encoded by the gene *CYP11A1*) and CYP17A1, an enzyme complex capable of 17α-hydroxylation and 17,20-lyase activity, but low levels of 17α-ketoreductase (AKR1C3, also known as 17BHSD5) and 3BHSD, thus resulting in the production of high levels of DHEA, but without significant progression to T. Similarly, the testis expresses all of these enzymes at high levels, but low levels of ARO; therefore, the main output of the testis is T. Finally, the ovary expresses all of these enzymes, including ARO, leading to a significant production of E_2 during the follicular phase of the menstrual cycle.

The expression of these enzymes, however, is not limited to the classical endocrine organs themselves. In fact, expression of these enzymes has been documented in liver, kidney, skin, adipose tissue, pancreas, lung, heart, brain, spleen, muscle, intestine, and mammary gland (Simard et al., 1991; Zhao et al., 1991; Martel et al., 1994). Therefore, many peripheral organs are capable of synthesizing their own hormones *de novo* from cholesterol, or converting circulating steroids (such as DHEA or T) to steroids further along the steroidogenic pathway, a phenomenon known as intracrinology (Labrie et al., 1988; Labrie 1991). In fact, Labrie and colleagues estimate that up to 50% of active androgens in men are derived within target tissues, and in women, up to 75% of active estrogens before menopause and 100% of active estrogens after menopause are produced in this manner (Labrie et al., 1998; Labrie et al., 2001). Therefore,

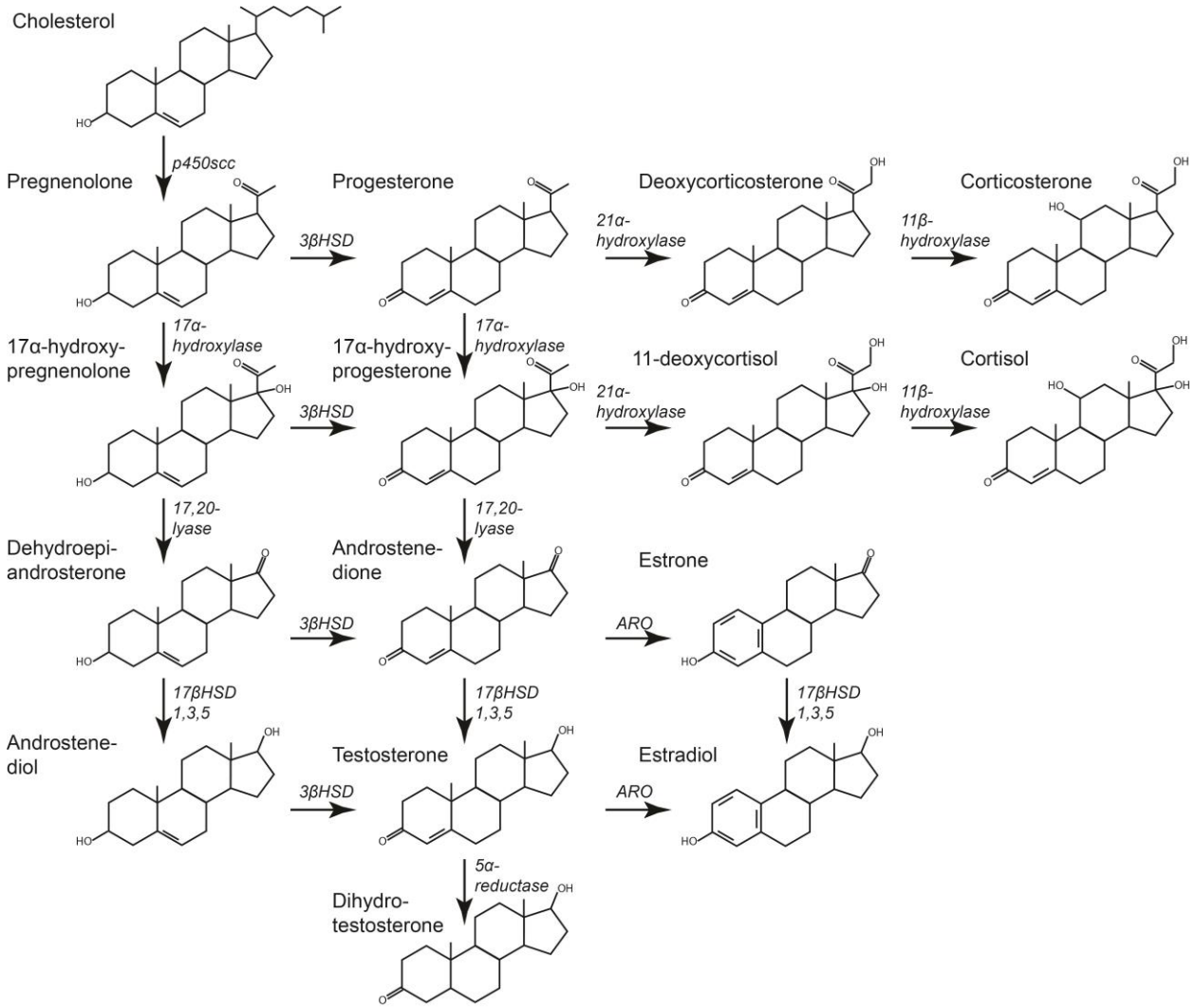


Figure I.4. Hormone synthesis pathway: synthesis of testosterone, estradiol, and cortisol from cholesterol. The synthesis of active steroids from cholesterol is a complex web, with the overall outcomes determined by the presence and relative abundance of steroidogenic enzymes. Cholesterol is metabolized to pregnenolone by p450scc (encoded by the *CYP11A1* gene) and can be converted to either progesterone by 3BHSD, or to DHEA by the combined 17α-hydroxylase and 17,20-lyase capabilities of CYP17A1. DHEA can then be metabolized to T by 3BHSD and a family of aldo-ketoreductase enzymes referred to as 17BHSDs (the form of which found in the brain is 17BHSD5, encoded by the gene *AKR1C3*). Of note is the opposing action of 17BHSD2 and 17BHSD4 (not shown) which convert androstenediol back to DHEA, or T back to androstenedione. Finally T can be converted to either DHT by 5α-reductase, or E_2 by ARO.

in examining the impact of gonadal steroids on functional domains such as cognition, it is important to consider not only the classical sources of these hormones, but the potential of the brain itself to contribute active estrogens and androgens to its own hormonal milieu.

5. Impact of circulating hormones on cognitive performance in rodents and humans

5.1 Rodent studies of hormones and cognition

5.1.1 Estrogen

Studies of learning and memory in mice and rats have found deficits induced by the loss of E_2 via ovariectomy in both spatial (Daniel et al., 1997; Bimonte and Denenberg, 1999; Fry et al., 2007) and working memory (Wide et al., 2004; Daniel et al., 2006) tasks, an effect which is rescued by E_2 replacement. E_2 is also sufficient to rescue age-induced cognitive deficits in reproductively senescent rodents (Gibbs, 2000; Frick et al., 2002b; Frye et al., 2005). *In vitro* studies suggest E_2 enhances memory by increasing acetylcholine (Bohacek et al., 2008; Gibbs and Aggarwal, 1998; Wu et al., 1999; Frick et al., 2002a) and glutamate (Nilsen and Brinton, 2002; Yokomaku et al., 2003) signaling, increasing synaptic excitability (Wooley 2007), enhancing hippocampal neurogenesis (Oremerod et al., 2004; Galea et al., 2006), and increasing dendritic spine density in the HPC (Wooley and McEwen, 1994) and PFC (Hao et al., 2006; Hao et al., 2007). E_2 can also serve as a powerful neuroprotectant (Dubal and Wise, 2001; McClean and Nuñez, 2008; Amtul et al., 2010) and may protect cognitive brain areas from the buildup of oxidative stressors in advanced age (Moorthy et al., 2005), ischemia (Zhang et al., 1998), and injury (Brown et al., 2009).

5.1.2 Testosterone

Because T does not decline as precipitously with age as does E_2, less research has been devoted to the involvement of T with changes in male cognitive performance with age. However, studies of T deprivation have found cognitive deficits induced by gonadectomy that can be reversed by T supplementation (Kritzer et al., 2001; Edinger and Frye, 2004). Additionally, loss of T results in a significant loss of synapses in the hippocampus, an effect that is rescued by either exogenous administration of T or its more active metabolite, dihydrotestosterone (Leranth et al., 2003). Finally, aromatase can convert T to E_2, activating estroge-driven cognitive effects if sufficient levels of the enzyme aromatase are locally expressed.

5.1.3 DHEA

While a receptor specific to DHEA or DHEAS has not been isolated, rodent studies have observed a number of celluarl effects of the steroid that are associated with improved memory. DHEA/S has been shown to antagonize the androgen receptor and agonize estrogen receptor β; thus, it may exert some of the same positive actions as E_2 (Chen et al. 2005). Also, DHEA/S may affect synaptic plasticity in the hippocampus through antagonism of the $GABA_A$ receptor (Majewska 1992), facilitating long-term potentiation via NMDA stimulation (Mellon and Griffin 2002; Chen et al. 2006), and enhancing glutamate release during learning (Lhullier et al. 2004). DHEA/S also significantly protects the CNS against the effects of cortisol by blocking its suppression of neurogenesis (Karishma and Herbert, 2002). Additionally, DHEA/S has been observed to be neuroprotective against oxygen-glucose deprivation (Kaasik et al. 2001), oxidative stress (Bastianetto et al. 1999; Kumar et al. 2008), and excitotoxicity (Kimonides et al.

1998; Mao and Barger 1998), as well as effective at enhancing cell survival, proliferation, and neurogenesis (Karishma and Herbert 2002; Suzuki et al. 2004). In line with this evidence, *in vivo* studies have shown an anti-amnestic effect of DHEAS (Flood et al. 1988) as well as an anti-aging effect on cognition in rodents (Flood and Roberts 1988).

Although DHEA/S supplementation studies have been performed both *in vitro* and in rodents, in many cases endogenous neurosteroidogenesis was uncontrolled. Therefore, the observed effects may have been mediated by endogenous conversion of DHEA/S to E_2. This is certainly plausible given that E_2 supplementation can produce many similar results (DeNicola et al. 2008). The proteins and enzymes necessary for the conversion of DHEA/S to E_2, as well as for the synthesis of DHEA/S from cholesterol (Fig. I.4), are present in a region-dependent manner in the rodent brain (Kohchi et al., 1998; Zwain and Yen 1999; Hojo et al. 2003; Gottfried-Blackmore et al. 2008), suggesting that this may be a valid mechanism of action. Interestingly, adrenalectomy combined with gonadectomy had no effect on the central levels of DHEA/S in rodents (Robel et al., 1987) and suppression of adrenal activity with dexamethasone had no effect on DHEA/S levels in the nonhuman primate brain (Corpechot et al., 1981), suggesting that the hormone is indeed synthesized *de novo* from cholesterol in the brain. Indeed, locally produced E_2 has been shown to have significant impacts on hippocampal synaptic plasticity in rodent models (Kretz et al., 2004; Rune and Frotscher, 2005; Mukai et al., 2006), and the neuroprotective effect of DHEA is at least in part mediated by its metabolism to E_2 (Veiga et al., 2003). It is important to note, however, that while similar neurosteroidogenesis has been suggested in primates and humans (Robel et al., 1987), this pathway has yet to be investigated in detail.

5.3 Human studies of hormones and cognition

5.3.1 Estrogen

Due to promising evidence from rodent aging studies, much research has been conducted on the effects of various E supplementation paradigms on cognition in postmenopausal women. Indeed, immediate E replacement in young women following ovohysterectomy can reverse cognitive deficits associated with ovarian E_2 loss (Sherwin et al., 1988; Farraq et al., 2002; Rocca et al., 2011), showing that E_2 has significant effects on learning and memory outside of the influence of advanced age. Some evidence suggests that HT involving E may attenuate age-related cognitive decline (Løkkegaard et al. 2002; Sherwin 2007b), particularly in women at risk for developing AD (Hu et al. 2006; Yue et al. 2007). These results remain controversial, however, as other HT studies, including that of the Women's Health Initiative, have shown null effects or negative influences of HRT on cognitive decline (Craig et al. 2005; Lethaby et al. 2008). Many postulate that these discrepancies may be due to patient age of HT initiation relative to menopause, as in both humans and rodents this variable seems to determine the direction of E's effect on cognition (Kang et al. 2004; Daniel et al. 2006; Sherwin 2007c; Bohacek et al. 2008).

5.3.2 Testosterone

Circulating T levels show an age-related decline in men (Plymate et al., 1989; Feldman et al., 2002; Kaufman and Vermeulen 2005), and aged men with higher levels of endogenous T exhibit greater cognitive performance (Barret-Connor et al., 1999; Yaffe et al., 2002; Muller et al., 2005). Additionally, T supplementation in men with low endogenous T levels has been shown to improve certain aspects of cognitive function (Janowsky et al., 2000; Kenny et al.,

2002; Beer et al., 2006). Although the exact mechanism is unclear, it is possible that the beneficial effects of supplementation are mediated by conversion of T to E_2. This rationale is based on the observation that men undergoing androgen deprivation therapy experience cognitive deficits that can be rescued by E_2 supplementation (Holland et al., 2011), and that aromatization of T to E_2 appears to be necessary for some of the cognitive benefits of T supplementation (Cherrier et al., 2005).

5.3.3 DHEA

Because of the strong pro-cognitive and anti-aging effects of DHEA/S previously observed in rodents, attempts have been made to examine the efficacy of DHEA/S supplementation in elderly humans. There is general agreement that DHEA supplementation can exert beneficial effects on mood and well-being in populations with adrenal insufficiency (Arlt et al. 1999; Bloch et al. 1999; Hunt et al. 2000) and depression (Wolkowitz et al. 1999; Schmidt et al. 2005), and that it shows some benefit in a primate model of Parkinson's disease (Bélanger et al. 2006); this suggests that DHEA supplementation might also enhance cognitive functions in the elderly. Additionally, low circulating DHEA/S levels are thought to play a role in the development of AD (Weill-Engerer et al. 2002) and in some domains of memory impairment (van Niekerk et al. 2001; Davis et al. 2008), again, supporting the hypothesis that DHEA/S supplementation may improve cognition in the elderly. So far, however, clinical studies of DHEA/S supplementation have failed to provide convincing evidence in support of this hypothesis. While individual studies have reported beneficial outcomes in young men (Alhaj et al., 2006) and postmenopausal women (Stangl et al 2011), the size and clinical significance of these effects were minimal. The vast majority of studies of DHEA replacement on healthy

elderly populations, either acute or chronic (up to 12 months) supplementation, have failed to show a clinically significant benefit in memory with treatment (Wolf et al. 1997, 1998; Wolf and Kirschbaum 1999; Arlt et al. 2001; Grimley Evans et al. 2006; Kritz-Silverstein et al. 2008). Additionally, some studies have observed a negative effect of DHEA treatment on memory (Wolf et al. 1998; Parsons et al. 2006). DHEA supplementation has also shown no benefit in the treatment of AD (Wolkowitz et al. 2003).

6. The nonhuman primate: choosing the most appropriate animal model for human endocrine aging research

While nonhuman primate research involves many costs not experienced in rodent research, including financial burdens of housing, costly veterinary care, longer lifespan (and thus a more significant time commitment when studying aging effects), and sociopolitical concerns, the rhesus macaque shares many endocrine characteristics with the human making it an ideal model for aging research. The above review of endocrine impacts on aging shows that E_2 and T have cognitive benefits that are seen across species; however, the effects of DHEA supplementation in rodents do not translate to humans. This discrepancy may arise from pronounced differences between rodent and human endocrine systems. Macaques, however, demonstrate endocrine profiles and mechanisms similar to humans, and also experience a menopause and adrenopause that is similar to those seen in aging humans.

As previously discussed, the key event in reproductive senescence in the female rodent is a decline in GnRH signaling from the hypothalamus (Lloyd et al., 1994; Wise et al., 2002), while in women menopause is currently believed to be induced by the depletion of ovarian follicles (Richardson et al., 1987; Richardson and Nelson, 1990) and a decline in hypothalamic GnRH

and E_2 responsivity (Weiss et al., 2004; Shaw et al., 2009; Zhang et al., 2011). This research has been strengthened by data from the nonhuman primate (Gore et al., 2004; Kim et al., 2009; Appt et al., 2010). Additionally, human (and nonhuman primate) menopause is associated with a sustained loss of E_2, while the rodent estropause can result in persistently low or high levels of E_2 (Mobbs et al., 1984; Everett, 1989). Therefore, the nonhuman primate is a much more appropriate model for aging with respect to endocrine changes in the female. Using a surgical model of menopause in female rhesus macaques, researchers have shown that cyclic E and P_4 supplementation is able to rescue cognitive deficits induced by ovariectomy and aging (Lacreuse et al., 2002; Rapp et al., 2003), just as E therapy rescues ovohysterectomy-induced cognitive deficits in humans (Sherwin 1988; Farraq et al., 2002; Rocca et al., 2011).

More importantly, rodents and humans differ dramatically in HPA physiology. While DHEAS is the most abundant circulating steroid in the human, it is all but absent in the circulation of mice and rats, due to a lack of CYP17A1 activity in the adrenal gland. Therefore, rodents lack the observed age-related decrease in DHEA/S production, and thus DHEA/S "replacement" may not be as physiologically relevant in a rodent model as in a primate or human model (Wolf and Kirschbaum, 1999). Not surprisingly, rhesus macaques have an adrenal gland structure and function similar to humans (Conley et al., 2004; Nguyen and Conley, 2008; Abbott and Bird 2009) and demonstrate a corresponding age-related decrease in circulating DHEAS concentrations (Urbanski et al., 2004; Downs et al., 2008), and therefore are a much more appropriate model for adrenal androgen supplementation in late life. Despite this, no studies have yet been published on the effects of supplementary DHEA/S in the macaque.

Finally, the rhesus macaque is an ideal model for healthy, normal aging with mild cognitive decline, as these animals show age-related deficits in cognition without the

development of dementia or Alzheimer's pathology (Tayebati, 2006; Woodruff-Pak DS, 2008). The ability to train and test monkeys longitudinally using tasks more similar to human assessments than rodent tasks also makes them an ideal model for translational research in cognitive aging. Additionally, studies of E supplementation in aged macaques show a significant positive effect on cognition as measured by the variable delayed response task (Figure I.5, personal communication, M. Neuringer), showing that macaques, like humans, are sensitive to the cognitive-enhancing effects of hormones in old age.

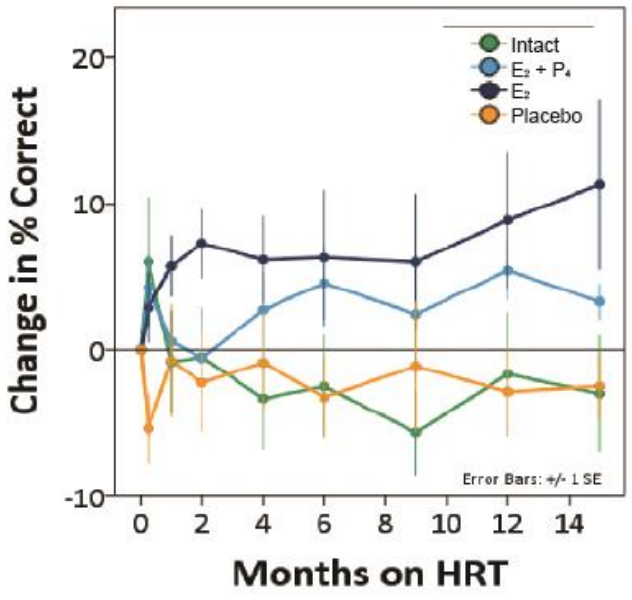


Figure I.5. Delayed response performance in aged female rhesus macaques treated with estradiol or estradiol plus progesterone. Aged female rhesus macaques were ovariectomized and treated with a silastic capsule releasing either a cholesterol placebo, continuous E_2, or continuous E_2 with oral cyclic P_4. An age-matched intact group of female macaques was included as an additional control group, though this group varied in menopausal status. Animals received hormone treatment with longitudinal cognitive testing that included a delayed response task for 14 months. Monkeys that were treated with either E_2 alone or E_2 combined with P_4 showed a significant increase in performance at the 15 second delay from baseline, which was maintained throughout the study; however, ovariectomized and intact control animals maintained baseline levels of performance. These data indicate a significant positive effect of E_2 replacement therapy in a model of menopause on visuospatial working memory.

7. Summary

This dissertation seeks to show the importance of using not only the relevant animal model, but the relevant and specific hormone supplementation paradigms to fully understand the complex endocrine interactions seen in human aging. Specifically, while the source of "pro-

cognitive" hormones is classically thought to be the gonads, whose decline in production greatly correlates with cognitive deficits, we aim to show that the HPA axis and even the brain itself can contribute greatly to the hormonal inputs to cognitive areas of the brain. While clinically it may be simple and convenient to administer single supplementary hormone to address issues such as declining memory, concentration, affect, bone density, muscle mass, sexual health, skin elasticity, digestive health or many other domains and tissues that suffer with age, the closely intertwining endocrine systems of the human physiology make it important to consider overlapping hormonal systems and their interactions for complete health into the later years.

Part A

Central steroidogenesis and cognition

Chapter 1: Steroidogenic potential of the rhesus macaque brain

Portions of this chapter have been published previously in:

Sorwell KG, Kohama SG, Urbanski HF (2012) Perimenopausal regulation of steroidogenesis in the nonhuman primate. Neurobiol Aging. *33:1487.e1-e13.*

1. Introduction

Human aging is associated with several physiological and cognitive changes, but the underlying etiology is poorly understood. Many of these age-associated disorders and pathologies, such as osteoporosis, are even more pronounced in females, due to the marked decrease of circulating E_2 concentrations that occurs around the time of menopause. Consequently, common therapies developed for postmenopausal women involve E replacement. In other target tissues, such as the brain, ovarian steroids are known to increase synaptic plasticity (Brann et al., 2007) and may improve cognition in postmenopausal women (Fillit et al., 1986; Tang et al., 1996). However, due to potential health risks associated with E-based HRT (Manson et al., 2003), there is need for safer alternative therapies to help alleviate postmenopausal disorders, such as cognitive decline. One potential alternative therapy that may be safer than E therapy involves the adrenal steroids DHEA and DHEAS, both of which decrease with age in humans (Labrie et al., 1997). Importantly, administration of these steroids to old mice has shown promise in restoring cognitive function to a level observed in young animals (Flood and Roberts, 1988; Markowski et al., 2001). However, as mouse and rat adrenal glands do not produce measurable levels of circulating DHEA/S, they may not be ideal models for human aging. In humans, high baseline levels of DHEA/S are associated with increased longevity in

men, and in elderly women they have been associated with better cognitive performance (Davis et al., 2008; Sanders et al., 2010). As DHEA/S treatment carries fewer risks than estrogen replacement, such increased breast cancer and cardiovascular risk (Labrie et al., 2003; Panjari et al., 2009), supplementation with this hormone may represent a relatively safe alternative therapy compared with traditional forms of HT. Despite promising associations with cognition, however, clinical studies in the elderly have failed to detect significant cognitive benefits of DHEA/S supplementation (Grimley-Evans et al., 2006). The reason for this is unclear.

One potential mechanism responsible for the pro-cognitive effects observed in rodents involves local conversion of DHEA/S to E_2 in the brain. This phenomenon is a process known as intracrine conversion, or the conversion of a circulating prohormone to an active hormone that acts locally in an auto- or paracrine manner (Labrie, 1991). This steroid synthesis pathway (Figure I.4) involves the actions of the following enzymes, all of which are expressed in the rodent brain (Mellon and Griffin, 2002): sulfyl transferase (SULT2B1) and steroid sulfatase (STS) convert DHEA to DHEAS and vice versa, respectively, while 17BHSD5, 3BHSD, and ARO are the primary enzymes involved in the central conversion of DHEA to E_2. This locally produced E_2 can have significant effects on hippocampal spine density and synapse frequency *in vivo*, suggesting metabolism of DHEA to E_2 is a likely mechanism of the observed cognitive effects of DHEA (Hajszan et al., 2004; Hirshman et al., 2004; Rune and Frotscher, 2005). Additionally, it has been observed the GnRH signaling directly on the hippocampus can increase local E_2 synthesis, resulting in increased spine density (Prange-Kiel et al., 2008). Although well established in rodents, no studies have directly examined this steroidogenic mechanism in humans or nonhuman primates. Thus, it is possible that an inability to convert DHEA/S to E_2 in the aged human brain underlies the lack of cognitive efficacy observed in clinical studies of

DHEA/S supplementation. Additionally, an age-related decline in the *de novo* central synthesis of DHEA/S from cholesterol, without the contribution of peripheral hormone precursors, and a resulting loss of central E_2 may add to the cognitive effects of the loss of peripheral E_2 and serve as a potential target for therapeutic intervention.

The aim of the studies presented in this chapter is to demonstrate the potential of the nonhuman primate brain, particularly in the cognitively-important hippocampus, to synthesize E_2, either from circulating DHEA/S or *de novo* from cholesterol. This is achieved through identification of mRNA and protein expression of steroidogenic genes in the hippocampus and PFC. The results indicate that the nonhuman primate does indeed possess the machinery necessary to synthesize its own hormones for local action and suggests this pathway may be a potential target for cognitive intervention.

2. Materials and Methods

2.1 Experimental animals

This study was performed using tissue samples obtained from rhesus macaques (*M. mulatta*), maintained at the ONPRC. The animals were maintained on a 12:12: light:dark cycle and were fed LabDiet High Protein Monkey Chow (LabDiet Inc., St. Louis, MO) twice daily, supplemented with fresh fruits and vegetables. Animal care was provided by the ONPRC DCM in accordance with the *NRC Guide for the Care and Use of Laboratory Animals*, and the experiments were approved by the OHSU Institutional Animal Care and Use Committee.

2.2 Identification of steroidogenic enzyme RNA expression

Rhesus macaque tissues were obtained through the ONPRC Tissue Distribution Program. Qualitative RT-PCR analysis of steroidogenic enzyme gene expression was performed on subdissected brain regions (amygdala, hippocampus, medial basal hypothalamus [MBH], and PF Cortex) from long-term (over 1 year) ovariectomized adult rhesus macaques. RNA samples from rhesus macaque adrenal gland and testis were used as positive controls, while water was used as a negative control. The reaction mixtures contained 22.5 μL of SuperMix, 0.5 μL of 25 μM specific forward and reverse primers, and 1 μL of cDNA, using the Platinum PCR SuperMix kit (Invitrogen, Carlsbad, CA). The RT-PCR primer sequences for the various genes and transcript variants are shown in Appendix A. Reactions were run for 25 to 35 cycles with annealing temperatures between 56° C and 60° C.

2.3 Identification of steroidogenic enzyme protein expression

Perfusion-fixed rhesus macaque tissues were obtained through the ONPRC Tissue Distribution Program. Hippocampi were sectioned at 30 μm on a microtome and stored in a 50%:30%:20% ratio of 0.05 M sodium phosphate:ethylene glycol:glycerol antifreeze solution at 20° C until use. Sections were first washed in phosphate buffer, then incubated in a series of 3% hydrogen peroxide followed by 10% normal goat serum. Samples were incubated overnight at room temperature with primary antibody. The primary antibodies used were raised against the rhesus macaque or human ARO (rabbit polyclonal, 1:500; Novus Biologicals, Littleton, CO), 3BHSD2 (rabbit polyclonal, 1:500; Aviva Systems Biology, San Diego, CA), 17BHSD5 (rabbit polyclonal, 1:500; Invitrogen, Carlsbad, CA), CYP17A1 (goat polyclonal, 1:500; Sigma, St. Louis, MO), GnRH (provided by Dr. Henryk Urbanski, 1:1000), NeuN (mouse monoclonal,

1:1000; Millipore, Billerica, MA), and GFAP (mouse monoclonal, 1:4000; Millipore) proteins. This was followed by incubation with the appropriate biotinylated secondary antibody (goat, rabbit, or mouse, from Vector Laboratories, Burlingame, CA) for one hour at room temperature. Sections were then incubated with an avidin/biotin-based peroxidase compound (Vectastain Elite ABC Kit, Vector Laboratories) and then stained with diaminobenzidine and 3% hydrogen peroxide. Sections were washed, mounted, and dehydrated using ethanol and xylene.

3. Results

3.1 Steroidogenic mRNA is expressed in multiple areas of the rhesus macaque brain

As shown in Figure 1.1, mRNA expression was observed for each steroidogenic enzyme examined, though at qualitatively different levels. Expression of *3BHSD* was particularly high in the PFC, while the amygdala was the highest expresser of *17BHSD5* and *ARO*. Of the five isoforms of *17BHSD*, only types 4 and 5 were expressed in all brain areas. *17BHSD3*, the isoform classically ascribed to the testis, was not expressed in any of the examined brain areas.

3.2 Steroidogenic proteins are expressed in the rhesus macaque hippocampus

Immunohistochemistry of rhesus macaque hippocampus is shown in Figure 1.2. Staining of CYP17A1 (the enzyme that synthesizes DHEA from pregnenolone), 3BHSD, and 17BHSD5 was observed in neurons, while ARO staining was seen in both neurons and astrocytes. Interestingly, GnRH was also expressed in the hippocampus. The "beads on a string" GnRH fibers typical of the hypothalamus were observed just outside the hippocampus, and neurons within the hippocampus were also stained positively.

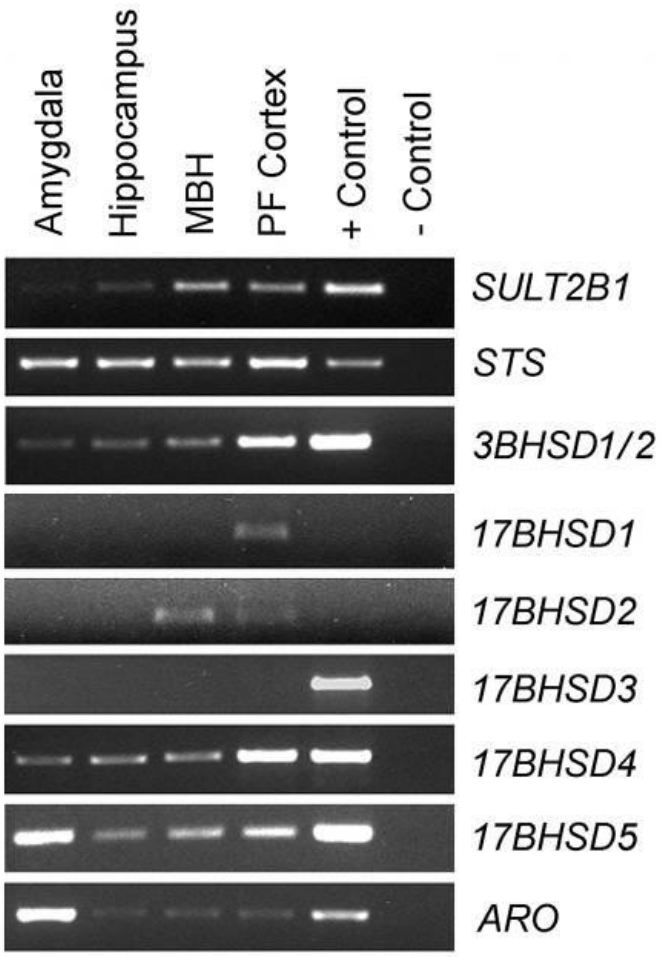
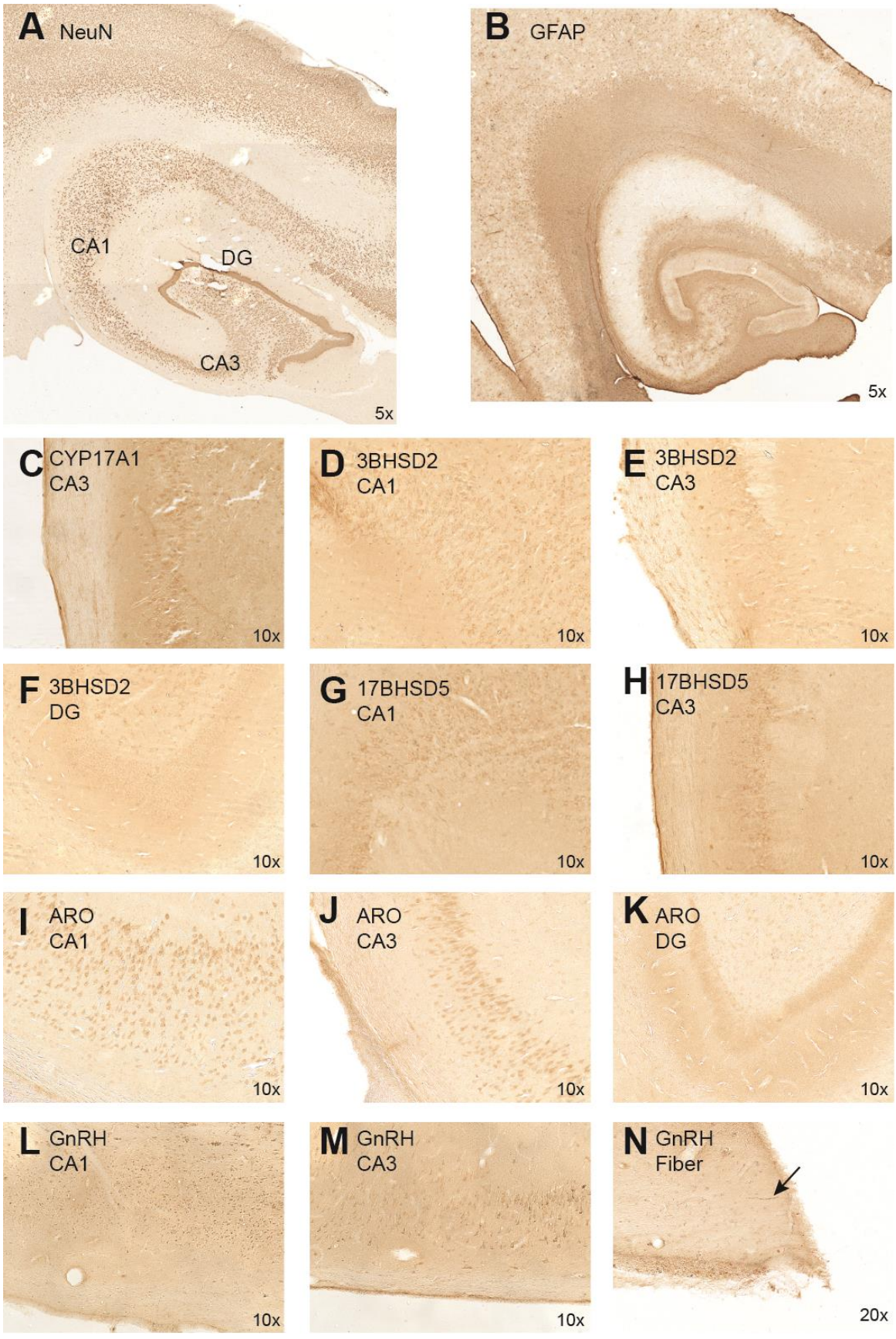


Figure 1.1. Expression of steroidogenic enzyme mRNA in multiple areas of the rhesus macaque brain. RNA from the amygdala, HPC, MBH, and PFC underwent RT-PCR for *SULT2B1, STS, 3BHSD,* five forms of *17BHSD* (1-5), and ARO. Expression of *SULT2B1, STS, 3BHSD,* and *ARO* was observed in all brain areas. The only two forms of *17BHSD* observed in all areas were types 4 and 5. *17BHSD1* was seen only in the PFC, while *17BHSD2* was expressed in the MBH and PFC. *17BHSD3,* the isoform associated with the testis, was expressed in the positive control tissue but was not expressed in any brain area examined.

4. Discussion

The attenuation of circulating DHEAS levels during aging has the potential to influence physiological functions through at least three mechanisms. First, the decrease in circulating DHEAS levels results in a lower DHEAS:cortisol ratio, which is likely to potentiate the negative effects of cortisol in the central nervous system (Ferrari and Magri, 2008). Second, the decrease in DHEAS levels may represent a potential loss of humoral circadian cues and thereby contribute to disruption of sleep-wake cycles (George et al., 2006; Pawlikowski et al., 2002) and resulting impairment of cognitive function. It has been recently found that increased perturbation of 24-


A NeuN
CA1
DG
CA3
5x
B GFAP
5x
C CYP17A1
CA3
10x
D 3BHSD2
CA1
10x
E 3BHSD2
CA3
10x
F 3BHSD2
DG
10x
G 17BHSD5
CA1
10x
H 17BHSD5
CA3
10x
I ARO
CA1
10x
J ARO
CA3
10x
K ARO
DG
10x
L GnRH
CA1
10x
M GnRH
CA3
10x
N GnRH
Fiber
20x

1.2. Expression of steroidogenic proteins in the rhesus macaque hippocampus. (Previous page) Neuronal (NeuN) and glial (GFAP) staining in complete hippocampi are shown for reference in panels A and B. Steroidogenic enzymes were expressed throughout the HPC, as shown in panels C-G. CYP17A1 exhibited light staining throughout the HPC, most prominently in the CA3 (C). 3BHSD2 was expressed in the CA1 (D), CA3 (E) and dentate gyrus (DG, F). 17BHSD5 was expressed in CA1 (G) and CA3 (H). ARO was expressed in CA1 (I), CA3 (J), and DG (K). GnRH was expressed in neurons in CA1 (L) and CA3 (M). Beaded GnRH fibers, typical of expression in the hypothalamus, were also observed in the white matter surrounding the HPC (N).

hour activity-rest cycles in old female rhesus macaques is negatively correlated with performance in a cognitive spatial maze task (Haley et al., 2009), and so it is plausible that a marked attenuation of the circadian plasma DHEAS rhythm plays a causal role. Although a link between DHEA/S and sleep-wake cycles has been suggested, the causality of this is complicated by concurrent changes in cortisol; thus, more studies are needed to investigate this relationship. Third, while DHEA/S itself has many neurobiological effects that may underlie cognition (reviews: Wolf and Kirschbaum, 1999 and Webb et al., 2006), DHEA and DHEAS are key substrates in the synthesis of E_2, which exerts a major influence on neurons (Brann et al., 2007).

The data from the current study demonstrate that the genes associated with the enzymatic conversion of DHEA/S to E_2 are all expressed within brain, and notably in the hippocampus, however, it is important to note that the current study provides only indirect evidence of central steroidogenesis. Consequently, it is plausible that the adrenal gland contributes to the maintenance of cognitive function via local intracrine conversion of DHEA/S to E_2. Moreover, an age-related decline in the availability of these sex-steroid precursors is likely to potentiate the negative impact of the menopausal loss of E_2 production by the ovaries.

Of interest in the current study is the identification of GnRH-positive fibers and neurons in and around the rhesus macaque hippocampus, consistent with the hypothesis of GnRH mediation of steroidogenesis in the hippocampus discussed by Fester et al. (2012). In rodents,

low levels of GnRH applied to hippocampal cultures induced the synthesis of E_2 through an aromatase-dependent mechanism, resulting in increased spine density. The identification of GnRH fibers in the macaque hippocampus, combined with expression of CYP17A1, 3BHSD, 17BHSD5, and ARO, suggest this effect may also be seen in nonhuman primates.

Early rodent studies reported promising effects of DHEA/S supplementation on cognition (Flood and Roberts, 1988; Roberts et al., 1987), particularly in aged rodents (Farr et al., 2004; Flood et al., 1988; Markowski et al., 2001). Additionally, observations in elderly humans identified a positive correlation between endogenous DHEA/S and certain aspects of cognitive ability (Davis et al., 2008; Sanders et al., 2010), particularly when analyzed in relation to cortisol (Kalmijn et al., 1998). While a similar association study in rhesus macaques failed to find a correlation between cognition and DHEA (Herndon et al., 1999), this study did not take into consideration circulating cortisol levels or the underlying 24-hour modulation of DHEA/S, which may have obscured an association with cognition. Despite promising correlations, most studies of DHEA/S supplementation in elderly humans have failed to replicate the effects seen in rodents, and robust improvements in cognition have yet to be reported (Grimley-Evans et al., 2006).

What, then, may account for this discrepancy between the rodent and human literature? One possibility may stem from differences between rodent and primate adrenal endocrine systems. For example, most rodents have nearly undetectable circulating levels of DHEA/S (Baulieu, 1998), making any exogenous dose of DHEA/S highly supraphysiological. In contrast, adult macaque monkeys do have high circulating DHEA/S levels and also show a marked age-related decline, similar to humans (Downs et al., 2008; Lupien et al., 1996). Because of these species differences, studies of DHEA treatment in rodents might not be translatable to humans

and nonhuman primates. Additionally, modulation of this pathway during aging through changes in enzyme expression may impact the ability to perform the intracrine conversion of DHEA to E_2, a possibility since DHEA supplementation has been shown to have positive effects, albeit small, on cognition in healthy young men (Alhaj et al., 2006). If the rhesus macaque is a valid model for cognitive decline and the inefficacy of DHEA supplementation in humans, it is to be expected that DHEA supplementation will have no effect on memory in aged macaques. Importantly, however, detailed analysis of the effects of age and hormone supplementation on cellular and molecular aspects of cognitive areas of the brain in the light of cognitive performance in the macaque is much more practical than in the human.

Chapter 2: Effects of the precursor hormone dehydroepiandrosterone on cognition in pre- and perimenopausal rhesus macaques

Portions of this chapter have been published previously in:

Sorwell KG, Kohama SG, Urbanski HF (2012) Perimenopausal regulation of steroidogenesis in the nonhuman primate. Neurobiol Aging. *33:1487.e1-e13.*

1. Introduction

As demonstrated in the previous chapter, the rhesus macaque hippocampus expresses the necessary enzymes for conversion of DHEA to E_2. If the hippocampus is capable of synthesizing E_2, DHEA supplementation should take advantage of this pathway and result in a local increase in E_2 and cognitive improvement; however, in humans, DHEA supplementation has no significant effect on memory in the elderly despite increased levels of circulating DHEA/S (Grimley-Evans et al., 2006). If we are to use the rhesus macaque as a model for age-related cognitive impairment, it is vital to replicate this effect and examine the steroidogenic pathway to identify the reason for the disparity between rodent and human literature.

This study aims to validate the rhesus macaque as a model for age-related cognitive and adrenal decline by (1) establishing that the rhesus macaque undergoes dramatic reductions in circulating DHEAS with age, as seen in humans, and (2) evaluating the cognitive effects of DHEA supplementation in aged animals. As most studies of DHEA supplementation have been performed in postmenopausal women, we hypothesized that the lack of effect of DHEA on cognition may be due to the delayed start of supplementation. DHEAS levels peak in young adulthood (Rainey et al., 2002), and by the age of menopause, levels have been greatly reduced

for an extended period. These women may also have passed the critical period for positive E effects on cognition, as E replacement long after menopause fails to impact cognition and its underlying neural mechanisms in the same manner of E replacement immediately after reproductive senescence (Sherwin, 2007a; Daniel and Bohacek, 2010). As a result, any locally-produced E_2 of DHEA/S origin is rendered inefficient. Thus, in order to increase the chance of a cognitive-enhancing effect of DHEA in the macaque, we began supplementation during pre- and peri-menopause.

2. Materials and methods

2.1 Experimental animals

This study was performed using female rhesus macaques (*M. mulatta*) maintained at the ONPRC. The animals were maintained on a 12:12: light:dark cycle and were fed LabDiet High Protein Monkey Chow (LabDiet Inc., St. Louis, MO) twice daily, supplemented with fresh fruits and vegetables. Due to physical constraints imposed by subclavian intravenous catheterization, animals used in the 24-hour sampling experiment were housed singly, while animals used in the DHEA supplementation study were housed in pairs. Animal care was provided by the ONPRC DCM in accordance with the *NRC Guide for the Care and Use of Laboratory Animals*, and the experiments were approved by the OHSU Institutional Animal Care and Use Committee.

2.2 Twenty-four– hour plasma cortisol and DHEAS measurements

This experiment involved a total of 16 adult female rhesus macaques that were maintained indoors under a 12:12 light:dark schedule (lights on at 0700 hours). Daily menses records and biweekly plasma E_2 and P_4 measurements were used to characterize the reproductive

neuroendocrine status of these animals, as previously described (Downs and Urbanski, 2006). Accordingly, the animals were divided into the following four groups: young adult (5-12 years, *n* = 5), middle-aged (12-20 years, *n* = 4), old premenopausal (21-25 years, *n* = 4), and old perimenopausal (21-25 years, *n* = 3) animals. In the latter group, the animals showed either an elongated (> 30 days) or highly irregular menstrual cycles, but had not yet attained menopause (total cessation of cyclicity). To obtain detailed 24-hour hormone profiles, each animal was fitted with a subclavian vein catheter and connected to a remote blood sampling system, as previously described (Urbanski et al., 1997; Urbanski, 2011), enabling hourly blood samples to be remotely collected from non-sedated animals, from 0700 hours to 0700 hours on the following day. Blood samples were collected in EDTA-coated borosilicate glass tubes, centrifuged at 4° C, and the plasma supernatant stored at -20° C until assay for cortisol and DHEAS.

For each hormone, group mean values were determined from the overall mean of the individual hormone values spanning the entire 24-hour sampling period. Group maximum hormone values were determined by first identifying the maximum and adjacent values, spanning 5 hours, for an individual and taking the mean of those individual maximum values. Group amplitude values were determined by first calculating the minimum, based on 5 adjacent values, and then calculating half the difference between the maximum and minimum values. Between-group differences in mean, maximum, and amplitude were analyzed (Mann-Whitney *U* test) for the cortisol and DHEAS concentrations, and also for the DHEAS:cortisol ratio.

2.3 Hormone supplementation

Eight aged female monkeys, classified by circulating reproductive hormones and menstrual cycles as premenopausal (22-26 years old at baseline, mean age of 23.2 ± 0.44 years),

were supplemented with oral DHEA (5 mg) hidden in a marshmallow treat at 0745 hours daily for three months. This dose was chosen to achieve a peak plasma DHEAS levels of 160-400 ng/mL 1-2 hours after administration, followed by a gradual decline throughout the day. The study lasted a total of nine months, with supplementation provided in the first three and last three months, with a three-month washout period in between.

2.4 Cognitive training and testing

A detailed outline of training and testing timelines is shown in Figure 2.1. As both tasks involved a computer touchscreen, animals were first trained to touch a target in the home cage, followed by acclimation to a testing box containing a touchscreen computer monitor and target training on the screen. In the DMS task, animals were trained to acknowledge a picture displayed in the center of the touchscreen. After a delay (5, 30, 60, 120, or 240 seconds), the previously shown picture and a novel picture were displayed randomly on the left and right sides of the screen. The animal indicated its selection by touching a picture. Correct responses (selecting the previously shown picture) were rewarded with a small palatable food item. In the VDR task, animals were presented with a red square on either the left or right side of the screen. After a delay (1, 5, 15, or 30 seconds), the animal was shown squares on the left and right side of the screen. Correct responses (selection of the square on the previously shown side) were rewarded with a small palatable food item. Representative trials from each task are shown in Figure 2.2. Animals were required to reach a criterion of 85% accuracy at the 5 second delay in each task before advancing to the testing battery.

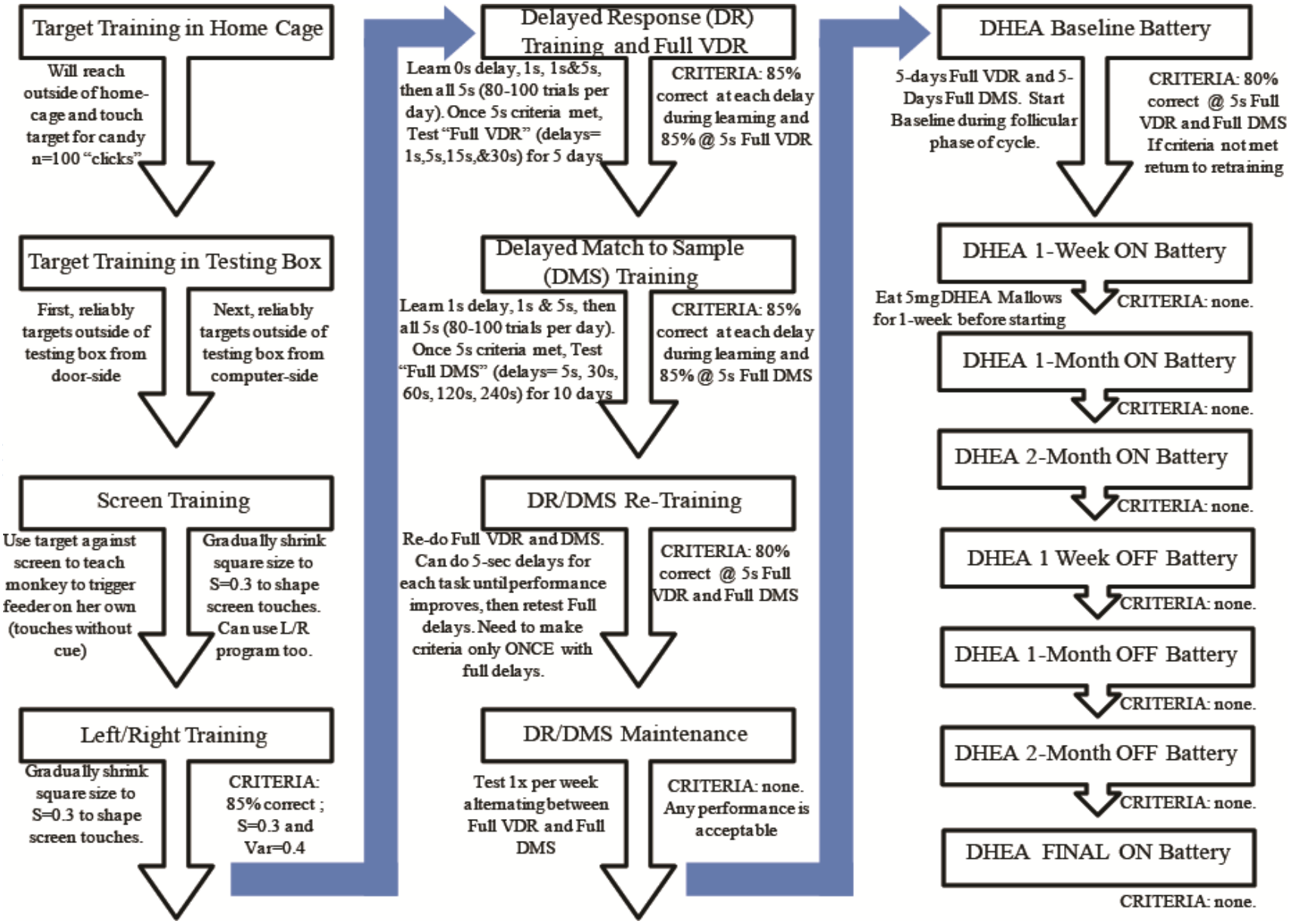

Target Training in Home Cage
Will reach outside of home-cage and touch target for candy n=100 "clicks"
Target Training in Testing Box
First, reliably targets outside of testing box from door-side
Next, reliably targets outside of testing box from computer-side
Screen Training
Use target against screen to teach monkey to trigger feeder on her own (touches without cue)
Gradually shrink square size to S=0.3 to shape screen touches. Can use L/R program too.
Left/Right Training
Gradually shrink square size to S=0.3 to shape screen touches.
CRITERIA: 85% correct ; S=0.3 and Var=0.4
Delayed Response (DR) Training and Full VDR
Learn 0s delay, 1s, 1s&5s, then all 5s (80-100 trials per day). Once 5s criteria met, Test "Full VDR" (delays= 1s,5s,15s,&30s) for 5 days
CRITERIA: 85% correct at each delay during learning and 85% @ 5s Full VDR
Delayed Match to Sample (DMS) Training
Learn 1s delay, 1s & 5s, then all 5s (80-100 trials per day). Once 5s criteria met, Test "Full DMS" (delays= 5s, 30s, 60s, 120s, 240s) for 10 days
CRITERIA: 85% correct at each delay during learning and 85% @ 5s Full DMS
DR/DMS Re-Training
Re-do Full VDR and DMS. Can do 5-sec delays for each task until performance improves, then retest Full delays. Need to make criteria only ONCE with full delays.
CRITERIA: 80% correct @ 5s Full VDR and Full DMS
DR/DMS Maintenance
Test 1x per week alternating between Full VDR and Full DMS
CRITERIA: none. Any performance is acceptable
DHEA Baseline Battery
5-days Full VDR and 5-Days Full DMS. Start Baseline during follicular phase of cycle.
CRITERIA: 80% correct @ 5s Full VDR and Full DMS If criteria not met return to retraining
DHEA 1-Week ON Battery
Eat 5mg DHEA Mallows for 1-week before starting
CRITERIA: none.
DHEA 1-Month ON Battery
CRITERIA: none.
DHEA 2-Month ON Battery
CRITERIA: none.
DHEA 1 Week OFF Battery
CRITERIA: none.
DHEA 1-Month OFF Battery
CRITERIA: none.
DHEA 2-Month OFF Battery
CRITERIA: none.
DHEA FINAL ON Battery
CRITERIA: none.

Figure 2.1. Experimental timeline of training, testing, and DHEA supplementation. (Previous page) Eight female macaques completed the training and testing for the DHEA supplementation study. Animals were trained to perform two touchscreen tasks to a criterion of 80% correct at a short (5 second) delay before undergoing testing at longer delays. Each testing battery consisted of five days of VDR testing and ten days of DMS testing. Animals completed one Overall Baseline battery before supplementation began. During DHEA supplementation (ON), animals underwent testing batteries starting at one week, one month, and two months after supplementation began. Following the cessation of supplementation, animals completed a final battery while receiving DHEA.

Each testing battery consisted of five days of VDR testing (20 trials at each delay for a total of 80 trials per day) and ten days of DMS testing (ten trials per delay for a total of 50 trials per day). One battery was completed before DHEA administration (overall baseline). Additional batteries began one week, one month, and two months after the start of DHEA administration. Additional testing batteries were begun one week, one month, and two months after the cessation of DHEA supplementation. A final testing battery was completed with the reintroduction of DHEA supplementation beginning one week after completion of the last unsupplemented battery.

2.5 Blood sampling

Baseline samples from before supplementation were also assayed for LH, FSH, AMH, and DHEAS. Blood samples were collected twice a week throughout the cognitive study to monitor E_2 and P_4 levels. Blood was collected into EDTA-coated borosilicate glass tubes, centrifuged at 4° C, and the plasma supernatant stored at -20° C until assay.

2.6 Hormone assays

Cortisol, DHEAS, E_2, and P_4 were measured using electrochemiluminescence (ECL) with the Elecsys 2010 Platform (Roche Diagnostics, Indianapolis, IN, USA). FSH was measured by

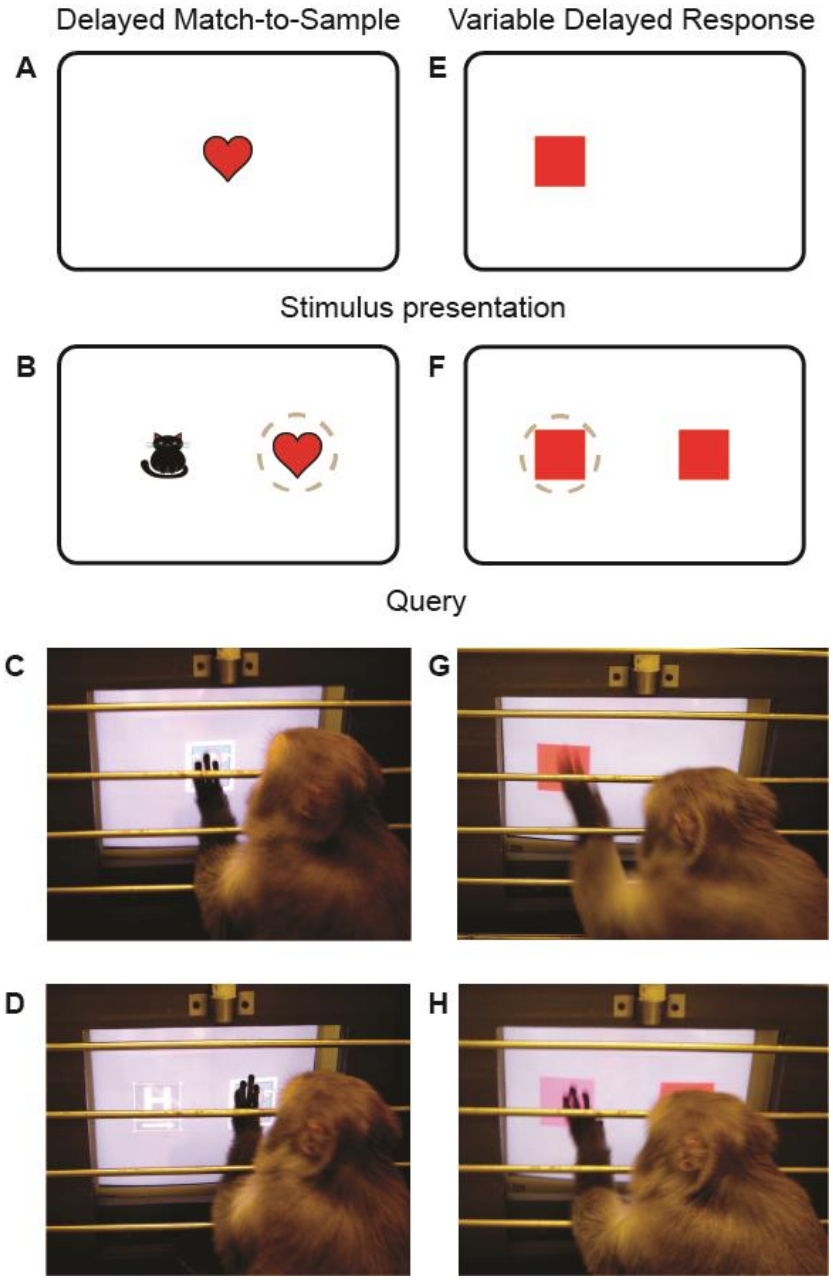


Figure 2.2. Examples of trials in delayed match to sample and delayed response touchscreen tasks. In the DMS task, monkeys were shown a picture at the center of a touchscreen (A and C). Acknowledgment of the picture by touching the screen was followed by a delay (5, 30, 60, 120, or 240 seconds), after which monkeys were presented with the same picture as well as a novel picture randomly displayed on the left and right sides (B and D). A correct response, indicated by touching the previously-shown picture, resulted in a food reward dropped into a hopper in front of the screen. Each day of testing consisted of ten trials at each delay in a random order. In the VDR task, animals were shown a red square on either the left or right side of the touchscreen (E and G). Acknowledgment of the picture was followed by a delay (1, 5, 15, or 30 seconds). In the query phase, red squares were presented on both the left and right sides of the screen. A correct response indicated by touching the square on the side shown in the stimulus presentation phase resulted in a food reward. Each day of testing consisted of ten trials at each delay in a random order.

RIA using antirecombinant monkey FSH (National Institute of Diabetes and Digestive and Kidney Diseases). LH was measured using a mouse Leydig cell bioassay involving RIA for T (Ellinwood and Resko, 1980). AMH was measured using a commercially-available AMH ELISA kit (Diagnostic Systems Laboratories, Inc., Webster, TX). Intra- and interassay coefficients of variation were less than 10% for each assay.

2.7 Characterization of hormonal status

Blood samples collected at baseline were used to characterize the cognitively profiled animals by hormonal status. All animals were cycling regularly at the beginning of training; however, by the time testing criteria were met, some animals showed signs of peri-menopause. Plasma levels of E_2, P_4, LH, FSH, DHEAS, and AMH were analyzed and each animal was scored by rank (Table 2.3), with a rank of 1 corresponding to the highest levels of E_2, P_4, DHEAS, and AMH and the lowest levels of LH and FSH. These rank scores were then added to form a rank total score. The four lowest rank total scores were categorized as "regular" and the four highest rank total scores were categorized as "irregular." These scores were compared with E_2 and P_4 levels throughout the study. All animals categorized as "regular" demonstrated regular menstrual cyclicity throughout the study, while all animals categorized as "irregular" had at least one missed or elongated cycle over the course of the study (representative menstrual cycles are shown in Figure 2.4), demonstrating the validity of this composite hormonal status score.

Table 2.3 Animals were characterized by hormonal status using circulating levels of LH, FSH, E_2, P_4, AMH, and DHEAS collected at baseline, before DHEA supplementation. Each animal was ranked within the group, with a rank of 1 assigned to the more youthful levels of each hormone. This corresponded to higher levels of E_2, P_4, AMH, and DHEAS, and lower levels of LH and FSH. These ranks were then summed to form a rank total. The lowest four rank totals were assigned to the "regular" group, while the highest four rank totals were assigned to the "irregular" group. These characterizations corresponded to the menstrual cyclicity of the animals, with all animals assigned to the "regular" group demonstrating normal menstrual cycles throughout the study, while all animals assigned to the "irregular" group displayed at least one elongated or missed menstrual cycle during the study.

Table 2.3. C

Animal ID	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank	Value	Rank		**Characterization**
15323	0.74	2	0.44	1	71.17	2	1.89	1	1.70	3	48.00	1	10	Regular
15405	0.68	1	0.56	2	60.05	4	1.06	4	4.87	1	25.00	7	19	Regular
18397	1.09	4	0.71	4	68.46	3	1.43	3	0.87	5	39.67	3	22	Regular
18398	1.52	5	1.76	6	38.50	7	1.94	1	1.32	4	45.20	2	25	Regular
20119	0.87	3	0.70	3	46.34	6	0.89	7	2.69	2	25.00	8	29	Irregular
14349	1.60	6	0.82	5	57.33	5	1.00	5	0.39	6	28.40	5	32	Irregular
14879	2.02	7	3.29	8	98.00	1	0.94	6	0.22	8	35.40	4	34	Irregular
17538	2.79	8	2.49	7	31.33	8	0.79	8	0.32	7	27.80	6	44	Irregular

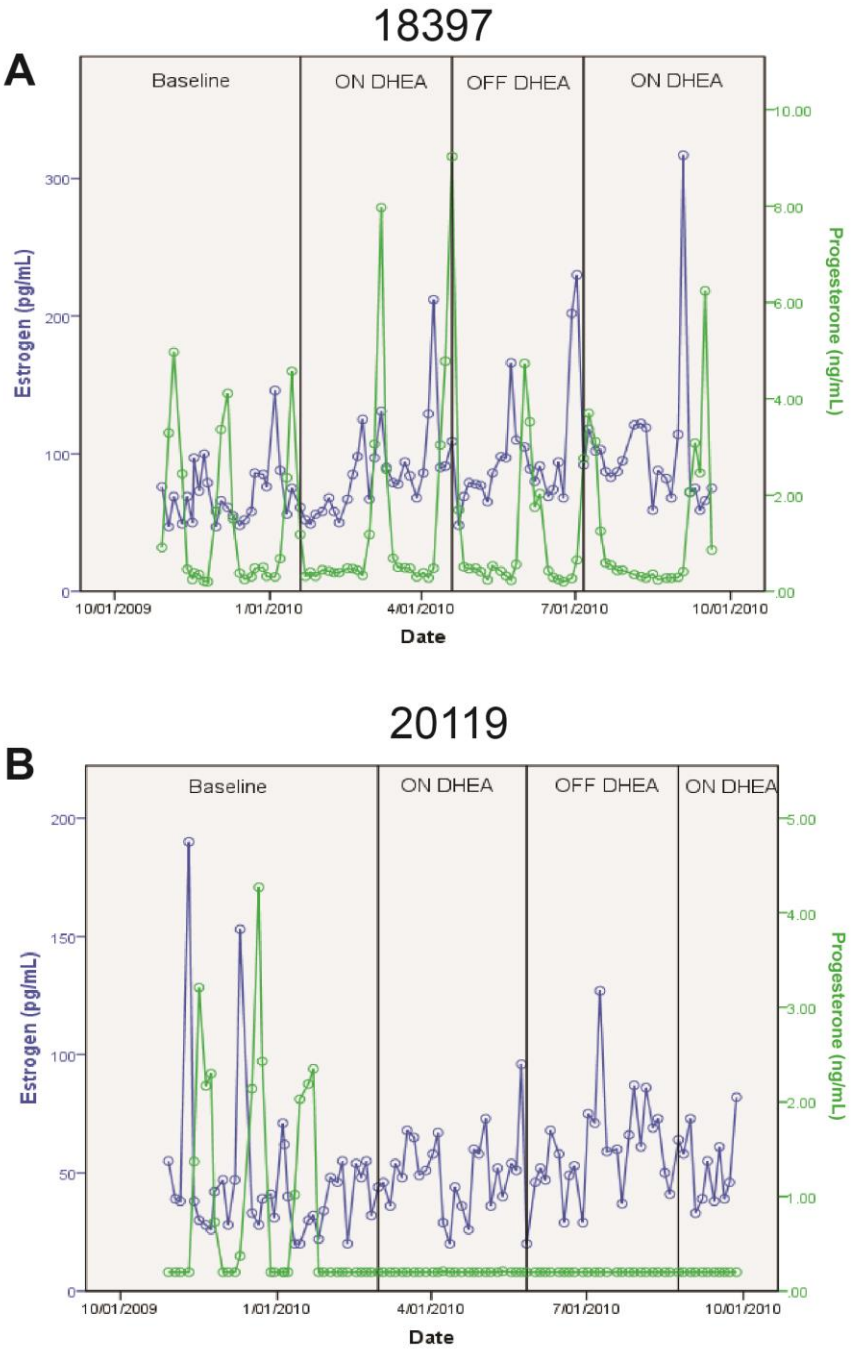


Figure 2.4. Representative menstrual cycles of regularly-and irregularly cycling aged female rhesus macaques. Menstrual cycles throughout the study are shown for two animals, one classified by the method presented in Table 2.3 as regular (A, animal 18397) and one classified as irregular (B, animal 20119). The regular animal (A) demonstrates typical menstrual cyclicity, characterized by a P_4 peak in the luteal phase and E_2 peaks in the luteal and follicular phases. The irregular animal (B) demonstrates P_4 peaks at the study baseline, but undetectable P_4 levels. While the E_2 levels do not show the cyclic pattern typical of a young regularly cycling animal, levels are still above those seen in post-menopausal animals (Downs and Urbanski, 2006).

2.8 Statistical analysis of cognitive data

Scores for each battery were calculated as percent correct for the five days of VDR (50 trials per delay) and ten days of DMS (100 trials per delay). Raw scores obtained from the Overall Baseline battery were used to assess the impact of hormonal status on cognitive performance. To examine the effect of DHEA administration on cognitive performance, a more complex measure was used. As all animals demonstrated a practice effect throughout the study, with performance improving with repetition of testing, a measure of study phase improvement was used. The percent improvement in score from the first (Baseline) battery of each phase (starting one week after the beginning and cessation of DHEA administration) to the last battery of each phase (starting two months after the beginning and cessation of DHEA administration) was calculated and is referred to as "percent performance of baseline." As roughly equivalent levels of improvement were seen in each phase of the study, this manipulation normalized performance to the beginning of each study phase. Due to time constraints, order of testing, with three batteries on DHEA and three batteries off DHEA, was not counter-balanced.

These improvement scores were analyzed with a repeated-measures ANOVA, with treatment and delay as within-subjects factors. An additional repeated-measures ANOVA was performed with treatment and delay as within-subjects factors and hormonal status as a between-subjects factor. Where the assumption of sphericity is not met as determined by Mauchly's test of sphericity, the Greenhouse-Geisser ε is used as a correction. In all figures, data are presented as mean ± SEM.

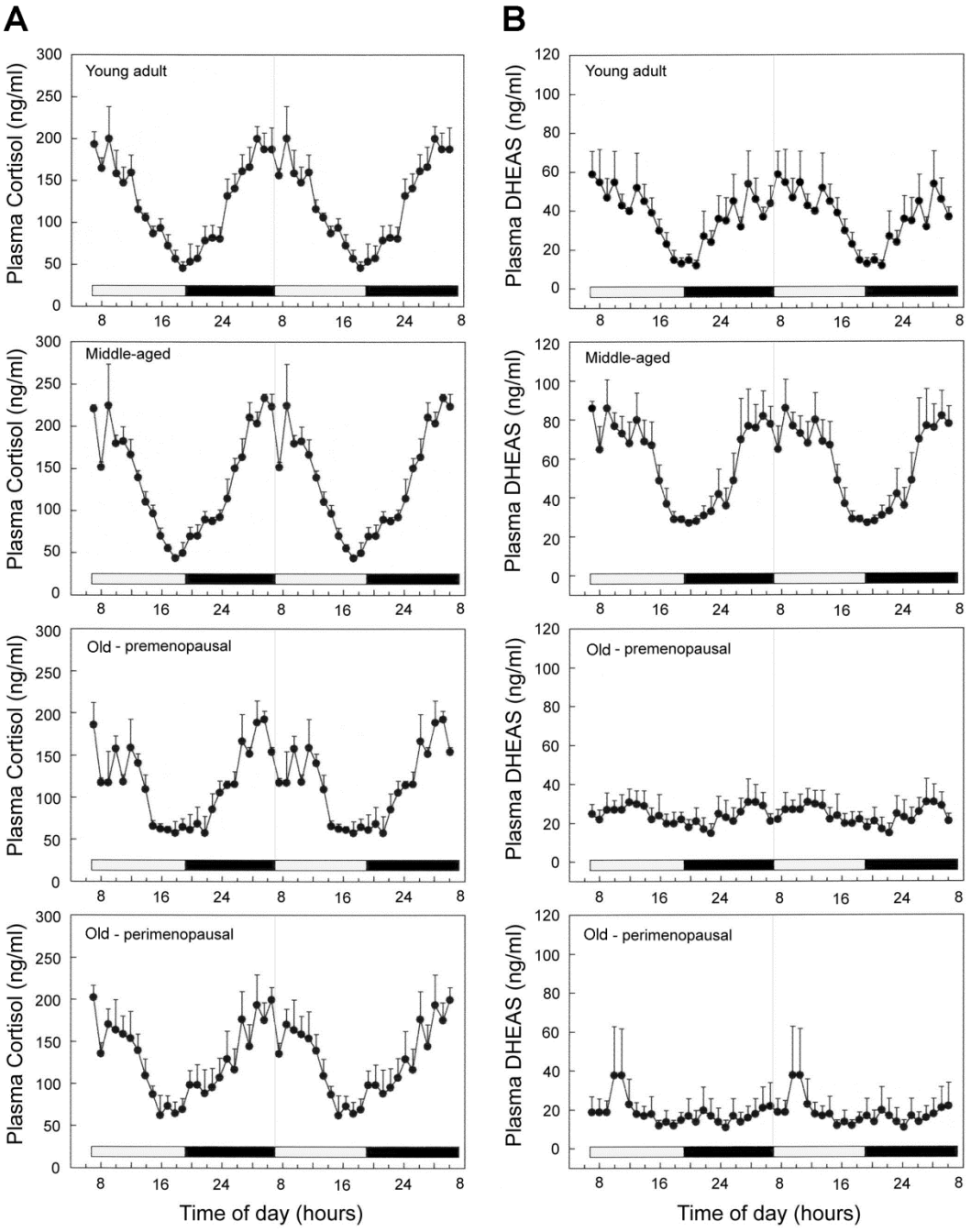


Figure 2.5. DHEAS declines with age in female nonhuman primates. 24-hour profiles of circulating cortisol and DHEAS are shown in young adult, middle-aged, aged premenopausal, and aged perimenopausal female rhesus macaques. The data are presented as mean + standard error of the mean (SEM), and to aid in the visualization of the night and day variations in hormone concentrations the values have been double plotted; the horizontal white and black bars on the abscissa correspond to the 12-hour light:12-hour day day-night lighting regimen. The panels show cortisol (A) and DHEAS (B) profiles from young adult (n = 5), middle-aged (n = 4), old premenopausal (n = 4), and old perimenopausal (n = 3) animals.

3. Results

3.1 Twenty-four hour plasma DHEAS and cortisol profiles

Due to possible interactions between the adrenal and gonadal steroid systems during aging (Crawford et al., 2009; Lasley et al., 2002; Pluchino et al., 2005), we examined adrenal hormone rhythms in relation to menopause in female rhesus macaques. Twenty-four-hour plasma profiles for cortisol and DHEAS are depicted in Figure 2.5 (panels A and B, respectively). In young adult and middle-aged female rhesus macaques, both hormones showed clear 24-hour rhythms with peak levels occurring in the morning around the time of lights on and a nadir just before the time of lights off. Both old animal groups continued to show a robust cortisol rhythm, whereas the DHEAS rhythm was highly attenuated; mean DHEAS levels were not significantly different between the premenopausal and perimenopausal macaques ($p > 0.05$). Statistical analysis of hormone levels between the combined young group (age range: 5–12 years) and combined old group (age range: 21–24 years) showed no age-related change in the plasma cortisol rhythm based on overall mean, maximum, or amplitude level, while plasma DHEAS rhythm showed a significant age-related decrease in each of these parameters (overall mean, $p < 0.01$; mean maximum, $p < 0.05$; mean amplitude, $p < 0.001$). Importantly, these data demonstrate that the onset of adrenopause in rhesus macaques precedes the onset of menopause.

3.2 Effect of DHEA on cognitive performance

Figure 2.6 presents percent performance of baseline at each delay in the VDR task when animals were untreated and receiving daily oral DHEA supplementation. Values greater than

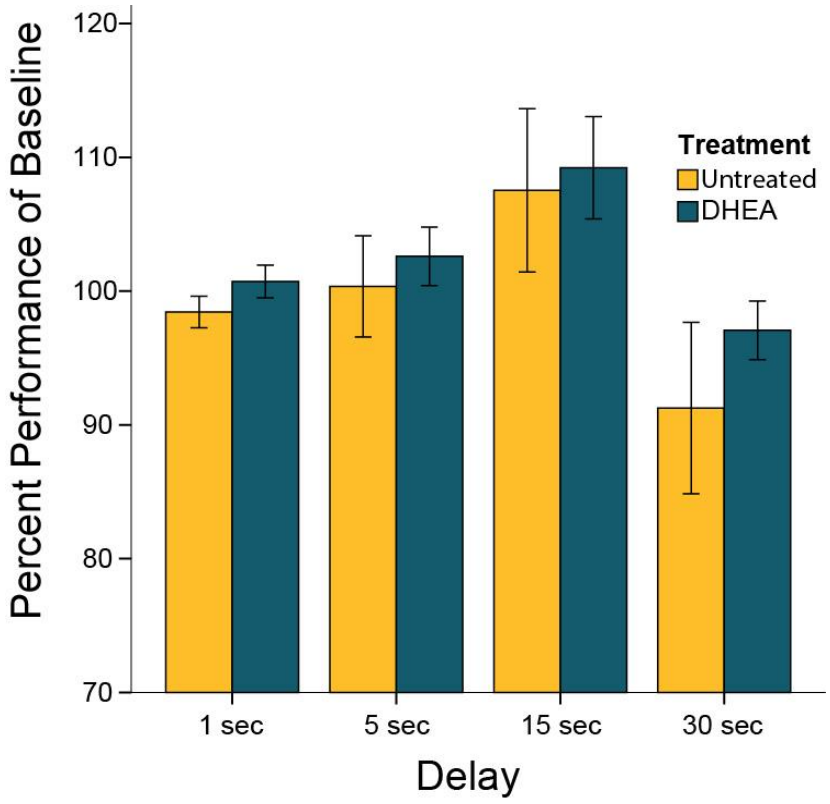


Figure 2.6. Delayed response performance in aged female rhesus macaques treated with DHEA. Performance in the VDR task is presented as percent performance of baseline, essentially the ratio of performance of the third battery of each testing phase to the performance of the first battery in each testing phase. This manipulation of the data corrected for the practice effect observed that resulted in higher scores in all animals as the study progressed. No significant treatment effect was observed, indicating no increase in performance with daily DHEA supplementation. Sec, seconds.

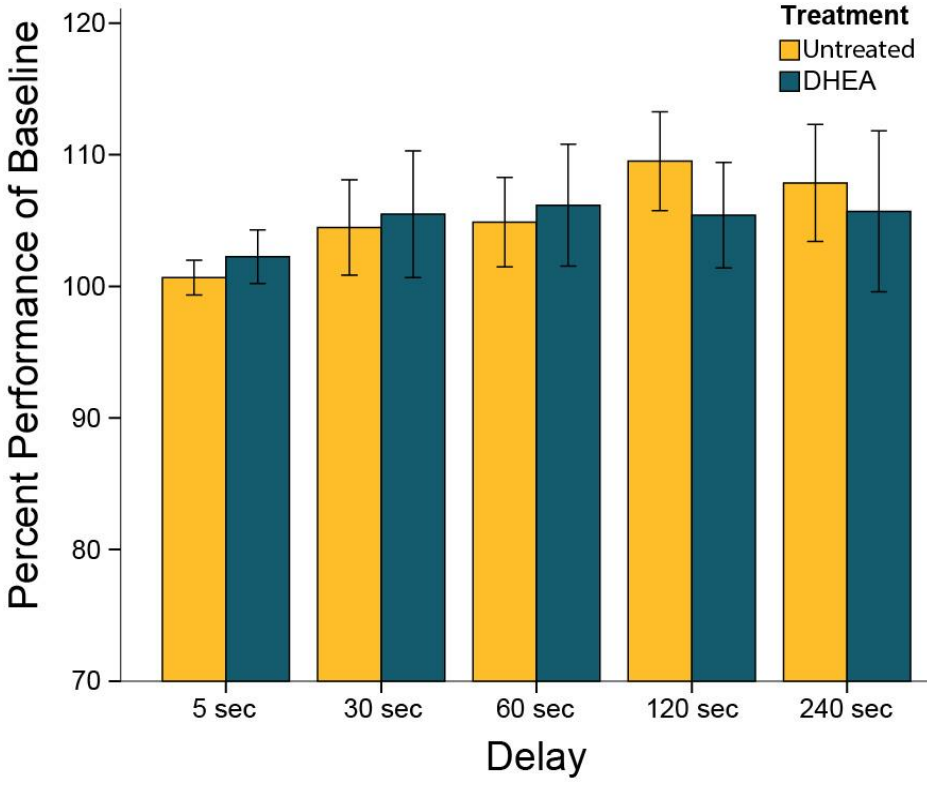


Figure 2.7. Delayed match to sample performance in aged female rhesus macaques treated with DHEA. Performance in the DMS task is presented as percent performance of baseline, essentially the ratio of performance of the third battery of each testing phase to the performance of the first battery in each testing phase. This manipulation of the data corrected for the practice effect observed that resulted in higher scores in all animals as the study progressed. As observed in the VDR task, no significant treatment effect was observed, indicating no increase in performance with daily DHEA supplementation. Sec, seconds.

100% indicate a significant improvement in performance over each three month testing phase, demonstrating a practice effect on performance and validating the use of a measure relative to baseline performance at the beginning of each testing phase. A repeated-measures ANOVA revealed no significant effects of delay ($p > 0.05$), no significant effects of treatment ($p > 0.05$), and no significant delay-by-treatment interactions ($p > 0.05$). Thus, DHEA supplementation did not affect performance in this task.

Figure 2.7 shows percent performance of baseline at each delay in the DMS task. Similarly to the VDR task, no significant effects of treatment, delay, or delay-by-treatment interactions on performance were observed ($p > 0.05$).

3.3 Effect of hormonal status on cognitive performance

While all animals began the training phase of the study demonstrating regular cyclicity, four of the eight animals began to cycle irregularly by the time of the Overall Baseline. Quantitation of hormone status using circulating E_2, P_4, LH, FSH, and AMH levels is shown in Table 2.3. This method of characterization corresponded to observed menstrual cyclicity throughout the testing phases of the study, with all animals classified as "regular" demonstrating typical menstrual regularity, while all animals classified as "irregular" missed at least one menstrual cycle during the testing phases. Representative cycles of a "regular" and "irregular" animal are shown in Figure 2.4.

This development allowed us to classify cognitive performance as a factor of hormonal status. First, to determine an overall effect of status on cognitive performance, we examined performance differences at the Overall Baseline battery (Figure 2.8). In VDR (Figure 2.8A), there was a significant within-subjects effect of delay ($F = 63.211$, $p<0.001$), showing that

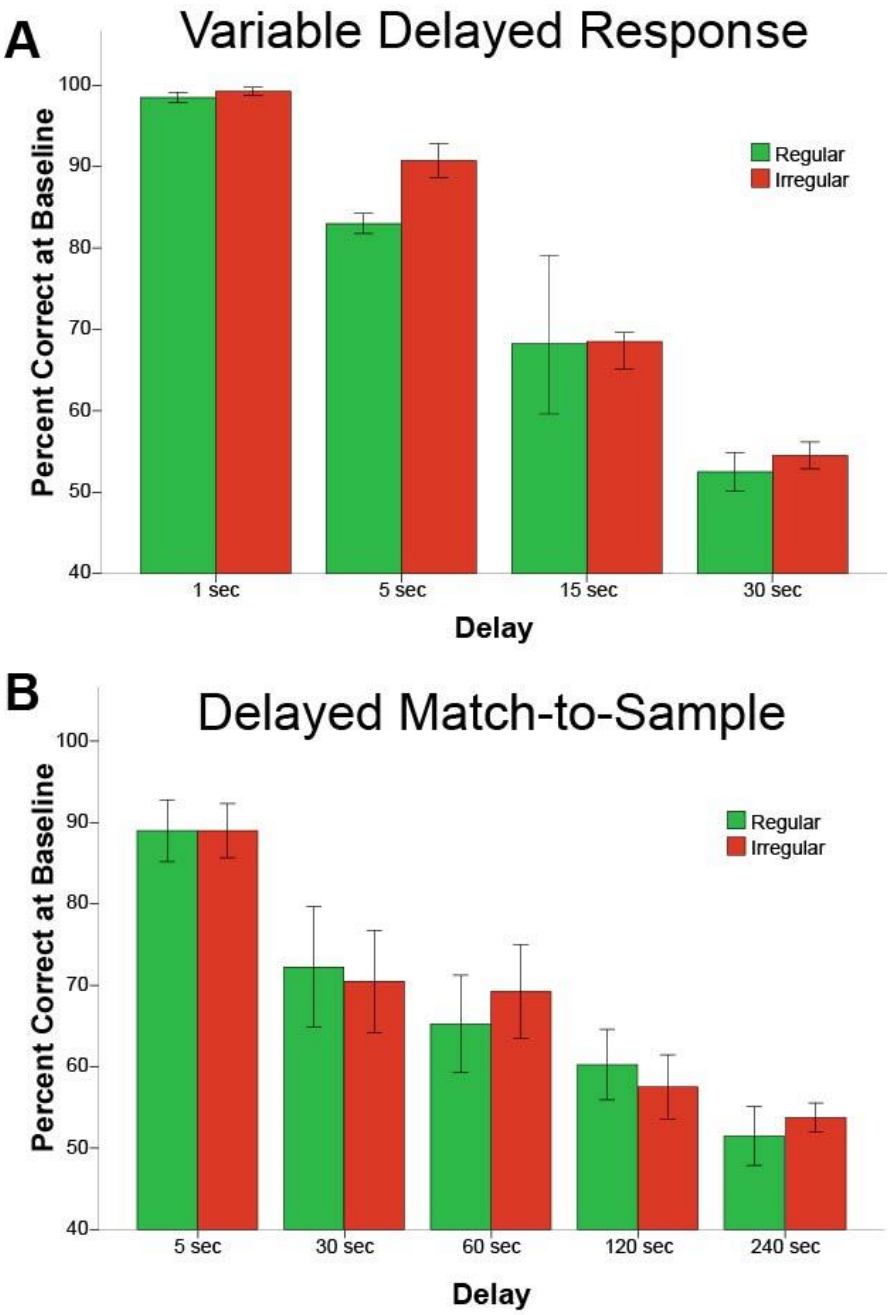


Figure 2.8. Hormonal status does not impact cognitive performance at baseline. Animals were categorized as regular (n = 4) or irregular (n = 4) cyclers based on hormone samples collected at the Overall Baseline (see Table 2.3). Performance at each delay during the Overall Baseline battery was assessed to investigate potential effects of cyclicity on cognitive performance before any animals were supplemented with DHEA. While there was a significant effect of delay (VDR, $F = 63.211$, $p<0.001$;DMS Greenhouse-Geisser $\varepsilon = 10.621$, $p = 0.008$), indicating that the trials were more difficult at longer delays, there were no effects of overall hormonal status or status-by-delay interactions. Sec, seconds.

animals performed worse at longer delays. There was no significant effect of hormonal status, and no significant status-by-delay interaction, thus, regular and irregular animals performed equally at baseline. Similar results were observed in the DMS task. There was a significant effect of delay (assumption of sphericity not met, Greenhouse-Geisser $\varepsilon = 10.621$, $p = 0.008$), but no significant effect of status or status-by-delay interaction.

To determine if reproductive hormonal status mediates cognitive response to DHEA supplementation, the percent performance of baseline measure was grouped by hormonal status as a between-subjects factor. The results for the VDR task are shown in Figure 2.9. Repeated-measures analysis revealed no significant difference in the effect of cycle status (2.9A and 2.9B), and no significant effect of treatment (2.9C and 2.9D). Thus, regular and irregular cyclers responded roughly equally to DHEA supplementation. While there does seem to be a slight affect at the 5 second delay, with regular cycles performing better than irregular cyclers when treated with DHEA, this was not statistically significant. Also, as this is a relatively short delay and not very cognitively demanding, any effect may simply be due to an impact on attention, not overall memory *per se*.

This analysis was also completed for the DMS task, as shown in Figure 2.10. Again, we observed no primary effect of hormonal status on performance or significant interaction of hormonal status with delay or treatment.

4. Discussion

The current study tested the hypothesis that early intervention with DHEA supplementation in aged female rhesus macaques would improve cognitive performance. This was tested using initially-premenopausal female rhesus macaques with the aim of providing

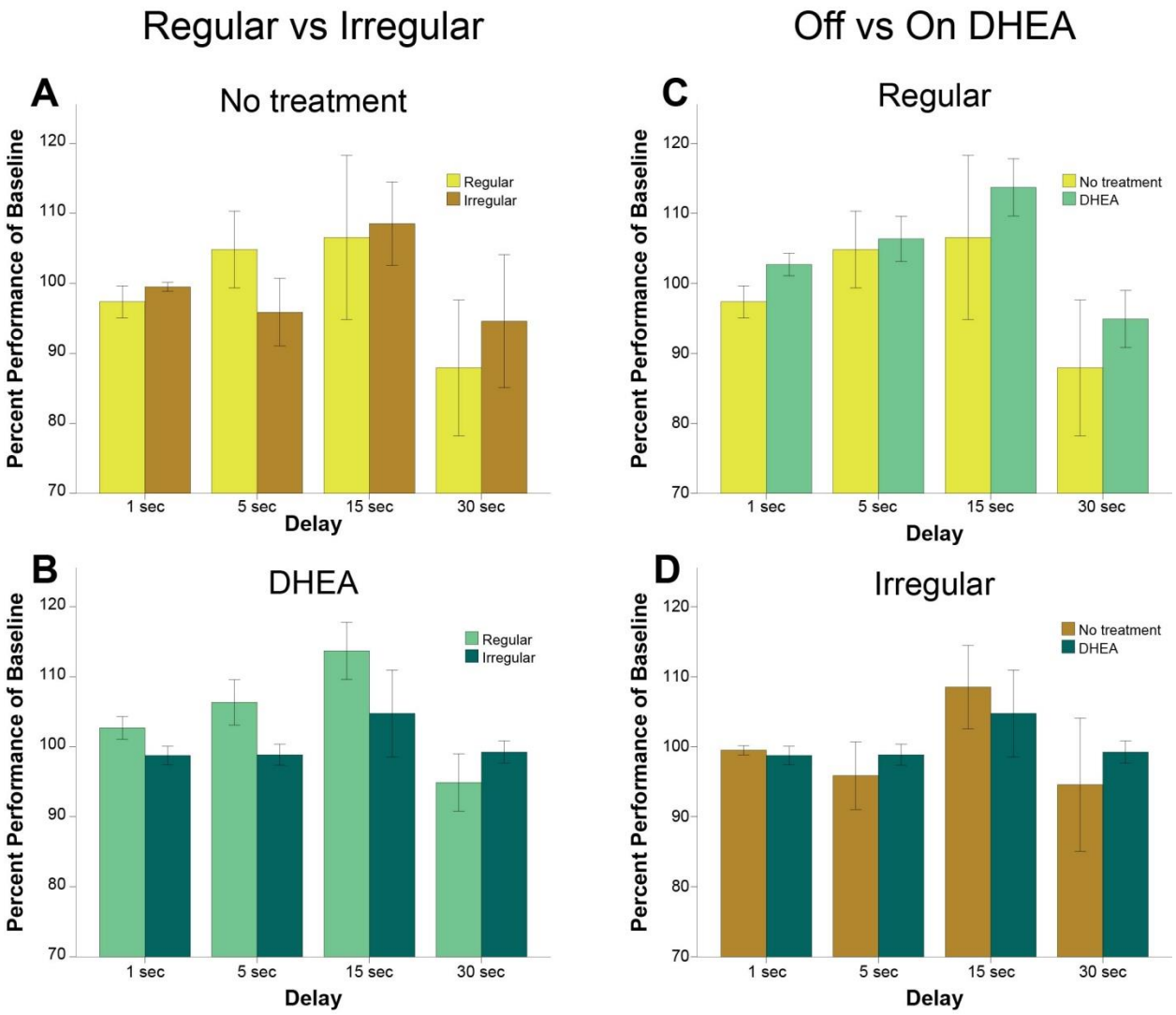


Figure 2.9. Delayed response performance with respect to hormonal status. Analysis of performance in the VDR task was repeated with the grouping of animals by menstrual cyclicity. A repeated-measures ANOVA with treatment and delay as within-subjects factors and status as a between subject factor yielded no significant effects of treatment, delay, or status on performance, as well as no significant interactions. While it appears that irregular animals performed better on DHEA at the 5 second delay, this was not significant due to variation. Sec, seconds.

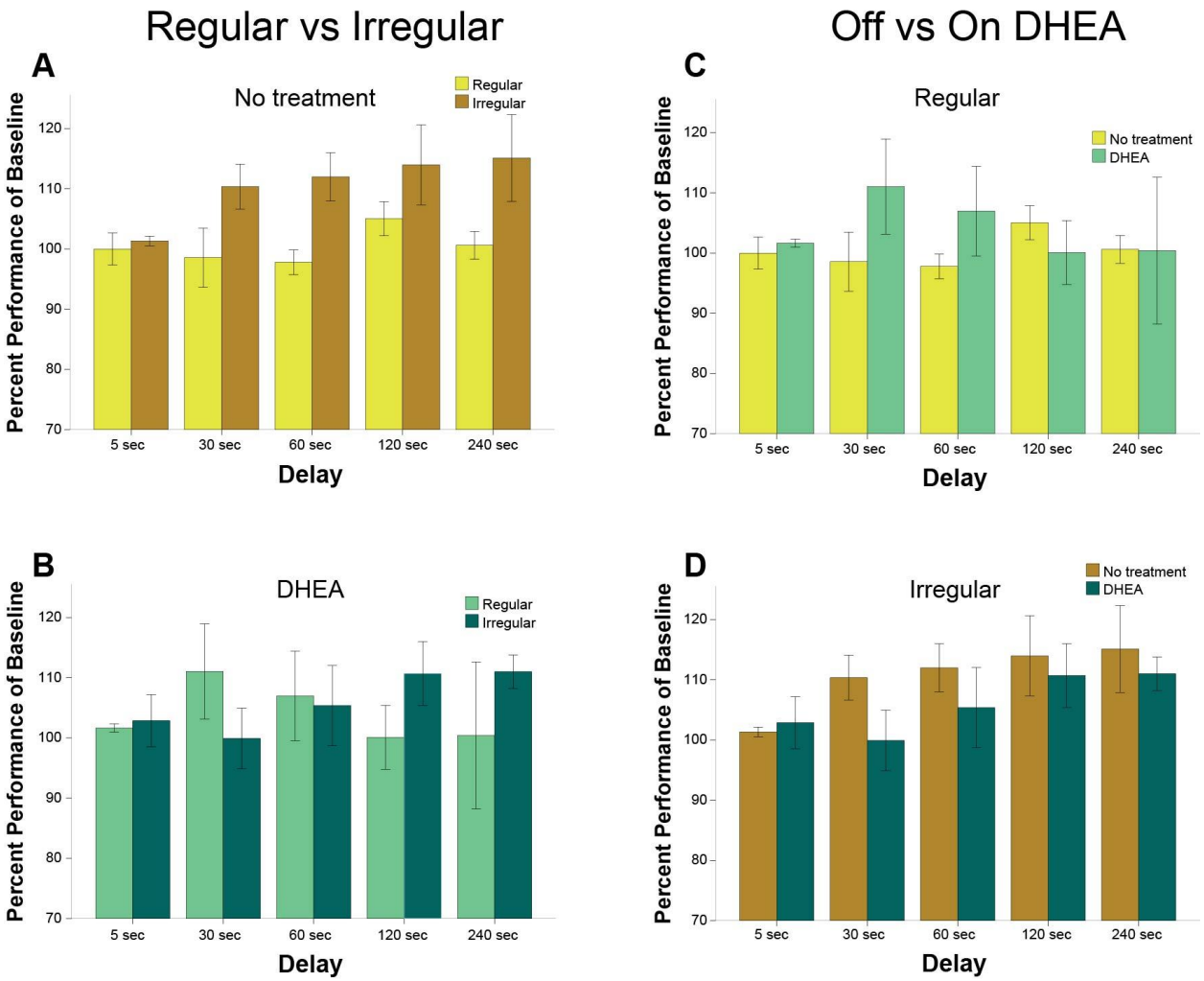


Figure 2.10. Delayed match to sample performance with respect to hormonal status. Analysis of performance in the DMS task was repeated separating animals by menstrual cyclicity. A repeated-measures ANOVA with treatment and delay as within-subjects factors and status as a between subject factor yielded no significant effects of treatment, delay, or cycle status on performance, as well as no significant interactions. Sec, seconds.

hormonal intervention within the critical period of E action (Sherwin, 2007a). Despite the relatively early treatment in our animals, initiating supplementation in pre- and peri-menopause as opposed to postmenopausally as seen in human studies, DHEA failed to increase cognitive performance on the DMS and VDR tasks, both tests sensitive to hippocampal and prefrontal cortical manipulations (Eichenbaum 2002). Additionally, there was no significant difference between pre-menopausal and peri-menopausal animals on either baseline performance or effect of DHEA treatment. While this does not rule out effects of the more complete loss of ovarian steroids seen in post-menopause, it does suggest that the cognitive deficits in aged female macaques occur later in the reproductive lifespan than examined in the current study.

The lack of effect seen in this study furthers the question of why DHEA supplementation is effective in age-related cognitive decline in rodents and not humans. However, as the rhesus macaque can be maintained in a controlled environment with the potential for extensive *in vivo* and postmortem studies, it offers a platform in which to further investigate this discrepancy. As noted in Chapter 1, modulation of the steroidogenic pathway throughout aging may impede the brain's ability to synthesize E_2 from DHEA, an effect that can be investigated through post-mortem histological means.

Though our experimental design was aimed at an earlier intervention than is typically used in humans in order to avoid an extended period of E deprivation, this design may have inadvertently resulted in the opposite effect: any cognitive impact of DHEA may have been masked by the E_2 levels in these animals. Though irregularly cycling animals demonstrated lower circulating levels of E_2, these levels were still above those seen in post-menopause (Downs and Urbanski 2006) and may have still been sufficient to support cognitive performance. Thus, a ceiling effect may have been reached by the endogenous circulating E_2, making any extra

locally-synthesized E_2 resulting from the DHEA supplementation superfluous, as animals may have already been performing at maximal levels.

Further studies will investigate the hypothesis that modulation of the central steroidogenic pathway renders DHEA supplementation in humans and nonhuman primates ineffective, as well as the effects of DHEA supplementation on circulating E_2 and central gene expression.

Chapter 3: Changes in central steroidogenic enzymes with age and mechanisms for the inefficacy of DHEA supplementation in humans and nonhuman primates.

Portions of this chapter have been published previously in:

Sorwell KG, Kohama SG, Urbanski HF (2012) Perimenopausal regulation of steroidogenesis in the nonhuman primate. Neurobiol Aging. *33:1487.e1-e13.*

1. Introduction

Thus far it has been demonstrated that the rhesus macaque hippocampus and PFC express the enzymatic machinery necessary to convert circulating DHEA to E_2, but that supplementation of DHEA in old female macaque, like in elderly humans, has no discernible effect on memory. The question now arises if the aged brain performs this conversion *in vivo* in old age. Indeed, there is some evidence that DHEA can be pro-cognitive in humans, as young men treated with the hormone show improvements in cognitive performance (Alhaj et al., 2006); however, this effect is not seen in older men (Wolf et al., 1997). Also, studies in primates demonstrate maintained central DHEA following prolonged adrenal suppression by dexamethasone, despite drastically reduced levels of circulating DHEA (Robel et al., 1987). Perhaps, then, age is the mitigating factor in DHEA's effect on cognition in humans. To test this hypothesis, we analyzed expression of steroidogenic genes in the hippocampi of rhesus macaques of various ages, with the prediction that age would be associated with decreased expression of steroidogenic enzymes.

While direct quantitation of E levels in brain tissue of nonhuman primates has previously proven difficult due to high lipid concentrations and relatively low levels of the hormone, recent

work in the lab of Dr. Laszlo Prokai in the Department of Molecular Biology and Immunology at the University of North Texas Health Center in Fort Worth, Texas has resulted in the development of a derivitization protocol that greatly increases the sensitivity of liquid chromatography/mass spectrometry (LC/MS-MS) assays for estrogens (Szarka et al., 2013). This process involves the conjugation of a dansyl group to carbons 2 and 4 of the aromatic ring of E_2 and estrone (E_1), an estrogen that can impair cognitive function (Gleason et al., 2005; Barha and Galea, 2010). Dr. Prokai graciously offered his assistance and expertise to allow us to compare the central levels of E_2 and E_1 between young, old DHEA supplemented, and old DHEA-naïve female rhesus macaques, allowing us to test the hypothesis that aged animals have lower levels of E_2 in cognitive brain areas, and that DHEA increases local concentrations of E_2.

2. Materials and Methods

2.1 Experimental animals

This study was performed using tissue samples obtained from rhesus macaques (*M. mulatta*), maintained at the ONPRC. The animals were maintained on a 12:12: light:dark cycle and were fed LabDiet High Protein Monkey Chow (LabDiet Inc., St. Louis, MO) twice daily, supplemented with fresh fruits and vegetables. Animal care was provided by the ONPRC DCM in accordance with the NRC Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the OHSU Institutional Animal Care and Use Committee.

2.2 Tissue collection for qRT-PCR

Hippocampi from 38 male and female rhesus macaques (ages 8–32 years) were collected at necropsy between 0900 hours and 1500 hours; following saline perfusion and subdissection,

they were quickly immersed in liquid N_2 and stored frozen at -80° C for later analysis. These samples were divided into the following three age groups: adult (8-17 years, n = 16; 7 females and 9 males), aged (18-24 years, n = 13; 8 females and 5 males), and oldest old (25-31 years, n = 9; 6 females and 3 males).

2.3 Quantitative real-time PCR

TaqMan (Applied Biosystems, Carlsbad, CA, USA) qRT-PCR was used to quantify hippocampal gene expression during aging. Random-primed reverse transcription was performed, and cDNA was diluted 1:3 and analyzed in triplicate. The PCR reaction mixtures contained 5 µL of TaqMan Universal PCR Master Mix, 0.3 µL of each specific forward and reverse primers (300-nM final concentration), 0.25 µL of specific probe (250-nM final concentration), 2.15 µL of water, and 2 µL of cDNA. The reaction sequence included 2 minutes at 50° C, 10 minutes at 95° C, and 50 cycles of 15 seconds at 95° C, followed by 1 minute at 60° C. Automatic baseline and threshold levels were determined by ABI sequence detection system software (version 2.2.1; Applied Biosciences, Carlsbad, CA, USA). Standard curve analysis was used to convert critical threshold values into relative RNA concentrations and final expression values were expressed as a ratio relative to the arithmetic mean of the relative RNA concentrations of three reference genes, *ALG9*, *GAPDH*, and *RPL13A*, of each respective sample. This combination of housekeeping genes was used to provide a more stable control than a single reference gene, as slight changes in housekeeping genes may be seen with certain conditions or treatments (Noriega et al., 2010). Real-time primer and probe sequences for the various genes and transcript variants are shown in Appendix A. Of the isozymes of 17BHSD, type 5 was chosen because of its confirmed expression in the brain and its primary direction of

action, namely the conversion of androstenedione to testosterone (Labrie et al., 2000; Luu-The and Labrie, 2010; Moeller and Adamski, 2009). Group means were compared using one-way ANOVA followed by Tukey's HSD with significance set at $\alpha = 0.05$.

2.4 Tissue collection for LC/MS hormone analysis

Tissue from the eight aged female rhesus macaques treated with DHEA described in Chapter 2 were used for the analysis of central levels of steroid hormones. Additionally, five young adult (9-13 years, average age 12.6 ± 0.73 years) female rhesus macaques and seven DHEA-naïve aged (21-26 years, average age 23.9 ± 0.63 years) female rhesus macaques were used to assess the effects of DHEA age and DHEA treatment, respectively, on central hormone levels. Monkeys used in the supplementation study received 5 mg of DHEA orally on the morning of necropsy. Animals were humanely euthanized by exsanguinations under ketamine sedation (15-25 mg/kg i.m.) followed by pentobarbital sodium (25-30 mg/kg i.v.). Brains were flushed with 0.9% saline and the hippocampi and dorsolateral PFC were immediately subdissected and flash frozen in liquid N_2 upon removal. Samples were stored at -80 C until assay.

2.5 Hormone analysis

Samples were analyzed by the laboratory of Dr. Laszlo Prokai according to methods previously established (Szarka et al., 2013). Briefly, brain tissue samples were homogenized in pH 7.4 phosphate buffer with a 100 pg internal standard at 20% weight/volume and estrogens were extracted with ethyl acetate. The samples were then centrifuged at 2500 rpm for 10 minutes to separate the organic layer, and subsequently evaporated under a nitrogen stream. To

increase the sensitivity of the assay, as central E_2 levels were expected to be very low, the samples were then derivatized by adding 30 µL of 1mg/mL dansyl chloride (Dns-Cl) in acetonitrile and 20 µL of 100mM aqueous sodium bicarbonate and incubating at 60° C for 10 minutes. The samples were then run though a Kinetex Phenyl-Hexyl (PK-PH) column (Phenomenex, Torrance, CA) at 30° C with a flow rate of 0.4 mL/min and analyzed for E_1 and E_2 by LC/MS-MS. Plasma samples from each animal were run simultaneously. Results were analyzed using a two-way ANOVA for group effect (DHEA, young control, and old control) and sample effect (plasma vs HPC) followed by Tukey's HSD with $\alpha = 0.05$. Statistical trends were defined as $0.05 < p < 0.10$.

3. Results

3.1 Effect of age on hippocampal expression of steroidogenic genes

To examine the effect of age on central steroidogenesis, real-time PCR was performed on hippocampal samples from male and female rhesus macaques aged 8 to 32 years, to quantify potential age-related changes in the expression of genes associated with sex-steroid synthesis (Figure 3.1). No statistically significant differences or trends were detected in gene expression between the males and females (Mann-Whitney *U*-test). Consequently, gene expression results from both sexes were combined to increase statistical power. A significant effect of age was observed in expression of *CYP17A1* ($F = 6.132$, $p = 0.006$), with the old animals demonstrating significantly lower expression than the adult ($p = 0.011$) and aged ($p = 0.009$) animals. *3BHSD* also showed a significant effect of age on expression ($F = 5.759$, $p = 0.007$), with old animals expressing significantly lower levels of the gene than adult animals ($p = 0.008$).

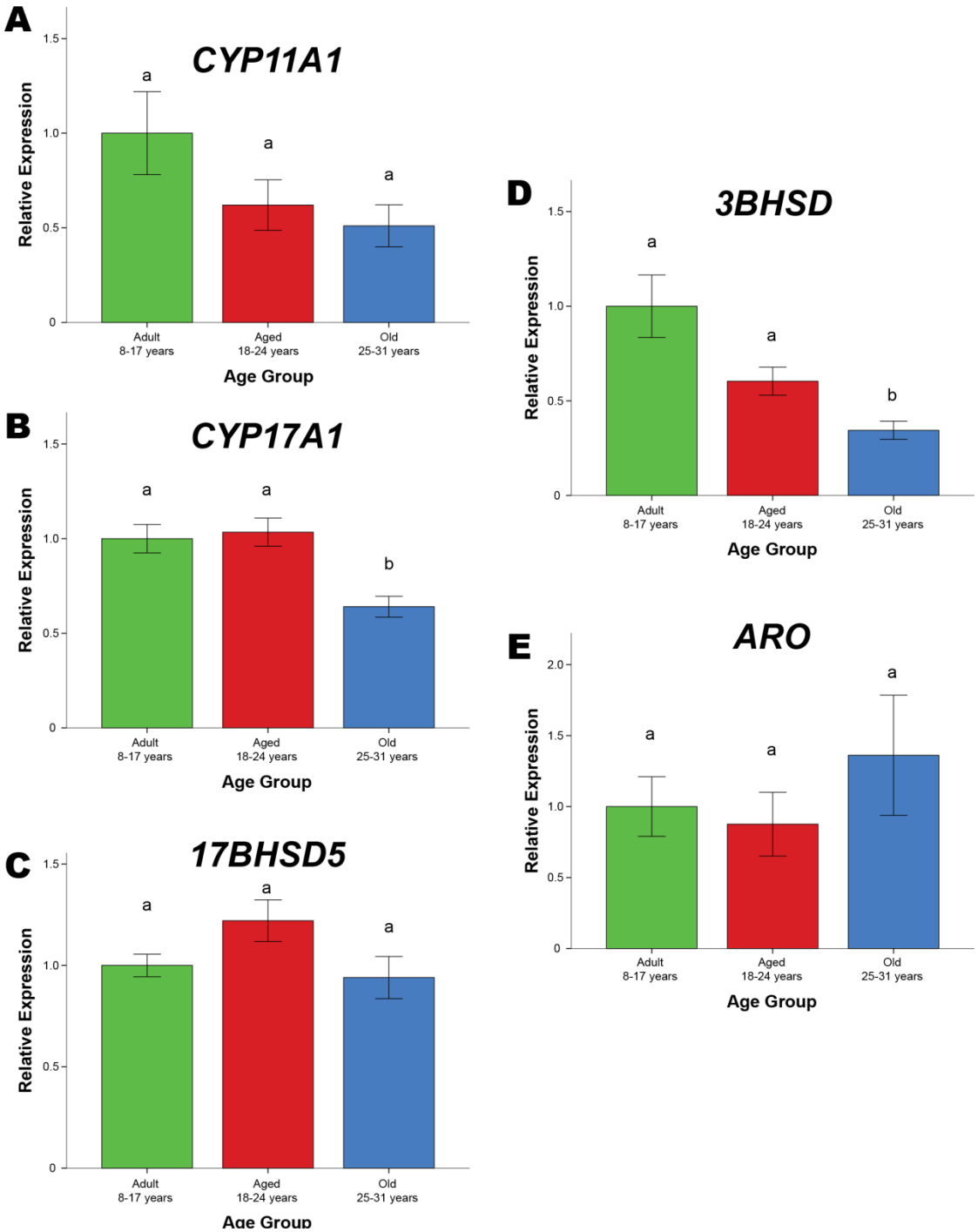


Figure 3.1. Steroidogenic enzyme expression in the aging rhesus macaque hippocampus. Steroidogenic enzyme expression in the hippocampi of adult (8-17 years, n = 16), aged (18-24 years, n = 13) and old (25-31 years, n =) was analyzed using qRT-PCR and is presented as relative mean expression ± SEM. This revealed a significant age effect on expression of *CYP17A1* ($F = 6.132$, $p = 0.006$) and *3BHSD* ($F = 5.759$, $p = 0.007$). Statistical significances were limited to the old group. Different letters above bars indicate significant differences ($p < 0.05$)

3.2 Effects of age and DHEA supplementation on central levels of E_2 and E_1

3.2.1 Estrone concentrations in the plasma and hippocampus

One-way ANOVA revealed a trend for group differences in E_1 concentrations in the plasma (Figure 3.2A; $F = 3.282$, $p = 0.062$), with a trend for higher levels in the young as compared to control ($p = 0.095$) and DHEA ($p < 0.076$) animals. A trend was also observed in E_1 concentrations in the hippocampus ($F = 3.022$, $p = 0.075$), driven by a trend for higher E_1 in young animals as compared to DHEA-treated animals ($p = 0.095$). Overall, E_1 concentrations were significantly higher in the HPC than in the plasma (ANOVA $F = 57.844$, $p < 0.001$).

3.2.2 Estradiol concentrations in the plasma and hippocampus

Plasma E_2 was significantly different between groups (Figure 3.2B; $F = 14.718$, $p < 0.001$), with higher levels in young as compared to old control ($p = 0.001$) and DHEA-treated animals ($p < 0.001$). No significant group differences were observed in HPC E_2 concentrations. There was no overall significant difference between E_2 levels in the plasma and in the HPC, despite a significant difference between plasma and HPC E_2 in the young group (post hoc Student's *t*-test, $t = 4.156$, $p = 0.004$). This lack of effect is likely due to large variation in HPC E_2 in the DHEA-treated animals.

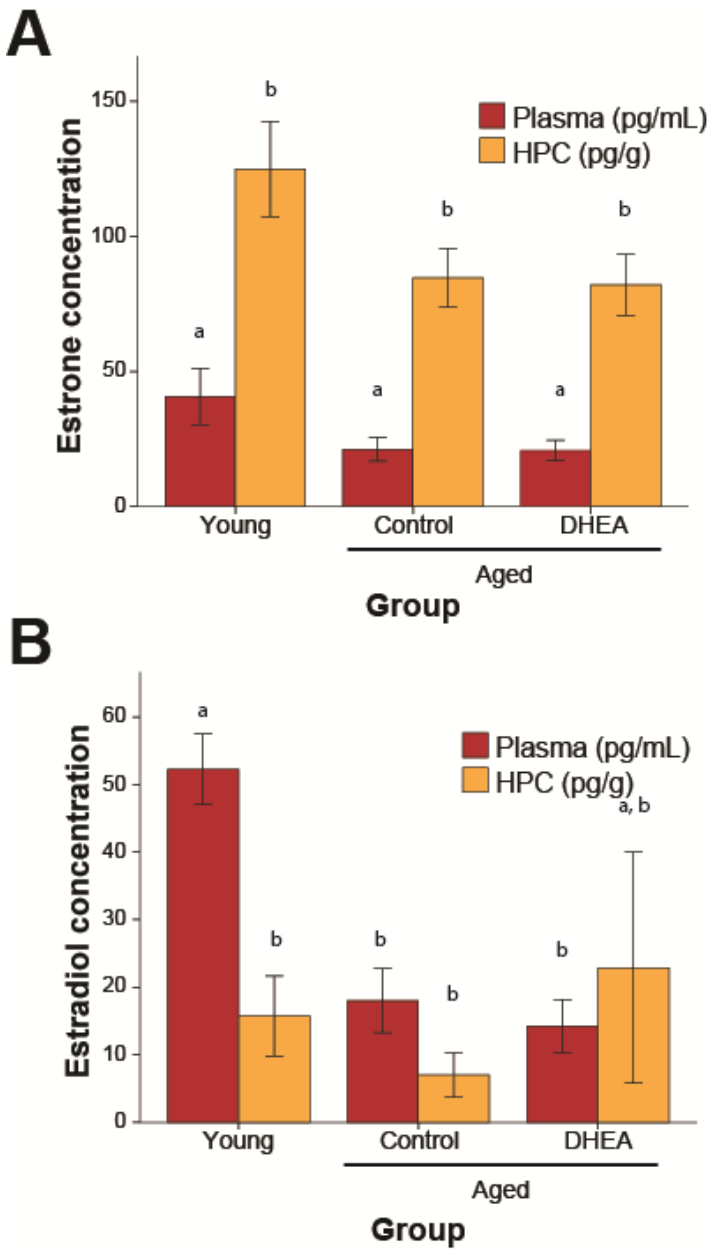


Figure 3.2. Evaluation of estrone and estradiol concentration in the plasma and hippocampus of young and old female rhesus macaques. E_1 and E_2 were extracted from plasma and HPC of young, DHEA naïve aged, and DHEA-treated aged female rhesus macaques. E_1 was significantly higher in the HPC than in the plasma overall (A). E_2 was significantly higher in the plasma of young animals than in either group of old animals (B). Different letters above bars indicate significant differences ($p < 0.05$)

4. Discussion

Although the ovaries are the predominant source of circulating E_2 in women, it has been estimated that up to 75% of active E_2 is derived locally through intracrine metabolism of DHEA/S (Labrie, 1991); therefore, plasma E_2 levels alone may be poor indicators of central E_2. For example, one study of HRT in young OVX rhesus macaques found cognitive benefits for old, but not young, monkeys (Hao et al., 2007), possibly because young animals were able to

synthesize sufficient E_2 locally *de novo* or from adrenal DHEA/S precursors. Similarly, in postmenopausal women circulating DHEA/S has been positively correlated with cognition (Davis et al., 2008; Sanders et al., 2010), perhaps due to a greater ability to produce local E_2 as compared with women with low circulating DHEA/S. In rodents, locally produced E_2 is neuroprotective (Juhász-Vedre et al., 2006) and can have effects on neuronal function (Kretz et al., 2004; Prange- Kiel et al., 2003; Rune and Frotscher, 2005) and plasticity (Prange-Kiel et al., 2009), even beyond that of exogenous E_2. An inability to perform this intracrine conversion, especially in old age, may explain the cognitive inefficacy of DHEA supplementation in humans. The current study supports this hypothesis. Not only are the key steroidogenic enzyme-encoding genes clearly expressed in the primate brain as shown in Chapter 1, but also the expression levels of *CYP17A1* and *3BHSD* show an age-related decline. Such changes are expected to result in decreased central synthesis of E_2, as CYP17A1 is necessary for DHEA synthesis and 3BHSD is necessary for conversion of DHEA to testosterone (see Figure I.4). Also, while expression of *CYP11A1*, *17BHSD5*, and *AROMATASE* was similar across the three age groups, it should be emphasized that all of our postmortem hippocampal tissues were collected during the daytime between 0900 hours and 1500 hours; many genes have a 24-hour pattern of expression with a peak occurring during the night (Lemos et al., 2006; Urbanski et al., 2009), and so we cannot exclude the possibility that some genes may show age-related changes that are evident only during the night. Together, the data highlight the potential involvement of DHEA/S in maintaining elevated E_2 concentrations within the brain, and show how enzymatic changes could contribute to the etiology of age-associated pathologies.

This study also examined the *de novo* steroidogenic potential of the macaque HPC by quantifying expression of genes involved in the conversion of cholesterol to DHEA throughout

aging. While the current study does not measure steroidogenic enzyme activity directly, *in vitro* studies show significant correlation between mRNA expression and activity of steroid-producing enzymes in human tissues (Speirs et al., 1998; Puche et al., 2002). Although neurosteroidogenesis has been well established in rodents (Zwain and Yen, 1999), the underlying mechanism may be different in primates because of significant input of DHEA/S of adrenal origin. Some evidence suggests, however, that the NHP brain is in fact capable of *de novo* neurosteroid synthesis (Robel et al., 1987; Schumacher et al., 2003). Our observation that the brain, and specifically the HPC, expresses all of the enzyme-encoding genes necessary for sex steroid biosynthesis suggests that primates are able to centrally synthesize DHEA/S *de novo*. Further, expression of *CYP17A1*, the enzyme responsible for conversion of pregnenolone to DHEA, was significantly lower in the oldest group of animals studied, suggesting the ability of the HPC to synthesize steroids declines with advanced age. Interestingly, a decline in activity of this same enzyme in the adrenal gland is thought to be the underlying cause of age-related declines in circulating DHEA/S (Liu et al., 1990); therefore, the current results may reflect parallel age-related changes in steroid synthesis in the brain and periphery. Based on the expression of key enzyme-encoding genes, these results suggest that *de novo* synthesis of DHEA/S, as well as conversion of adrenal DHEA/S to E_2, is feasible within the primate HPC, and that changes in the expression of these genes may contribute to age-associated cognitive decline.

We also show here that DHEA supplementation did not increase E_2 levels in the HPCs of our animals. Interestingly, despite a significantly higher concentration of E_2 in the plasma of young animals, E_2 in the HPC did not differ by age. While it is possible the dose of DHEA used in this study (5 mg) was too low to sustain elevated DHEA in the circulation or brain, this lack

of difference between young and aged animals suggests a higher dose would also not elevate central E_2. Further, E_1 concentrations were significantly higher in the brain than plasma when all animals were pooled together, though no group differences were seen. Importantly, the ratio of E_1:E_2 was higher in the HPC than the plasma, and absolute levels of E_1 were higher in the brain as well. This may be due to either a sequestration of E_1 in the HPC or preferential synthesis of E_1 over E_2. Greater concentrations of E_1 than E_2 indicate a relative lack of 17BHSD5 (or AKR1C3) activity (see Figure I.4) preventing the conversion of E_1 to E_2, or a relatively high level of 17BHSD2 or 17BHSD4 activity acting to convert E_2 to E_1. As E_1 lacks some of the beneficial effects of E_2 (McClure et al., 2013) and can even impair learning and memory (Barha and Galea, 2010; Engler-Chiurazzi et al., 2012), these relative levels could have significant impacts on cognition.

We suggest that multiple neuroendocrine events can contribute to aspects of cognitive decline that are mediated by the loss of steroids during aging, including: (1) loss of gonadal E_2 at the time of menopause leads to a direct loss of central E_2 availability; (2) the decline in circulating levels of E_2 precursors, namely DHEA and DHEAS, results in reduced intracrine E_2 synthesis in the brain; (3) decreased brain expression of steroidogenic enzymes results in less potential for conversion of the diminished levels of DHEA/S to E_2; and (4) a combined loss of peripheral and local *de novo* DHEA/S production results in loss of the protective effects of the hormone itself, without conversion to E_2 (Flood et al., 1999; Mao and Barger, 1998; Rhodes et al., 1997).

Part A Summary: The cognitive benefits of hormone therapy in the female are complicated by multi-level systems interactions

Previous work in female rhesus macaques has shown a significant pro-cognitive effect of estradiol treatment postmenopause; however, due to possible complications of traditional estrogen therapy in humans, it is vital to search out new interventions to combat cognitive decline. While DHEA supplementation shows promise in rodent literature, this effect has not been translated successfully to humans. The previous three chapters have discussed the absence of an effect of DHEA supplementation on cognition in the aged female rhesus macaque and have explored a potential mechanism, finding that the steroidogenic potential of the HPC, an area of the brain involved in both tasks used to evaluate memory in our DHEA-supplemented monkeys, decreases with age, as determined by quantitation of steroidogenic enzyme expression. Thus, the pro-cognitive interaction between the HPA and HPG axes is lost as monkeys, and presumably humans, become older.

The following chapters will further discuss the interactions between the HPA and HPG axes, examining the effects of the manipulation of one axis on the output of the other. This will shed light on the importance of maintaining a whole-body perspective in hormonal interventions, as the manipulation of one system invariably influences the other.

Part B

Role of endocrine interactions in cognition

Chapter 4: Central and peripheral endocrine interactions in models of female hormone therapy

Portions of this chapter have been published previously in:

Sorwell KG, Kohama SG, Urbanski HF (2012) Perimenopausal regulation of steroidogenesis in the nonhuman primate. Neurobiol Aging. *33:1487.e1-e13.*

1. Introduction

Thus far, we have shown that, despite evidence of positive E_2 effects on cognition in aged females and the expression of the enzymatic machinery necessary to convert DHEA to E_2 in the brain, DHEA supplementation does not improve cognitive ability in aged female rhesus macaques. This lack of effect may be due to an age-related decrease in the expression of the enzymes necessary for this intracrine conversion, as old animals (over the age of 25) express significantly lower levels of *3BHSD* in the HPC than younger animals. This age-related change in steroidogenic gene expression is intriguing, and as the animals used to collect these data were not hormonally characterized, one potential mechanism for this change in gene expression is the changing hormonal milieu as animals age. If this is the case, a combined treatment of E_2 and DHEA supplementation may be a viable option. As DHEA opposes the growth of breast cancer tissue (López-Marure et al., 2011; Rovito et al., 2013) and may have positive effects on cardiovascular health (Sanders et al., 2010) and lowers ischemia risk (Jiménez et al., 2013), this addition to traditional HRT may mitigate the negative side effects associated with E supplementation (Davison and Davis, 2003; Renoux and Suissa, 2011; Chlebowski et al., 2013). In fact, a combination therapy including DHEA and E_2 has recently been shown to have a

number of beneficial effects on overall health in perimenopausal women, though cognition was not tested in these individuals (Stephenson et al., 2013).

To investigate the effects of different hormone paradigms on steroidogenic gene expression, we used qRT-PCR of the HPC and dorsolateral PFC of female rhesus macaques treated with short-term E_2 replacement. Additionally, given evidence for interaction between the HPA and HPG axes in perimenopausal women (Pluchino et al., 2005; Crawford et al., 2009) suggesting a compensatory mechanism between circulating E_2 and DHEAS, combined with the potential for conversion of DHEA to E_2 in the circulation, we measured the effect of DHEA supplementation over the course of our cognitive testing study described in Chapter 2 on circulating E_2, with the hypothesis that DHEA supplementation contributes to circulating E_2. We also investigated the relationship between cognitive performance of the animals previously described and steroid-related gene expression in the HPC and PFC to identify genes involved in more favorable cognitive performance in the monkey.

2. Materials and methods

2.1 Experimental Animals

Animals used in this study were rhesus macaques (*M. mulatta*), maintained at the ONPRC. The animals were maintained on a 12:12: light:dark cycle and were fed LabDiet High Protein Monkey Chow (LabDiet Inc., St. Louis, MO) twice daily, supplemented with fresh fruits and vegetables with water available *ad libitum*. Animal care was provided by the ONPRC DCM in accordance with the *NRC Guide for the Care and Use of Laboratory Animals*, and the experiments were approved by the OHSU Institutional Animal Care and Use Committee.

2.2 Hormone supplementation

2.2.1 Short-term E_2 supplementation

Twelve female rhesus macaques (9.4 ± 0.3 years) were used to assess the effects of HRT on hippocampal steroidogenic gene expression. Each animal underwent bilateral OVX and was used on average 6.7 ± 0.6 months later. At this time, eight animals were implanted with subcutaneous Silastic capsules containing E_2; 15 days later, four of these animals received subcutaneous Silastic implants containing P_4. Four animals were implanted with an empty silastic capsule as a placebo. This experimental design has been used previously to mimic circulating hormone levels of the late follicular (in the E_2-only group, referred to as the E group) and midluteal (in the E_2 plus P_4 group, referred to as the EP group) phases (Kohama and Bethea, 1995). To confirm the efficacy of the implants, both sex steroids were assayed using the Elecsys 2010 Platform (Roche), as previously described in Chapter 2. The animals were sacrificed 28 days after receiving the initial subcutaneous implant and hippocampi and dorsolateral prefrontal cortices were frozen in liquid N_2 for later analysis.

2.2.2 DHEA supplementation

DHEA treatment began, animals were cognitively tested, and E_2 was assayed as described previously in Chapter 2. Plasma E_2 samples were collected twice every week throughout the study. To avoid excessive variation due to the E_2 peak during the menstrual cycle, all values over 100 pg/mL were dropped and means and standard errors were determined for the remaining "Background E_2" values over the course of the Overall Baseline and each one-month testing battery. These data were analyzed using a repeated-measures ANOVA, with a

within-subjects factor of time and a between-subjects factor of hormonal status, as determined in Chapter 2.

2.3 Tissue processing and quantitation of RNA

Tissue was collected, processed, and analyzed for mRNA expression as described in Chapter 3. In the short-term HRT paradigm, mRNA levels are expressed relative to the mean of the OVX group. Following conversion of RNA to cDNA using the RT^2 First Strand Kit (SABiosciences, Qiagen, Valencia, CA) samples from the hippocampi and dorsolateral PFCs of the DHEA-supplemented animals were run on custom RT^2 Profiler PCR Array plates (SABiosciences) using the provided kit. Genes selected for inclusion on this plate were chosen for their involvement in either steroidogenesis (i.e., steroidogenic enzymes or transport proteins such as steroidogenic acute regulatory protein [StAR]) or steroid responses (i.e., steroid receptors and intracellular signaling proteins involved in the estrogen response). Additional samples were run on plates containing genes involved in the GABA and glutamate systems. These data are presented in Appendix B. All genes were normalized to a composite of the three housekeeping genes, *ALG9*, *GAPDH*, and *RPL13A*. Lists of all genes examined are provided in Appendix A.3 and A.4.

2.4 Cognitive characterization of DHEA-supplemented animals and correlation with gene expression

To allow for correlations between gene expression and cognitive performance, animals were classified based on their scores on the 15-second delay in the VDR task and the 60-second delay in the DMS task during the final testing battery. These specific delays were chosen as they

demonstrated the widest range in performance among the animals. The final battery was chosen for analysis as this allowed animals to learn and practice the task as much as possible after meeting the baseline criteria. Animals were ranked based on their mean performance at this delay, and ranks were correlated with gene expression using Kendall's τ b. The level of significance was defined as $\alpha = 0.05$.

3. Results

3.1 Effect of DHEA supplementation on circulating estradiol

Mean background E_2 throughout the course of the DHEA supplementation study is shown in Figure 4.1. A main effect of status was observed, with regular animals demonstrating higher levels of E_2 ($p < 0.05$). While E_2 appears to increase with time, this factor was not significant as determined by repeated-measures ANOVA. When animals are grouped by menstrual cyclicity, although mean circulating E_2 in regular animals was greater than irregular animals, due to variation this interaction is not statistically significant.

3.2 Hippocampal expression of steroidogenic genes after short-term HRT

In humans, circulating DHEAS levels increase transiently during menopause (Crawford et al., 2009; Lasley et al., 2002) and decrease in response to HRT (Pluchino et al., 2005). To examine the possibility of a similar central mechanism of hormonal steroidogenesis regulation in the brain, we quantified steroidogenic gene transcripts following short-term (one month) HRT. Subcutaneous E_2 implants in the E group yielded an average plasma concentration of 118 ± 6.7 pg/ml E_2 over the 28 days of treatment. Implants in the EP group yielded E_2 levels at an average of 130 ± 9.5 pg/ml over 28 days and P_4 levels of 3.8 ± 0.9 ng/ml over the last 14 days of

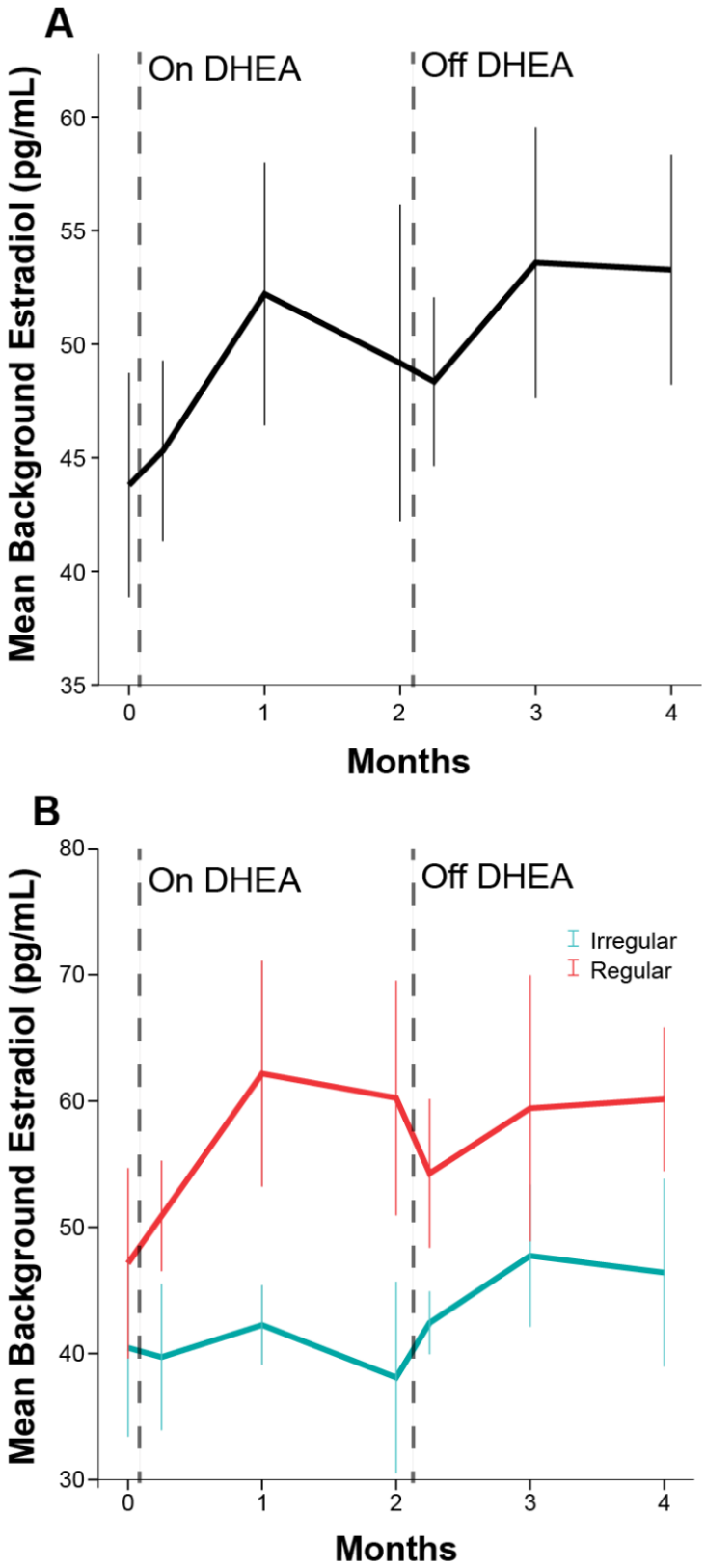


Figure 4.1. Circulating levels of estradiol following DHEA supplementation in pre- and peri-menopausal aged female rhesus macaques. E_2 levels were determined twice a week throughout the study. To avoid variation due to the periovulatory E_2 peak, all values over 100 pg/mL were removed from the analysis. Remaining values were binned for each testing battery. Data are presented as mean ± SEM. Circulating E_2 did not increase within all animals (A) over the course of the study. When separated by menstrual cycle status (B), regular cyclers had greater levels of circulating E_2, as expected due to the method of hormonal characterization used. Again, no significant effect of treatment was observed on circulating E_2 levels.

treatment. E_2 and P_4 levels in the OVX animals were undetectable (i.e., <5 pg/ml, and <0.03 ng/ml, respectively).

To investigate changes in gene expression that occur in response to HRT, real-time PCR was conducted on whole hippocampal RNA extracts obtained from female rhesus macaques after OVX, OVX + E_2 treatment (E), and OVX + E_2 + P_4 treatment (EP). The data are normalized to an index of housekeeping genes consisting of *ALG9, GAPDH,* and *RPL13A*, and are expressed relative to the mean of the OVX group (Figure 4.2). No significant between-treatment differences or trends toward significance were seen in *CYP11A1*, *3BHSD1/2*, or*17BHSD5*. ANOVA revealed a significant inhibitory effect of E and EP hormone treatment on expression of *CYP17A1* and *ARO*. Contrast analysis revealed individual group differences between OVX and both hormone treatment groups; specifically, there was a significant decrease in expression of *CYP17A1* and *ARO* following either hormone treatment. Individual group comparisons revealed no significant difference in gene expression between E and EP groups, but a significant decrease with E_2 treatment in expression of *SULT2B1* and *ARO* and a significant decrease in *CYP17A1, SULT2B1,* and *ARO* with E_2 and P_4 treatment.

3.3 Correlations between cognitive performance and steroid-related gene expression

No significant differences were observed between regular and irregular cyclers on gene expression in either the HPC or PFC and therefore the groups were collapsed for correlation analysis. Results of the Kendall's τ b test for correlation between cognitive performance and steroid-related gene expression in the HPC and PFC are shown in Table 4.3. In the HPC, more favorable cognitive performance in the DMS task was correlated with lower levels of expression of *HSD11B1* (the gene encoding 11β-hydroxysteroid dehydrogenase, which synthesizes cortisol

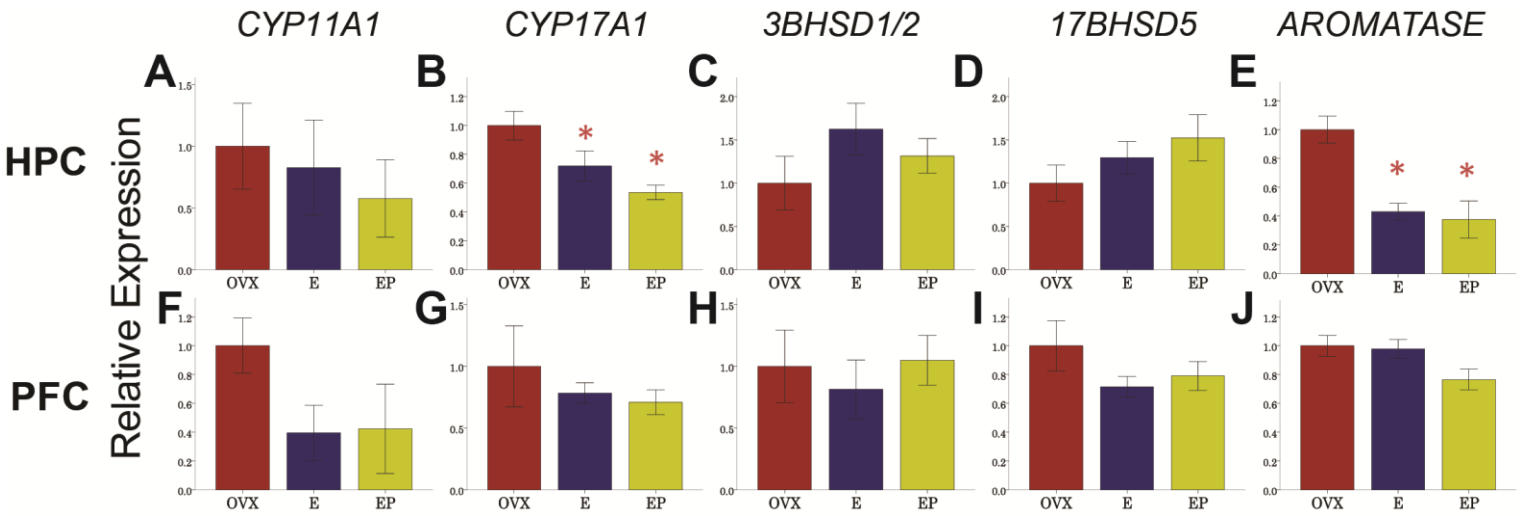


Figure 4.2. Regulation of central steroidogenic enzyme expression by short- term estradiol or estradiol plus progesterone treatment. mRNA was analyzed from the HPC and PFC of ovariectomized female monkeys treated with empty capsules (OVX), E_2 (E), or E_2+ P_4 (EP) (n = 4 per group). Statistical differences from the OVX group as determined by ANOVA followed by Tukey's HSD are indicated by asterisks. HRT significantly decreased *CYP17A1* and *ARO* expression in the HPC No significant effects of HRT were observed in gene expression in the PFC. Data are presented as mean ± SEM. Asterisks indicate statistically significant difference ($p < 0.05$) from the OVX group.

from cortisone), *MC4R* (melanocortin 4 receptor), *MCHR1* (melanin-concentrating hormone receptor 1), and *NR3C1* (glucocorticoid receptor). More favorable performance in the VDR task was associated with higher levels of expression of *AKR1C3* (17BHSD5), *ESR2* (estrogen receptor β), *MAP2* (microtubule-associated protein 2), and *STAR* (steroidogenic acute regulatory protein, the first protein necessary for synthesis of steroids from cholesterol), and with lower levels of *SRD5A1* (5α-reductase type 1, enzyme synthesizing DHT from T).

In the PFC, greater performance in the DMS task was correlated with higher levels of expression of *AKR1C3*, and with lower levels of expression of *GNRHR2* (GnRH receptor 2), *MAPK1* (mitogen-activated protein kinase 1, also referred to as ERK2), *MCHR1*, *SIGMAR1* (the sigma receptor, a known target of DHEA), and *STAR*.

Table 4.3. Steroidogenic and steroid-related gene expression in the hippocampus and prefrontal cortex correlated with cognitive performance

Hippocampus						Prefrontal Cortex		
DMS			VDR			DMS		
	Kendall's			Kendall's			Kendall's	
Gene	τ b	p value	Gene	τ b	p value	Gene	τ b	p value
HSD11B1	-0.473	0.010	AKR1C3	0.500	0.039	AKR1C3	0.400	<0.001
MC4R	-0.473	0.005	SRD5A1	-0.643	<0.001	GNRHR2	-0.546	0.006
MCHR1	-0.473	0.010	STAR	0.429	0.014	MAPK1	-0.473	0.010
NR3C1	-0.327	0.031				MCHR1	-0.691	0.003
						SIGMAR1	-0.546	<0.001
						STAR	-0.473	0.017

Table 4.3. Animals were ranked by their score in the final Off-DHEA battery on the 60 second delay in the DMS task and the 15 second delay in the VDR task. These ranks were then correlated with relative gene expression using Kendall's τ b. Only significant correlations are presented. See text for definition of gene names.

4. Discussion

In the current study of aged female macaques, we observed no significant increase in circulating E_2 with DHEA supplementation, contrary to what is expected according to the hypothesis that DHEA is a significant source of active estrogens. We expected E_2 to increase, as DHEA is a precursor; however, this lack of change in circulating E_2 does not necessarily indicate that E_2 levels within various peripheral tissues were not increased by the treatment. Labrie and colleagues (1998) estimate that in premenopausal women, roughly 75% of active estrogens in tissues are produced locally; thus, a detailed analysis of E_2 concentrations in multiple tissues on and off DHEA treatment would be necessary to assess the true effect of DHEA supplementation on E_2 exposure. As this experiment was a within-subjects design, it was not feasible to perform these analyses in the current study. Additionally, circulating DHEA and E_2 have been shown to be inversely correlated during the menopausal transition and E supplementation, with lower E_2 corresponding to short-term increases in DHEA and vice versa (Crawford et al., 2009; Pluchino et al., 2005). Thus, ovarian E_2 may be downregulated by an increase in E_2 derived from exogenous DHEA.

Consistent with the inverse relationship between circulating levels of adrenal and gonadal steroids seen in perimenopausal women (Lasley et al., 2002; Crawford et al., 2009; Pluchino et al., 2005), we observed a compensatory effect of short-term E_2 replacement of young OVX animals on steroidogenic enzyme levels in the HPC. When circulating levels of E_2 were replaced, animals expressed significantly lower levels of *CYP17A1* and *ARO*. This suggests a titrating feedback mechanism, with the regulation of gene expression within the HPC controlling its own exposure to E_2 from three potential sources: the circulation, metabolism of DHEA or T, and *de novo* synthesis. Interestingly, this effect was not seen in the PFC, where expression of all

enzymes was statistically equal across groups. This is consistent with the E_2's tendency to improve prefrontal cortical domains of memory, such as verbal memory, to a greater extent than purely hippocampal memory (Wolf et al., 1999; Keenan et al., 2001; Maki et al., 2001; Krug et al., 2006): If the PFC does not compensate for higher levels of circulating E_2 and continues synthesizing its own E_2, either from a prohormones or *de novo*, it will be exposed to more net E_2 than an area that does compensate its steroidogenesis.

This compensatory mechanism could explain the lack of a negative effect of OVX on performance of E-sensitive cognitive tasks in young monkeys, as they may still produce sufficient DHEA/S for intracrine neurosteroidogenesis (Hao et al., 2007). Similar end-product inhibition is seen in peripheral tissues, with E_2 acting via estrogen receptor alpha (ERα) in ovarian follicles to downregulate transcription of *CYP17A1* and ultimately to reduce local steroidogenesis (Taniguchi et al., 2007). Interestingly, while the present study demonstrates a downregulation of aromatase following E_2 treatment, other tissue cells exhibit a positive feedback mechanism between E_2 and aromatase. For example, activation of ERα increases aromatase gene expression through the I.1 promoter in placenta (Kumar et al., 2009) and the I.f promoter in mouse hypothalamic cells (Yilmaz et al., 2009). This discrepancy may be explained by the tissue-specificity of these promoter regions, or by variations in regulation with E_2 dosing and timing, as hypothalamic cells exhibit decreased aromatase expression 12 hours after E_2, but increased aromatase after 24 hours.

In light of this evidence of end-product inhibition, the loss in circulating DHEA and E_2 in old animals would be expected to result in increased expression of steroidogenic genes. However, the data presented in Chapter 3 suggest an inability to increase synthesis with this loss of negative feedback. There is evidence of age-related promoter damage that leads to

downregulation of certain genes (Lu et al., 2004), which may explain the lack of steroidogenic regulation in the oldest animals. Additionally, as local E_2 synthesis has been shown to be regulated by gonadotropin-releasing hormone (GnRH) input to the HPC independent of LH and FSH (Prange-Kiel et al., 2008, 2009; Rosati et al., 2011), a dysregulation of GnRH signaling, or reduced GnRH responsiveness with age or OVX, may further modulate steroidogenic enzyme expression. The present observation that exogenous E_2 inhibits neurosteroidogenic enzyme expression in young animals is consistent with a previous study demonstrating E_2 treatment of ovariectomized rhesus macaques results in a decrease in GnRH pulse amplitude (Mizuno and Terasawa, 2005); thus, peripheral or exogenous E_2 may suppress local hippocampal steroidogenesis via GnRH suppression.

The results of correlations between cognitive performance and central gene expression yielded interesting results regarding the role of steroidogenic and steroid-related genes in cognition. In the HPC, performance in the DMS task was negatively correlated with *HSD11B1*, suggesting that the local synthesis of cortisol may negatively impact cognition. This is particularly interesting, as no direct evidence of central cortisol production in the nonhuman primate has yet been published. Also surprisingly, expression of the glucocorticoid receptor was negatively correlated with performance, as downregulation of glucocorticoid receptor in the HPC is associated with hypercortisolemia and cognitive impairment (McEwen 2002). However, studies of aging and memory in mice demonstrate a significant impact of central corticosterone production on memory, particularly throughout aging. Expression of HSD11B1 is associated with greater cognitive impairment with age (Holmes et al., 2010; Holmes and Seckl, 2006), and inhibition or knockout of HSD11B1 maintain spatial memory throughout aging to a greater extent than wild-type controls (Yau et al., 2007; Sooy et al., 2010), and this mechanism has been

shown to be due to a greater activation of mineralocorticoid receptors than glucocorticoids receptors (Yau et al., 2011). Therefore, the downregulation of these two genes may work in concert to protect the HPC from stress-induced memory impairments.

Also of note are the significant correlations between HPC gene expression and cognitive performance in the VDR task. Greater expression of *AKR1C3* (the gene encoding 17BHSD5) and *STAR* were associated with better performance, suggesting that an ability to synthesize hormones *de novo* and an ability to metabolize DHEA into T can confer cognitive benefit. Consistent with the hypothesis that local production of E_2 increases memory performance, lower levels of *SRD5A1* (encoding 5α-reductase type 1) were associated with better VDR performance. Lesser expression of this enzyme would shunt the steroidogenic pathway away from synthesizing DHT from T, resulting in more T potentially being aromatized to E_2. Thus, despite a lack of effect of DHEA on cognitive performance, these data suggest that changes in local steroid synthesis do play an important role in declining cognitive ability in aged female macaques, and thus targeting the activity and expression of steroidogenic enzymes, rather than replacing active steroids or their precursors, may prove to be a valuable target for the treatment and prevention of age-related cognitive decline.

Chapter 5: Adrenal interactions in male rhesus macaque

Portions of this chapter have been published previously in:

Sorwell KG, Garten J, Renner L, Weiss A, Garyfallou VT, Kohama SG, Neuringer M, Urbanski HF (2012) Hormone supplementation during aging: how much and when? Rejuv Res. *15:128.*

1. Introduction

Up to this point, we have only investigated HRT effect on cognition in female rhesus macaques; however, age-related declines in T are seen in men (Harman et al., 2001; Feldman et al., 2002) and can be reversed with HT (Moffat 2005; Janowsky et al., 1994; Janowsky et al., 2000). As ARO does not appear to decline with age (Chapter 3), supplementation with T may increase local levels of E_2 even in the elderly. In fact, aromatization of T to E_2 has been shown to be necessary for some of the pro-cognitive effects of T in men (Cherrier et al., 2005). As it is not clinically feasible to supplement aged women with high levels of T alone due to potential unwanted side effects, we chose to further investigate the effects of HT by using a model of male aging and physiological hormone supplementation, with the hypothesis that intracrine conversion of T to E_2 can improve cognitive function in the aged male. Also, while a number of studies have reported interactions between the HPA and HPG axes in women (Crawford et al., 2009; Pluchino et al., 2005 Lasley et al., 2002), this literature is lacking in males.

We developed a novel oral hormone supplementation paradigm to further investigate the efficacy of steroid treatment on cognition in the aged macaque. We used aged male rhesus macaques to avoid the confounding effect of the variable E_2 concentrations observed in the menstrual cycle, and to take advantage of the lack of age-related decrease in ARO observed in

Chapter 3. Like humans, old male macaques have reduced circulating T levels (Schlatt et al., 2008; Sitzmann et al., 2013). While T supplementation in men is commonly prescribed to address a number of age related issues (Gooren 2003; Krause et al., 2005), the clinical formulation of this treatment is advised to be administered in the morning; however, T has a pronounced circadian rhythm resulting in maximum levels at night (Schlatt et al., 2008; Sitzmann et al., 2013). Thus we supplemented macaques with oral T in the evening to fully reproduce the natural physiological rhythms of the hormone. Also, to address the effects of combined bioidentical HT, we additionally supplemented these animals with DHEA. Again, this supplementation was administered at the physiologically relevant time, with animals receiving DHEA in the morning.

The current study addresses the interactions between the HPG and HPA axes in the periphery of the male, a phenomenon that has not yet been studied in either humans or nonhuman primates. The surprising results from this study further strengthen the idea of a multiple-level approach to endocrine supplementation in aging.

2. Materials and methods

2.1 Experimental animals

The study used adult (~7 years, n = 4) and old (~21 years, n = 9) rhesus macaques (*M. mulatta*), and was approved by the Institutional Animal Care and Use Committee. The animals were cared for by the ONPRC DCM in accordance with the *NRC Guide for the Care and Use of Laboratory Animals*, and were caged singly indoors under controlled environmental conditions: 24° C temperature; 12-h light, 12-h dark photoperiods (lights on at 0700 h). Monkey chow was

provided twice daily and was supplemented with fresh fruit and vegetables; fresh drinking water was available *ad libitum.*

2.2 Androgen supplementation

A series of hormone paradigms were examined, with administration times and doses chosen to replicate the endogenous circadian peaks of T and DHEA. Animals were treated with hormones for five days prior to each blood sampling session to allow serum steroid levels to equilibrate. DHEA (10 mg/ml; Sigma-Aldrich, St. Louis, MO, USA), testosterone (T, 120 mg/ml; Sigma-Aldrich), and dihydrotestosterone (DHT, 5-10 mg/ml; Sigma-Aldrich) were suspended in sesame oil and mixed individually with melted chocolate. Sesame oil was used to bypass testosterone metabolism by the liver by increasing absorption through the lymphatic system (Amory and Bremner, 2005; Amory et al., 2006), as testosterone has a very high rate of clearance. Chocolates were kept refrigerated at 4° C until the time of administration. Four supplementation paradigms were performed (Table 5.1). In the last experiment, T was administered in the morning to examine a possible role of time of day on the steroid response to T. To protect animals against excessive blood sampling, it was not feasible to repeat all hormone combinations at all doses. Animals were monitored at the time of steroid supplementation to ensure each ate the entire treat in a timely manner; if the treat was refused, an equivalent dose was administered via a drug-soaked cookie or prune. The doses used and times of administration for each experiment are provided in Table 5.1.

Table 5.1. Steroid supplementation paradigms

Experiment	Time 0700 h	1000 h	1900 h
Baseline	-	-	-
1	DHEA 0.04 mg/kg	DHEA 0.04 mg/kg	T 12 mg/kg
2	DHEA 0.10 mg/kg	DHEA 0.05 mg/kg	-
3	DHEA 0.10 mg/kg	DHEA 0.05 mg/kg	DHT 5-10 mg/kg
4	T, 12 mg/kg	-	-

Table 5.1. The times and doses of the four androgen supplementation paradigms are shown. Due to limitations on blood sampling, not all combinations of hormones were able to be tested at all doses. DHEA was dissolved in sesame oil, while T was suspended in sesame oil, and steroids were mixed into chocolates for oral supplementation. Animals were monitored to ensure the entire dose was eaten in a timely manner.

2.3 Remote blood sampling

Cortisol and DHEA/S both demonstrate a circadian pattern of release (Downs et al., 2008). Therefore, to gain meaningful insights regarding sex-related or age-related hormone differences it was necessary to collect blood samples from each animal serially across an entire 24-h period. To achieve this with minimal disruption of the animals, each monkey was surgically implanted with a subclavian vein catheter, leading to a remote blood sampling system in an adjacent room, as previously described (Urbanski, 2011). Blood samples were collected into EDTA-coated borosilicate glass tubes every hour for a complete 24-hour cycle. The samples were centrifuged at 4° C, and the plasma was stored at -20° C until assay for cortisol and DHEAS. The 24-hour serial blood sampling procedure was performed once on each animal, except for the androgen-supplemented old animals, which were re-sampled after each of the 5-day androgen supplementation tests, performed approximately 1 month apart.

2.4 Hormone assays

Serial blood samples were assayed for cortisol and DHEAS using electrochemiluminescence (ECL) with the Elecsys 2010 Platform (Roche Diagnostics, Indianapolis, IN, USA) as described in chapter 2.

3. Results

3.1 Combined testosterone and DHEA supplementation does not increase circulating cortisol

Both DHEA and cortisol are secreted by the adrenal gland in response to adrenocorticotropic hormone (ACTH) from the hypothalamus. In order to further examine where testosterone is having an effect to increase DHEAS, we also assayed a series of 24 h blood samples from our DHEA and T combined supplementation paradigm for cortisol. If androgen activation at the level of the brain increases ACTH secretion, cortisol would also increase at the time of T supplementation. However, as shown in Figure 5.2A, T did not increase circulating cortisol. A repeated measures ANOVA with time as a within-subjects factor and group (old control, open circles; old androgen-treated, closed circles; and young, open squares) as a between subjects factor revealed a significant effect of time ($F = 56.758$, $p < 0.001$), group ($F = 5.438$, $p = 0.028$), and group-by-time interaction ($F = 2.093$, $p < 0.001$). The significant effect of group was driven by differences between the old control and old androgen-treated animals, with androgen treatment associated with significant reductions in circulating cortisol at 1000, 1500, 2100, 2200, 2300, 2400, and 0300 hours ($p < 0.05$). There was no significant difference between circulating cortisol in the young animals as compared to either the old control or old androgen-treated animals.

3.2 Testosterone administration significantly increases circulating DHEAS in a manner replicating the DHEAS rhythm of young males

Supplementation with DHEA resulted in a significant increase in circulating DHEAS in old males shortly after administration (Figure 5.2B-D, filled circles). Interestingly, although

DHEAS levels decline throughout the day, they again began to increase shortly after administration of T at 1900 h (Figure 5.2B). This increase was sustained throughout the night, resulting in higher DHEAS levels than baseline in the morning before DHEAS had been administered.

The supplementation paradigm was modified to further explore this phenomenon. If DHEAS rose throughout the night in the absence of exogenous T, we could conclude that this is a possible priming effect, by which exogenous DHEA stimulates the adrenal glands to produce more DHEAS for the following day. However, as shown in Figure 2C, DHEAS remained low throughout the night when no T was administered. A repeated-measures ANOVA with Greenhouse-Geisser correction for differences in sphericity was performed, comparing DHEAS between 1900 hours and 700 hours in animals receiving both T and DHEA as described versus animals receiving DHEA in the morning but no T at 1900 hours. This test revealed a significant effect of time ($F = 4.978$, $p = 0.026$), treatment ($F = 20.786$, $p = 0.003$), and treatment-by-time interaction ($F = 10.640$, $p = 0.002$). Thus, it appears that T itself increases circulating DHEAS.

Testosterone is only two steps beyond DHEA in the steroidogenic pathway (Figure I.4), and our dose of T needed to be very high in order to increase circulating levels to those of young animals. Therefore, we hypothesized that some T could have been back-converted to DHEAS, resulting in our observed gradual increase in circulating DHEAS. To address this, we replaced T with its more stable metabolite, DHT. If our administered T was being converted back to DHEAS, this treatment would not increase circulating DHEAS. However, this supplementation paradigm also resulted in an increase in circulating DHEAS above baseline levels starting at 1900 hours, resulting in a hormone pattern nearly identical to that of young control animals. A repeated measures ANOVA between the baseline and DHT-supplemented animals revealed a

significant effect of group (F = 12.944, p = 0.009), with DHT treatment resulting in an increase in circulating DHEAS starting at 1900 h. Taken together, these data suggest that androgen receptor activation, either at the level of the hypothalamus or the adrenal gland, increases DHEAS production in the male rhesus monkey.

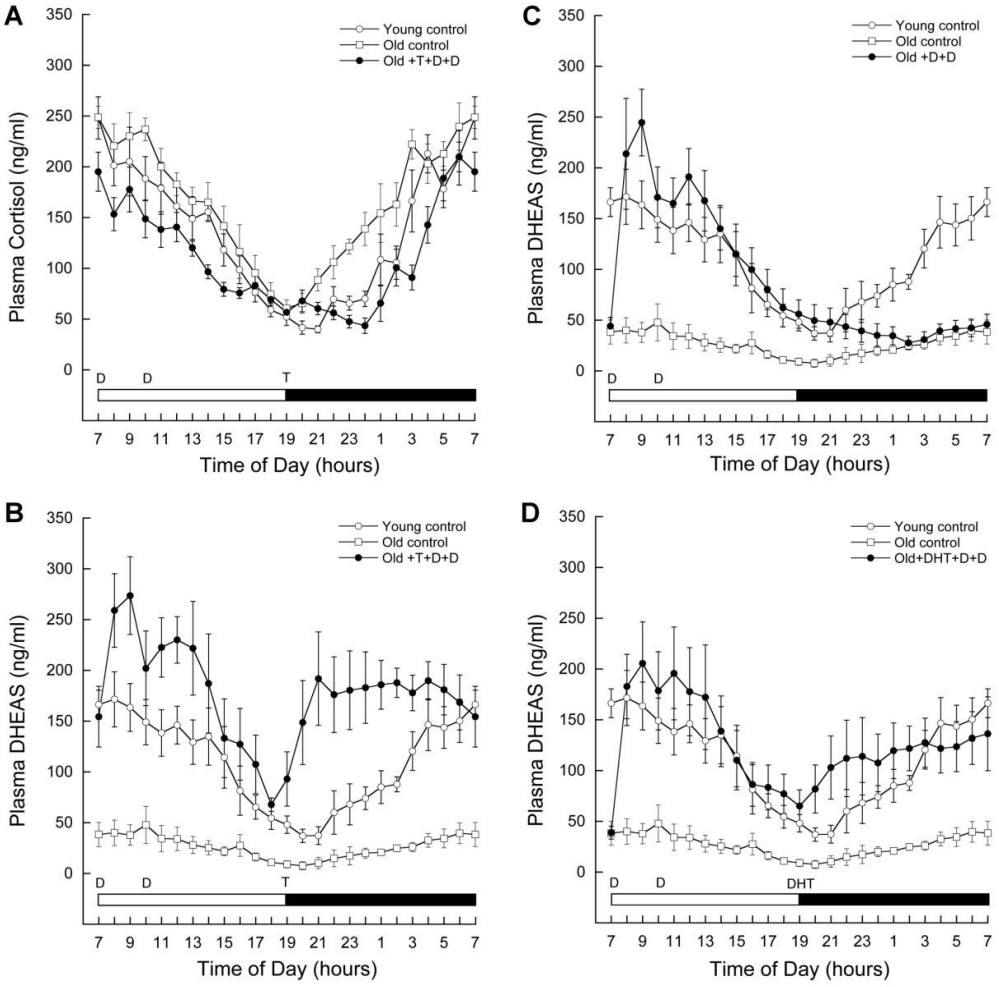


Figure 5.2. Testosterone supplementation significantly increases circulating DHEAS, but not cortisol, in aged male rhesus macaques. Serial blood samples were remotely collected from young (~7 year, n=4) and old (~21 years, n=4) males, as well as old males exposed to 5 days of various androgen supplementation paradigms (~21 years, n=5 in each supplementation paradigm). The samples were subsequently assayed for cortisol (A) and/or DHEAS (B-D). Each data point represents the mean, and vertical lines indicate the SEM. Time of day is indicated along the abscissa, and periods of light and darkness are represented by white and black bars, respectively. In each panel, the times of oral androgen administration are depicted as follows: D = DHEA (0.04-0.10 mg/kg body weight), T = testosterone (12 mg/kg body weight), DHT = 5α-DHT (5-10 mg/kg body weight). For reference the same DHEAS profiles from the young (open circles) and old controls (open squares) are depicted in panels B-D. Closed circles indicate hormone levels following (A, B) two doses of morning DHEA and one dose of evening T, (C) two doses of DHEA only, and (D) two doses of morning DHEA and one dose of evening DHT. The data demonstrate that androgen supplementation at an appropriate time of day can restore 24-hour circulating DHEAS levels in old males rhesus macaques. Importantly, they also demonstrate an unexpected stimulatory action of gonadal androgens on DHEAS. Statistical analysis is presented in the text.

3.3 Morning testosterone supplementation increases DHEA at a physiologically inappropriate time

As clinically-prescribed T supplementation is usually suggested to be administered in the morning, we examined the effect of morning T supplementation on circulating DHEAS levels in a small subset (n = 2) animals. Due to sampling limitations in old animals reached by repeated blood draws, young animals were used in this pilot study. As shown in Figure 5.3, T administration at 0700 h increased plasma DHEAS above levels seen at baseline, extending the DHEAS peak throughout the day.

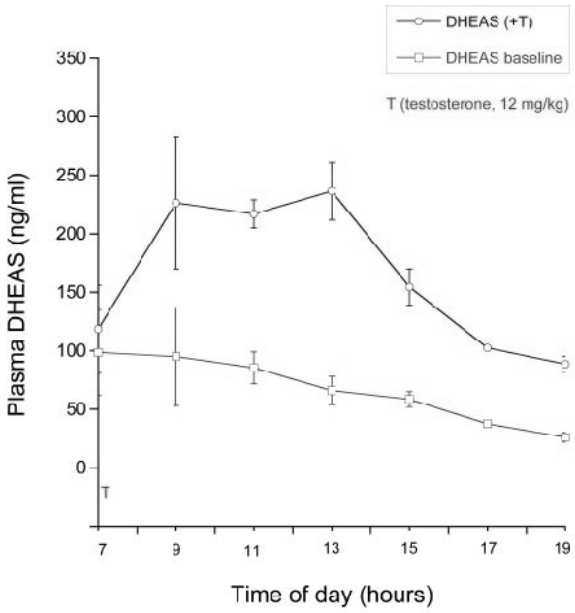


Figure 5.3. Testosterone supplementation increases circulating DHEAS regardless of time of administration. In a pilot study to assess the effect of T administration at a non-physiological time on DHEAS, two young male rhesus macaques were supplemented with 12 mg/kg T at 0700 h. This dose was sufficient to increase circulating DHEAS above baseline throughout the afternoon.

4. Discussion

This study examined the peripheral interactions between adrenal and gonadal hormones by monitoring plasma cortisol and DHEAS during a variety of androgen supplementation paradigms. Interestingly, we found that the administration of T resulted in an increase in circulating DHEAS. When T was given to old males in the evening to mimic the maximum levels found in young males, DHEAS gradually increased throughout the night in a pattern

similar to that seen in young males. This gradual increase combined with exogenous supplementation of DHEA in the morning resulted in a DHEAS profile that mimicked youthful levels. A proposed mechanism for this interaction is shown in Figure 5.4, with androgen receptor activation in the ZR potentiating the response of the ACTH receptor. Although we cannot rule out the possibility that our androgen supplementation paradigms acted further up in the HPA axis, it is unlikely that CRH or ACTH were affected as we saw no stimulatory effect of androgen on cortisol; like DHEA/S, cortisol production is stimulated by ACTH, but it is secreted primarily from the ZF rather than the ZR. Therefore, the observed effect is likely due to actions in the adrenal gland, specifically in the ZR. Also, because DHT administration also increased circulating DHEAS, this appears to be an androgen-receptor-mediated phenomenon.

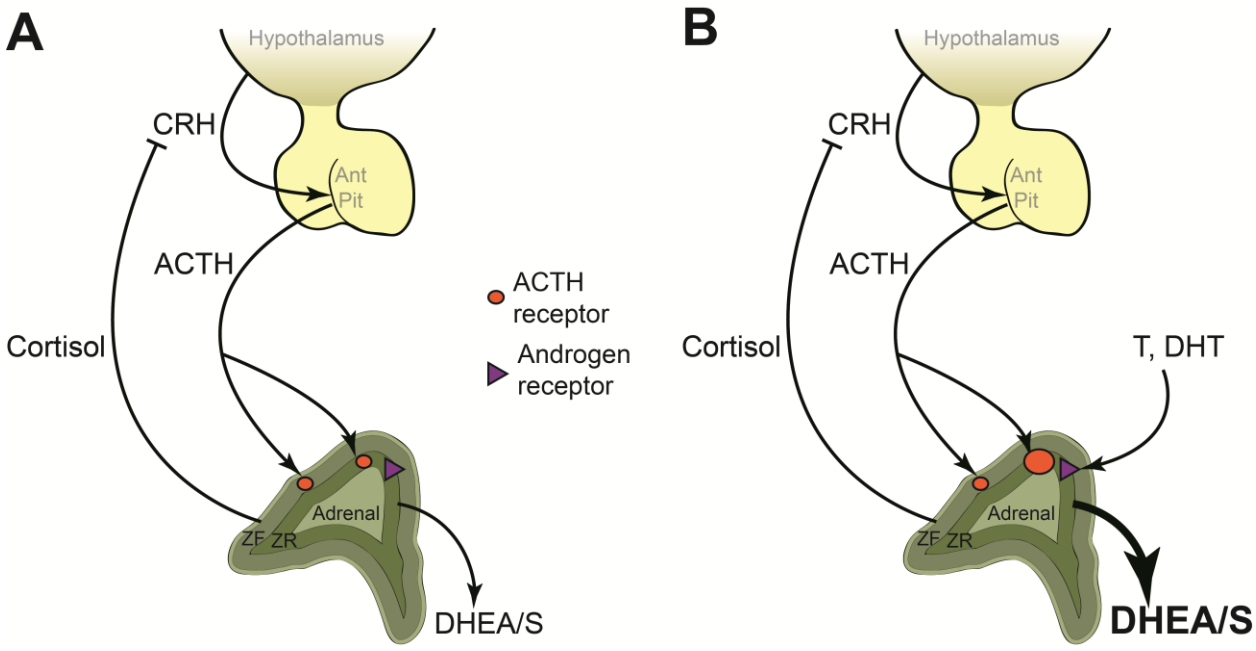


Figure 5.4. Proposed mechanism for the interaction between the male HPG and HPA axes. The hypothesized interaction between gonadal and adrenal hormones is shown. We propose that activation of the androgen receptor in the ZR potentiates the activation of the ACTH receptor, resulting in enhanced secretion of DHEA/S (B). This mechanism explains the results observed in the current study, as well as the sex differences in DHEA/S secretion, with males exhibiting significantly higher levels of DHEAS.

While this mechanism is interesting from the perspective of male aging, it also may shed light on sex differences in adrenal hormones, as women tend to have lower levels of DHEAS than men. If testosterone increases DHEAS by potentiation of the ACTH receptor, as has been suggested (Polderman et al., 1994), the higher levels of T in men may explain the higher levels of DHEAS, despite roughly equal circulating ACTH in men and women (Arva et al., 2000; Pasquali et al., 2002; Keenan et al., 2009; Sanchez et al., 2010; DeSantis et al., 2011).

Finally, these interactions emphasize the importance of focusing on the entire endocrine system in HRT paradigms, rather than the HPG or HPA axes alone. As these systems appear to interact significantly, both in the male as seen here and in the female as is observed during the menopausal transition and E-based HRT (Crawford et al., 2009; Pluchino et al., 2005 Lasley et al., 2002), the manipulation of one steroid may have significant and important primary effects on its own levels, but may also have an effect on other steroids, be it compensatory—as in the female—or facilitatory—as in the male.

Chapter 6
Sex differences in endocrine interactions

1. Introduction

The adrenal steroids DHEA and DHEAS are the most abundant hormones in the human and nonhuman primate circulation; however, levels of DHEA/S greatly differ between males and females despite no obvious differences in adrenal structure, and previously-published evidence suggests some factor outside the adrenal gland controls the greater secretion of DHEA/S in males (Fearon et al., 1998). The most clear-cut explanation for this difference may be the drastically different levels of sex hormones in males and females. In fact, previous work in perimenopausal women has shown that decreasing levels of estrogens correspond to increases in DHEAS (Lasley et al., 2011; McConnell et al., 2012), whereas estrogen replacement paradigms decrease DHEA (Pluchino et al., 2005). As DHEA/S is a precursor to estradiol (Labrie 1991), this may reflect a compensatory interaction between the HPG and HPA axes. This interaction of higher sex steroid levels dampening adrenal steroids, may be limited to females, as males with much higher levels of T, a precursor of estradiol, have greatly higher circulating DHEA/S. Also, as seen in Chapter 5, T supplementation induces increases in circulating DHEAS; thus, the effects of the primary gonadal hormones on adrenal DHEAS production differ dramatically between the sexes.

The current study sought to confirm the gender difference between circulating DHEAS in the rhesus macaque by examining expression of steroidogenic and steroid-related genes in the male and female adrenal gland. The results support the model of T enhancement of DHEAS interaction presented in Chapter 5.

2. Materials and methods

2.1 Experimental animals

The study used adult rhesus macaques (*M. mulatta*), and was approved by the Institutional Animal Care and Use Committee. The animals were cared for by the ONPRC DCM in accordance with the *NRC Guide for the Care and Use of Laboratory Animals*, and were caged indoors under controlled environmental conditions: 24° C temperature; 12-h light, 12-h dark photoperiods (lights on at 0700 h). Monkey chow was provided at 0800 h and 1500 h and was supplemented with fresh fruit and vegetables; fresh drinking water was available *ad libitum.* Ten adult (11-12 years) male and eight (11-12 years) female rhesus macaques were used to assess sex differences in circulating DHEAS levels. As described in previous chapters, these animals were maintained on a remote blood sampling system to monitor 24-hour rhythms of DHEAS and cortisol.

2.2 Tissue extraction and RNA quantitation

Six male and six female (~19 years) flash frozen adrenal glands were retrieved from the rhesus macaque aging archive resource at the ONPRC. Whole adrenal glands were homogenized and RNA was extracted using the Qiagen RNeasy Maxi Kit (Qiagen, Valencia, CA, USA). RNA was converted to cDNA using the RT^2 First Strand Kit (SA Biosciences, Qiagen, Valencia, CA). Samples were then loaded onto a custom PCR array plate from SA Biosciences, with genes chosen for involvement in steroid synthesis and steroid response. Genes included on the arrays are listed in Appendix A.3 and A.4. Two genes (CYB5R1 and CYB5R2) were not available for the PCR arrays and were analyzed separately using qRT-PCR (primer and probe sequences are provided in Appendix A). Quantitative PCR was performed with an Applied Biosystems

7900HT thermocycler (Invitrogen, Grand Island, NY, USA) and CT values were assessed using ABI sequence detection system software (version 2.2.1). The delta-delta CT method (Livak and Schmittgen, 2001) was used to transform CT values into linear form for statistical analysis. Following transformation to linear form as described above, relative gene expression was determined first by normalization to a set of three housekeeping genes (ALG9, GAPDH, and RPL13A) (Noriega et al. 2010). To perform a sex comparison, the average relative expression level of each gene for each sex was arbitrarily normalized to the average value in the males, thus defining the male expression level for each gene as 1. Student's *t*-tests were then performed, with sex as the independent variable and relative expression as the dependent variable. Twenty-two genes were identified as genes of interest (see Table 6.2), therefore to protect against Type I error, a Bonferroni adjustment was used to set the level of significance at α=0.0023.

3. Results

3.1 Male rhesus macaques display significantly higher levels of DHEAS than females

Cortisol and DHEAS showed well-defined 24-hour plasma profiles, both in the males and females (Figure 6.1). The plasma levels rose gradually during the night and reached a peak in the morning at about the time when the lights came on. Although mean cortisol levels were similar between the two sexes, mean and maximum DHEAS levels were significantly higher in males than in females ($p < 0.01$, Student's *t*-test.)

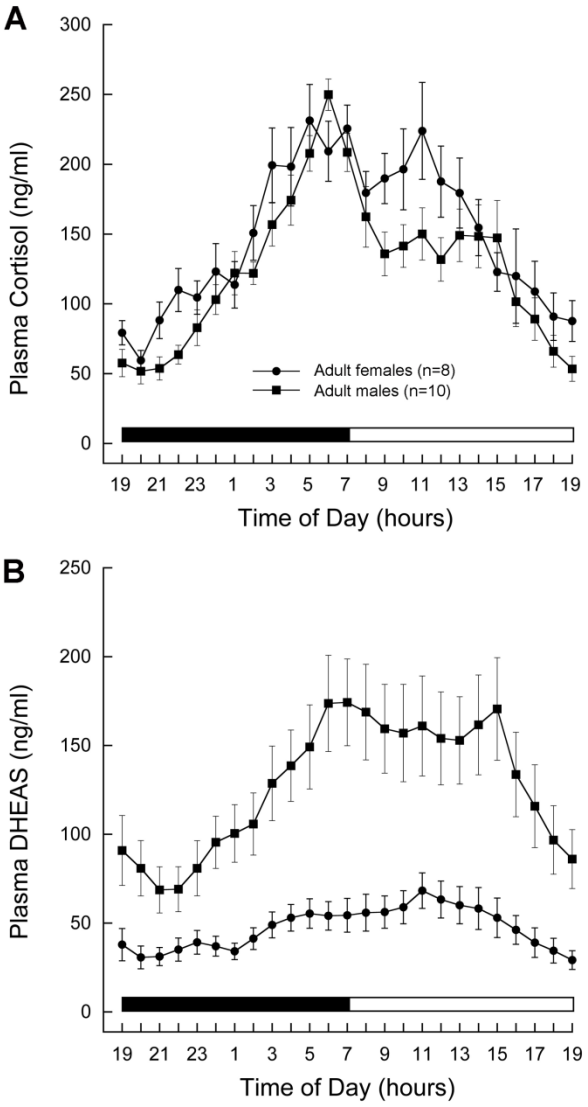


Figure 6.1. Twenty-four hour plasma rhythms in adult male and female rhesus macaques demonstrate a significant sex difference in DHEAS, but not cortisol. Twenty-four-hour plasma profiles of adrenal steroids in male and female rhesus macaques. Serial blood samples were remotely collected from adult (11-12 years) male (n=10) and female (n=8) animals, and assayed for cortisol (A) and DHEAS (B). Each data point represents the mean, and vertical lines indicate the SEM. Time of day is indicated along the abscissa, and periods of darkness and light are represented by black and white bars, respectively. In both sexes, cortisol and DHEAS levels rose gradually during the night and reached peak levels around the time of lights on in the morning. Mean cortisol levels were similar in the two sexes, whereas mean DHEAS levels were significantly higher in males than in females ($p < 0.01$, Student's *t*-test.)

3.2 Lack of sex difference in adrenal steroidogenic gene expression

Genes were chosen for inclusion on a custom PCR array based on their involvement in the steroidogenesis of DHEA, T, and E_2, as well as for responsiveness to hormones (Table 6.2). These arrays were used to evaluate gene expression in male and female rhesus macaque adrenal glands to determine if there are any baseline differences in steroidogenic potential. No genes approached a significant difference in expression between males and females, even before the Bonferonni correction for multiple comparisons.

4. Discussion

Research on adrenal sex differences in humans and nonhuman primates is limited, but some interesting observations have been made. The current finding that DHEAS differs dramatically between males and females corroborates the human literature (Labrie 1991), but to date no theories as to the mechanism of this difference have been adequately described. The differences in aging profiles of adrenal and gonadal hormones further complicates any potential interactions, as estrogen in females drops precipitously at the time of menopause, DHEA/S in both sexes declines slowly and consistently starting in the third decade (Labrie 2010), and testosterone in males decreases very slowly, gradually, and to a much lesser extent than other hormones (Harman et al., 2001; Feldman et al., 2002; Schlatt et al., 2008; Sitzmann et al., 2013).

While much work is yet to be done to study differences between male and female adrenal glands, early work suggests that the difference in adrenal output is not due to underlying physiological or genetic differences, but is influenced by testosterone stimulation of the adrenal glands. While sex differences in both circulating DHEA/S and adrenal physiology are seen in the marmoset, with respect to both size of the ZR and expression of steroidogenic genes, these differences are not observed in humans. In the marmoset, females secrete significantly more DHEA/S than males due to increased adrenal zonation and expression of cytochrome b5 (Pattison et al., 2007), while in humans the male and female ZR are similar in both size and cytochrome b5 expression (Dharia et al., 2005). Also, cultured human male and female adrenal glands respond with identical levels of DHEA secretion when stimulated by ACTH (Fearon et al., 1998). However, females receiving long-term treatment with T also show an increased release of DHEA in response to ACTH stimulation as compared to controls (Polderman et al.

Table 6.2. Male and female adrenal glands do not differ in steroidogenic enzyme expression

Gene Name	Encoding protein	Fold difference	Standard error	p value	Significance
AKR1C3	17β-hydroxysteroid dehydrogenase type 5	1.00	0.3	0.997	NS
AKR1C4	3α-hydroxysteroid dehydrogenase type 1	1.35	1.42	0.81	NS
AR	Androgen receptor	1.27	0.16	0.121	NS
TSPO	Translocator protein	1.20	0.16	0.225	NS
CYB5R1	Cytochrome b5 reductase 1	0.70	0.35	0.411	NS
CYB5R2	Cytocrhome b5 reductase 2	0.95	0.21	0.81	NS
CYP11A1/LOC708065	p450 side chain cleavage enzyme	1.07	0.19	0.289	NS
CYP17A1	Cytochrome p450 17A1	1.22	1.12	0.287	NS
CYP19A1	Cytochrome p450 19A1 (aromatase)	2.26	0.17	0.521	NS
CYP21A2	Cytochrome p450 21A2	0.89	0.2	0.161	NS
ESR1	Estrogen receptor α	1.31	0.21	0.223	NS
ESR2	Estrogen receptor β	0.73	0.25	0.597	NS
GNRH1	Gonadotropin releasing hormone type 1	0.86	0.25	0.594	NS
GNRH2	Gonadotropin releasing hormone type 2	1.14	0.26	0.429	NS
HSD11B1	11β-hydroxysteroid dehydrogenase type 1	0.79	0.25	0.223	NS
HSD3B2	3β-hydroxysteroid dehydrogenase type 2	1.33	0.28	0.713	NS
MC2R	ACTH receptor	0.90	0.21	0.503	NS
SRD5A1	5α-reductase type 1	1.15	0.24	0.878	NS
SRD5A2	5α-reductase type 2	0.96	0.21	0.207	NS
STAR	Steroidogenic acute regulatory protein	0.70	0.23	0.85	NS
STS	Steroid sulfatase	1.04	0.7	0.35	NS
SULT2A1	Sulfyl transferase type 2A1	1.25	0.16	0.671	NS

Table 6.2. qRT-PCR was performed on steroidogenic and steroid-related genes in male and female adrenal glands. Data are presented as fold difference of females as compared to males. No significant differences between males and females were observed for any gene examined.

1994; Polderman et al., 1995), suggesting that hormonal input from the HPG axis can manipulate adrenal output. Our current PCR results show no difference in steroidogenic gene expression or expression of steroid receptors, consistent with the above study; thus it is our hypothesis that the higher levels of DHEA/S seen in male humans and nonhuman primates is due to higher levels of T increasing the sensitivity of the ZR to ACTH, consistent with the proposed mechanism of interaction between HPA and HPG axes presented in Chapter 5.

One interesting endogenous manipulation of androgen interactions can be seen in the case of polycystic ovarian syndrome (PCOS), in which women experience high levels of T. A subset of women with PCOS with high levels of DHEA/S demonstrate increased DHEAS production when stimulated with ACTH (Moran et al. 2004). High levels of testosterone have in fact been implicated as a potential cause of PCOS (Zhou et al., 2005; Nisenblat and Norman, 2009); however, the impact of increased testosterone on circulating DHEA/S in women has yet to be studied extensively.

The current study, combined with the results presented in Chapter 5, suggest significant interactions between adrenal and gonadal interactions account for the sex difference observed in circulating DHEA/S in both humans and rhesus macaques. This further complicates the concept of HTs in the elderly, as men and women may respond differently to certain treatments based on endogenous levels of circulating hormones.

Part B Summary: Endocrinology as a whole-body integrator of aging physiology

In females, E_2 and DHEA/S appear to have a compensatory relationship which may account for some of the null effects seen in steroid supplementation paradigms in postmenopausal women. While we did not observe a regulation of circulating E_2 by DHEA supplementation, this may be masked by the conversion of supplemented DHEA such that E_2 of ovarian origin decreased but E_2 derived from supplemented DHEA increased. We did, however, observe a regulation of steroidogenic enzyme expression with E_2 treatment, such that increasing peripheral E_2 was associated with a decrease of central synthesis of steroids as well as the intracrine conversion of DHEA to E_2. Expression of several steroidogenic and steroid-regulatory genes were significantly correlated with cognitive performance, consistent with the hypothesis that locally produced E_2 contributes positively to learning and memory.

The current study of rhesus males demonstrates the opposite effect: exogenous T appears to increase serum levels of DHEAS in both old and young animals. This interaction could possibly result in a snowball effect of low T on markers of aging such as cognitive decline and frailty, as hypoandrogenism may result in lower levels of DHEAS. Thus, our double-androgen replacement paradigm demonstrates the importance of considering both the HPG and HPA axes in models of hormone therapy. This mechanism may also help to explain the sex differences observed in adrenal output of DHEA, which does not appear to be related to steroidogenic gene expression in the adrenal gland.

General Conclusion

Summary

The work presented herein elucidates a number of novel interactions betweenthe HPA and HPG hormonal axes and suggests that these interactions may influence cognition during aging. While DHEA supplementation in aged female macaques did not have significant cognitive effects, it was observed that cognitive performance was correlated with genes involved in steroidogenesis and the steroid response in the HPC and PFC. While the lack of cognitive effect may seem discouraging, this in part validates the rhesus macaque as a model for human cognitive aging, as a number of DHEA supplementations in elderly humans have shown no effects on memory. We show that this inefficacy may be due to a decrease in steroidogenic gene expression in the HPC, and thus targeting the steroidogenic pathway rather than merely replacing deficient hormones may prove to be a useful target for cognitive intervention. Also, a variation of traditional HRT using bioidentical E_2 induced significant changes in steroidogenic gene expression in the HPC, demonstrating a compensatory mechanism that may titrate local E_2 concentrations.

We have also demonstrated a novel effect of T in the male rhesus macaque, with T supplementation significantly increasing circulating DHEAS concentrations, an effect that we have isolated to the adrenal gland; however, there were no differences in steroidogenic gene expression in male and female adrenal glands, particularly in expression of cytochrome b5, the accessory protein that defines DHEA production in the adrenal gland. Hence, we propose that the sex difference in circulating DHEA is due to the higher circulating levels of T in males than in females, and a sensitivity of ACTH receptors in the ZR to androgen stimulation.

Future directions

The current studies are the first of their kind conducted in the rhesus macaque. While a large number of studies of neurosteroidogenesis have been performed in the rodent, the translational potential of these studies is questionable. This is due in part to the rodent adrenal gland, which does not produce significant levels of circulating DHEA/S, so that local synthesis of E_2 *de novo* may have a more significant impact on cognition than in humans, who can synthesis local E_2 from adrenal DHEA/S. Moreover, all of the genes necessary to synthesize E_2 from cholesterol are present in the HPC of the rhesus macaque. The additional identification of genes that are associated with more favorable cognition, particularly *HSD11B1*, *SRD5A1,* and *STAR*, offer valuable starting points for further investigating the role of intracrine sources of steroids on memory. This model, therefore, offers a rich opportunity to expand on the rodent literature. Future work in this regard should include the investigation of the effects of T supplementation on steroidogenic gene expression in the brain to parallel the HT studies presented here.

This work has demonstrated the importance of considering whole-body physiology in treating age-related decline. Nonetheless investigation must be completed to assess the full impact of single or combination bioidentical hormone therapy in the nonhuman primate as a model for human aging. Particularly, we have shown the importance of supplementing physiological hormones at physiologically relevant times. The administration of T in the morning, as is recommended to men prescribed T supplementation, resulted in an increase in DHEAS outside of its normal circadian rhythm. This is only one of many biological measures that vary throughout the day, and it will be important to assess the effects of time-appropriate

and time-inappropriate supplementation on factors such as sleep, attention, digestion, and immune function.

Appendix A: RT-PCR and qRT-PCR primers and probes

Table A.1 Primer sequences used in RT-PCR

Gene name	GenBank accession ID	Nucleotide sequences (5′ – 3′)
STS	XM_001088752	Forward AGGACAGGATCATTGATGGACG
		Reverse TGGCAAAGCATCCATTGGA
SULT2B1	XM_001111839	Forward CGCCCAGCTAATTTGTGTCCT
		Reverse TCAGAGCCTTGGTCCCTCTTCT
3BHSD1/2 (v.1–3)	XM_001113873	Forward CCACACGGTGACATTGTCAAAT
		Reverse CCCACATGCACATCTCTGTCAT
17BHSD1	NM_001047132	Forward GACCCATCCCAGAGCTTCAAA
		Reverse TGCGTTACACACCAGCACGT
17BHSD2	XM_001111794	Forward TCACATAACTCAGGCTGCCTCC
		Reverse CCATCCAGTTCCACAGCTGC
17BHSD3 (v.2)	XM_001105829	Forward AGGCCCTGCAAGAGGAATATAGAG
		Reverse CCTGACCTTGGTGTTGAGCTTC
17BHSD4 (v.1,3)	XM_001087837	Forward GTGGATCTTGCACCAACATCTGG
		Reverse CTCTGGCCTTCAGCCTGCCAC
17BHSD5 (v.1,2)	XM_001104543	Forward GAAGTAAAGCTTTGGAGGTCT
		Reverse CCATCGTTTGTCTCGCTGAGA
AROMATASE	XM_001082665	Forward CCAGCAGACCCAGGACTCTAAA
		Reverse CCAGGACCTGGTATTGAGGATG

Table A.2. qRT-PCR primer and probe sequences

Gene name	GenBank accession ID	Nucleotide sequences (5′ – 3′)
CYP11A1	XM_001096506	6FAM– TGCAGTGGCACTTGTA–MGB-NFQ
		Forward GAGGACATCAAGGCCAACGT
		Reverse TTCAGGTTGCGTGCCATCT
CYP17A1	NM_001040232	6FAM– TGAAGAAGAAGCTCTACGAGGA–MGB-NFQ
		Forward CCTTCCTGCTGCACAATCCT
		Reverse ACGGTTACGGTCACTGATGGTT
STS	XM_001088752	6FAM– CCAGTGCGACAGAGAAAAACAGGATAAGAGA–MGB-NFQ
		Forward CCTTCCTCCGGCCTGTCT
		Reverse AGCTTTGCCACATGCATCTG
SULT2B1	XM_001111839	6FAM– TTCTTCAGCTCCAAGGCCAAGGTGATC–MGB-NFQ
		Forward CAGTACAGCCCTCGCCTCAT
		Reverse GGGTTGCGGCCCATGT
3BHSD1/2 (v.1–3)	XM_001113873	6FAM– CATTGATGTCTTTGGTGTCACTCA–MGB-NFQ
		Forward AGGACGTCTCGGTCGTCATC
		Reverse GAGCTGGGTACCTTTCACATTGA
17BHSD5 (v.1,2)	XM_001104543	6FAM– CAGAAGCCGTGCGTGTGGATGG–MGB-NFQ
		Forward TGGAGGGCTTTGCTGAAGTCT
		Reverse GGTCCAGTCACCAGCATACAGA
AROMATASE	XM_001082665	6FAM– AATGCATGGACTTTGCCACTGAGTTGATTTT–MGB-NFQ
		Forward TAGCAGAAAAAAGACGCAGGATT
		Reverse CGTCAGGTCACCTCGTTTCTC
CYB5R1	NM_001257476.2	6FAM– TTCGGAGGRCCCGCC –MGB-NFQ
		Forward CTGGCTGTGGGCTCCTACTT
		Reverse TCGTCTTGTCCAGCAGTCGTA
CYB5R2	XM_002799661.1	6FAM– ACCCTGGACAGGCC–MGB-NFQ
		Forward CAGACCAGTTCGACCTGTGGTA
		Reverse GGAAGGTGCTCCTTGATCATG
GAPDH	XM_001105471	6FAM– TGAGCACCAGTGGTCTCCTCCGACT–MGB-NFQ
		Forward AAGGGCATCCTGGGCTACA
		Reverse GAAGAGTGGGTGTCGCTGTTG
ALG9	XM_001106180	6FAM– ACTGTCTTCCTGTTCGGG–MGB-NFQ
		Forward AACAGTGCCACAGAGCGAGAA
		Reverse CGATACCGCCTGGAGCACTA
RPL13A	XM_001115079	6FAM– CCAGGCAGTGACAGCCACCTTGG–MGB-NFQ
		Forward TCACGAGGTTGGCTGGAAGT
		Reverse GATCTTGGCTTTCTCCTTCCTCTT

Table A.3. Genes included on RT^2 Custom Array CAPQ11099

Gene Symbol	Encoding protein	Refseq #	RT^2 Catalog Number
GABBR1	Gamma-aminobutyric acid (GABA) B receptor, 1	XM_001097474	PPQ11071
GABBR2	Gamma-aminobutyric acid (GABA) B receptor, 2	XM_001110194	PPQ16906
GABRA1	Gamma-aminobutyric acid (GABA) A receptor, alpha 1	XM_001086287	PPQ00199
GABRA2	Gamma-aminobutyric acid (GABA) A receptor, alpha 2	XM_001100685	PPQ07712
GABRA3	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	XM_001099995	PPQ12491
GABRA4	Gamma-aminobutyric acid (GABA) A receptor, alpha 4	XM_001101231	PPQ07792
GABRA5	Gamma-aminobutyric acid (GABA) A receptor, alpha 5	XM_001109231	PPQ12880
GABRA6	Gamma-aminobutyric acid (GABA) A receptor, alpha 6	XM_001086065	PPQ03096
GABRB1	Gamma-aminobutyric acid (GABA) A receptor, beta 1	XM_001099985	PPQ12482
LOC696767	Gamma-aminobutyric acid (GABA) A receptor, beta 2	XM_001085849	PPQ02927
GABRB3	Gamma-aminobutyric acid (GABA) A receptor, beta 3	XM_001109060	PPQ12826
GABRE	Gamma-aminobutyric acid (GABA) A receptor, epsilon	XM_001093865	PPQ08233
LOC719723	Gamma-aminobutyric acid receptor subunit gamma-1-like	XM_001114347	PPQ17987
LOC697248	Gamma-aminobutyric acid receptor subunit gamma-2-like	XM_001087838	PPQ03267
LOC720390	Gamma-aminobutyric acid receptor subunit gamma-3-like	XM_001115799	PPQ18396
GABRP	Gamma-aminobutyric acid (GABA) A receptor, pi	XM_001093483	PPQ05859
GABRQ	Gamma-aminobutyric acid (GABA) receptor, theta	XM_001093973	PPQ08608
GABRR1	Gamma-aminobutyric acid (GABA) receptor, rho 1	XM_001095237	PPQ05595
GABRR2	Gamma-aminobutyric acid (GABA) receptor, rho 2	XM_001095465	PPQ05683
GABRR3	Gamma-aminobutyric acid (GABA) receptor, rho 3	XM_001087158	PPQ04218
GAD1	Glutamate decarboxylase 1 (brain, 67kDa)	XM_001082995	PPQ00300
GAD2	Glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)	XM_001101800	PPQ00299
GLS	Glutaminase	XM_002798995	PPQ69041
GRIA1	Glutamate receptor, ionotropic, AMPA 1	XM_001111339	PPQ14377
GRIA2	Glutamate receptor, ionotropic, AMPA 2	XM_001095129	PPQ00240
GRIA3	Glutamate receptor, ionotrophic, AMPA 3	XM_001088327	PPQ04049
GRIA4	Glutamate receptor, ionotrophic, AMPA 4	XM_001101031	PPQ08968
GRIK1	Glutamate receptor, ionotropic, kainate 1	XM_001100491	PPQ08899
LOC713545	Glutamate receptor, ionotropic kainate 3-like	XM_001111351	PPQ13986
GRIK4	Glutamate receptor, ionotropic, kainate 4	XM_001106948	PPQ09363
GRIK5	Glutamate receptor, ionotropic, kainate 5	XM_001086640	PPQ02143

GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	XM_001117773	PPQ00275
LOC715349	Glutamate [NMDA] receptor subunit epsilon-1-like	XM_001105525	PPQ15188
GRIN2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	XM_001088140	PPQ04163
GRIN2C	Glutamate [NMDA] receptor subunit epsilon-3-like	XM_002800617	PPQ69042
LOC717945	Glutamate [NMDA] receptor subunit epsilon-4-like	XM_002808205	PPQ19647
GRIN3A	Glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	XM_001107918	PPQ16174
SLC1A1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	XM_001085339	PPQ01633
SLC1A2	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	XM_001115008	PPQ00254
SLC32A1	Solute carrier family 32 (GABA vesicular transporter), member 1	XM_001089139	PPQ03989
SYP	Synaptophysin	XM_001106095	PPQ15438
GFAP	Glial fibrillary acidic protein	XM_001102095	PPQ13601
ALG9	Asparagine-linked glycosylation 9, alpha-1,2-mannosyltransferase homolog (S. cerevisiae)	XM_001106241	PPQ12085
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	XM_001105471	PPQ00249
RPL13A	Ribosomal protein L13A	XM_001115079	PPQ00210

Table A.4. Genes included on RT^2 Custom Array CAPQ11114

Gene Symbol	Encoding Protein	Refseq #	RT^2 Catalog Number
ACHE	Acetylcholinesterase	NM_001128088	PPQ13873
ADRA1A	Adrenergic, alpha-1A-, receptor	NM_001114733	PPQ13318
AKR1C3	Aldo-keto reductase family 1, member C3	XM_001104469	PPQ12623
AKR1C4	Aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4)	XM_001118631	PPQ19604
APOE	Apolipoprotein E	XM_001104482	PPQ14705
AR	Androgen receptor	NM_001032911	PPQ00190
BDNF	Brain-derived neurotrophic factor	XM_001089568	PPQ05954
TSPO	Translocator protein-like	XM_001108482	PPQ12798
CNR1	Cannabinoid receptor 1 (brain)	NM_001032825	PPQ00045
CREB1	CAMP responsive element binding protein 1	XM_001107192	PPQ10897
CREBBP	CREB binding protein	XM_001095225	PPQ08001
LOC708065	Cholesterol side-chain cleavage enzyme, mitochondrial-like	XM_001096506	PPQ10458
CYP17A1	Cytochrome P450c17	NM_001040232	PPQ00416
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	XM_001082665	PPQ00420
CYP21A2	Cytochrome P450, family 21, subfamily A, polypeptide 2	XM_001114240	PPQ16257
DRD1	Dopamine receptor D1	NM_001206975	PPQ69038
DRD2	Dopamine receptor D2	XM_001085571	PPQ01110
ESR1	Estrogen receptor 1	XM_001097228	PPQ00295
ESR2	Estrogen receptor 2 (ER beta)	XM_001101433	PPQ00014
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	XM_001106343	PPQ15536
GFAP	Glial fibrillary acidic protein	XM_001102095	PPQ13601
GNRH1	Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)	XM_001107290	PPQ00303
GNRH2	Gonadotropin-releasing hormone 2	NM_001034202	PPQ00323
GNRHR	Gonadotropin-releasing hormone receptor	XM_001109168	PPQ12712
GNRHR2	Gonadotropin-releasing hormone II receptor	NM_001032842	PPQ00065
GPER	G protein-coupled estrogen receptor 1	XM_001084531	PPQ02827
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	XM_001110531	PPQ00218
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	XM_001113717	PPQ13432
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A	XM_001083407	PPQ01664
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	NM_001032966	PPQ00302
HTR2C	5-hydroxytryptamine (serotonin) receptor 2C	XM_001101997	PPQ10516
KYNU	Kynureninase-like	XM_002798863	PPQ69039
MAP2	Microtubule-associated protein 2	XM_001109205	PPQ12253

MAPK1	Mitogen-activated protein kinase 1	XM_001089600	PPQ04161
MAPK3	Mitogen-activated protein kinase 3-like	XM_002802443	PPQ68666
MC2R	Melanocortin 2 receptor (adrenocorticotropic hormone)	XM_001090666	PPQ06000
MC4R	Melanocortin 4 receptor	XM_001088600	PPQ04181
MCHR1	Melanin-concentrating hormone receptor 1	NM_001032885	PPQ00132
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	XM_001097126	PPQ09241
NR3C2	Nuclear receptor subfamily 3, group C, member 2	XM_001099855	PPQ08303
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	XM_001107264	PPQ11559
PGR	Progesterone receptor	XM_001095317	PPQ00294
SIGMAR1	Sigma non-opioid intracellular receptor 1	XM_001096077	PPQ05417
SLC18A2	Solute carrier family 18 (vesicular monoamine), member 2	XM_001095699	PPQ05874
SLC1A1	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	XM_001085339	PPQ01633
SLC1A2	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	XM_001115008	PPQ00254
SLC6A2	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	NM_001033022	PPQ00307
SLC6A3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	NM_001032826	PPQ00046
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	NM_001032823	PPQ00043
SNPH	Syntaphilin	XM_001112820	PPQ15315
SRD5A1	Steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	XM_001083405	PPQ01744
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)	XM_001105329	PPQ08884
STAR	Steroidogenic acute regulatory protein	XM_001090472	PPQ04777
STS	Steroid sulfatase (microsomal), isozyme S	XM_001088752	PPQ10281
SULT2A1	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	XM_001113439	PPQ16626
SYP	Synaptophysin	XM_001106095	PPQ15438
TAC3	Tachykinin 3	XM_001115535	PPQ14287
TDO2	Tryptophan 2,3-dioxygenase-like	XM_002804253	PPQ69040
ALG9	Asparagine-linked glycosylation 9, alpha-1,2-mannosyltransferase homolog (S. cerevisiae)	XM_001106241	PPQ12085
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	XM_001105471	PPQ00249
RPL13A	Ribosomal protein L13A	XM_001115079	PPQ00210

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