AGE-RELATED CHANGES IN THE RHESUS MONKEY HYPOTHALAMUS

By

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Abstract: The hypothalamus plays a key role in maintaining homeostatic functions that change during aging, like reproduction and sleep-wake activity. Using a nonhuman primate animal model, which has the benefit of age-related hormone changes similar to those of humans as well as being diurnal and showing consolidated sleep patterns, I have assessed age versus hormone-related changes in the arcuate (ARC) nucleus and the suprachiasmatic nucleus (SCN) that control reproduction and sleep-wake activity, respectively. I have previously observed marked estrogen-dependent changes within the ARC of rhesus macaques during aging, which suggest that these neurons retain the capacity to respond to sex-steroids similar to young neurons (Eghlidi et al. 2010), but the male ARC has been largely unstudied in the context of reproductive aging. I will also present data on age and sex-steroid related alterations in another hypothalamic nucleus, the SCN, which controls age-related changes in sleep-wake activity rhythms. Using models of reproductive aging in females and males, I have attempted to elucidate the underlying molecular and cellular changes that are associated with reproductive function and perturbed circadian physiology in the elderly. In Chapter I, I performed an in-depth analysis of the effect of hormone therapy in the ARC nucleus of aging female rhesus monkeys using quantitative real-time PCR and PCR Array Plates. Using these quantitative approaches, I determined that the progesterone receptor and many other reproductive-related genes are still responsive to estrogen treatment, despite aging. In Chapter II, I characterized the organization and age-related decrease in output signals and a core-clock modifier protein in the SCN, and showed that hormone therapy did not have an effect on protein expression. In Chapter III, I show that the male ARC and SCN are resistant to attenuated testosterone levels during aging, however, I found significant

that clock genes and steroid receptors in the ARC and SCN have diurnal expression pattern and more highly expressed at night. In conclusion, the differences in reproductive aging between men and women relate to key differences in gene and protein expression in hypothalamic nuclei. In females, the ARC plays a central role in mediating estrogen feedback onto the brain and the dramatic loss of sex-steroids at the time of menopause have major consequences on reproductive-related gene expression. In males, the SCN and ARC have rhythmic gene expression in elderly animals, which may have implications for changes in behavioral rhythms in older males. Finally, genes in the ARC of females were particularly responsive to hormone therapy, which suggests that there may be benefits of using estrogen supplementation to treat physiological changes related to reproductive function and activity patterns in older women.

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General Introduction

Many physiological processes are impacted by aging, including reproductive function, circadian hormone patterns, and sleep-wake activity. Loss of reproductive hormones, age-related changes in 24-hour hormone profiles, and disrupted activity patterns have all been linked to age-associated diseases, as well as behavioral and cognitive deficits. One area of research that has been well studied for the treatment of age-related pathologies involves hormone therapies (HT) for women and men. In particular, hormone supplementation has been used to treat osteoporosis and hot flashes in menopausal women and other age-related pathologies in both males and females, such as behavioral and cognitive impairment, and decline of immune function (Messaoudi et al. 2011, Gore & Hayes 2015).

The hypothalamic-pituitary-gonadal (HPG) axis is conserved among mammals and controls development, reproductive function, and changes during aging (Goodman 2015). In both males and females, the HPG axis initiates and coordinates reproductive maturity at the time of puberty (Foster & Hilleman 2015, Plant et al. 2015, Prevot 2015). Later in life, reproductive decline occurs and this has varying effects on the HPG axis depending on the species and sex of the animal (Downs & Wise 2009, Gore & Hayes 2015)

In women, reproductive senescence typically occurs near middle-age as ovarian follicles are depleted, estrogens and progesterone are no longer released into the bloodstream, and reproductive function is lost. This also results in major alterations in the brain, in particular, within the hypothalamic arcuate (ARC) neurons that control the function of the anterior pituitary gland (Rance et al. 2009, Eghlidi et al. 2010, Gore &

Hayes 2015). The male reproductive axis is much more stable during aging. However, there is a dampened and disorderly LH pulse amplitude, enhanced pituitary sensitivity to GnRH antagonist treatment, and a reduction in the number of Leydig cell number with age (Kaufman et al. 1991, Veldhuis et al. 1992, Pincus et al. 1996, Mulligan et al. 1999, Takahashi et al. 2005, Veldhuis et al. 2005). These gradual, and less-extreme, changes within the HPG axis result in attenuated androgen levels and weakened reproductive function in the testes (Harman et al. 2001, Gore & Hayes 2015).

Another nucleus that has been strongly linked to the aging process is the suprachiasmatic nucleus (SCN), which coordinates timed daily rhythms in sleep-wake activity and 24-hour hormone profiles (Urbanski & Sorwell 2012, Saper 2013). Age-related changes in sleep-wake activity near the time of menopause have been reported (Guidozzi 2013, 2015) and there are circadian changes in testosterone rhythms in elderly males (Bremner et al. 1983). Lastly, both older females and males have perturbed physiological and behavioral cycles that have been well documented in the literature (Hofman & Swaab 2006). In the following sections I review what is known about reproductive aging and hormone therapy (HT) in females, reproductive aging in males, and circadian aging in men and women.

Aging and Hormone Therapy in Females

Menopause is defined as the last menstrual period and marks the end of reproductive function for women. Initially, there is an increase in follicle-stimulating hormone release just prior to the onset of irregular menstrual cycles during the

perimenopausal transition (Rance 2009). Irregular menstrual cycles occur as ovarian follicles are depleted and levels of inhibin, 17β-estradiol and progesterone decline. Loss of sex-steroid negative feedback onto hypothalamic neurons impacts the central nervous system and periphery in menopausal women (Rance 2009). Finally, postmenopause is defined as one year without a menstrual cycle (Soules et al. 2001). Outlined here, are the major characteristics of the transition to menopause, its central and systemic consequences, the hallmark clinical trials used to assess the benefits of HT on post-menopausal health consequences, advances in HT for menopausal women, and current animal models of menopause and HT.

The three primary changes during menopause are 1) ovarian failure, 2) loss of circulating sex-steroids, and 3) altered feedback within the HPG axis (Rance 2009). The rate at which ovarian follicle numbers decline with age has not been adequately characterized or quantified, but menopause is initiated when follicle numbers fall below a critical threshold (Faddy et al. 1992). During the menopausal transition women experience irregular menstrual cycles and lower circulating levels of inhibin, 17β-estradiol, and P (Harlow et al. 2012). At the later stages of menopause, in the absence of ovarian steroidogenesis, circulating 17β-estradiol and progesterone drop to castrate levels. The anterior pituitary increases release of gonadotropins, luteinizing hormone (LH) and follicular stimulating hormone (FSH), in response to the loss of 17β-estradiol and progesterone negative feedback onto the pituitary and hypothalamus. Neurons in the hypothalamic ARC that respond to 17β-estradiol in females include the kisspeptin, neurokinin B, and dynorphin A (known as KNDy neurons). With regard to menopause, these neurons are responsible for the negative feedback effects of 17β-estradiol on

gonadotropin releasing hormone's control of pituitary gonadotrophs and subsequent inhibition of LH and FSH. Kisspeptin (encoded by *KISS1*) and neurokinin B (encoded by *NKB*) are still responsive to the negative feedback effects of 17β-estradiol treatment, despite aging, in rhesus monkeys (Eghlidi et al. 2010, Eghlidi & Urbanski 2015). Thus, the negative feedback effects of 17β-estradiol are preserved in animals despite aging, suggesting that HT may have benefits on the central nervous system even in elderly women (Gore & Hayes 2015).

As reviewed above, ovarian failure disrupts the activity in the ARC and pituitary, which has detrimental consequences on the HPG axis. Centrally, the most common side-effects of menopause are hot flashes and sleep disturbances. Altered thermoregulatory response is a defining property of menopause in women, and recent studies have shown that hot flashes appear to be a result of changing circulating 17β-estradiol levels and KNDy neuron feedback onto inhibitory GABA neurotransmission in thermosensitive neurons of median preoptic area (Mittelman-Smith et al. 2012). More recently, research has also concentrated on the relationship between menopause and perturbed sleep. Estrogen treatment has been shown to improve sleep quality in postmenopausal women (Guidozzi 2013). While the basic science experiments for these human studies are lacking, we do know that the SCN expresses very few estrogen receptors (ERs) (Vida 2008) and it is likely that 17β-estradiol-sensitive nuclei that project to the SCN, like the mPOA and BNST, may act as conduits for 17β-estradiol's action on the SCN during menopause (De La Iglesia 1999). While hot flashes and sleep disturbance are serious side-effects of menopause, the physiological consequences, like heart disease, stroke, hormone-associated cancers, as well as

learning and memory deficits, were the targets of early clinical studies aimed at addressing the benefits of estrogen treatment in postmenopausal women.

In the early nineties the Women's Heath Initiative (WHI), a set of clinical trials using HT in post-menopausal women, was conducted by the National Institute of Health to investigate the physiological effects of menopause in women. The study compared placebo, estrogen, or estrogen and progesterone supplementation in post-menopausal women. The goal of the large study was to address a number of health consequences of menopause that were of interest at the time (i.e., cancer, osteoporosis, cardiovascular health, and cognition) and the potential benefits of treating with sex-steroids. However, the study was halted after only five years when women in the HT groups were showing higher incidence of breast cancer, heart disease, and other negative side effects relative to the placebo group (Nelson et al. 2002, Rossouw et al 2002).

The WHI had strengths in that it was a prospective study designed to look at potential benefits of HT on physiological and cognitive health in post-menopausal women. The study also had a large sample size and utilized commonly prescribed hormones therapies, Premarin and Prempro, at fixed doses, 0.625 mg of conjugates equine estrogen (CEE) and 0.625 CEE plus 2.5 mg of medroxyprogesterone acetate (MPA), respectively (The Writing Group for the Women's Health Initiative, 1998). In addition to assessing negative physiological consequences of menopause, the WHI memory study, which looked at the effect of HT on global cognitive function using the modified version of the Mini-Mental State Exam (MMSE), showed that CEE and CEE and MPA treated women's performance on tests of memory had not improved relative

to untreated controls. Furthermore, postmenopausal women aged 65 years or older that received CEE and MPA were at an elevated risk of developing dementia due to mild cognitive decline compared to placebo treated control subjects (Rapp et al. 2003, Shumaker et al. 2003). Additional follow-up studies revealed that the CEE treated group alone, which was comprised of hysterectomized women, did not perform worse than untreated controls, but showed no benefit from the CEE therapy on cognitive performance (Espeland 2004, Shumaker et al. 2004).

In light of these negative findings, new theories were developed that speculated that there may be a temporal window to benefit from CEE and MPA treatment that was not accurately assessed in the WHI study. The women in the WHI were between the ages of 53 and 79 with the average age ~73 years old and most of these women had been post-menopausal for a number of years. It was thought that perhaps the body and brain became desensitized to the effects of sex-steroids during the span of time between becoming postmenopausal and the initiation of HT. Another possibility is that women without estrogen in their system for 20+ years developed other health problems that estrogen had no effect on. In light of the findings from the WHI, it was proposed that there may be a critical window for initiating HT, which was closer to the time of their final menstrual period, in order to benefits from hormones (Brinton 2004, Maki 2006, Sherwin 2006). Support for this concept came from multiple lines of evidence including animal studies and clinical trials showing that HT appeared to be very effective in women treated near their final menstrual period (Maki, Maki 2006, Morrison et al. 2006).

The limitations of the experimental design in the WHI are now being addressed by a new clinical study, the Kronos Early Estrogen Protection Study (KEEPS). KEEPS,

headed by Dr. Mitchell Harman, is using a younger cohort of women (aged 42-58 years old) than was used in the WHI, and at the onset of the experiment women are no less than 6 months and no more than 36 months past their last menstrual period (Harman et al. 2005). This is in contrast to the women in the WHI who were far past their final menstrual period and may have been unresponsive to sex-steroids. Secondly, the KEEPS study used a lower dose of CEE than was used in the WHI. In response to the Nurse's Health Initiative, where they found an increased risk of stroke in women treated with the same dose used in the WHI (0.625 mg of CEE), the KEEPS study chose a lower dose of 0.4 mg. Furthermore, they also used an additional route of administration for sex-steroid therapy by delivering 17β-estradiol via a transdermal patch. The KEEPS study also tested the hypothesis that progesterone treatment may need to be delivered at a lower dose or in a cyclic manner as it may have prevented the benefits of CEE, potentially by competing for ERs or inducing progesterone receptors. In particular, MPA has been shown to negate the beneficial effects of CEE on cardiovascular health in humans and non-human primate models, whereas the natural and micronized form of progesterone does not (The Writing Group for the PEPI Trial 1995, Minshall et al. 1998a, Minshall et al. 1998b, Rossouw et al. 2002,). Thus, a smaller dose of micronized progesterone was given 12 days a month to mimic the hormone environment in young cycling women (Harman et al. 2005). Finally, the study used physiological markers indicative of menopause to screen subjects by confirming that all the women had low circulating 17β-estradiol levels (< 40 pg/ml) and high levels of FSH (735 ng/ml). While the WHI shed a negative light on HT the KEEPS study has started to provide some evidence that HT improves some parameters of behavior. The most convincing result

provided so far is the beneficial effects to affective behavior (Gleason et al. 2015). Women who received 4 years of 0.45 mg oral CEE plus 200 mg MPA per day showed improvements in depression and anxiety symptoms. This was in contrast to women that received placebo patches and pills and a third group of women that received transdermal patches that delivered 50 μg 17β-estradiol plus 200 mg of MPA per day. Similar to the WHI, the KEEP study showed that cognitive performance did not improve in women treated with hormones near the time of their last menstrual period.

The neuroprotective benefits of a class of drugs, selective estrogen receptor modulators (SERMs), are now being explored for their effects on the physiological consequences of menopause. Since the mechanism of action of sex-steroids on their target tissues can vary, the change in response of receptors in different tissues following menopause may require a more targeted form of treatment that we can address pharmacologically. SERMs are a novel treatment method by which ER activation is promoted or reduced in a tissue specific manner. For example, Raloxifene is a benzothiophene derivative that antagonizes estrogenic activity in the breast and mammary gland to prevent cancer cell proliferation (Clemens et al. 1983, Wakeling et al., 1984). In addition, Raloxifene mimics estrogen's action to prevent osteoporosis by protecting bone mineral density and decreasing serum cholesterol levels (Jordan et al. 1987, Black et al. 1994, Delmas et al. 1997).

SERM have the ability to bind to ERs in a tissue specific manner. For example, Raloxifene binds ERs in the bones and antagonizes ERs in breast tissues via controlling the formation of ERα and ERβ homodimers as well as ERαβ heterodimerization, preventing their binding to estrogen response elements in the DNA-binding domain

(Kuiper & Gustafsson 1997, Heringa 2003). Secondly, SERMs can regulate conformational changes of ERs on ligand binding, such that the SERM receptor complex is a continuum of unique conformations that confer their role as either analogous to estrogen or antiestrogenic (Riggs & Hartmann 2003). Finally, SERMs have the ability to cause differential expression and binding of coregulator proteins, such as the recruitment of corepressors to ER target promoters in the mammary cells (Shang & Brown 2002, Riggs & Hartman 2003).

In animal models, sex-steroid treatment continues to improve age-related deterioration in brain structure, physiological, and cognitive function (Garcia-Segura & Balthazart 2009). While much of this body of literature has been conducted in rodent models, the use of the non-human primate provides some advantages over studies in rodents and will also be discussed.

Studies on the neuroendocrine changes in age-associated reproductive quiescence in mice have shown that they too exhibit a decrease in the number of ovarian follicles during reproductive aging (Bellino & Wise 2003). However, only about one quarter of aged female mice or rats show complete reproductive quiescence similar to postmenopausal women, while the remaining three quarters spontaneously transition to an anovuatory phase of constant estrus characterized by high serum 17β-estradiol and low progesterone levels followed by persistent diestrus (Finch et al. 1984). Furthermore, there is an additional population of neurons located in the anteroventral periventricular (AVPV) region in the rodent brain that has not been shown to serve the same function in primates (Downs & Wise 2009). These neurons express kisspeptin and respond to 17β-estradiol positive feedback to the ARC by initiating the preovulatory

LH surge (Smith et al. 2005, Adachi et al. 2007). AVPV kisspeptin neurons also show an age-related decline in expression prior to reproductive inactivity in rodents, representing another difference between rodents and humans during reproductive aging (Downs & Wise 2009, Neal-Perry et al. 2009, Lederman et al. 2010).

Given the dissimilarities between rodent models and humans in many key features of menopause, the non-human primate may be a more suitable model for studying the impact of reproductive aging on the human brain and body. Aged female rhesus macaques have the same hormone profiles as menopausal women (Downs & Urbanski 2006) and display the same neuroendocrine changes in the brain due to the loss of sex-steroid negative feedback onto the hypothalamus following ovarian failure (Nichols et al. 2005). The neurons that respond to sex-steroids in primates are located in the ARC nucleus, which is functionally the same region as the infundibular population of neurons located in the human hypothalamus (Rance 2009). Similarly, some non-human primates naturally go through menopause, although it should be mentioned that this takes place very late in their life span and many non-human primate studies use a surgically induced model of menopause by bilaterally removing the ovaries (Eghlidi & Urbanski 2015). The high cost of non-human primate research may be prohibitive in some cases, but the translational benefits are often substantially better when compared to using rodent models.

More recently, Oregon National Primate Research Center (ONPRC) neuroscientists (Drs. Henryk Urbanski, Steve Kohama, and Martha Neuringer) designed experiments to utilize the non-human primate model to study the critical theory hypothesis, which suggests that hormone therapy may be more beneficial if

administered near the time of the final menstrual period (Maki 2006). The animals were divided into four groups, young intact regular cyclers, old intact irregular cyclers, bilaterally ovariectomized (OVX), and OVX +17β-estradiol (E) treated animals (Figure 1). The animals that were OVX were allowed to recover for 2 months and then the OVX + E group received subcutaneous silastic implants containing crystalline 17β-estradiol, which produced circulating levels of around 200 pg/ml, similar to the late follicular phase of the menstrual cycle, for all 4 years of the study (Knobil 1999). The animals were part of the experiment for about 4 years and during this time ONPRC neuroscientists were able to measure physiological and behavioral responses to HT and conduct post-mortem tissue molecular studies, some of which are presented in Chapters I and II of this Dissertation.

Animal studies have provided us with models to test the benefits of HT on the consequences of menopause and have provides the bases to assess pharmacological therapies prior to clinical trials in human. Both human and animal studies demonstrate that menopause has consequences on physiological and cognitive health. Furthermore, there is more evidence suggesting that careful control of the temporal (critical period hypothesis) and spatial (SERMs and SARMS) delivery of hormone therapies may protect against the negative health consequences associated with the WHI.

Male Reproductive Aging

Reproductive aging in males is less dramatic than menopause in females and is characterized by a gradual decline and a dampened 24-hour rhythm of testosterone (T),

variable changes within the HPG axis, and little to no change in gametogenesis (Bremner et al. 1983, Tenover et al. 1988, Winters & Atkinson 1997, Harman 2001, Veldhuis et al. 2004, Sartorius & Nieschlag 2010). In this section, I review a current definition of aging in males, the impact it has centrally and in the periphery, the use of HT in males, and animal models of male aging.

There is a common belief that males undergo an andropause similar to their female counterparts that go through menopause. However, that does not appear to be the case because the reproductive axis of males is largely unaffected by aging. On one hand, there is a gradual decrease in testosterone levels and other hormones; such as dehydroepiandrosterone (DHEA), melatonin, and growth hormone, that have been associated with age-related health problems, such as skeletal and muscle deterioration, and cognitive decline (Moffat et al. 2002, Liverman & Blazer 2004, Gore and Hayes, 2015). On the other hand, hormones like FSH and LH are released normally or exhibit slight increases despite aging, and are not associated with age-related decline (Zirkin & Chen 2000, Hardy & Schlegel 2004). The variable changes in steroidogenesis and reproductive stability of males during aging have made a clinical diagnosis of reproductive decline difficult (Morales et al. 2000). Simply put, male reproductive aging is associated with a decrease in circulating testosterone levels that has been associated with physiological, behavioral, and cognitive consequences in elderly men. However, this decrease in sex steroid levels is much more gradual and less extreme than that observed in postmenopausal women.

T levels in men begin to decline around the age of 30 and gradually decrease as men get older (Hardy & Schlegel 2004). Harman and colleagues assessed age-related

changes in serum T and sex hormone binding globulin (SHGB) in the Baltimore Aging Study (Harman et al. 2001). They collected serum samples from 890 men over 6 months and reported decreased serum T and increased SHGB as men got older. In this study, serum T decreased by 0.111 nmol/l/year or 3.17 ng/dl in longitudinal and cross-sectional comparisons, respectively. Another large study of age-related changes in male hormone levels was the Massachusetts Male Aging Study. A cross-sectional analysis of a cohort of 1,700 men showed that there was about a 1.2% decrease in free T per year, whereas total T decreased 0.4% per year of age in a cross-sectional analysis. A follow up experiment revealed that total T decreased by 0.8% per year, whereas free and albumin-bound T decreased about 2% per year in cross-sectional analyses (Feldman et al. 2002). The study also reported that DHEA, DHEAS, cortisol, and estrone decreased, whereas DHT, LH, FSH, and prolactin gradually increased. At baseline, these men were between 40-70 years old and hormone data was documented yearly for about a decade.

Decreased T levels is the result of altered steroidogenesis in the testes, but there have also been reports of changes in gonadotropin signaling. In general, there is reduced secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which results in declining LH secretion from the anterior pituitary gland (Gore & Hayes 2015). However, there have been somewhat mixed results on the changes in LH levels in aging men. Studies have reported both small to no increase in LH (Zirkin & Chen 2000, Feldman et al. 2002, Hardy & Schlegel 2004, Gore & Hayes 2015). In aging men with hypogonadotropic hypogonadism, circulating LH levels are suppressed only following T therapy (64 years or older) (Winters & Atkinson 1997). These data suggest

that the systems involved in the negative feedback effects of T are sensitized with age and may explain the discordant results on altered LH levels during aging. While FSH levels have been reported to increase with age as a result of decreased negative feedback of inhibin from the testes, overall, there appears to be more variable effects on the HPG-axis in aging men as compared to aging women (Zirkin & Chen 2000, Gore & Hayes 2015).

As mentioned earlier, age-related changes in circulating T levels yielded mixed results that were likely due to the diurnal expression pattern of its release over the 24-hour day (Bremner et al. 1983, Tenover et al. 1988). Studies conducted in the morning have reported that T levels decreased in elderly males (Vermeulen et al. 1972). Another study, conducted in the afternoon, showed that T levels remained stable despite aging (Baker et al. 1976). Eventually, an experiment that measured T levels across the 24-hour day revealed that T has a nocturnal peak and the circadian pattern of T was lost with age (Marrama et al. 1982, Bremner et al. 1983, Tenover et al. 1988, Cooke et al. 1993). In summary, the male reproductive axis never makes it to an andropause as women do a menopause, however, T and other androgens, DHEA, and DHEAS, do show a partial age-related decline in men (Feldman et al. 2002).

There are some examples of how the reproductive axis of men resembles that of women and can influence age-related diseases and behavior. First, the HPG axis in males is organized similarly to that of females. The KNDy neurons are located in the ARC nucleus and project to GnRH neurons, which control the release of LH and FSH from the anterior pituitary, as described in rodent studies (Winters & Atkinson 1997). Gonadotropins circulate through the blood stream and drive steroidogenesis and sperm

production in the testes (Gore & Hayes 2015). Although male reproductive aging is just beginning to be studied at a systems level, there is mounting evidence that dysregulation in the HPG axis may play a role in age-related changes in reproductive function and health. First, T loss has been associated with altered skeletal and muscle function (Liverman & Blazer 2004). Changes in bone density were a big part of the WHI and appear to be as much a concern for elderly men as women. Second, memory performance declines with age and correlates with changes in serum free T level in elderly men (Moffat et al. 2002). Finally, Alzheimer's disease (AD) has been directly linked to declining testosterone levels with age. Elderly men with AD had lower T levels compared to controls, and the decrease occurs before cognitive impairment becomes clearly established (Rosario 2006).

A meta-analysis of placebo controlled experiments on T therapy revealed that it may have a variety of health benefits in men (Liverman & Blazer 2004). T therapy was found to improved metabolic, skeletal, cognitive, behavior, and sexual function in men. However, the WHI study disclosed several adverse side-effects of HT in women. Similarly, there are data to suggest that T therapy in men may also have some negative health consequences. The available information on the effects of T therapy in men suggests that there are consequences on health parameters associated with prostate cancer and cardiovascular health. Slightly alarming, T therapy delivered at 10-20 mg/day in older men has been linked to elevated risk for prostate cancer (Bhasin et al. 2003, Liverman & Blazer 2004). Furthermore, elevated levels of serum T, and not other hormones like DHEA, have been directly associated with prostate cancer in men from the Baltimore Longitudinal Study of Aging (Parsons et al. 2005). Similarly, a recent

meta-analyses showed that hemoglobin and hemacrit were elevated in adult men that received T therapy (Fernandez-Balsells et al. 2010). There is the potential for elevated hemoglobin and hemacrit levels to impair circulation and contribute to abnormal blood clotting. Furthermore, the available studies did not assess the long-term consequences of T therapy on health and there is potential for these side-effects to lead to more serious health conditions following long-term use of T. Future studies on T therapy should look carefully for side-effects on cardiovascular health, especially in subject that have a history of heart disease.

Overall, the use of T therapy for aging men is way ahead of the scientific data on the benefits and risks of its use. In 2002, there were more than 1.75 million prescriptions for T-related therapies for men, which was one third more than the previous year (Livermann & Blazer 2004). There have been very few studies on T therapy in men and the duration of these experiments were short with varying end measurements and inadequate controls (Liverman & Blazer 2004).

When it comes to studying age-related changes in males there are various animal models that have shown potential. For reproductive aging experiments, the rodent models offer the benefit of being short-lived species, less expensive to maintain for long periods of time, and less genetically diverse, which allows for more controlled experimental use. For these reasons, we have learned a lot about male aging from: 1) the Brown Norway rat that ages similarly to men, and 2) rodent experiments on the effects of androgens on the hypothalamus.

The Brown Norway rat, which recapitulates the primary symptoms associated with reproductive aging in men, is perhaps the best rodent model of male reproductive

aging. Brown Norway rats have altered steroidogenesis in the testes and decreased circulating T levels and they have stable LH levels as they get older (Zirkin & Chen 2000, Syntin et al. 2001, Chen et al. 2002). While LH levels remain stable, there are changes in LH pulse amplitude similar to reports in humans (Chen et al. 2002). In vitro experiments in the Brown Norway rat have reported a reduction in GnRH-release by hypothalamic neurons (Wang et al. 2002). Similar to reports in female studies, GnRH neurons are expressed in the hypothalamus of the aging Brown Norway males (Witkin 1987). However, a reduction in the inputs to GnRH neurons in males has been shown using electron microscopy (Witkin 1987).

In mice, recent studies on the effects of T on the HPG-axis have demonstrated that they share a similar organization to females, where sex-steroids coordinate gonadotropin release through KNDy-related pathways. T is rapidly converted into estradiol via aromatase and then acts through ERs, or it is converted to 5α-dihydrotestosterone (DHT) via 5α-reductase and then acts through ARs, both of which are located on KNDy expressing neurons in the ARC. Studies using gonadectomized (GDX) male mice have shown that the latter pathway appears to be mainly a male feature (Smith et al. 2005, Navarro et al. 2011).

The non-human primate model has more recently been used to study male aging and offers the benefits of having similar neural correlates of physiological functions to humans, a long life span with diurnal sleep-wake activity patterns similar to humans. Moreover, non-human primates can also be maintained in a more controlled experimental environment. In male monkeys, T has been shown to have negative feedback effects on GnRH-release within the hypothalamic-pituitary axis and

gonadotropins synchronize reproductive function and steroidogenesis in the testes (Plant & Dubey 1984, Plant 1986). Similarly, T treatment in castrated adult male monkeys reinstates negative feedback to *KISS1* in the ARC and suppresses LH release from the anterior pituitary (Shibata et al. 2007). Nevertheless, GDX male models do not recapitulate the hormone environment of aging males that still have plenty of circulating sex-steroids despite aging.

There has been very little work on age-related changes in T levels in male monkeys (Gore and Hayes 2015). More recently, studies from Dr. Henryk Urbanski's lab have used remote blood sampling to measure serum hormone profiles across the 24-hour day in aging male rhesus monkeys. Similar to the discovery of Bremner et al. in 1983, Dr. Urbanski observed a 24-hour rhythm in T levels in adult males that was perturbed in elderly male monkeys (Urbanski & Sorwell 2012). The 24-hour blood sampling revealed a clear age-related decline in the average T and DHEAS levels between adult and aged animals (Urbanski & Sorwell 2012, Urbanski et al. 2014).

Dr. Urbanski's most recent work has focused on the impact of hormone supplementation in aging male monkeys. First, he designed an androgen supplementation paradigm for elderly male monkeys to receive a nighttime dose of T and two morning doses of DHEA to mimic the 24-hour hormone profiles found in healthy young males (Urbanski et al. 2014). This paradigm, significantly improved 24-hour hormone profiles in aged males as compared to baseline (Urbanski et al. 2014). The most striking differences were found in the 24-hour hormone profiles of DHT and estradiol, which T is converted into (Urbanski et al. 2014). This experiment was later expanded to include young intact, old intact, and old intact animals receiving T and

DHEA for 6 months in order to study physiological and behavioral parameters and molecular changes in the brain (Figure 2). Some of the most recent molecular data from this experiment will be presented in Chapter III of this Dissertation.

Male aging is best characterized by a subtle decline and dampening of 24-hour hormone rhythms, particularly in T. Whether or not HT has any benefit to health and brain function remains to be determined. Recently, T and DHEA therapy in aged male rhesus monkeys improved working memory and it remains to be determined if this same benefit would occur in elderly men receiving HT (Unpublished data, presented at Society for Neuroscience Meeting, 2015).

Changes in Circadian Behavior during Aging

It is clear that sleep, like nutrition and exercise, is a critical factor for healthy living. As we age, there is a deterioration in circadian rhythms, disrupted sleep, changes in sleep-wake activity, and reduced functioning during the daytime (Bliwise 1993, Myers & Badia 1995, Youngstedt et al. 2001, Huang et al. 2002). In more recent years, there has been debate as to whether women and men age similarly, with regard to activity and sleep patterns. One investigator has provided evidence suggesting that women experience sleep disturbance twice as often as men with advanced age (Guidozzi 2013, Guidozzi 2015). It may be that the differences in reproductive aging between men and women play a role in age-related changes in circadian behavior. Here, I present the information on known changes in the central clock mechanism in the brain, how 17β-estradiol influences clock mechanisms in females, and how T influences clock

mechanisms in males. Together, these studies provide compelling evidence that there are clear differences in how sex-steroids influence the clock mechanism of females versus males

In mammals, the timing system is coordinated by a hierarchical network made up of a self-sustained “master” pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral oscillators located in nearly every cell in the body (Welsh 2010, Albrecht 2012). The SCN synchronizes extra-SCN oscillators primarily though 1) neuropeptide signaling factors (Antle & Silver 2005) and 2) humoral (Reddy et al. 2007, Sellix et al. 2012, Gerber et al. 2013) and neural signals (Vujovic et al. 2008). Furthermore, peripheral clocks can exert influence over the phase of other peripheral oscillators, such as the adrenal gland over the core-clock rhythm in the liver (Albrecht 2012).

Currently, our knowledge about the molecular clock mechanisms in mammals is based on studies in rodent models. The core-clock genes cycle in a 24-hour coupled transcriptional/translational feedback loop consisting of stimulatory, inhibitory, and modifier genes, and their protein products that together take approximately 24-hours to cycle (Reppert & Weaver 2002). The core-clock genes in the cycle consist of *BMAL1* (Brain and Muscle ARNT-Like 1/ARNTL) and *CLOCK* (Circadian Locomotor Output Cycles Kaput) that heterodimerize to form the positive “arm”, which binds to E-box motifs located on the genes in the inhibitory “limb” made up of Period (PER1,2) and Cryptochrome (CRY1,2) (Reppert & Weaver 2002, Ko &Takahashi 2006). Inhibitory feedback by PER and CRY proteins block DNA binding by BMAL1 and CLOCK, which results in reduced PER and CRY proteins to allow BMAL1 and CLOCK to resume

transcription of *PER* and *CRY*. Together with posttranslational modification of the basic feed-back loop by Casein Kinases (CKIδ,ε), RAR-Related Orphan Receptors (RORα,β,γ) and Nuclear Receptor Subfamily 1, Group D (REV-ERBα,β) of the rodent cycle has a period of approximately 24-hours (Schibler 2007).

The circuitry that controls sleep-wake activity is primarily located in the hypothalamus. The SCN receives information about the time of day from ganglion cells in the retina that project to the SCN via the retinohypothalamic tract. There are two principle subregions of the SCN in mammals, the core that contains VIP neurons in ventrolateral region right above the optic chiasm and the shell AVP neurons that are located in the dorsomedial region in sheathing the core (Saper 2013). Synchronously generated circadian activity depends on VIP signaling within the SCN (Maywood et al. 2006). The VIP neurons send projections to VPAC2 receptors on AVP neurons in the shell and together the core and shell regulate circadian behavior. VIP coordinates timing via VPAC2 receptors that are expressed throughout the SCN as well as in other nuclei, to maintain robust circadian rhythms and photic-entrainable responses to the environmental changes (An et al. 2012, 2013).

Age-related changes in physiological rhythms are associated with deterioration in the output signals of the master oscillator in the SCN. In rodents, aging results in desynchrony of peripheral oscillators from the SCN (Davidson et al. 2008) and a shift in the phase and amplitude of core-clock genes (Valentinuzzu et al. 1997, Weinert et al. 2001, Kolker et al. 2003, Wyse & Coogan 2010, Ando et al. 2010, Duncan et al. 2013). Moreover, multiple health consequences associated with aging (i.e., perturbed sleep-wake activity, AD, and Huntington's disease) have also been linked to the loss of a

hierarchical coordination of the timing system (Hastings et al. 2008, Bass & Takahashi 2010, Delezie & Challet 2011).

The SCN also coordinates timing via a multitude of other output signals, like prokineticin 2 (PK2), neuropeptide Y (NPY), and calbindin (CALB). So far, there is little information about the neurons that make up the SCN of non-human primates. Urbanski and colleagues recently described the organization and 24-hour expression pattern of PK2 mRNA in the rhesus monkey SCN (Burton et al. 2016). Additionally, Moore has described the organization VIP, AVP, and NPY in the macaque SCN, however these descriptions were observational versus quantitative (Moore 1993). With regard to aging, a temporal shift and decrease in VIP expression over the 24-hour day between young and old animals has been described in rodents and in only one nocturnal primate species, the mouse lemur (Kawakami et al. 1997, Krajnak et al. 1998, Aujard et al. 2006). The expression of other populations of neurons, like calbindin (CALB) neurons, remain stable despite aging, but decreased protein expression in the nucleus has been reported in the mouse lemur (Cayetanot et al. 2007).

While age-related changes in clock mechanisms and physiological rhythms have been described, little is known about how differences in reproductive aging between men and women play a role in the desynchronization of these processes during aging. In older women, the profound hormonal changes that occur near the time of menopause are likely to contribute (Woods & Mitchell 2010, Guidozzi 2013). There is molecular data to suggest that 17β-estradiol may influence timing mechanisms for example, genetic manipulations of core-clock genes significantly alter the estrous cycles of rodents (Chappell et al. 2003, Miller et al. 2004). There have also been studies showing that

various estrogen therapies can improve sleep quality in postmenopausal women (Scharf et al. 1997, Moe et al. 1999, Moe et al. 2001, Guidozzi 2013, Guidozzi 2015). The attenuation of sex-steroids near the time of menopause may be related to age-associated uncoupling of peripheral clocks to the SCN 1) by changing input on the SCN, either directly or through extra-SCN brain areas that feedback onto the SCN and 2) by loss of action at local oscillators. Because the SCN of rodents and humans express very few ERs (Kruijver & Swaab 2002, Vida et al. 2008) estrogen-sensitive nuclei that project to the SCN, like the mPOA and BNST, may act as conduits for 17β-estradiol's action on the SCN (de la Iglesia et al. 1999). Finally, sex-steroid can influence local oscillators in extra-SCN brain and peripheral tissues to help reentrain local core-clock rhythms in those tissues (Perrin et al. 2006, He et al. 2007, Nakamura et al. 2010).

While the SCN of females does not appear to express many steroid receptors, the SCN of males does. There have been studies in rodents, monkeys, and humans showing expression of the androgen receptor (AR) in the SCN of males, which suggests that T may exert its effects directly on the SCN (Rees & Michael 1982, Zhou et al. 1994, Wu et al. 1995, Fernandez-Guasti et al. 2000). In rodents, the AR is primarily expressed throughout the core region of the SCN where VIP and GRP neurons are located (Karatsoreos et al. 2007). Furthermore, gonadectomized male mice exhibit changes in activity rhythms and response to light stimulus that are reinstated upon T or DHT therapy (Karatsoreos et al. 2007, 2011). Finally, T has been shown to act on local oscillators in the prostate gland, a further example of the influence of hormones on core-clock mechanisms throughout the body (Kawamura et al. 2014).

In summary, there are age-related changes in behavioral and physiological rhythms and these changes are due to alterations in the underlying circadian system. How differences in reproductive decline between men and women affect the circadian system during aging requires further exploration. However, it is clear that in females, 17β-estradiol may exert some influence over circadian timing by acting on steroid receptors in nuclei like mPOA and BNST that feedback to the SCN, whereas T can act directly on the SCN to influence the master oscillator.

Summary

The effects of aging on the reproductive axis is very different between females and males. In elderly women, the loss of 17β-estradiol and progesterone at the time of menopause results in reproductive failure and has immediate effects on physiology, such as hot flashes, that may contribute to sleep disturbance. In elderly males, the subtle decrease and loss of diurnal T levels influences physiology and cognitive function. In the proceeding sections I build a base of knowledge on the interaction between aging, hormones, and locomotor activity patterns. I also show the need for carefully designed experiments to identify the differences in age-related changes in physiology using a systems level approach, in males and females. In the following Chapters, I test the hypotheses that the profound loss of 17β-estradiol near menopause has a significant effect on the brain in aged females and 17β-estradiol treatment will: 1) re-establish negative feedback effects to hypothalamic arcuate neurons, and 2) increase expression of core-output signals of the SCN in surgically postmenopausal

rhesus monkeys. I also hypothesize that: 1) androgen supplementation in males will have no effect on the arcuate neurons involved in reproductive function because age-related changes in testosterone levels do not appear to have significant effects on the HPG-axis or on LH release, and 2) androgen supplementation delivered cyclically will improve age-related changes in the SCN of males because those neurons express steroid receptors.

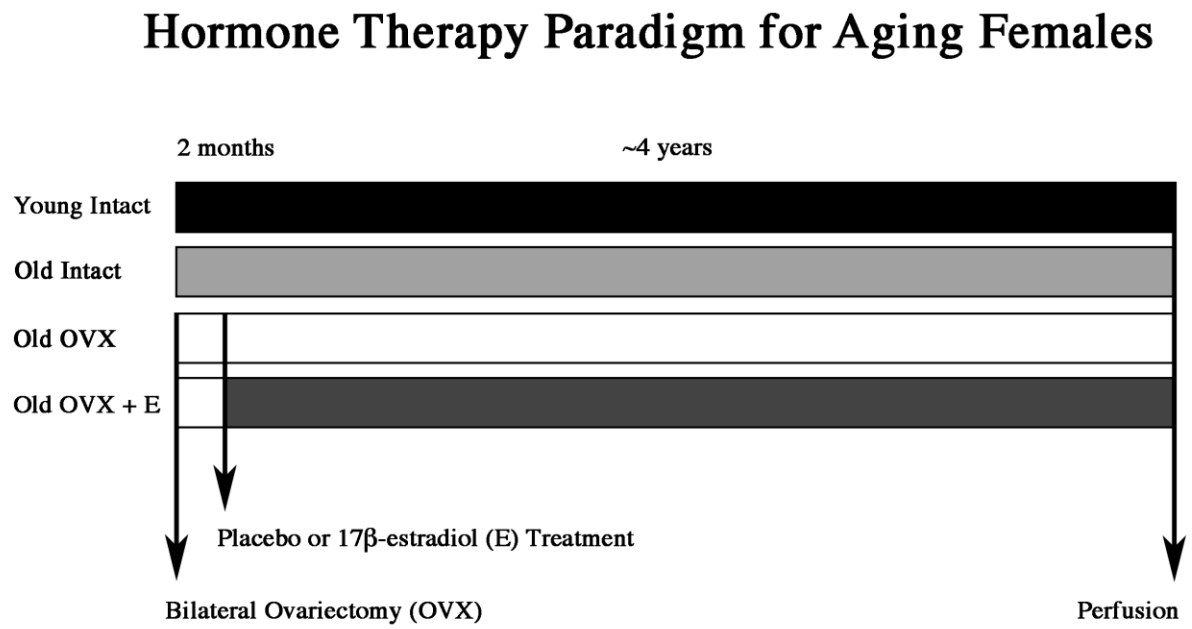

Figure 1. Hormone therapy paradigm in aging female rhesus monkeys.

The animals were divided into the following four groups: young ovary intact (Young Intact, n = 3), old ovary intact (Old Intact, n =3), ovariectomized (OVX, n = 7), and ovariectomized treated with 17β-estradiol (OVX + E, n = 5). The OVX animals recovered for 2 months prior to the initiation of 17β-estradiol treatment. The intact animals had variable E levels across the experiment, whereas animals that were OVX had very low levels of E and those treated with E displayed E concentrations clamped at consistently high levels.

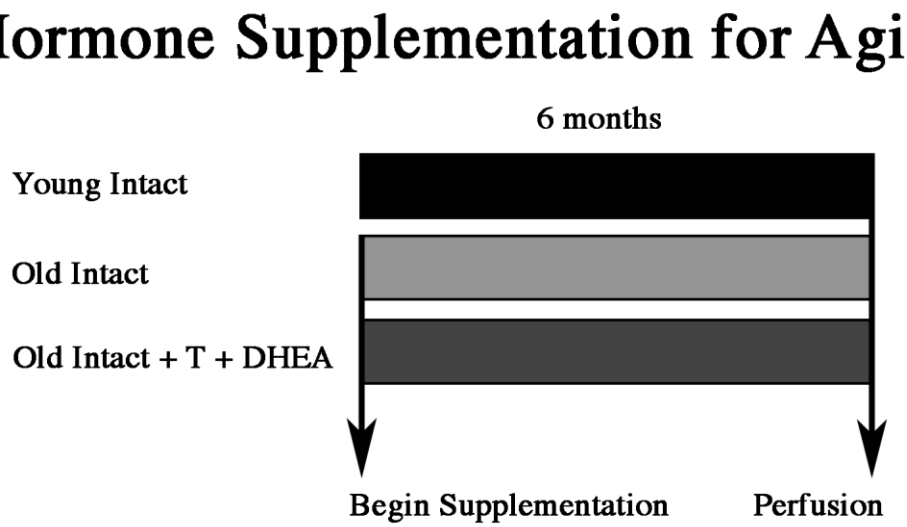

Figure 2. Hormone paradigm in aging male rhesus monkeys.

Hormone levels were measured across the 24-hour day in the following three groups: young intact males (n = 6), old intact males (n = 7), and old intact males receiving testosterone (T) and dehydroepiandrosterone (DHEA) (n = 6) diluted in sesame oil and delivered in a chocolate treat. Briefly, testosterone chocolate treats were administered in the evening and DHEA was provided at two time points in the morning. This method of delivery yielded 24-hour androgen levels similar to the pattern found in the young intact group (Urbanski et al. 2014).

Chapter 1:

Effects of age and estradiol on gene expression in the rhesus macaque hypothalamus

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ABSTRACT:

The hypothalamus plays a key role in mediating the effects of estrogen on many physiological functions, including reproduction, metabolism, and thermoregulation. We have previously observed marked 17β-estradiol (E)-dependent gene expression changes within the hypothalamus of rhesus macaques during aging, especially in the KNDy neurons of the arcuate-median eminence (ARC-ME) that produce kisspeptin, neurokinin B, and dynorphin A. Little is known, however, about the mechanisms involved in mediating the feedback from E onto these neurons. Methods: We used real-time PCR to profile age- and E-dependent gene expression changes in the rhesus macaque hypothalamus. Our focus was on genes that encode steroid receptors *(ESR1*, *ESR2*, *PGR*, and *AR)* and on enzymes that contribute to the local synthesis of 17β-estradiol (E; *STS, HSD3B1/2, HSD17B5,* and *CYP19A)*. In addition, we used RT^2 Profiler^{TM} PCR Arrays to profile a larger set of genes that are integral to hypothalamic function. Results: KISS1, KISS1R, TAC3, and NPY2R mRNA levels increased in surgically menopausal (ovariectomized) old females relative to age-matched ovariectomized animals that received E hormone therapy. In contrast, PGR, HSD17B, GNRH2, SLC6A3, KISS1, TAC3, and NPY2R mRNA levels increased after E supplementation. Conclusion: The rhesus macaque ARC-ME expresses many genes that are responsive to changes in circulating E levels, even during old age, and these may contribute to the cause of normal and pathological physiological changes that occur during menopause.

Introduction

Menopause in women and female rhesus macaques is associated with a marked decline in the production and secretion of 17β-estradiol (E) from the ovaries (Burger et al. 2002, Downs & Urbanski 2006, McKinlay et al. 2008). This attenuation of circulating E levels adversely impacts many physiological processes and is thought to play a major role in the etiology of age-related pathologies such as reproductive quiescence, hot flashes, and disrupted sleep-wake cycles (Mittelman-Smith et al. 2012, Sarti et al. 2005). Little is known, however, about the mechanisms involved in mediating the feedback from E onto neurons in the hypothalamus.

Evidence from human and nonhuman primate studies suggests that loss of circulating E can affect the cytoarchitecture of the arcuate region of the hypothalamus and alter the pattern of gene expression (Rance et al. 1990, Rance & Uswandi 1996, Abel & Rance 1999, Abel et al. 1999, Krajewski, Krajewski et al. 2003, Escobar et al. 2004). For example, using in situ hybridization, Rance et al. (1990) demonstrated a significant increase in the size of estrogen receptor (ER)-expressing neurons of postmenopausal compared to premenopausal women. ERα-expressing neurons in the arcuate-median eminence (ARC-ME) colocalize with kisspeptin (KISS1), neurokinin B (NKB), and dynorphin A (DYN) (referred to as KNDy) and exert a major influence on the neuroendocrine reproductive axis by modulating the secretion of gonadotropin-releasing hormone (GnRH). Moreover, KNDy neurons show marked changes in their pattern of gene expression after menopause or ovariectomy, and these changes can be blocked by exposure to exogenous sex steroids (Rance & Young 1991, Rometo et al. 2007, Rometo & Rance 2008, Rance 2009, Rance et al. 2010, Eghlidi et al. 2010).

Consequently, it is plausible that the influence of sex steroids on GnRH neuronal function is mediated by ERα, ERβ, and the progestin receptor, associated with the KNDy neural systems (Sullivan et al. 1995, Terasawa 1995, Van Look et al. 1997, Skinner et al. 1998, Wilson et al. 2002, Dorling et al. 2003, Peterson et al. 2003, Glidwell-Kenney et al. 2007,). Furthermore, the expression of these receptors may change, as animals show the characteristic menopausal decrease in circulating sex steroid levels, and may represent a mechanism by which the hypothalamus undergoes a compensatory increase in its sensitivity to sex steroids. Another possible menopause-associated compensatory mechanism could involve an increased expression of enzymes involved in the local intracrine synthesis of sex steroids from precursor steroids such as dehydroepiandrosterone (DHEA) (Labrie 1991, Sorwell & Urbanski, Labrie 2015). The circulating levels of DHEA and DHEA sulfate are especially pronounced in adult humans and nonhuman primates, and there is evidence that all of the key enzymes involved in DHEA-to-E conversion are expressed in the primate hypothalamus (Sorwell et al. 2012). This suggests that local synthesis of sex steroids may also be contributing to the hormone milieu of the hypothalamus.

The aim of the present study was to help resolve these issues by examining the effect of age and E treatment on gene expression in the ARC-ME of female rhesus macaques. These nonhuman primates show age-related hormonal changes similar to those in women, though late in their lifespan (Downs & Urbanski 2006, Gilardi et al. 1997, Walker & Herndon 2008), and thus they represent a pragmatic translational animal model in which to study the neuroendocrine mechanisms that contribute to healthy human aging. My primary focus was on genes that encode the main sex steroid

receptors (Pfaff et al. 1976, Bethea et al. 1996, Blurton-Jones et al. 1999, Mills et al. 2002, Simerly et al. 1990, Fernandez-Guasti et al. 2000): ERα (encoded by *ESR1*), ERβ (encoded by *ESR2*), the progestin receptor (encoded by *PGR*), and the androgen receptor (encoded by *AR*). In addition, we examined the expression of genes that play a key role in the intracrine conversion of DHEA to testosterone and E: steroid sulfatase (encoded by *STS*), 17β-hydroxysteroid dehydrogenase type 5 (encoded by *HSD17B5*), 3β-hydroxysteroid dehydrogenase types 1 and 2 (encoded by *HSD3B1/2*), and aromatase (encoded by *CYP19A1*). Our second goal was to profile the expression of a larger set of genes that contribute to the control of hypothalamus-mediated functions such as reproduction and metabolism. Our prediction was that the expression of hypothalamic genes would increase during aging, ensuring that the hypothalamus remains responsive to sex steroids even when the levels of these hormones in the circulation are attenuated.

Materials and Methods

Animals

Adult female rhesus macaques *(Macaca mulatta)* were cared for by the Division of Comparative Medicine at the Oregon National Primate Research Center (ONPRC) in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals, and used in a study approved by the Institutional Animal Care and Use Committee. The animals were housed indoors under controlled environmental conditions: 24°C temperature; 12-hour light, 12-hour dark photoperiods with lights on at 0700 h; regular meals at 0800 and 1500 h (ZT1 and ZT8) (Purina High Protein Monkey

Chow; Purina Mills, Inc., St. Louis, Mo., USA) supplemented with fresh fruit and vegetables, and fresh drinking water available ad libitum.

Design of Experiments

Effect of Aging

Experiment 1 used real-time PCR to compare mRNA levels between young (~13 years) and old (~26 years) ovary-intact animals.

Effect of E

Experiment 2 used real-time PCR to compare mRNA levels between young (~10 years) bilaterally ovariectomized (OVX) animals and young (~10 years) OVX animals that had been treated with E for ~1 month. This experiment also compared mRNA levels between old OVX controls and old OVX animals that had been treated with E for ~4 years. The two older groups of animals were ~21 and ~22 years of age at the onset of the study, respectively.

Effect of Menopause and Hormone Therapy

Experiment 3 used custom-made RT^2 Profiler^{TM} PCR Arrays (SABiosciences) to compare gene expression in the ARC-ME of young animals (~12 years) with that of old, surgically menopausal animals (old OVX) from Experiment 2, and also with the long-term estradiol-treated animals (old OVX + E).

Menstrual Cycle Status and Serum Hormone Concentrations

Experiment 1

The serum levels of E were 36.5 ± 6.3 pg/ml in the young gonad-intact animals and 62 ± 16.5 pg/ml in the old gonad-intact animals. All but 1 of these animals had progesterone (P) levels below the assay detection limit. One young animal showed elevated P concentrations (2.4 ng/ml) and was assumed to still be in the luteal phase of the menstrual cycle. Based on individual animal menstruation records, all 4 young intact animals were normal cyclers, and the old intact animals consisted of 2 cycling animals, 1 irregular cycler, and 1 postmenopausal animal.

Experiment 2

One month following ovariectomy, half of the animals in the young and old OVX groups were implanted subcutaneously with empty Silastic capsules (Dow Corning, Midland, Mich., USA), while the other half received capsules containing crystalline E (Steraloids, Wilton, N.H., USA), as previously described (Kohama & Bethea 1995); these were designed to last up to 1 year and to maintain circulating E levels between 100 and 200 pg/ml, which is similar to what is observed during the late follicular phase of the menstrual cycle. In the old OVX + E group, the E capsules were replaced annually to ensure sustained long-term delivery of the steroid for the entire duration of the study (~4 years); the untreated OVX animals maintained the same capsules throughout. Terminal serum E levels were undetectable in the young untreated OVX animals and had a mean level of 117 ± 7.0 pg/ml in the young OVX + E group. Serum E concentrations were measured at various time points across the four-year experiment in the old OVX animals to confirm that the target hormone concentrations were being maintained. The serum E levels in the old untreated OVX group were <30 pg/ml at all time points and

often fell below the limit of assay sensitivity. In the old OVX + E animals, the mean E concentration during the final two years of the study was 118.8 ± 15.9 pg/ml, and at the time of tissue collection it was 94.3 ± 20.5 pg/ml.

Experiment 3

At the time of postmortem tissue collection, the young intact animals had a mean serum E level of 58.9 ± 24.5 pg/ml and very low serum P levels (<0.3 ng/ml). These hormone levels are indicative of the follicular phase of the menstrual cycle. In one of these animals, however, the E level was considerably higher (128 pg/ml), suggesting that the animal was most likely in the late follicular phase. Note that the surgically menopausal animals used in experiment 3 were the same old OVX and old OVX + E animals from Experiment 2. The E and P assays were performed by the ONPRC Endocrine Technology and Support Core using a chemiluminescence-based automatic clinical platform (Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, Ill., USA). The sensitivity limits of these assays were 20 pg/ml and 0.2 ng/ml for E and P, respectively (Jensen et al. 2008), and the intra-assay and inter-assay coefficients of variation were all <15%.

RNA Isolation

All the animals had previously been involved in various cross-sectional aging studies; their hypothalami and terminal blood serum became available for postmortem analysis through the ONPRC Tissue Distribution Program. At the time of necropsy, the mean (± SEM) age of the gonad-intact young animals from experiment 1 was 12.8 ± 2.3 years (n

= 4), and the age of the old animals was 25.8 ± 0.4 years (n = 4). The animals from Experiment 2 were 9.7 ± 0.3 years old (young OVX; n = 4), 9.6 ± 0.8 years old (young OVX ± E; n = 4), 25.0 ± 1.7 years old (old OVX; n = 4), and 27.0 ± 0.9 years old (old OVX + E; n = 4). The age of the young animals from experiment 3 was 12.3 ± 0.9 years (n = 4). After sedation with ketamine (15–25 mg/kg, i.m.) and pentobarbital sodium (25–30 mg/kg, i.v.), a procedure consistent with the recommendations of the American Veterinary Medical Association's Panel on Euthanasia, each brain was flushed with 1 liter of 0.9% saline via a vascular catheter, and the hypothalamus was removed and preserved for ~2 weeks in RNAlater (Ambion, Austin, Tex., USA). A coronal slice encompassing the ARC-ME was dissected and stored frozen at -80°C. The boundaries for this tissue block included the exterior ventral edge of the ME, lateral cuts midway between the third ventricle and the optic nerve, an anterior cut along the posterior edge of the optic chiasma, a posterior cut just anterior to the mammillary bodies, and a cut 1 mm dorsal to the base of the third ventricle (i.e., based on stereotaxic coordinates, this represents the approximate border between the arcuate and the ventromedial hypothalamus). Subsequently, each ARC-ME block was individually homogenized using a PowerGen rotor-stator homogenizer (Fisher Scientific, Pittsburgh, Pa., USA), and RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, Calif., USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif., USA) was used to determine the quality of the RNA, and a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Mass., USA) was used to determine the concentration. For each sample, 1 µg of RNA was then converted to cDNA using random hexamers and the SuperScript III

First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, Calif., USA).

Gene Expression Profiling

The ARC-ME expression levels of genes encoding the nuclear sex steroid receptors *ESR1, ESR2*, *PGR*, and *AR* were determined using quantitative real-time PCR (qRT-PCR). Similarly, the ARC-ME expression levels of genes encoding the following steroidogenic enzymes were examined: *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1*. Finally, RT^2 Profiler^{TM} PCR Arrays were used to profile a more extensive set of genes in the ARC-ME during aging and after treatment with E.

Primers and Probes

Table 1 depicts nucleotide sequences of the primers and probes that were used for detection of the steroid receptor genes *(ESR1*, *ESR2*, *PGR*, and *AR)*, steroidogenic enzyme genes *(STS*, *HSD3B1/2, HSD17B5,* and *CYP19A1)*, and also two housekeeping genes *(ALG9* and *RPL13A)* that we have previously shown to be stably expressed in the rhesus ARC-ME across various sex steroid environments (Eghlidi et al. 2010, Noriega et al. 2010). The nucleotide sequences for these genes were designed using Primer Express 2.0 software [Applied Biosystems (ABI), Foster City, Calif., USA] and were based on NCBI rhesus macaque reference sequences. The primers and probes were purchased from Invitrogen Life Technologies and ABI, respectively. Reverse transcriptase-PCR (RT-PCR), using ARC-ME and ovarian RNA

samples (as positive controls), was used to validate each of the primer sets by producing amplicons of a predicted size.

TaqMan qRT-PCR

A 7900HT Fast Real-Time PCR thermal cycler and sequence detection system software (version 2.2.1; ABI) was used to obtain qRT-PCR data. Initially, pooled cDNA was used to create standard curves for each gene, and the experimental samples were subsequently diluted accordingly so as to fall within the linear part of the curve. The PCR mixtures contained 5 µl TaqMan Universal PCR Master Mix, 0.3 µl of each specific forward and reverse primer (300 nM final concentration), 0.25 µl of specific probe (250 nM final concentration), and 2 µl of cDNA. The reaction sequence included 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. Automatic baseline and threshold levels were determined by ABI sequence detection system software (Version 2.2.1.), and the final expression values were normalized to the arithmetic mean of two reference housekeeping genes, *ALG9* and *RPL13A.* Individual genes from Experiments 1 and 2 were examined together on the same 384-well optical plate. A negative control included the omission of cDNA templates from the reaction mixture.

RT^2 Profiler^{TM} PCR Array

Total RNA samples (0.5 µg) were reverse transcribed using the RT^2 First Strand Kit (Qiagen). Each RT-PCR reaction was performed in 25 µl of solution containing cDNA, 2 × RT^2 SYBR Green Mastermix, and RNase-free water, using custom-made RT^2

Profiler™ PCR Arrays (Custom PCR Arrays; Qiagen) and a QuantStudio™ 12K Flex thermocycler (Life Technologies, Grand Island, N.Y., USA). The RT-PCR reaction sequence included 10 min of incubation at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, 1 min at 60°C, and 15 s at 95°C. The relative gene expression was calculated using the ΔΔCt method, and the results are expressed with reference to the arithmetic mean of three reference housekeeping genes *(ALG9*, *GAPDH*, and *RPL13A)*.

Statistical Analysis

Paired Student's *t*-tests were used to compare differences in *ESR1*, *ESR2*, *PGR*, *AR*, *STS*, *HSD3B1/2*, *HSD17B5,* and *CYP19A1* expression between young and old gonad-intact animals (Experiment 1) and between OVX and OVX + E animals (Experiment 2). ANOVA followed by the Dunnett multiple-range test was used to assess between-group differences in expression for all other analyses (Experiment 3). Significance was considered at $P < 0.05$.

Results

The ovary-intact animals showed no detectable effect of age on the expression of genes encoding steroid receptors or steroidogenic enzymes in the ARC-ME (Table 2). In both young and old OVX animals, however, *PGR* expression was significantly enhanced by E supplementation (Fig. 1). Similarly, *ESR1* and *ESR2* expression was enhanced by E supplementation in the young (Fig. 1A) but not in the old (Fig. 1B) animals. In the OVX animals, there was no detectable effect of E supplementation on the expression of *AR* (Fig. 1) or on genes encoding steroidogenic enzymes (Fig. 2), except for a significant (*P*

< 0.05) decrease in *HSD17B5* expression in the old OVX + E animals relative to the untreated age-matched controls.

To corroborate and extend the findings from the real-time PCR experiments, we also performed gene expression analyses on RNA extracts obtained from young gonad-intact, old OVX, and old OVX + E animals, using custom-made RT^2 Profiler^{TM} PCR arrays. These three groups were selected to provide insights into ARC-ME gene expression during sexual maturity, after menopause, and after subsequent hormone therapy (HT). Our focus was on genes that encode steroid receptors, as well as genes associated with the control of the reproductive system, metabolism, and neurotransmission (Table 3). As expected, the array analysis revealed a significant decrease ($P < 0.01$) in *PGR* expression in old OVX animals and showed that this decrease could be blocked by long-term HT. The expression of *GNRH2* and *SLC6A3* (dopamine transporter, DAT) was also significantly enhanced by E ($P < 0.05$ and $P < 0.01$, respectively). In contrast, a significant increase in expression levels was observed for several genes associated with the KNDy neuronal system or metabolism (*KISS1*, $P < 0.01$; *KISS1R*, $P < 0.05$; *TAC3*, $P < 0.01$; *NPY2R*, $P < 0.05$), and in most cases the increase was reversed by E (KISS1, $P < 0.01$; TAC3, $P < 0.01$; NPY2R, $P < 0.05$).

Discussion

Previous studies of the rhesus macaque hypothalamus relied on RT-PCR and in situ hybridization to examine the expression of *ESR1*, *ESR2*, and *PGR* (Pau et al. 1998, Gundlah et al. 2000). Although these studies were essentially qualitative or semiquantitative, taken together their findings suggested that *ESR1* and *ESR2*

expression is unaffected by circulating E levels, whereas *PGR* expression is highly stimulated by E. In the present study, we used qRT-PCR, a more quantitative molecular approach, to corroborate these previous observations and to examine if these genes show age-associated changes in their expression. First, *ESR1, ESR2*, and *PGR* were all expressed in the ARC-ME region of the hypothalamus, thus confirming the results from previous studies. Despite their old age, however, we failed to detect a significant change in the expression of these genes in our old ovary-intact animals. This most likely stems from the fact that most of these animals had not completely entered menopause and their circulating E levels had not yet fallen to a sustained basal level. Consequently, to more closely mimic the human postmenopausal condition, we also examined age-related differences in the ARC-ME of young and old OVX animals and exposed some of them to supplementary E in order to gain insights into the influence of HT.

E HT had an age-related effect on *ESR1* and *ESR2* expression in OVX animals. While young OVX + E-treated animals expressed more ESR1 and ESR2 mRNA than did untreated age-matched controls, the old animals did not respond to E supplementation. Although the reason for this difference is unclear, one possibility is that the hypothalamus of older animals is itself less responsive to exogenous E. Another possibility is that the stimulatory effect of E on ESR1 and ESR2 mRNA is transitory and that it gradually decreases during long-term HT. In contrast to *ESR1* and *ESR2*, *PGR* expression was highly stimulated by E regardless of age. It should be emphasized that the two major isoforms of the progestin receptor *(PGR-A* and *PGR-B)* show different expression patterns (Vegeto et al. 1993, Richer et al. 2002) and independent functions (Duffy et al. 1997, Mulac-Jericevic et al. 2000, Stouffer 2003). In the present study,

however, we amplified both isoforms of *PGR* and thus cannot establish which form plays the dominant physiological role in the primate hypothalamus. Expression of the androgen receptor in the female rhesus macaque hypothalamus has not previously been described, but in the present study we found *AR* to be highly expressed in the female hypothalamus and to be unaffected by circulating E levels. This result is consistent with, and complements, the finding from a previous study in which a ribonuclease protection assay showed no effect of testosterone on the hypothalamic expression of *AR* in male rhesus macaques (Abdelgadir et al. 1999). Taken together, the quantitative data from the present study clearly establish that gonadal nuclear steroid receptors are highly expressed in the female rhesus macaque hypothalamus, and that circulating E levels can significantly modulate progestin receptor gene expression even in old animals.

Recently, Naugle et al. (2014) used immunohistochemistry to examine the effects of long-term E treatment on *ERα* and *PGR* expression in the rhesus macaque ARC-ME. Semiquantitative analysis of the immunoreactive cells revealed no effect of E on the number and density of ERα-positive cells in old OVX females, which is in agreement with the mRNA findings from the present study. On the other hand, E also failed to significantly affect the number and density of PGR-positive cells, which does not agree with our mRNA findings or those previously reported by Bethea et al. (1996). It is unclear whether this discrepancy is a reflection of the relative insensitivity of the semiquantitative immunohistochemistry methodology or whether it indicates that E-induced *PGR* expression changes are not reflected at the translation level. Western blot analysis would help to resolve this issue in the future.

Given that the brain has an intrinsic capacity to synthesize sex steroids de novo (Labrie 1991, Gilardi et al. 2008, Sorwell & Urbanski 2010, Sorwell et al. 2012), it is plausible that the hypothalamus can also compensate for the menopausal loss of sex steroids by increasing its local production of these steroids. There are several key enzymes involved in the synthesis of testosterone and E in the brain, using DHEA as a precursor. In the present study, we examined the hypothalamic expression of *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1*. These genes encode key enzymes in the conversion of DHEA to testosterone and E and are expressed in the rhesus macaque hypothalamus (Sorwell et al. 2012). The findings from the current study corroborate these earlier observations. Moreover, they show that the expression of *HSD17B5* becomes enhanced in old animals when circulating levels of E are low, which may result in a compensatory increase in hypothalamic E levels due to enhanced intracrine synthesis from DHEA. On the other hand, circulating levels of DHEA (particularly in the sulfated form as DHEA sulfate) show a significant age-related decline (Sorwell et al. 2012, Sowell et al. 2014). Consequently, it is unclear if the expression of *STS*, *HSD3B1/2*, *HSD17B5*, and *CYP19A1* alone is sufficient to maintain high hypothalamic sex steroid levels during aging. Nevertheless, the results suggest that local steroid synthesis in the hypothalamus is likely to be preserved in older animals, provided that there is a sufficient amount of DHEA precursor available.

Other significant age-related gene expression changes have previously been observed in the human and nonhuman primate ARC-ME, especially in the KNDy neurons (Rance & Young 1991, Rometo et al. 2007, Rometo & Rance 2008, Rance 2009, Eghlidi et al. 2010, Rance et al. 2010). We observed the same increase in *KISS1*

and *TAC3* gene expression observed in postmenopausal women, and this increase could be blocked by the administration of exogenous E (Eghlidi et al. 2010). This suggests that the observed gene expression changes stemmed from an age-associated decline in circulating E levels, rather than from a sex steroid-independent aging mechanism. Although the physiological significance of the E-dependent changes in *KISS1*, *KISS1R*, and *TAC3* expression is unclear, these changes may serve to maintain homeostatic control of the reproductive neuroendocrine axis after sex steroid secretion from the ovaries has declined (Downs and Urbanski 2006). We also found that the expression of *NPY2R*, a central mediator of food intake, was suppressed following chronic E supplementation in old OVX animals (Koegler et al. 2005). *NPY2R* may mediate health consequences such as diabetes, obesity, and metabolic syndrome that can be improved by HT in postmenopausal women (Salpeter et al. 2006).

Analysis of the RT^2 Profiler^{TM} PCR arrays also established that most of the selected genes are stably expressed during reproductive aging and after HT. On the other hand, E supplementation was found to significantly stimulate the expression of *GNRH2.* We previously reported that E exerts a stimulatory action on *GNRH2* gene expression in the hypothalamus of young OVX animals (Densmore & Urbanski 2004, Urbanski 2012, Urbanski 2014). The finding that *GNRH2* expression in old OVX animals also responds to E suggests that this second form of primate GnRH may play an important role in mediating E-positive feedback to the reproductive neuroendocrine axis, as previously suggested (Urbanski 2012, Urbanski 2014). E supplementation was also found to stimulate the expression of *SLC6A3*, the gene that encodes the dopamine transporter. Voytko et al. (2009) reported that chronic E therapy did not have an effect

on dopaminergic activity in the ventral tegmentum and dorsolateral prefrontal cortex. However, the present results show that chronic E supplementation was able to significantly increase the expression of the dopamine transporter in arcuate neurons of aged OVX animals. The influence of estrogen on the dopaminergic system is less well understood, but it appears that chronic E treatment may facilitate reuptake of dopamine by presynaptic neurons or interact with prolactin release.

Menopause in women and female rhesus macaques is associated with attenuated circulating E concentrations (Burger et al. 2002, Downs & Urbanski 2006, McKinlay et al. 2008), which is thought to contribute to the development of many age-related pathologies. On the other hand, the results from the present study suggest that the hypothalamus retains its capacity to respond to sex steroids even in old age, and that the sensitivity of the hypothalamus to E shows some compensatory changes that may help to maintain local homeostasis.

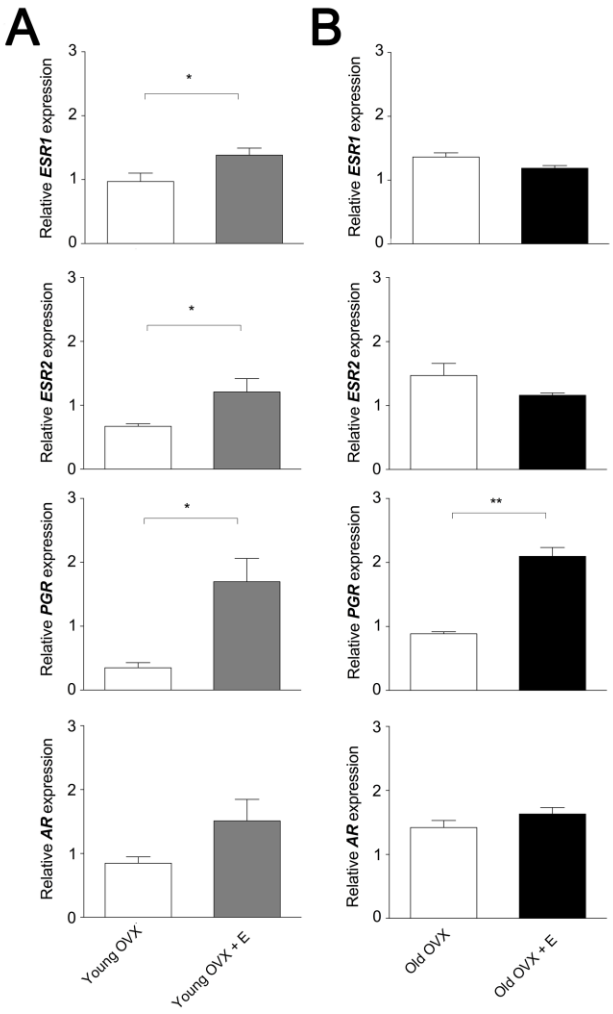

Fig. 1. Effect of E treatment on steroid receptor gene expression in the ARC-ME of OVX rhesus macaques. The animals either served as controls or were treated with E for ~1 month (young animals) or for ~4 years (old animals). Each bar represents the mean (± SEM) mRNA levels of 4 animals, normalized to the arithmetic mean of two housekeeping genes, *ALG9* and *RPL13A*. In the young animals (A), ESR1, ESR2, and PGR mRNA levels were significantly higher in the OVX + E group than in the OVX group, whereas AR mRNA levels were not significantly different. In the old animals (B), only PGR mRNA levels were significantly higher in the OVX + E group relative to the OVX group. * $P < 0.05$, ** $P < 0.01$ (paired Student's *t*-test).

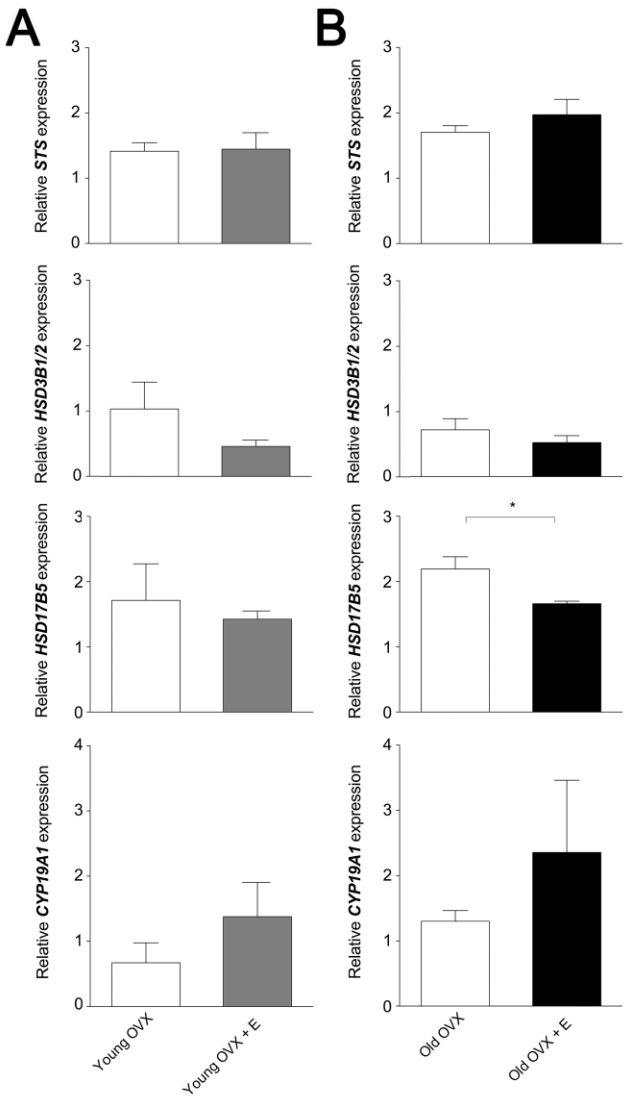

Fig. 2. Effect of E treatment on steroidogenic enzyme gene expression in the ARC-ME of OVX rhesus macaques. Each bar represents the mean (± SEM) mRNA levels of 4 animals, normalized to the arithmetic mean of two housekeeping genes, *ALG9* and *RPL13A*. In the young animals (A), there was no significant effect of E on any of the mRNA levels examined. In the old animals (B), HSD17B5 mRNA levels were significantly higher lower in the OVX + E group relative to the OVX group. * $P < 0.05$ (paired Student's *t*-test).

Table 1. TaqMan real-time PCR primers and probes

Target Genes	Sequences		GenBank Accession ID.
ESR1	F-	ACAAGATCACAGACACTTTGATCCA	XM_002803858
	R-	CCTGATGTGGGAGAGGATGAG	
	P-	AGGCCTGACCCTGCAG	
ESR2	F-	GATCGCTAGAACACACCTTACCTGTA	NM_001265821
	R-	GGTGCAACGGTTCCCACTAA	
	P-	AGAGACATTGAAAAGGAAG	
PGR	F-	CATGTCAGTGGGCAGATGCT	NM_001278456
	R-	TGCCACATGGTAAGGCATAATG	
	P-	CTGAATGAACAGCGGATG	
AR	F-	AAAATCCCACATCCTGCTCAA	NM_1032911
	R-	CAGGTCAAAAGTGAACTGATGCA	
	P-	TGCAGCCTATTGCGAGAG	
STS	F-	CCTTCCTCCGGCCTGTCT	XM_001088752
	R-	AGCTTTGCCACATGCATCTG	
	P-	CCAGTGCGACAGAGAAAAACAGGATAAGAGA	
SULT2B1	F-	CAGTACAGCCCTCGCCTCAT	XM_001111839
	R-	GGGTTGCGGCCCATGT	
	P-	TTCTTCAGCTCCAAGGCCAAGGTGATC	
3BHSD1/2	F-	AGGACGTCTCGGTCGTCATC	XM_001113873
	R-	GAGCTGGGTACCTTTCACATTGA	
	P-	CATTGATGTCTTTGGTGTCACTCA	
17BHSD5	F-	TGGAGGGCTTTGCTGAAGTCT	XM_001104543
	R-	GGTCCAGTCACCAGCATACAGA	
	P-	CAGAAGCCGTGCGTGTGGATGG	
AROMATASE	F-	TAGCAGAAAAAAGACGCAGGATT	XM_001082665
	R-	CGTCAGGTCACCTCGTTTCTC	
	P-	AATGCATGGACTTTGCCACTGAGTTGATTTT	
ALG9	F-	AACAGTGCCACAGAGCGAGAA	XM_001106180
	R-	CGATACCGCCTGGAGCACTA	
	P-	ACTGTCTTCCTGTTCGGG	
RPL13A	F-	TCACGAGGTTGGCTGGAAGT	XM_001115079
	R-	GATCTTGGCTTTCTCCTTCCTCTT	
	P-	CCAGGCAGTGACAGCCACCTTGG	

Reference sequences for each of the *Macaca mulatta* target genes can be accessed through GenBank accession IDs.

Effect of natural aging on steroid receptor and steroidogenic enzyme gene expression in the ARC-ME of young and old female rhesus monkeys.

Gene Symbol	Young Intact (n = 4)	Old Intact (n = 4)
ESR1	0.81 ± 0.25	1.32 ± 0.17
ESR2	0.86 ± 0.10	1.32 ± 0.20
PGR	0.44 ± 0.12	1.25 ± 0.43
AR	0.89 ± 0.17	1.48 ± 0.12
STS	1.39 ± 0.27	1.96 ± 0.26
HSD3B1/2	0.35 ± 0.15	0.53 ± 0.16
HSD17B5	1.28 ± 0.26	1.87 ± 0.20
CYP19A1	0.58 ± 0.36	1.57 ± 0.62

Values represent mean (± SEM) mRNA levels of young adult and old adult animals, normalized to *ALG9* and *RPL13A*.

No significant effect of age was found on any of the steroid receptor or steroidogenic enzyme transcripts with a Student's *t*-test ($P > 0.05$).

Custom RT2 ProfilerTM PCR-array in female rhesus monkey model of menopause

Gene Symbol	Young Intact			Old OVX			Old OVX + E			
Sex-steroid Receptors										
AR	1.00	±	0.13	0.91	±	0.10	1.02	±	0.07	
ESR1	1.00	±	0.11	1.19	±	0.06	0.90	±	0.07	
ESR2	1.00	±	0.18	1.22	±	0.13	1.08	±	0.14	
PGR	1.00	±	0.10 **	0.33	±	0.13	1.82	±	0.14	**
GnRH Signaling										
GNRH1	1.00	±	0.17	1.09	±	0.33	0.89	±	0.20	
GNRH2	1.00	±	0.15	0.59	±	0.07	3.31	±	1.01	*
KISS1	1.00	±	0.32 **	11.72	±	2.20	0.25	±	0.08	**
KISS1R	1.00	±	0.11 *	1.99	±	0.15	2.18	±	0.38	
PDYN	1.00	±	0.15	0.89	±	0.06	1.19	±	0.07	
TAC3	1.00	±	0.09 **	3.21	±	0.64	0.49	±	0.05	**
Hypothalamic Control of Metabolism										
AGRP	1.00	±	0.25	1.48	±	0.24	1.00	±	0.07	
INSR	1.00	±	0.16	1.27	±	0.25	1.12	±	0.07	
LEPR	1.00	±	0.09	1.23	±	0.17	1.12	±	0.27	
MC2R	1.00	±	0.32	0.37	±	0.11	0.69	±	0.43	
MC4R	1.00	±	0.29	0.62	±	0.07	0.71	±	0.11	
MCHR1	1.00	±	0.05	1.22	±	0.17	1.71	±	0.34	
NPY	1.00	±	0.53	0.81	±	0.40	0.40	±	0.05	
NPY1R	1.00	±	0.19	1.08	±	0.28	1.05	±	0.16	
NPY2R	1.00	±	0.15 *	1.53	±	0.12	1.04	±	0.12	*
NPY5R	1.00	±	0.12	1.05	±	0.17	1.31	±	0.17	
POMC	1.00	±	0.19	1.17	±	0.27	0.83	±	0.11	
Nervous System										
ACHE	1.00	±	0.10	1.17	±	0.04	1.13	±	0.10	
ADRA1A	1.00	±	0.14	0.80	±	0.03	0.73	±	0.21	
DRD1	1.00	±	0.10	0.89	±	0.08	0.82	±	0.09	
DRD2	1.00	±	0.21	0.82	±	0.11	1.02	±	0.22	
SLC18A2	1.00	±	0.22	0.88	±	0.08	1.07	±	0.26	
SLC6A3	1.00	±	0.34	0.62	±	0.06	4.52	±	0.74	**
SLC6A4	1.00	±	0.57	0.67	±	0.62	0.55	±	0.25	
GABBR1	1.00	±	0.06	0.99	±	0.02	1.08	±	0.10	
GABBR2	1.00	±	0.27	0.69	±	0.04	0.77	±	0.05	
GABRA1	1.00	±	0.23	0.75	±	0.03	1.01	±	0.07	
GABRA5	1.00	±	0.12	0.82	±	0.06	0.87	±	0.16	
HTR1A	1.00	±	0.14	0.71	±	0.06	0.86	±	0.15	
HTR2A	1.00	±	0.04	0.81	±	0.12	0.63	±	0.07	
HTR2C	1.00	±	0.12	1.35	±	0.15	1.20	±	0.06	

Expression profiles of selected genes in the arcuate-median eminence of young intact, ovariectomized (OVX), and OVX estrogen supplemented females (OVX + E). Changes in expression were calculated using ANOVA followed by the Dunnett post hoc test, * $P < 0.05$, ** $P < 0.01$.

Androgen receptor (AR), estrogen receptor 1 (ESR1), estrogen receptor 2 (ESR2), progesterone receptor (PR), acetylcholinesterase (ACTH), adrenergic, alpha-1A-, receptor (ADRA1A), agouti related protein homolog (AGRP), dopamine receptor D1 (DRD1), dopamine receptor D2 (DRD2), gamma-aminobutyric acid (GABA) B receptor, 1 (GABBR1),gamma-aminobutyric acid (GABA) B receptor, 2 (GABBR2), gamma-aminobutyric acid (GABA) A receptor, alpha 1 (GABRA1),gamma-aminobutyric acid (GABA) A receptor, alpha 5 (GABRA5), gonadotropin-releasing hormone 1 (luteinizing-releasing hormone) (GNRH1), gonadotropin-releasing hormone 2 (GNRH2), 5-hydroxytryptamine (serotonin) receptor 1A (HTR1A), 5-hydroxytryptamine (serotonin) receptor 2A (HTR2A), 5-hydroxytryptamine (serotonin) receptor 2C (HTR2C), insulin receptor (INSR), KiSS-1 metastasis-suppressor (KISS1), KISS1 receptor (KISS1R), leptin receptor (LEPR), melanocortin 2 receptor (adrenocorticotropic hormone) (MC2R), melanocortin 4 receptor (MC4R), melanin-concentrating hormone receptor 1 (MCHR1), neuropeptide Y (NPY), neuropeptide Y receptor Y1 (NPY1R) neuropeptide Y receptor Y2 (NPY2R), neuropeptide Y receptor Y5, prodynorphin (PDYN), proopiomelanocortin (POMC), solute carrier family 18 (vesicular monoamine) (SLC18A2), solute carrier family 6 (neurotransmitter transporter, noradrenalin) (SLC6A2), member 2, solute carrier family 6 (neurotransmitter transporter, dopamine (SLC6A3), member 3, solute carrier family 6 (neurotransmitter transporter, serotonin) (SLC6A4), alpha-1,2-mannosyltransferase (ALG9), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L13a (RPL13A).

Chapter 2:

Organization of the aging female rhesus macaque suprachiasmatic nucleus

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ABSTRACT:

The hypothalamus plays a key role in regulating reproductive function and sleep-wake activity, and there is evidence that these physiological processes undergo marked age-related changes. I previously showed that neurons in the arcuate nucleus (ARC) are responsive to 17β-estradiol (E) supplementation in a monkey model of menopause. I hypothesize that if the SCN exhibits age-related changes in protein expression, then SCN neurons may be responsive to E therapy in aged female rhesus monkeys similar to other hypothalamic nuclei, like the ARC. Methods: I used immunohistochemistry (IHC) to label the sub-compartments of the SCN, like vasoactive intestinal peptide (VIP) in the core and vasopressin (AVP) in the shell that together maintain synchronous output from the SCN to downstream nuclei. To gain insight into changes in the canonical clock mechanism during aging I measured core-clock modifier protein, nuclear receptor subfamily 1, group D, member 1 (NR1D1). First, I compared the effect of age on expression in the SCN between young intact (n = 3) and old intact (n = 3-4) monkeys. Second, I compared aged ovariectomized (OVX; n = 4), and aged OVX and E supplemented animals (OVX + E; n = 4) to determine if there were menopause-related changes in the SCN. Results: I found that aged animals showed significantly less VIP immunoreactivity in the SCN compared to young intact controls, regardless of whether or not they had been treated with E. Similarly, AVP immunoreactivity in the SCN decreased with age and did not respond to E supplementation. The number of NR1D1 neurons decreased during aging suggesting that the core-clock modifier mechanism was preserved, but

less NR1D1 protein was produced in aged animals. Conclusion: There are age-related reductions in neuropeptide synthesis in the SCN of female rhesus macaques, which appear to be unrelated to changes in circulating E levels.

Introduction: Hypothalamic nuclei show patterns of gene and protein expression during aging that relate to altered hormone levels and sleep-wake activity (Wang et al. 2015, Eghlidi et al. 2015). Three notable features of the aging hypothalamus include: 1) menopause-related increases in the expression of arcuate kisspeptin and neurokinin B (Eghlidi et al. 2010, Eghlidi & Urbanski 2015), 2) stable expression of vasopressin (AVP) and oxytocin in magnocellular neurons in the paraventricular and supraoptic nuclei (Fliers et al. 1985) as well as orexin neurons in the lateral hypothalamic area (Downs et al. 2007) and 3) decreased expression of output signals like vasoactive intestinal peptide (VIP) and AVP in the suprachiasmatic nucleus (SCN) (Roozendaal et al. 1987, Chee et al. 1988, Hofman 1997).

Currently, we know a lot about the age-associated sex-steroid dependent changes in hypothalamic arcuate kisspeptin, neurokinin B, and dynorphin A (KNDy) neurons of females. However, we know much less about the age-related changes in neurons of the SCN, especially in non-human primates. VIP neurons located in the core of the SCN project to cognate receptors located on AVP neurons in the shell to maintain synchronicity within the SCN (Saper 2013, An et al. 2013). During aging, a temporal shift and decrease in VIP and AVP expression has been described in rodents and in the mouse lemur, a short lived and nocturnal primate species, over the 24-hour day (Chee et al. 1988, Roozendaal et al. 1987, Kawakami et al. 1997, Krajnak et al. 1998, Aujard et al. 2006, Cayetanot et al. 2005, Aujard et al. 2006). In rats, marmosets, and rhesus monkeys there is no difference in the number of SCN neurons with age, however there is a decrease in VIP and AVP immunoreactive neurons, therefore only the protein expression in neurons of the SCN decrease with age and there is no neuronal loss

(Engelberth et al. 2014, Roberts et al. 2012, Sartin & Lamperti 1985). In terms of the genetic clock mechanism, neurons in the SCN remain rhythmic despite aging, but there are subtle changes in the expression of core-clock elements (Yamazaki et al. 2002, Weinert 2000, Weinert et al. 2001). It remains to be determined if factors that are not directly related to the oscillatory mechanism, like modifier transcription factors RAR-Related Orphan Receptors (RORα,β,γ) and Nuclear Receptor Subfamily 1, Group D (REV-ERBα,β or NR1D1), play a role in attenuated core-clock expression during aging.

The aim of the present study was to characterize the organization of the rhesus monkey SCN and to determine if there are changes in neuropeptide signals or the core-clock modifier protein NR1D1, during aging. Furthermore, we assessed whether these changes were sex-steroid dependent, and how the activity profiles in the aged animals relate to the expression patterns in the SCN. I used immunohistochemistry to characterize the expression of the primary output signals (i.e., VIP and AVP), and a core-clock modifier protein (NR1D1) in the female rhesus macaque SCN. There are relatively few steroid receptors in the SCN of females (Pfaff et al. 1976). Nevertheless, I controlled for menopause-related changes in the SCN by comparing old ovariectomized, and old ovariectomized E-supplemented females to see if sex-steroids influenced output signals in the SCN during aging. Finally, I compared the activity profiles in the aged animals to determine if the active animals had a pattern of expression in the SCN that was more similar to young animals.

Materials and Methods

Animals

Female rhesus macaques (*Macaca mulatta;* age range 8-28 years old) were cared for by the Division of Comparative Medicine at the Oregon National Primate Research Center (ONPRC) in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The animals were housed indoors under controlled conditions: 24° C temperature, 12-hour light, 12-hour dark photoperiods with lights on at 0700 h or Zeitgeiber time (ZT) 0; regular meals at 0800 h (ZT1) and 1500 h (ZT8) (Purina High Protein Monkey Chow; Purina Mills, Inc., St. Louis, MO) supplemented with fresh fruit and vegetables, and fresh drinking water available *ad libitum*. Post-mortem tissue and serum was collected between 1100 and 1300 h (ZT4-6). Female rhesus macaques were subjected to the following Institutional Animal Care and Use Committee-approved studies.

Design of Experiments

Experiment 1: Effect of age and hormone therapy on the SCN

Single-label immunofluorescence (IF) IHC was used to compare protein expression between young (~10 years, n = 3) and aged (~26 years, n = 4) ovary-intact animals

Experiment 2: Effect of age on core-clock expression in the SCN core versus shell neurons

Double-label IF-IHC for VIP or AVP with NR1D1 was used to compare core-clock gene expression in young (~10 years) and aged (~26 years) females.

Experiment 3: Effect of hormone therapy on the SCN

Single-label IF-IHC was performed on hypothalamic sections from old intact (n = 3), bilaterally ovariectomized (OVX) (n = 4), and old OVX (n = 4) females that received E supplementation for ~4 years.

Experiment 4: Activity in aged rhesus monkeys

Aged females were grouped based on activity profiles. Animals that had higher total activity for two weeks were deemed *active* (n = 4) and animals with significantly lower total activity became the *sedentary* (n = 4) group.

Menstrual Cycle Status and Serum Hormone Concentrations

Experiment 1 and 2

At the time of postmortem tissue collection, the young intact animals had a mean serum E level of 47 ± 10.6 pg/ml and very low serum P levels (<0.2 ng/ml). These hormone levels are indicative of the early follicular phase of the menstrual cycle. The serum levels of E were 76 ± 20.42 pg/ml in the old gonad-intact animals. All but 1 of these animals had progesterone (P) levels below the assay detection limit. One young animal showed elevated P concentrations (2.4 ng/ml) and was assumed to still be in the luteal phase of the menstrual cycle. Based on individual animal menstruation records, all 3 young intact animals were regular cyclers, and the old intact animals consisted of 2 cycling animals and 1 irregular cycler.

Experiment 3

One month following ovariectomy, half of the animals in the old OVX groups were implanted subcutaneously with empty Silastic capsules (Dow Corning, Midland, Mich., USA), while the other half received capsules containing crystalline E (Steraloids, Wilton, N.H., USA), as previously described (Kohama & Bethea 1995); these implants were designed to last up to one year and to maintain circulating E levels between 100 and 200 pg/ml, which is similar to what is observed during the late follicular phase of the menstrual cycle. However, these levels were higher than E levels typically observed over the majority of menstrual cycle. In the old OVX + E group, the E capsules were replaced annually to ensure sustained long-term delivery of the steroid for the entire duration of the study (~4 years); the untreated OVX animals maintained the same capsules throughout. Terminal serum E levels were undetectable in the young untreated OVX animals and had a mean level of 117 ± 7.0 pg/ml in the young OVX + E group. Serum E concentrations were measured at various time points across the four-year experiment in the old OVX animals to confirm that the target hormone concentrations were being maintained. The serum E levels in the old untreated OVX group were <30 pg/ml at all time points and often fell below the limit of assay sensitivity. In the old OVX + E animals, the mean E concentration during the final two years of the study was 118.8 ± 15.9 pg/ml, and at the time of tissue collection it was 94.3 ± 20.5 pg/ml.

Experiments 4

Note that the animals used in Experiments 4 are the same animals used in Experiment 1, 2, and 3. The E and P assays were performed by the ONPRC Endocrine Technology and Support Core using a chemiluminescence-based automatic clinical platform

(Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, Ill., USA). The sensitivity limits of these assays were 20 pg/ml and 0.2 ng/ml for E and P, respectively (Jensen et al. 2008), and the intra-assay and inter-assay coefficients of variation were all <15%.

Activity Analysis

Locomotor activity was recorded in aged female rhesus macaques (~26 years old) prior to tissue collection. Briefly, an Actiwatch accelerometer-based recorder (Philips-Respironics, Bend, OR) was attached to a collar and collected activity intensity data in 1-min epochs for 2 weeks. The data were then downloaded to a computer and analyzed using Sleepwatch, an activity scoring algorithm (Philips-Respironics), and the mean daytime and nighttime activity intensities were calculated for each individual, as previously described (Urbanski 2011).

Fluorescence Immunohistochemistry

Brains were flushed with 1 L of saline and hypothalamic blocks were immersion fixed in 4% paraformaldehyde (pH 7.6) for 1 week. Next, the blocks were transferred to a 20% sucrose in Phosphate Buffer solution for two days; transferred to 2% DMSO and 10% glycerol in 0.02 M KPBS (pH 7.6) for 1 day; then transferred to 2% DMSO with 20% glycerol in 0.02 M KPBS for 1 week. The blocks were then frozen in an isopentane bath and cooled in dry ice ethanol before storage at -80° C. Blocks were mounted on a frozen-stage sliding microtome using KPBS and sectioned at 25-µm; they were immediately transferred to a cryoprotectant solution and stored at −20 C until use. Single-label immunohistochemistry for VIP, AVP, and NR1D1 was performed as follows.

Floating tissue sections were removed from the cryoprotectant and washed in 0.05 m Tris Buffer (pH 7.6). Next, the sections were transferred to individual preheated 0.5 ml centrifuge tubes filled with 50 mM Sodium Citrate Buffer (pH 9.0) and then the tubes were submerged in a hot water bath for 30 min at 80 C (Jiao et al. 1999). After cooling the sections were rinsed in Tris Buffer and then incubated in Blocking Buffer (Tris Buffer + 3% normal donkey serum) for 1 h, to reduce background staining, and then incubated in a rabbit polyclonal anti-VIP (1:750; LifeSpan Biosciences, Seattle, WA), or rabbit polyclonal anti-AVP (1:2000; LifeSpan Biosciences, Seattle, WA), or monoclonal anti-NR1D1 (1:200; Lifespan Biosciences, Seattle, WA) for 24 h at 37° C. After washing in Tris Buffer, the sections were incubated for 1 h in goat fluorescent anti-rabbit or anti-mouse secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed and mounted on slides. To confirm antibody specificity, the protocol was repeated with the minimum concentration of primary antibody needed to obtain visible label for VIP (1:2000), AVP (1:3000) and NR1D1 (1:200) without and with the preadsorption of VIP, AVP and NR1D1 at a concentration of 100-300 μg/ml for 1 h, which was sufficient to eliminate label in the SCN. Sections from all of the experiments were mounted onto poly-L-lysine coated glass microscope slides and then processed with Autofluorescence Eliminator Reagent (Chemicon®**;** Temecula, CA). Briefly, once sections dried to the slides they were immersed in Tris Buffer for 5 min, 70% ethanol for 5 min, Autofluorescence Eliminator Reagent for 7 min, ethanol for 5 min and then cover slipped using ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA).

Confocal analysis

Immunofluorescence was imaged using a Leica SP5 confocal microscope with Acousto-Optical Beam Splitter. For VIP, AVP, and NR1D1, photomicrographs were taken at a 10x objective at 1024 x 1024-pixel resolution and sampling rate of 400 Hz for (Figure 1). For VIP and NR1D1, a montage of photomicrographic image was obtained using at x20 objective, 512 x 512-pixel resolution, and 400 Hz frequency, which enabled accurate counting of the number of VIP neurons and the expression of nuclear NR1D1, which were difficult to see at a lower magnification. In each animal the neurons were counted in the hemilateral SCN through five coronal sections at 150-μm intervals, spanning the rostral to caudal portion of the SCN. VIP or AVP and NR1D1 cells of the SCN were imaged using a 63x objective at 1024 x 1024-pixel resolution and sampling rate of 400 Hz, in representative young and old animals. Focal planes were 1 μm apart for x10 and x20 objective images and 0.5 μm apart for x63 objective images. Image stacks were analyzed using FIJI (Image J) software with the cell counter tool.

Statistical analysis

One-way ANOVA followed by post-hoc Newman-Keuls or Student's *t*-tests were used. Significance was considered at $P < 0.05$. Where applicable, a Bonferroni correction was used to correct for multiple comparisons.

Results

Effect of age on VIP, AVP, NR1D1 expression

The number of neurons positive for VIP, AVP, and NR1D1 expression was significantly lower in old intact compared with to the young intact animals ($P < 0.05$) (Fig. 1 and 2). When I examined the distribution of these proteins I found that VIP was primarily decreased in the rostral portion of the SCN (Fig. 2B), whereas AVP and NR1D1 were reduced in the caudal portion of the SCN (Fig. 2D and F) ($P < 0.01$). Lastly, in the young and old intact animals, VIP fibers projected to the subparaventricular zone (Fig. 3A and B) and appeared to be even more dense in the aged animals (Fig. 3B).

Clock protein expression in the core versus the shell of the SCN during aging

Using VIP as a marker of core and AVP as marker of shell neurons, I determine core-clock expression in the SCN between young and old animals in the daytime, when tissue was collected for all the animals. In the young and old animals, NR1D1 was not coexpressed with VIP signal in the core of the SCN (Fig. 4A and B). However, NR1D1 protein was coexpressed in the nucleus of AVP neurons in both young and old animals (Fig. 4C and D). Although no statistical analysis was performed on this portion of the data, NR1D1 expression appeared to be reduced in the AVP neurons of the aged animals (Fig. 4C and D).

Effect of hormone therapy on VIP, AVP, NR1D1 expression in aged monkeys

In Experiment 3, VIP, AVP, and NR1D1 neuron number did not differ between the experimental groups, regardless of the gonadal status (ovary-intact vs OVX) or long-term E hormone treatment (OVX + E) (Fig. 5 A, B, and C) ($P > 0.05$).

Age-related changes in activity rhythms between aged monkeys

After first determining that there was no treatment effect on activity in our aged-rhesus monkeys, it was apparent that the animals in the aged cohort displayed variable patterns of activity, regardless of their sex-steroid environment (Fig. 6A). Two phenotypes emerged from the dataset, one group that showed a high level of activity during the daytime (*active*) and another group that showed low daytime activity (*sedentary*) (Fig. 6B, $P < 0.05$). The nighttime activity patterns were similar in both groups (Fig. 6B, $P > 0.05$).

VIP, AVP, and NR1D1 expression in active versus sedentary monkeys

The level of activity in the aged animals did not correlate with the number of neurons expressing VIP, AVP, or NR1D1 ($P > 0.05$) (Fig. 7A, B, and C).

Discussion

Previous studies of the SCN have reported different age-related changes in VIP and AVP expression. For VIP, there are consistent reports using IHC and *in situ* hybridization showing both the protein and mRNA levels decrease with age in the SCN of rodents and also of a nocturnal primate species, the mouse lemur (Duncan et al. 2001, Kallo et al. 2004, Kawakami et al. 1997, Cayetanot et al. 2005). For AVP, there are reports of a significant age-related decrease in AVP protein in the SCN of rodent and of the mouse lemur (Roozendaal et al. 1987, Cayetanot et al. 2005). However, in humans there is only one report showing that there may be diurnal changes in the number of AVP neurons expressing protein in the SCN (Hofman & Swaab 1994). In this

study, VIP neurons formed a distinctive core population in the rostral portion of the SCN. VIP neurons were encapsulated by a shell of AVP neurons that extended up toward the paraventricular nucleus in the SCN of the hypothalamus, thus confirming the results from a previous supplemental publication on the organization of rhesus macaque SCN (R.Y. Moore). Also described here is that elderly female rhesus monkeys had an age-related decrease in both VIP and AVP protein expression in each neurons in the SCN. The age-related decrease in the VIP was especially significant in the rostral portions of the SCN, which contained the most VIP immunoreactive neurons. The decrease in AVP occurred through the entire SCN, but was most consistently decreased in the caudal portion of the SCN. In humans, one study showed that the expression of AVP in neurons of the SCN was lower as a function of the deviation from the weighted 24-h mean between young (< 50) and elderly subjects (> 50), specifically during the morning peak in expression, which coincides with the time of day when my tissue was collected (Hofman & Swaab 1994). In contrast, VIP neurons of humans do not show diurnal variation in any neuronal parameters (number, size, or density) (Hofman et al. 1996). Interestingly, in the mouse lemur, a short-lived nocturnal primate species, there is a 24-h pattern of VIP and AVP expression in the SCN that shows phase advancement for VIP and phase delay for AVP with advanced age (Cayetanot et al. 2005). While I cannot rule out the possibility that the results presented here are indicative of phase shifts in 24-h output of VIP and AVP immunoreactivity in the SCN, because rhesus monkeys have a more similar lifespan to humans, exhibit diurnal sleep-wake patterns, and the animals used in our study were more biologically equivalent to the ages described in the human port-mortem studies, it seems likely that there is an

overall decrease in the protein expression of primary output signals of the SCN with age in higher primates.

The expression of VIP in SCN cell bodies was lower in older animals, which was reflected in the decrease in VIP immunoreactive neuron number with age (Fig. 1A and Fig. 2A,B). However, more diffuse and punctate VIP fiber immunoreactivity, extending toward the subparaventricular zone of the hypothalamus, were observed in older animals (Fig 3B). One possibility is that VIP protein synthesis was phase advanced in the aged animals and if samples had been collected at an earlier time point then a similar concentration of VIP immunoreactivity in the cell bodies would be observed in the aged animals. It is also important to point out that the number of neurons in the SCN does not change with age in rhesus monkeys (Roberts et al. 2012). Therefore, it is likely that the expression levels of VIP, AVP, and NR1D1 was decreased in individual neurons, rather than fewer neurons producing the peptides, in older animals. Based on this study, neurons are still able to produce VIP protein despite aging and it is possible that downstream targets, like VPAC2, on AVP neurons are disrupted by this change in release. In the mouse and rat, the 24-hour rhythm in the number of VIP-expressing neurons is significantly dampened with age and coincides with a dampened rhythm in VPAC2 gene expression (Kalló et al. 2004). Therefore, altered communication between VIP and AVP neurons may be responsible for age-related changes in behavioral rhythms, rather than a complete loss of mRNA and protein production and rhythms *per se*.

In the literature, is unclear whether the genetic clock mechanism changes with age in the SCN. The lack of good antibodies to core-clock proteins, distinct 24-hour

rhythms that require multiple time points of sample collection, and low expression in the SCN compared to other brain regions and peripheral tissues (unpublished observation) in these signals make non-human primate studies of the core-clock feedback loop difficult. One core-clock antibody that our lab has had good success working with in the rhesus macaque is NR1D1 (Lemos et al. 2006, Sitzmann et al. 2010). Using the antibody to NR1D1, this study is first to describe the expression of a clock protein in output of the SCN. Interestingly, NR1D1 signal was expressed in in AVP neurons (between 1100-1300 h or ZT4-6) of young and old monkeys. Overall, NR1D1 decreased in expression with age and the pattern of decrease was most significant in the same plane as the shell population of AVP neurons. Future studies should use this method to describe the changes in expression pattern over the 24-h day to make links between core-clock rhythms and output signals of the SCN. It is especially important that there were still AVP neurons that expressed NR1D1 in the aged animals, which suggests that the core-clock-modifier mechanism may still be functional in aged monkeys.

A number of studies have attributed the deterioration of output signals found in the SCN during aging to the menopausal loss of sex-steroids (Engelberth et al. 2014). A previous study showed that short-term ovariectomy in adult rats attenuated VIP mRNA signal in the whole hypothalamus and short-term estradiol dibenzoate treatment increased VIP expression in OVX animals (Gozes et al. 1989). While the results of that study were in whole hypothalamus, the expression of output signals within the SCN of OVX and OVX hormone supplemented animals has not been described. In the present study, I did not find an effect of chronic E on VIP or AVP expression in old OVX + E females rhesus monkeys. Thus, the age-related changes in the number of neurons

expressing VIP or AVP in the SCN of females do not appear to be responsive to the long-term hormone therapy. Specifically, elevation of circulating E levels in aged female monkeys, through hormone supplementation, does not restore core-clock protein in the SCN or its signal output to levels observed in the young animals.

Recently, Wang (2015) and Lim (2014) showed correlations between at least one sleep-wake activity parameter and VIP expression in elderly adults, either with or without Alzheimer disease. I did not find an association between the number of VIP or AVP neurons and any of the activity parameters in aged monkeys. However, our animals were on a fixed light:dark cycle, which may account for the variation between the results found here compared to the recent human studies. Furthermore, Actiwatches only measure the intensity of locomotor activity, but provide no information about sleep architecture. Therefore, it is possible that some parameters of sleep quality that were not analyzed are associated with age-related changes in protein expression in the SCN of the non-human primate model (like sleep fragmentation or hours spent in sustained rest). In summary, the total activity did not correlate with the decrease in VIP, AVP, and NR1D1 protein signals in the SCN.

Aging in both men and women is associated with changing sleep-wake activity. Some studies have hypothesized that the altered circulating E levels, which are thought to contribute to many age-related pathologies, are involved in perturbed circadian rhythms with age (Guidozzi 2013, 2015, Engelberth et al. 2014). On one hand, the results from the current study show that aging results in clear changes in protein levels in the SCN of the non-human primate. On the other hand, my results suggest that the SCN does not respond to sex-steroids in old animals. Together, these data show that

VIP, AVP, and NR1D1 decrease with age and these changes are not sex-steroid dependent or associated with changes in daytime activity in old and postmenopausal female monkeys.

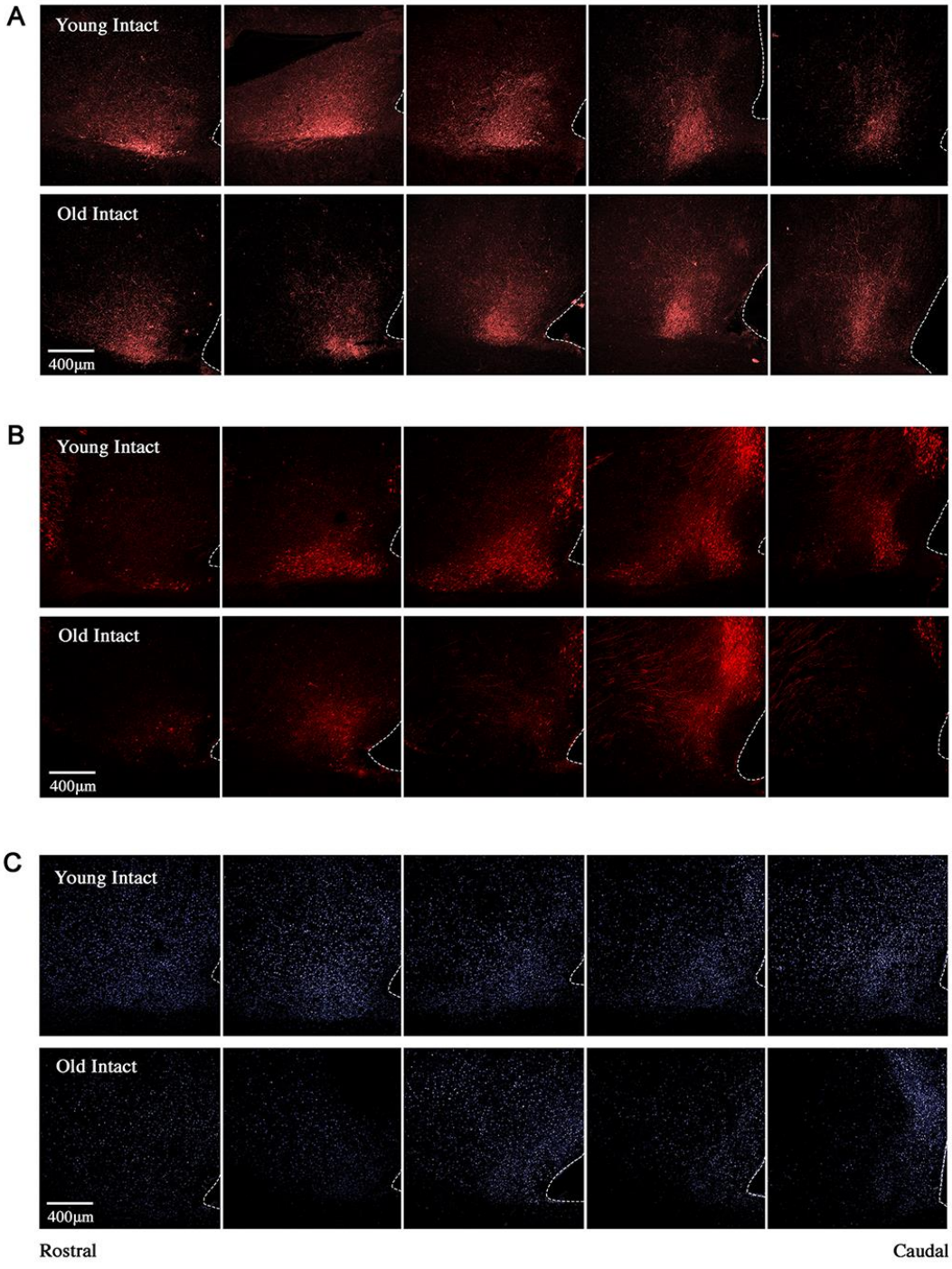

Figure 1. Age-related changes in immunoreactive VIP, AVP, NR1D1 in the SCN of female rhesus monkeys. A, B, and C show VIP, AVP, and NR1D1 expression in the same representative young and old ovary-intact animals.

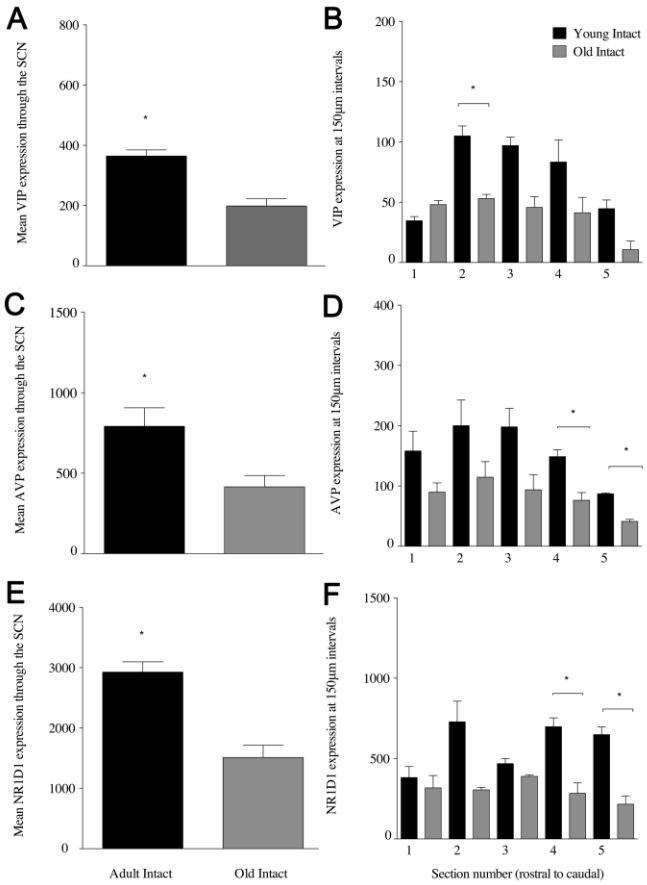

Figure 2. Expression of VIP, AVP, and NR1D1 immunoreactivity neurons in the SCN of young (~10 years) and old (~26 years) gonad-intact female rhesus monkeys. Overall mean number of neurons (± SEM) and distribution patterns of neurons are depicted in the left and right panels, respectively. A and B, number of neurons immunoreactive for VIP was significantly higher in the young animals compared to old animals and this difference was most consistently found in rostral portion of the SCN. C and D, AVP expression was significantly higher in young animals the difference was most significant in the caudal portion of the SCN. Finally, NR1D1 expression was lower in the aged animals and this was primarily in the caudal part of the SCN. A, C, and E, *, $P < 0.05$ (Student's *t*-test). B, D, and F, *, $P < 0.01$, (Student's *t*-test followed by Bonferroni correction).

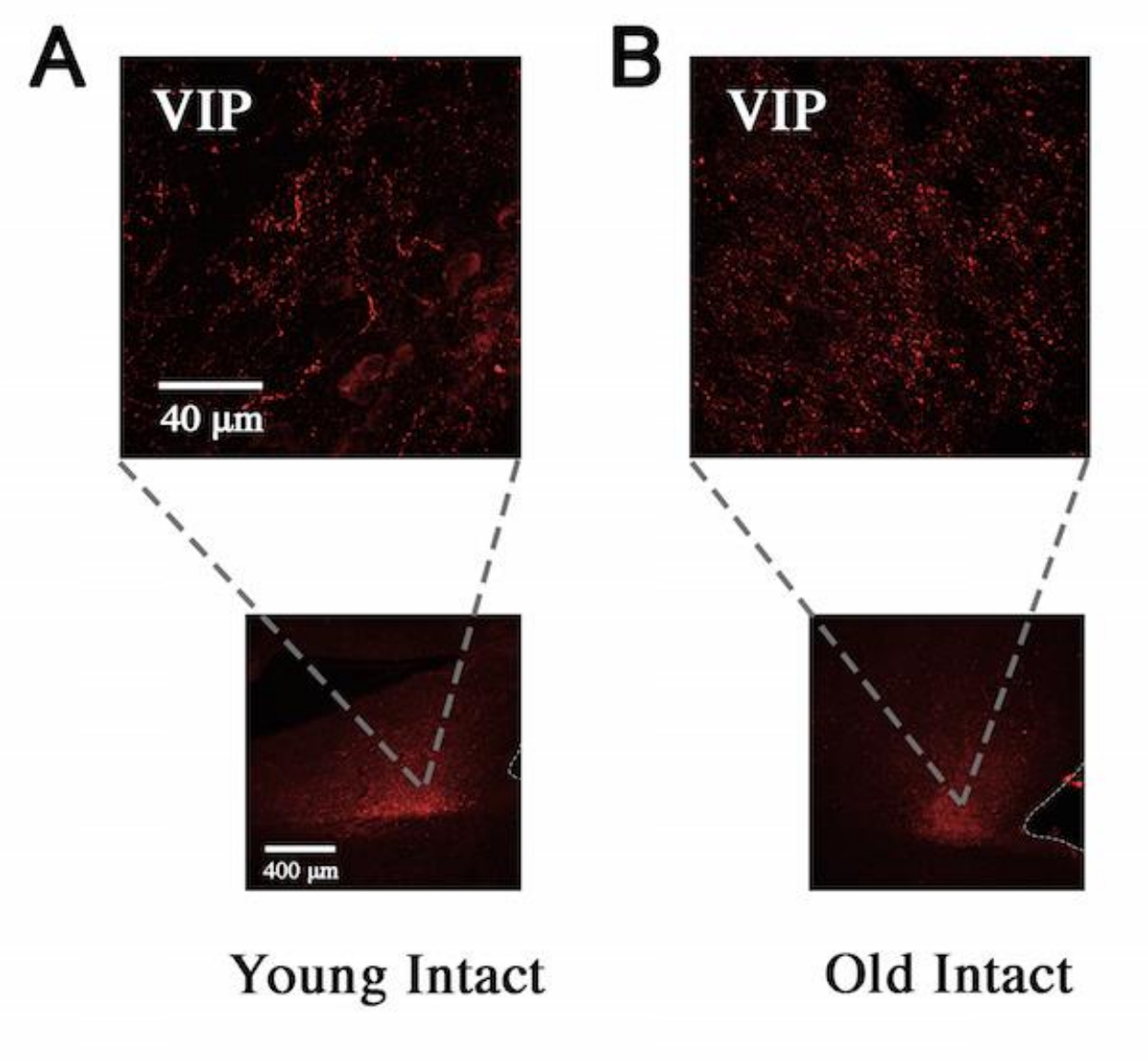

Figure 3. Age-related differences in VIP fiber immunoreactivity in the SCN of ovary-intact rhesus monkeys, as revealed by single-label IHC. More diffuse and punctate VIP fiber immunoreactivity projecting toward the subparaventricular zone in the older animals as compared to the young animals.

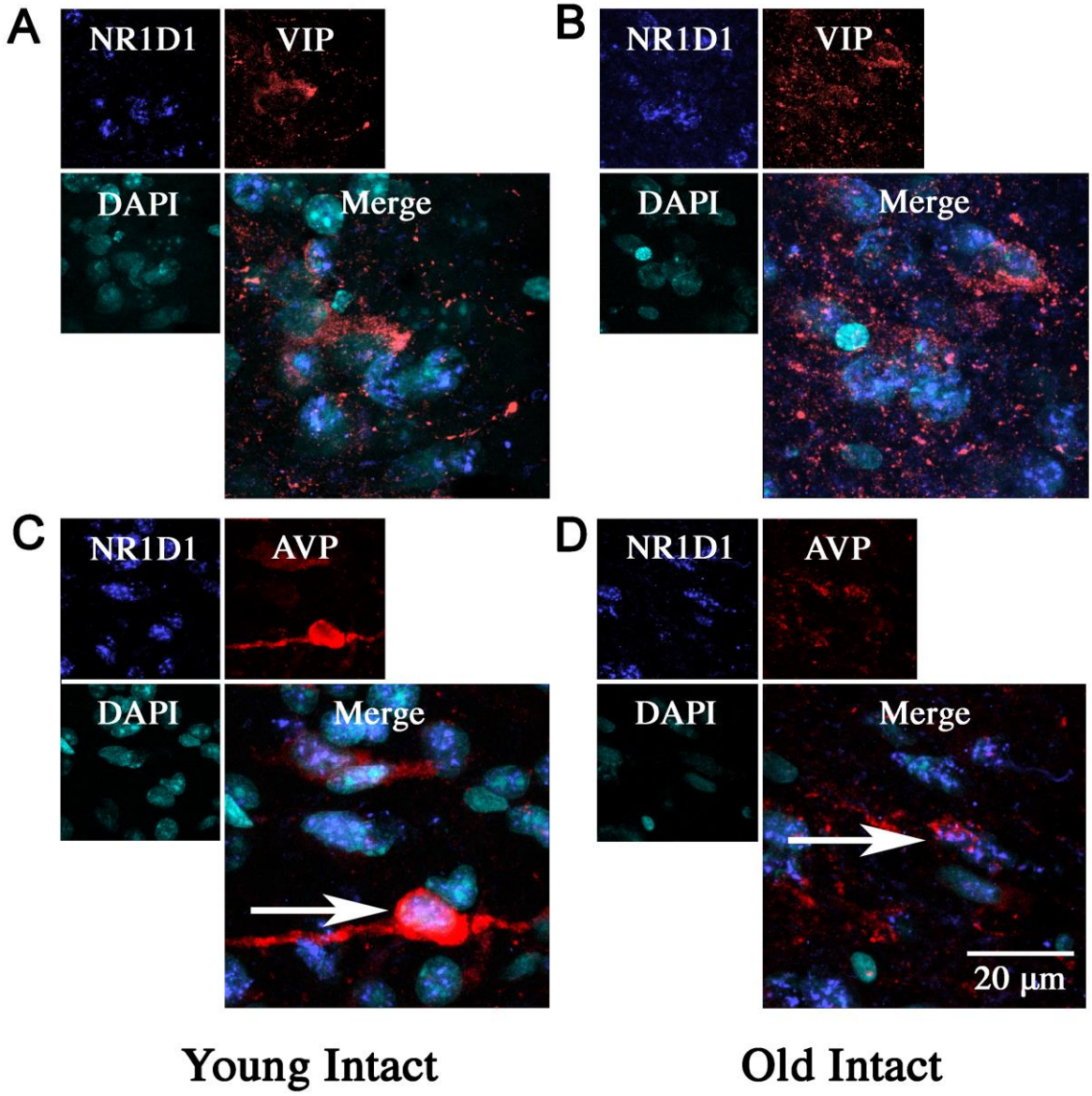

Figure 4. Age-related differences between VIP and AVP immunoreactivity in the SCN core and shell of ovary-intact rhesus monkeys, as revealed by double-label IHC. Expression of NR1D1 and VIP did not overlap in young (A) or old (B) animals in the core of the SCN. NR1D1 and AVP overlapped in both young (C) and old (D) animals in the shell of the SCN. Representative images from animals necropsies at 1000 h (ZT3).

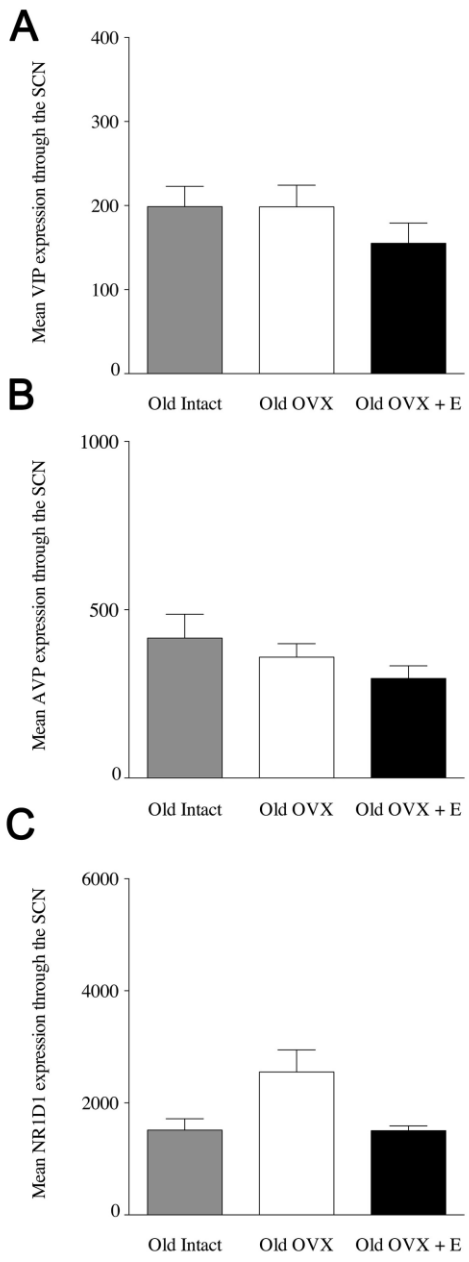

Figure 5. VIP, AVP, and NR1D1 immunoreactive neurons in the SCN of female rhesus monkeys and the effect of long-term E supplementation. The values represent the mean number of neurons (± SEM) from old (~26 years) animals that were intact (n = 3 or 4), OVX (n = 4), or OVX + E (n = 4) treatment. No differences in VIP, AVP, or NR1D1 expression were detected. $P > 0.05$ (ANOVA followed by Student-Newman-Keuls test).

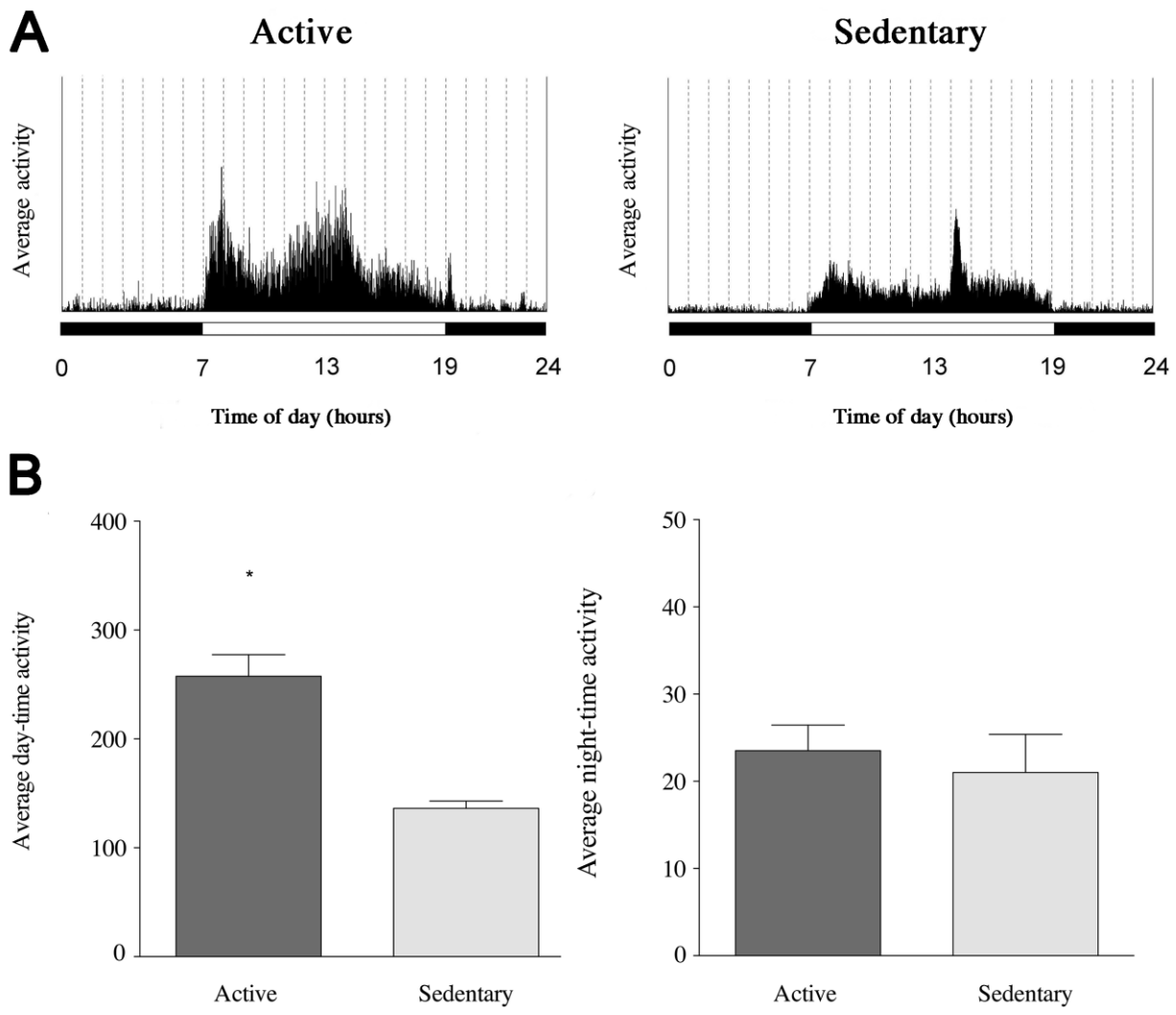

Figure 6. Activity patterns in aged female rhesus monkeys. A, Representative average 24-hour activity-rest patterns from aged female rhesus monkeys, illustrating two different activity phenotypes. B, *Active* animals had significantly more activity than *sedentary* animals over a two-week period near the time of tissue collection. There was no difference in average nighttime activity between *active* and *sedentary* animals. The values represent mean (± SEM). *, $P < 0.05$, (Student's *t*-test).

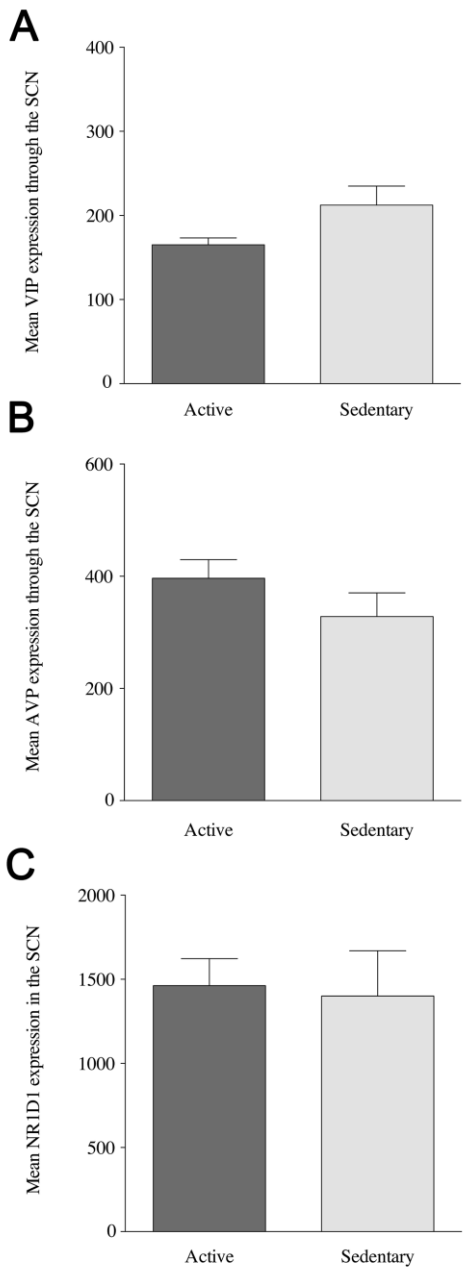

Figure 7. VIP, AVP, and NR1D1 immunoreactive neuron numbers in the SCN of *active* and *sedentary* old (~26 years) female rhesus macaques. The values represent the means (± SEM). There was no significant difference in VIP, AVP, or NR1D1 immunoreactivity in the SCN between *active* and *sedentary* animals. $P > 0.05$ (Student's *t*-test).

Chapter 3

Diurnal expression of clock and steroid receptor genes in the suprachiasmatic nucleus and arcuate nucleus of aged male rhesus macaques

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ABSTRACT:

Like men, aging male monkeys gradually lose circulating sex-steroids throughout the later part of their lifespan. However, little is known about the impact of changing hormone levels on the brain of aging men. Here, a monkey model of aging and hormone therapy (HT) was used to determine the impact of age and sex-steroid changes on the hypothalamus. Methods: An Affymetrix Rhesus Gene Chip 1.0 ST Array in the arcuate (ARC) and suprachiasmatic nucleus (SCN) of young intact (n = 6), old intact (n = 7), and old intact testosterone supplemented animals (n= 6), was used to assess age and sex-steroid-related changes on the hypothalamus. Results: In general, there were very few effects of age and no effect of HT on reproductive-related, clock, steroid receptor, or output signal genes in the ARC or SCN. Interestingly, many of the core-clock and core-clock modifier mRNA levels in the ARC and SCN displayed diurnal expression patterns in aged animals. Conclusion: Unlike gonad-intact female macaques that show reproductive changes in the ARC after menopause, reproductive-related mRNA expression in the ARC and even the SCN of gonad-intact males remains stably expressed despite aging. Interestingly, the data do suggest that there is a diurnal expression pattern of clock and steroid receptor genes in aged males in the ARC ad SCN. In particular, the androgen receptor was expressed at night when testosterone levels are elevated. Together, these data support the idea that sampling tissue at multiple time points may be necessary to detect age and hormone therapy benefits if the gene and protein have a 24-hour expression pattern.

Introduction:

Reproductive aging in males is characterized by a gradual decrease in circulating testosterone (T) that results in physiological and cognitive deterioration (Urbanski & Sorwell 2012, Urbanski et al. 2014, Gore & Hayes 2015). In females, the complete loss of circulating estrogen and progesterone (P) at the time of menopause results in clear alterations in the hypothalamic-pituitary gonadal (HPG)-axis. In particular, a population of neurons known as KNDy (Kisspeptin, Neurokinin B, and Dynorphin A), increase in expression with age and show decreased expression in response to hormone therapy in older female monkeys (Eghlidi et al 2010, Eghlidi & Urbanski 2015). However, there is very little information on the impact of aging and declining testosterone levels on hypothalamic neurons in males.

Originally, a comparison of T measurements between young and old males yielded mixed results. Studies in the morning reported that testosterone levels decreased while another experiment, conducted in the afternoon, showed that T levels remained stable despite aging (Vermeulen et al. 1972, Baker et al. 1976). Eventually, it was realized that the nocturnal peak and circadian pattern of T was attenuated with age in males (Marrama et al. 1982, Bremner et al. 1983). Similar to humans, male monkeys also show a change in the diurnal rhythms of androgens with age (Urbanski & Sorwell 2012, Urbanski et al. 2014). Specifically, the evening increase of circulating androgens decreases in older animals, and there is leveling out of serum T and 5-alpha-dihydrotestosterone (DHT) throughout the day (Urbanski et al. 2014). In addition to T, the serum levels of other hormones like dehydroepiandrosterone (DHEA) and estradiol (E) show reduced rhythmicity in old male monkeys (Urbanski et al. 2014).

There is very little information on how changing androgen levels impact the hypothalamus of elderly males. In gonadectomized male mice, T appears to exert its effects on the HPG axis by binding to the androgen receptor or by being aromatized to E and binding to estrogen receptors (ERs) on KNDy expressing neurons in the ARC (Smith et al. 2005, Navarro et al. 2011). In gonadectomized male monkeys and rodents, there is an increase in kisspeptin mRNA (*KISS1* and *kiss1*, respectively) in the ARC. In male monkeys, T treatment reduced this *KISS1* expression. Together these data suggest that altered negative feedback in the HPG axis of males is similar to that of postmenopausal females. However, it is unclear how relevant this is to male aging because, unlike post-menopausal females, males show a more gradual and less complete decline in circulating sex-steroid levels (Bremner et al. 1983). On the other hand, it is possible that nuclei outside of the HPG axis may be effected- due to declining sex-steroids during male aging. One possibility is that the SCN, which expresses androgen receptors that responds to T and DHT treatment, is effected by the age-related decline in circulating androgen levels. In terms of the genetic clock mechanism, neurons in the SCN remain rhythmic despite aging, but there are subtle changes in the expression of core-clock elements (Weinert 2000, Weinert et al. 2001, Yamazaki et al. 2002). It remains to be determined if factors that are not directly related to the oscillatory mechanism, like modifier transcription factors, Casein Kinases (CKIδ,ε), RAR-Related Orphan Receptors (RORα,β,γ) and Nuclear Receptor Subfamily 1, Group D (REV-ERBα,β), play a role in attenuated core-clock expression during aging. The overall aim of the present experiment was to: 1) determine how gene expression in the ARC changes during aging and in response to androgen supplementation and 2) test the

hypothesis that reproductive aging has little effect on the HPG axis of males, but impacts other nuclei, like the SCN, and that hormone therapy may help to maintain activity rhythms in aged males.

Materials and Methods

Animals

Six young adult (age range 7-12 years) and twelve old (age range 21-26 years) male rhesus macaques (*Macaca mulatta*) were cared for by the Division of Comparative Medicine at the Oregon National Primate Research Center (ONPRC) in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. The animals were housed indoors under controlled environmental conditions: 24° C temperature; 12-hour light, 12-hour dark photoperiods with lights on at 0700 relative hours (h) or Zeitgeiber time (ZT) 0; regular meals at 0800 and 1500 h (ZT1 and ZT8, respectively) (LabDiet High Protein Monkey Chow; LabDiet, Inc., St. Louis, MO) supplemented with fresh fruit, vegetables and candy, and with fresh drinking water available *ad libitum*. Animals were subjected to the following Institutional Animal Care and Use Committee-approved studies, which involved postmortem isolation of RNA from the ARC and SCN and gene profiling using the Affymetrix Rhesus Gene Chip 1.0 ST Array.

Design of Experiments

Effect of Aging and androgen supplementation

Experiment 1 used postmortem rhesus macaque brain tissue obtained through the ONPRC Tissue Distribution Program. The intact males had previously been involved in a non-human primate androgen supplementation study and comprised three groups: young intact (n = 6); old intact (n = 6 or 7); and old intact + hormone supplementation (n = 6). The six old males in the hormone supplementation group received T, administered orally; because T has previously been shown to undergo significant first pass metabolism by the liver, following oral administration, the T was first dissolved in sesame oil at 120 mg/ml and then mixed into a chocolate, 12 grams, and administered before bedtime. T was administered prior to bedtime to mimic the nighttime peak in androgen levels found in healthy young males (Urbanski et al. 2015). In addition, they also received the two morning doses of DHEA, dissolved in sesame oil and mixed with chocolate (20 mg/ml). Serum, collected across the 6-month experiment in all three groups of animals, was frozen and assayed for T, DHEA, and estradiol (Amory & Bremner 2005, Armory et al. 2006, Urbanski et al. 2015).

24-hour changes in gene expression in aged animals

Experiment 2 examined whether gene expression differs between the day and night, we compared ARC-ME and SCN gene expression at 1000 h (n = 4) with that of 2200 h (n = 4) (ZT3 and ZT15, respectively). The animals were old intact male macaques, and the 2200 h (ZT15) tissue collection group had been maintained under the same 12-hour light, 12-hour dark photoperiods as described above. During the nighttime tissue collections, the animals' eyes were covered to prevent the impact of light exposure on mRNA expression in post-mortem tissue experiments.

RNA extractions

The ARC was bilaterally subdissected (as previously described in Eghlidi et al. 2010) and the SCN was subdissected based on its position relative to the third ventricle and optic chiasm. RNA was isolation from the whole ARC and SCN using the RNeasy Plus Universal Mini Kit (Qiagen) with two QIAcube Automation System (Qiagen). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to determine the quality of the RNA, and a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) was used to determine the concentration.

Gene Microarray

Microarrays were performed by the OHSU Gene Profiling Shared Resource. Briefly, target labeling was performed using total RNA samples that were prepared for array hybridization by synthesizing cDNA using the Ovation Pico WTA RNA Amplification System v.2 (NuGEN Technologies). Array hybridization and processing was performed by injecting the hybridization cocktail into a cartridge containing the GeneChip Rhesus Gene 1.0 ST array (Affymetrix) containing 37,292 gene-level probe sets. Arrays were incubated, washed, and stained on a GeneChip Fluidics Station 450 (Affymetrix) using the Hybridization Wash and Stain kit (Affymetrix). Arrays were scanned using the GeneChip Scanner 3000 7G with an autoloader (Affymetrix). Each array file was then analyzed using the Affymetrix® Command Console™ (AGCC) v.3.1.1 software, and expression analysis was performed using Affymetrix® Expression Console™ Software 1.4. Probe cell intensity files (CEL) for each of the experimental samples were uploaded into the Affymetrix® Expression Console™ Software 1.4, and a multi-array analysis

incorporating all the samples of the study group was performed using the Robust Multi-array Average (RMA)-Sketch normalization and summarization algorithm for all exon transcripts. Probes that were the most highly expressed or showed age, treatment, or diurnal expression differences are reported in Tables 1-6.

TaqMan qRT-PCR

QuantStudio™ 12K Flex thermocycler (Life Technologies, Grand Island, NY). Initially, pooled cDNA was used to create standard curves for each gene, and the experimental samples were subsequently diluted accordingly so as to fall within the linear part of the curve. The PCR mixtures contained 5 µl TaqMan Gene Expression Master Mix, 0.3 µl of each specific forward and reverse primer (300 nM final concentration), 0.25 µl of specific probe (250 nM final concentration), and 2 µl of cDNA. The RT-PCR reaction sequence included 10 min of incubation at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, 1 min at 60°C, and 15 s at 95°C. Automatic baseline and threshold levels were determined by QuantStudio™ 12K Flex thermocycler detection system software, and the final expression values were normalized to *RPL13A.* KISS1, NKB, NPAS2, and RPL13A were examined on 384-well optical plate. A negative control included the omission of cDNA templates from the reaction mixture.

Statistics

In experiments 1, an ANOVA was used to assess between-group differences in expression. For experiments 2, Student's *t*-tests were used to assess differences

between groups. Significance was considered at $P < 0.05$ following an False Discovery Rate correction for multiple comparison.

Results

ARC Aging and Hormone Treatment

Age and treatment differences in reproductive-related gene expression in the ARC nucleus between young intact (n= 6), old intact (n = 7), and old intact androgen supplemented (n = 6) males were assessed. No differences in clock, GnRH signaling, or steroid receptor mRNA levels were found in the ARC during aging or in response to hormone therapy in males ($P > 0.05$). Real-time PCR to *TAC3* showed the same constitutive expression pattern between treatment groups. Probes to *KISS1* were not present on the microarray chip. However, like *TAC3*, real-time PCR of *KISS1* expression showed no significant effects of age or hormone therapy using real-time PCR.

Diurnal Pattern of Gene Expression in the ARC

The possibility that diurnal differences in gene expression may have accounted for the lack of differences found in the reproductive genes during aging and hormone therapy was considered (Eghlidi et al. 2010). To examine whether there were day and night differences in gene expression, the ARC genes of old intact males at 1000 h (n = 7) and 2200 h (n = 5) (ZT3 and ZT15, respectively) were compared. Many of the genes in the ARC displayed a diurnal expression pattern and some of them were more significantly expressed at night, including *AR, BMAL1, CSNK1E, NPAS2, NR1D1* ($P < 0.05$). *NPAS2*

expression was also confirmed using real-time PCR and showed the same increase during the evening compared to the daytime in the SCN ($P < 0.05$). Finally, *KISS1* did not show a diurnal expression in the ARC using real-time PCR (data not shown).

SCN Aging and Hormone Treatment

There were no other changes in clock-related, SCN output signal, or steroid receptor mRNA in the SCN during aging or androgen supplementation ($P > 0.05$).

Gene Expression Changes in the SCN at 1000 and 2200 h (relative to 0700 h lights on) in Old Animals

AR, *ESR1*, and *NPAS2* were significantly higher during the night than during the day ($P < 0.05$). *NPAS2* expression was also confirmed using real-time PCR and showed the same increase during the evening compared to the daytime in the SCN ($P < 0.05$).

Discussion

Like men, aged male rhesus monkeys gradually lose androgen rhythms as they age (Urbanski et al. 2014, Hardy & Schlegel 2004, Harman et al. 2001, Feldman et al. 2002, Brenner et al. 1983, Vermeulen et al. 1972). For this reason, they represent a pragmatic animal model for studying neuroendocrine changes in aging males. In this study, I showed that the male ARC KNDy gene expression was resistant to the gradual decline in circulating androgens during aging. This observation is consistent with my earlier findings that the profound changes in E and P at the time of menopause contribute to the age-associated neuroendocrine alteration in the ARC nucleus of old

female rhesus monkeys (Eghlidi et al. 2010, Eghlidi & Urbanski 2015). On the other hand, there were significant effects of age and androgen supplementation on the SCN of males that suggest males may benefit from hormone therapy.

In this study, the animals received T and DHEA in a cyclical treatment paradigm meant to mimic the 24-hour hormone profiles of young male animals (Bremner et al. 1983, Corona et al. 2013, Urbanski et al. 2015). Serial blood samples were collected across the 24-hour day to ensure that hormone levels were maintained in the treatment group. First, of all the genes involved in gonadotropin signaling that were examined in the ARC none of them showed a significant age-related change in expression. This suggests that the most changes in testosterone rhythms during aging have very little effect on the steroid feedback mechanism within the HPG axis during aging. T exerts its effects on the ARC KNDy neurons by binding to the ARs, either directly or after conversion to DHT, or by being aromatized to E and binding to ERs in the male ARC (Smith et al. 2005, Navarro et al. 2011). In an earlier study of these male monkeys, the levels of E were measured over the 24-hour day and T and DHEA treatment reinstated the evening rise of both DHT and estradiol in aged male animals (Urbanski et al. 2014). The lack of change in KNDy and steroid receptor gene expression between the young and aged androgen supplemented group suggests that the steroid negative feedback effects to KNDy neurons is preserved in aged males.

In the ARC, AR and ESR1 expression were more highly expressed at night. T can be converted to either DHT or E, and act through these steroid receptors, respectively. Both T and E are higher at night even in aged males and so perhaps had we assessed the benefits of hormone therapy in tissue collect at night we may have

detected a difference. Furthermore, the treatment did not have an effect on KNDy gene expression, which support the idea that this system is only perturbed when there is a substantial loss of circulating sex-steroids, as is the case in post menopausal females. Overall, the lack of change during aging in the ARC supports the idea that modest changes in testosterone levels during aging did not affect the HPG-axis, because there was no change in KNDy neuronal gene expression

Using gene microarray analysis, we have previously observed that many genes have a clear 24-hour pattern of expression even outside of the SCN (Urbanski et al. 2009, Urbanski 2011, Lemos et al. 2006). For this reason, I was concerned that the collection of tissue at a single time point (i.e., during the morning at ZT3) may have prevented me from detecting changes in certain genes even in the ARC. Clock genes were stably expressed despite aging and hormone therapy in the SCN and ARC. However, there were differences in the diurnal expression patterns of many of these genes, especially, in ARC. Interestingly, *NPAS2* was more highly expressed at night in the SCN and ARC. While CLOCK typically forms a heterodimer with BMAL1, NPAS2 can compensate for the loss of CLOCK in the brain (DeBruyne et al. 2007, DeBruyne et al. 2007). Therefore, *NPAS2* was likely increasing to dimerize with *BMAL1* in the absence of *CLOCK* expression in the aged animals to help preserve the function of the core-clock mechanism in the SCN and ARC. There were a number of genes that has a diurnal in the ARC and not the SCN. *BMAL1*, *CSNK1E*, and *NR1D1* (*REV-ERBα*) were more highly expressed at night. CSNK1E phosphorylates Period proteins to aid in their breakdown and can phosphorylate and activate BMAL1 (Dunlap 1999, Whitmore et al. 2000, Eide & Virshup 2001, Eide et al. 2002, Eide et al. 2005, Eide et al. 2005). *BMAL1*

was similarly expressed in young and old animals at the 1000 h (ZT3), but the increase in *CSNK1E* in the aged animals suggests that the core-clock feedback loop required further modifications. *BMAL1* was more highly expressed at night in aged animals, so it is possible that *CSNK1E* modifies *BMAL1*, and collecting tissues in the evening may have revealed some age-related interaction between these genes. Finally, *NR1D1*, a core-clock modifier that can suppress BMAL1 expression had a diurnal rhythm in the aged males and suggests that the positive limb of the core-clock feedback need to be suppressed in the evening in the ARC (Schibler 2007). Finally, since all of these genes were more highly expressed at night it is possible that I was unable to detect changes in these genes in response to androgen supplementation because I was looking at the suboptimal time of day.

In females, we found that *KISS1R*, the receptor to kisspeptin, exhibited a diurnal expression pattern and was more highly expressed during the daytime. Another study revealed a similar finding in the GT1-7 cell line, which showed E treatment elicited a circadian expression profile in *Kiss1r in vitro* (Tonsfeldt et al. 2011). I did not find any diurnal variation in the expression of *KISS1R* in the male ARC nucleus, which suggested that there may be subtle differences in the GnRH mediated signaling in the ARC of males, either in old age, or due to sex differences in the organization of these this pathway between males and females.

Recently, Chen et al. (2016) used microarray analyses to identify rhythmic transcripts in human prefrontal cortex (PFC) tissue during aging. Using two PFC regions (Brodmann's areas 11 and 47) from 146 subjects, the authors identified genes that were rhythmically expressed in both tissues and transcripts that showed an age-related

changed in rhythmicity. Interestingly, a number of genes gained rhythmicity with age and the authors propose that there may be a mechanism in the brain that compensates for a decline in core-clock function. On one hand, these results complement my interpretation of the changes in canonical clock and modifier gene expression in the SCN and ARC of old animals during day versus night. Namely, that *NPAS2* was likely increasing to compensate for the loss of *CLOCK* rhythm and core-clock modifier *CSNK1E* increased to aid in the breakdown of other core-clock genes that may have been desynchronized as a result of aging. On the other hand, the results presented by Chen and colleagues also suggest that some of the genes that were rhythmically expressed in the old gonad-intact males in my study may not be rhythmic in young intact males. It will be important in future experiments to examine young intact SCN and ARC samples, at multiple time points, to determine whether the genes that displayed a diurnal rhythm in older males has a similar diurnal pattern of expression in young animals. Therefore, I cannot rule out the possibility that some of the diurnal gene expression patterns that I observed in the aged males might also be present in young animals.

The majority of physiological function and behaviors have a rhythmic component (Hastings et al. 2008, Kyriacou & Hastings 2010, Urbanski 2011, Hastings & Goedert 2013, Schroeder & Colwell 2013, Bailey & Silver 2014). Age-related changes in the 24-h release of hormones are likely to play a significant role in many of these functions and may contribute to the etiology of age-related pathologies (Scheer et al. 2009, Downs & Wise 2009). This is especially important in men and aged male monkeys, with age-associated attenuation of T and DHEA rhythms (Plymate et al. 1989, Feldman et al.

2002, Luboshitzky et al. 2003, Kaufman and Vermeulen 2005, Page et al. 2006, Schlatt et al. 2008, Bremner et al. 2010, Urbanski et al. 2014). While perturbed hormone rhythms in men do not result in a clear andropause, they may still exert effects on male physiology and behavior (Morales et al. 2000, Handelsman & Liu 2005, Downs & Urbanski 2006, Rance 2009).

Overall, the findings in this study are in agreement with previous literature that the SCN of males is sensitive to androgen treatment, and further indicate that androgen treatment may help to synchronize the core-clock mechanisms in the SCN (Karatsoreos et al. 2007, Iwahana et al. 2008, Karatsoreos et al. 2011, Butler et al. 2012). The primary change I observed was a decrease in CLOCK gene expression within the SCN, which has been described previously in at least one rodent species (Kolker et al. 2003). No alteration in the KNDy system was observed, suggesting that there are no central effects of male aging on the HPG axis.

Affymetrix GeneChip Rhesus Gene 1.0 ST Array in the Male ARC																	
Gene Symbol		**Probe Set ID**	**Young Intact**			**Old/ Young**	**Old Intact**			**Old Supp/ Young**	**Old Intact Supplemented**			**Old Supp/Old**	***P*-value**	**FDR *P*-value**
Clock genes																
BMAL1	ARNTL (aryl hydrocarbon receptor nuclear translocator like)	13656832	6.21	±	0.11	-1.0	6.15	±	0.07	1.1	6.24	±	0.04	1.1	0.7262	0.8958
BMAL2	ARNTL2 (aryl hydrocarbon receptor nuclear translocator like 2)	13617047	5.64	±	0.05	1.0	5.70	±	0.11	1.1	5.76	±	0.12	1.1	0.7084	0.8958
CLOCK	Clock circadian regulator	13752327	8.16	±	0.06	-1.1	8.10	±	0.06	-1.1	8.15	±	0.02	1.0	0.6243	0.8946
CRY1	Cryptochrome circadian clock 1	13627793	5.92	±	0.11	-1.1	5.83	±	0.04	1.1	5.98	±	0.06	1.2	0.3775	0.8258
CRY2	Cryptochrome circadian clock 2	13655873	5.70	±	0.05	1.1	5.80	±	0.11	1.0	5.73	±	0.06	-1.1	0.6921	0.8958
CSNK1E	Casein kinase 1 epsilon	13614725	5.97	±	0.11	-1.1	5.97	±	0.08	-1.0	5.99	±	0.06	1.0	0.9888	0.9888
DEC1	Deleted in esophageal cancer 1	13666246	3.34	±	0.12	-1.1	3.19	±	0.13	-1.1	3.11	±	0.12	-1.0	0.4664	0.8749
NPAS2	Neuronal PAS domain protein 2	13640655	6.57	±	0.07	-1.0	6.61	±	0.07	-1.1	6.54	±	0.09	-1.1	0.8599	0.9405
NR1D1	Nuclear receptor subfamily 1 group D member 1	13679414	6.13	±	0.10	1.0	6.00	±	0.09	-1.0	6.02	±	0.03	-1.0	0.5495	0.8749
NR1D2	Nuclear receptor subfamily 1 group D member 2	13708515	8.19	±	0.06	-1.1	8.08	±	0.06	1.0	8.23	±	0.11	1.1	0.4180	0.8606
PER1	Period circadian clock 1	13676737	4.93	±	0.07	-1.0	4.95	±	0.07	-1.0	4.93	±	0.05	1.0	0.9611	0.9888
PER2	Period circadian clock 2	13637104	6.28	±	0.05	-1.0	6.29	±	0.05	1.0	6.32	±	0.05	1.0	0.8887	0.9426
PER3	Period circadian clock 3	13580448	8.21	±	0.06	-1.0	8.06	±	0.10	-1.0	8.11	±	0.10	-1.0	0.5241	0.8749
RORA	RAR related orphan receptor A	13780419	7.18	±	0.03	1.1	7.17	±	0.08	-1.1	7.02	±	0.04	-1.2	0.1545	0.5668
RORB	RAR related orphan receptor B	13663781	6.28	±	0.07	1.1	6.30	±	0.05	1.1	6.33	±	0.05	-1.0	0.8538	0.9405
RORC	RAR related orphan receptor C	13599134	5.51	±	0.07	-1.1	5.46	±	0.05	1.0	5.49	±	0.07	1.1	0.8427	0.9405
GnRH Signaling																
GNRH1	Gonadotropin-releasing hormone 1	13790870	6.94	±	0.18	1.1	6.82	±	0.14	1.2	7.07	±	0.30	1.0	0.7422	0.8958
GNRH2	Gonadotropin-releasing hormone 2	13606683	5.59	±	0.12	-1.1	5.51	±	0.09	-1.0	5.64	±	0.08	1.0	0.6390	0.8946
KISS1R	KISS1 receptor	13690401	4.02	±	0.03	1.0	4.10	±	0.06	1.1	4.22	±	0.15	1.1	0.3521	0.8216
OPRK1	Opioid receptor, kappa 1	13791718	6.50	±	0.12	1.0	6.66	±	0.12	1.2	6.84	±	0.10	1.2	0.1450	0.5668
PDYN	Prodynorphin	13612278	6.49	±	0.13	-1.0	6.37	±	0.10	-1.1	6.27	±	0.19	-1.1	0.5813	0.8845
TAC3	Tachykinin 3	13625903	6.49	±	0.20	-1.1	6.39	±	0.26	-1.6	5.87	±	0.16	-1.4	0.1315	0.5668
TACR3	Tachykinin receptor 3	13757467	4.20	±	0.17	1.1	4.17	±	0.10	-1.2	3.88	±	0.09	-1.3	0.1619	0.5668
Steroid receptors																
AR	Androgen receptor	13806207	5.91	±	0.05	-1.1	5.81	±	0.10	-1.0	5.91	±	0.06	1.1	0.5499	0.8749
ESR1	Estrogen receptor 1	13827480	5.97	±	0.10	-1.2	5.70	±	0.10	-1.2	5.72	±	0.06	1.0	0.1039	0.5668
ESR2	Estrogen receptor 2	13784393	4.42	±	0.03	1.1	4.54	±	0.11	1.1	4.54	±	0.08	1.0	0.5132	0.8749
PGR	Progesterone receptor	13658473	7.13	±	0.10	-1.2	6.86	±	0.07	-1.3	6.72	±	0.06	-1.1	0.0091	0.3180

Table 1. mRNA levels in the arcuate nucleus of young adult, old adult and old androgen supplemented male monkeys. There were no significant effects of age or androgen supplementation on clock, GnRH signaling, or steroid receptor genes. Values represent raw means (± SEM). $P > 0.05$ (One-way ANOVA). The magnitude of change between Old/ Young, Old Supplemental/ Young, and Old Supplemental/ Old were generated using Affymetrix® Transcriptome Analysis Console (TAC) Software, which normalized variability within each group before calculating the fold change.

Affymetrix GeneChip Rhesus Gene 1.0 ST Array in the Male ARC					
Gene Symbol	**Probe Set ID**	**Old Intact AM**	**Old Intact PM**	**Old PM/AM**	***P*-value**	**FDR *P*-value**
Clock genes						
BMAL1	13656832	6.15 ± 0.07	6.83 ± 0.12	1.6	0.0021	***0.0256***
BMAL2	13617047	5.70 ± 0.11	5.53 ± 0.11	-1.1	0.3059	0.5244
CLOCK	13752327	8.10 ± 0.06	8.07 ± 0.06	-1.0	0.8025	0.8501
CRY1	13627793	5.83 ± 0.04	6.54 ± 0.20	2.1	0.0210	0.1192
CRY2	13655873	5.80 ± 0.11	6.01 ± 0.07	1.1	0.1525	0.4483
CSNK1E	13614725	5.97 ± 0.08	6.37 ± 0.06	1.3	0.0035	***0.0314***
DEC1	13666246	3.19 ± 0.13	2.91 ± 0.10	-1.3	0.1205	0.4338
NPAS2	13640655	6.61 ± 0.07	7.32 ± 0.06	1.7	0.0000	***0.0007***
NR1D1	13679414	6.00 ± 0.09	6.39 ± 0.06	1.3	0.0062	***0.0443***
NR1D2	13708515	8.08 ± 0.06	7.81 ± 0.12	-1.1	0.0823	0.3293
PER1	13676737	4.95 ± 0.07	4.92 ± 0.12	1.0	0.8265	0.8501
PER2	13637104	6.29 ± 0.05	5.82 ± 0.14	-1.3	0.0260	0.1192
PER3	13580448	8.06 ± 0.10	7.75 ± 0.04	-1.3	0.0265	0.1192
RORA	13780419	7.17 ± 0.08	7.31 ± 0.13	1.0	0.3909	0.5471
RORB	13663781	6.30 ± 0.05	6.50 ± 0.15	1.0	0.2802	0.5044
RORC	13599134	5.46 ± 0.05	5.32 ± 0.15	-1.1	0.4103	0.5471
GnRH Signaling						
GNRH1	13690401	6.82 ± 0.14	7.10 ± 0.37	-1.0	0.5082	0.6534
GNRH2	13757467	5.51 ± 0.09	5.21 ± 0.19	1.5	0.2003	0.4754
KISS1R	13606683	4.10 ± 0.06	4.15 ± 0.08	-1.5	0.6631	0.7626
OPRK1	13625903	6.66 ± 0.12	6.73 ± 0.11	1.1	0.6779	0.7626
PDYN	13612278	6.37 ± 0.10	6.71 ± 0.19	1.2	0.1619	0.4483
TAC3	13791718	6.39 ± 0.26	6.10 ± 0.48	-1.3	0.6134	0.7615
TACR3	13790870	4.17 ± 0.10	4.25 ± 0.30	-1.1	0.8046	0.8501
Steroid receptors						
AR	13806207	5.81 ± 0.10	6.45 ± 0.11	1.6	0.0015	***0.0256***
ESR1	13827480	5.70 ± 0.10	6.07 ± 0.26	1.3	0.2509	0.4754
ESR2	13784393	4.54 ± 0.11	4.43 ± 0.07	-1.1	0.3961	0.5471
PGR	13658473	6.86 ± 0.07	6.39 ± 0.26	-1.4	0.1438	0.4483

Table 2. Diurnal gene expression in the ARC of old gonad intact rhesus macaques. Brains were collected at either 1000 or 2200 h relative to lights on at 0700 h (ZT3 and ZT15, respectively). Values represent means (± SEM). *AR, BMAL1, CSNK1, NPAS2, NR1D1* were significantly higher in the ARC at night ($P < 0.05$), but no other significant effects were found on the expression of clock, reproductive-related, or steroid receptor transcripts. ($P > 0.05$; Student's *t*-test; FDR correction for multiple comparisons). Fold changes between PM/AM were generated using Affymetrix® TAC Software.

Affymetrix GeneChip Rhesus Gene 1.0 ST Array in the Male SCN																
Gene Symbol		Probe Set ID	Young Intact			Old/ Young	Old Intact			Old Supp/ Young	Old Intact Supplemented			Old Supp/Old	*P*-value	FDR *P*-value
Clock genes																
BMAL1	ARNTL (aryl hydrocarbon receptor nuclear translocator like)	13656832	6.43	±	0.10	-1.0	6.36	±	0.05	1.1	6.50	±	0.08	1.1	0.4213	0.8766
BMAL2	ARNTL2 (aryl hydrocarbon receptor nuclear translocator like 2)	13617047	5.87	±	0.12	-1.1	5.66	±	0.09	-1.0	5.67	±	0.08	1.1	0.2630	0.8766
CLOCK	Clock circadian regulator	13752327	8.20	±	0.07	-1.1	8.11	±	0.03	1.0	8.27	±	0.04	1.1	0.1210	0.8766
CRY1	Cryptochrome circadian clock 1	13627793	6.01	±	0.08	-1.1	5.90	±	0.06	-1.0	6.00	±	0.06	1.1	0.4653	0.8766
CRY2	Cryptochrome circadian clock 2	13655873	5.70	±	0.09	1.2	5.85	±	0.13	1.1	5.79	±	0.09	-1.1	0.6261	0.8766
CSNK1E	Casein kinase 1 epsilon	13614725	5.94	±	0.07	1.2	6.10	±	0.03	1.0	5.84	±	0.14	-1.2	0.1687	0.8766
DEC1	Deleted in esophageal cancer 1	13666246	3.10	±	0.12	1.2	3.39	±	0.15	1.0	3.20	±	0.12	-1.2	0.2908	0.8766
NPAS2	Neuronal PAS domain protein 2	13640655	6.49	±	0.08	1.1	6.66	±	0.07	-1.0	6.51	±	0.05	-1.1	0.1748	0.8766
NR1D1	Nuclear receptor subfamily 1 group D member 1	13679414	6.09	±	0.05	1.0	6.05	±	0.06	-1.0	6.08	±	0.03	-1.0	0.7710	0.9126
NR1D2	Nuclear receptor subfamily 1 group D member 2	13708515	7.90	±	0.12	-1.0	7.87	±	0.04	1.0	7.92	±	0.08	1.0	0.9367	0.9643
PER1	Period circadian clock 1	13676737	4.87	±	0.07	1.0	4.85	±	0.12	1.0	4.88	±	0.08	-1.0	0.9785	0.9785
PER2	Period circadian clock 2	13637104	6.38	±	0.05	-1.1	6.27	±	0.03	-1.1	6.30	±	0.07	1.0	0.3524	0.8766
PER3	Period circadian clock 3	13580448	7.80	±	0.05	1.1	7.83	±	0.09	1.0	7.81	±	0.05	-1.0	0.9246	0.9643
RORA	RAR related orphan receptor A	13780419	7.58	±	0.07	-1.1	7.53	±	0.08	1.1	7.61	±	0.11	1.1	0.8113	0.9126
RORB	RAR related orphan receptor B	13663781	6.49	±	0.09	1.0	6.50	±	0.05	1.0	6.57	±	0.08	1.0	0.7263	0.9126
RORC	RAR related orphan receptor C	13599134	5.35	±	0.11	-1.0	5.25	±	0.10	-1.0	5.31	±	0.07	-1.0	0.7870	0.9126
SCN output signals																
AVP	Arginine vasopressin	13612355	6.75	±	0.16	1.4	6.81	±	0.26	1.1	7.06	±	0.11	-1.2	0.4837	0.8766
AVPR1A	Arginine vasopressin receptor 1A	13626231	3.06	±	0.20	-1.2	3.16	±	0.28	1.2	3.39	±	0.11	1.4	0.5384	0.8766
AVPR1B	Arginine vasopressin receptor 1B	13589155	4.63	±	0.04	-1.0	4.60	±	0.12	1.0	4.75	±	0.07	1.1	0.4405	0.8766
AVPR2	Arginine vasopressin receptor 2	13808675	5.21	±	0.12	1.0	5.26	±	0.12	-1.2	5.02	±	0.13	-1.2	0.3862	0.8766
VIP	Vasoactive intestinal peptide	13748448	5.61	±	0.42	2.0	6.61	±	0.17	1.6	6.24	±	0.14	-1.2	0.0606	0.8766
VIPR1	Vasoactive intestinal peptide receptor 1	13737851	7.01	±	0.04	1.1	6.96	±	0.08	1.2	7.11	±	0.06	1.0	0.2372	0.8766
VIPR2	Vasoactive intestinal peptide receptor 2	13737837	4.80	±	0.10	-1.1	4.85	±	0.13	1.1	4.99	±	0.20	1.1	0.6676	0.8987
Steroid receptors																
AR	Androgen receptor	13806207	5.53	±	0.12	-1.2	5.32	±	0.09	1.0	5.49	±	0.10	1.3	0.3331	0.8766
ESR1	Estrogen receptor 1	13827480	5.31	±	0.08	-1.1	5.12	±	0.04	-1.1	5.19	±	0.19	1.1	0.5601	0.8766
ESR2	Estrogen receptor 2	13784393	4.38	±	0.09	1.1	4.45	±	0.08	1.0	4.44	±	0.08	-1.0	0.8293	0.9126
PGR	Progesterone receptor	13658473	6.50	±	0.22	-1.4	6.18	±	0.20	-1.5	5.94	±	0.18	-1.1	0.1702	0.8766

Table 3. Effects of age and hormone supplementation on the mRNA in the suprachiasmatic nucleus of young adult, old adult, and old androgen supplemented male rhesus macaques. There were no significant effects of age or androgen supplementation on clock gene expression, SCN output signals, or steroid receptors in the SCN. Values represent means (± SEM). $P > 0.05$ (One-way ANOVA). Fold changes between Old/ Young, Old Supplemental/ Young, and Old Supplemental/ Old were generated using Affymetrix® TAC Software.

Affymetrix GeneChip Rhesus Gene 1.0 ST Array in the Male SCN									
Gene Symbol	**Probe Set ID**	**Old Intact AM**			**Old Intact PM**			**Old PM/AM**	***P*-value**	**FDR *P*-value**
Clock genes										
BMAL1	13656832	6.43	±	0.05	6.67	±	0.10	1.3	0.0314	0.1560
BMAL2	13617047	5.67	±	0.09	5.61	±	0.07	-1.0	0.6683	0.8378
CLOCK	13752327	8.14	±	0.03	8.08	±	0.07	-1.1	0.6942	0.8378
CRY1	13627793	5.94	±	0.06	5.95	±	0.10	1.0	0.7204	0.8405
CRY2	13655873	5.80	±	0.13	6.24	±	0.09	1.4	0.0357	0.1560
CSNK1E	13614725	6.06	±	0.03	6.11	±	0.09	1.0	0.9080	0.9080
DEC1	13666246	3.32	±	0.15	3.00	±	0.04	-1.3	0.0431	0.1677
NPAS2	13640655	6.66	±	0.07	7.15	±	0.03	1.5	0.0004	***0.0037***
NR1D1	13679414	6.06	±	0.06	6.32	±	0.10	1.2	0.0528	0.1682
NR1D2	13708515	7.93	±	0.04	7.80	±	0.05	-1.0	0.3138	0.5780
PER1	13676737	4.83	±	0.12	4.92	±	0.07	1.0	0.6235	0.8378
PER2	13637104	6.29	±	0.03	6.15	±	0.05	-1.1	0.0791	0.2307
PER3	13580448	7.82	±	0.09	7.82	±	0.05	-1.0	0.8600	0.9080
RORA	13780419	7.56	±	0.08	7.47	±	0.09	-1.1	0.6569	0.8378
RORB	13663781	6.49	±	0.05	6.52	±	0.12	1.0	0.8867	0.9080
RORC	13599134	5.23	±	0.10	5.18	±	0.06	-1.1	0.5838	0.8378
SCN output signals										
AVP	13612355	6.74	±	0.26	7.27	±	0.33	1.2	0.3131	0.5780
AVPR1A	13626231	3.25	±	0.28	3.76	±	0.12	1.8	0.0970	0.2612
AVPR1B	13589155	4.58	±	0.12	4.55	±	0.13	-1.2	0.7835	0.8846
AVPR2	13808675	5.18	±	0.12	5.05	±	0.08	-1.2	0.1902	0.4437
VIP	13748448	6.56	±	0.17	6.24	±	0.82	1.2	0.6823	0.8378
VIPR1	13737851	6.96	±	0.08	7.31	±	0.11	-1.0	0.0316	0.1560
VIPR2	13737837	4.83	±	0.13	4.67	±	0.12	1.3	0.3128	0.5780
Steroid receptors										
AR	13806207	5.33	±	0.09	5.98	±	0.13	1.3	0.0031	***0.0216***
ESR1	13827480	5.13	±	0.04	5.54	±	0.04	1.1	0.0001	***0.0008***
ESR2	13784393	4.41	±	0.08	4.59	±	0.09	-1.0	0.2627	0.5746
PGR	13658473	6.21	±	0.20	6.33	±	0.12	-1.1	0.5456	0.8303

Table 4. Diurnal expression of core-clock genes in the SCN of gonad-intact male macaques. Values represent means (± SEM). AR, ESR1, and NPAS showed significantly different levels of expression between 1000 and 2200 relative to lights on at 0700 h (ZT3 and ZT15, respectively) ($P < 0.05$; Student's *t*-test; FDR correction for multiple comparisons). Fold changes between PM/AM samples were generated using Affymetrix® TAC Software.

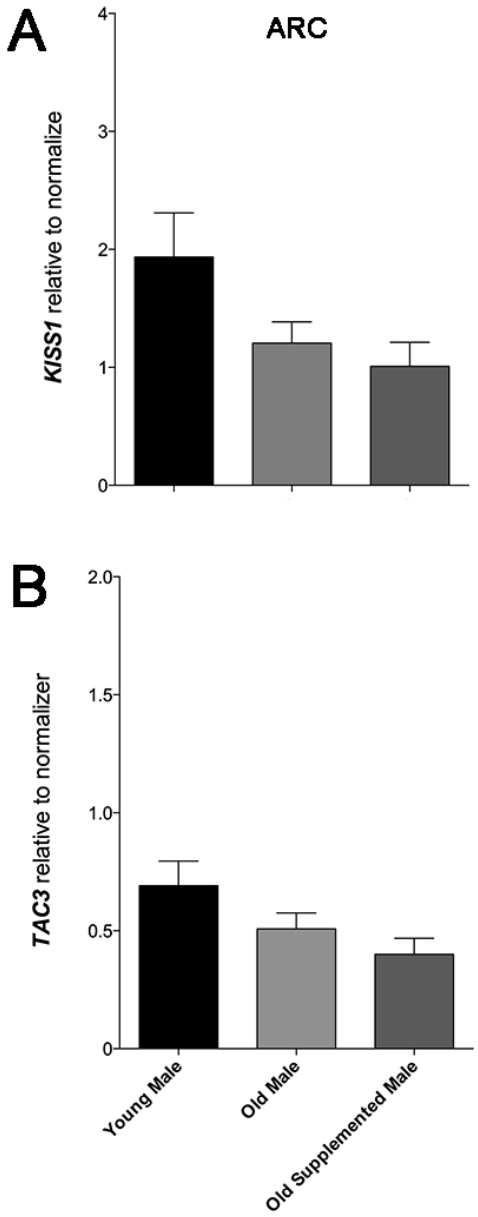


Figure 1. Effect of age and androgen supplementation on *KISS1* and *TAC3* mRNA in male monkeys. The values represent the means (± SEM). *KISS1* and *TAC3* were unaffected by age or androgen supplementation. $P > 0.05$; ANOVA.

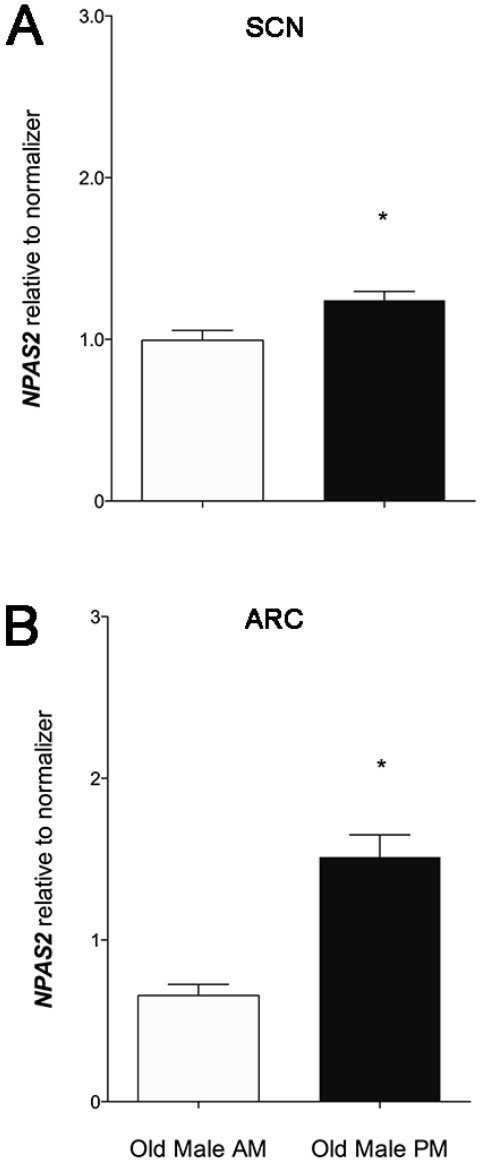


Figure 2. Diurnal gene expression in the ARC and SCN of aged gonad-intact male macaques. Values represent the means (± SEM). *NPAS2* was significantly higher in the evening ($P < 0.05$), no significant ($P > 0.05$; Student's *t*-test).

General Discussion

Aging is a complex process that involves molecular, cellular, and systemic level changes that result in altered homeostatic function. Reproductive decline and perturbed circadian rhythms, such as disrupted 24-h hormone profiles and changes in sleep-wake activity, have been associated with disease, behavioral, and cognitive deficits that eventually lead to death. The goals of this dissertation were to: 1) study the hypothalamus during aging and in response to HT, and 2) associate these changes with perturbed physiology. I showed that KNDy gene expression was resistant to the gradual decline in circulating androgens during aging in the male ARC, while the profound changes in 17β-estradiol and progesterone at the time of menopause contribute to the age-associated neuroendocrine alteration in the ARC nucleus of old female rhesus monkeys (Eghlidi et al. 2010, Eghlidi & Urbanski 2015). On the other hand, there were significant effects of age and androgen supplementation on the core-clock and modifier genes of males that suggest males may benefit from hormone therapy. Finally, there were differences in age and sex-steroid dependent changes in output signals and clock modifier signals between males and females that may be related to the different techniques used or sex differences.

One of the primary brain regions effected by aging is the hypothalamus. The ARC nucleus, primarily in females, exhibits age-related increases in gene and protein expression, as well as neuronal hypertrophy (Rance 2009, Eghlidi 2010, Eghlidi & Urbanski 2015). KNDy neurons control reproductive function and also influence other physiological functions, such as mediating 17β-estradiol's effects on vasomotor control and hot flashes. In contrast to females, the ARC KNDy gene expression in males

appears to remain intact despite aging. Moreover, there is an age-related deterioration in neurons of the SCN in both males and females. The SCN of females has an age-related decrease in VIP, AVP, and core-clock protein expression and 17β-estradiol therapy administered right after OVX did not improve the expression of these proteins in the female SCN. Finally, the SCN and ARC of males show diurnal expressed patterns of steroid receptors and core-clock genes despite aging. Since all of these genes were more highly expressed at night the study will require further analysis to determine if T therapy in males has benefits on gene expression in the ARC and SCN of aged animals.

The ARC nucleus in females exhibits a unique age-related increase in gene expression that is the largest change we observe in the aging non-human primate brain. The increase in kisspeptin and neurokinin B are a direct response to the loss of 17β-estradiol feedback from the ovaries onto the hypothalamus because the gene expression of these neuropeptides are suppressed following hormone supplementation (Eghlidi et al. 2010, Eghlidi & Urbanski 2015). The majority of women experience vasomotor symptoms during menopause that can affect their quality of life. More recently KNDy systems have been implicated in hot flashes and may be therapeutic targets for alleviating this prevalent symptom of menopause. One study showed that hot flashes are a result of changing circulating 17β-estradiol levels on KNDy neuron feedback onto inhibitory GABA neurotransmission in thermosensitive neurons of median preoptic area (Mittelman-Smith et al. 2012). Very recently, eight healthy postmenopausal women between the ages of 48-60 years old with hot flashes were treated with a kappa opioid agonist to evaluate the role of KNDy neurons in hot flashes.

These women received a placebo, standard dose, or a low dose of pentazocine/naloxone in a randomized order. In both the standard and low dose groups, hot flashes were significantly reduced (Oakley et al. 2015). These data suggest a role for OPRK1 in hot flashes, which differs from the findings of Mittleman-Smith and colleagues, which suggested that vasomotor control of hot flashes was through the TAC3R, or the receptor to NKB (Mittleman-Smith et al. 2012, Rance et al. 2013). Together, these recent studies suggest that KNDy neurons may be novel therapeutic targets for the treatment of hot flashes in menopausal women.

KNDy neurons have also been implicated in 17β-estradiol mediated metabolic function in females. A population of neurons that coexpress catecholamines and NPY in the brainstem send projections to KNDy neurons in ARC of rodents (True et al. 2011). The finding that *NPY2R* expression increase with age and was suppressed with hormone supplementation in the AR, the same pattern as *KISS1* and *NKB* expression, suggests that *NPY2R* may mediate 17β-estradiol effects on brainstem regulation of metabolic function (Eghlidi and Urbanski, 2015).

In females, there was a significant decrease in NR1D1, VIP, and AVP protein during aging. However, in the males there was no changes in *NR1D1*, *VIP*, or *AVP* mRNA during aging. One possible explanation for this is that the two studies used different techniques. The study in females used immunohistochemistry to quantify neuron number at intervals through the SCN and it is possible that the total amount of protein was not significantly different between the two groups. The male experiments tested differences in mRNA in the entire SCN. It is possible that the total protein levels in the females were not different for NR1D1, VIP, and AVP, which could have been

shown using Western Blot analysis. Another possibility is that there are translational differences in the expression of the mRNA and protein. Previously, we found differences in protein and mRNA levels for microtubule associated protein 2 (MAP-2), synaptophysin (SYN), and apolipoprotein E (APOE) in the prefrontal cortex, hippocampus, and amygdala of rhesus monkeys using western blots and real-time pcr. In the hippocampus, higher expression of synaptophysin and apolipoprotein E protein levels and not mRNA was associated with better performance in a spatial maze, for example (Hayley et al. 2012). Another possibility is that the pattern and amplitude of expression over the 24-hours may vary for mRNA and protein in these signals and in the male study we did not look at the right time of day to find differences at the mRNA level.

One limitation of the current set of experiments was that I did not find a significant association between activity patterns, aging, and SCN protein expression in females. First, the cost of non-human primate work prohibits a comprehensive analysis of circadian changes. Ideally, young and old animals would have been collected at multiple time points across the 24-hour day to evaluate neurochemical changes during aging. Second, the appropriate EEG experiments to validate the sleep data from the actograms would need to be performed in order to compare other parameters like sleep fragmentation. Third, other analysis of actigram data that has shown significant changes during aging, like scale-invariant patterns of activity that are reduced during aging in humans, should be assessed (Hu et al. 2009). While this technique has not yet been validated for non-human primates, future studies may benefit from using this robust approach to identify changes in activity rhythms using accelerometer-based activity

data. Fourth, the core-clock mechanism and output signals of the SCN are part of a complex neurocircuit that involves many downstream nuclei and it is possible that other systems in the sleep-wake circuitry are associates with the change in day-time activity in aged rhesus monkeys. Fifth, there may be an optimal time-window to study the underlying neurochemical changes in the SCN and perturbed activity patterns during aging that were not captured in the age group studied here. Utilizing samples collected across the 24-hour day, quantifying other sleep-wake activity parameters, studying the sleep-wake circuitry rather than localized function in the SCN, and even determining if there is specific time-window where neurochemical changes are associated with perturbed sleep-wake activity during aging may help to elucidate the underlying neurochemistry involved in age-related changes in activity rhythms.

17β-estradiol treatment has been shown to improve sleep quality in postmenopausal women (Guidozzi 2013, Guidozzi 2015). However, 17β-estradiol therapy did not have any effect on the expression of the primary output signals in the SCN, such as VIP or AVP. Similarly, 17β-estradiol therapy did not significantly change the expression of NR1D1, a core-clock modifier protein. One nucleus that has recently been implicated in age-related changes in sleep-wake activity is the ventrolateral preoptic area, which is involved in the initiation and maintenance of sleep (Saper et al. 2010, Lim et al. 2014). Both ESR1 and ESR2 are expressed in the VLPO, and so it is a likely to be one site of 17β-estradiol action (Shughrue et al. 1992, Shughrue et al. 1997, Shughrue et al. 1998, Shyghrue & Mechenthaler 2001). During spontaneous sleep, 17β-estradiol prevents activity in the sleep-promoting VLPO (Hadjimarkou et al. 2008). Brain infusions of prostaglandin or adenosine promotes sleep by increasing activity in the

VLPO. E suppressed lipocalin-type prostaglandin D2 synthase and adenosine A2A receptor mRNA expression (Mong et al. 2003, Mong et al. 2003, Hadjimarkou et al. 2008). This suggests that blocking this system would suppress sleep, while increasing arousal (Mong et al. 2003) Finally, OVX rats have decreased FOS expression in the VLPO and E therapy increases FOS expression in this nucleus (Peterfi et al. 2004). Perhaps the VLPO is the 17β-estradiol sensitive nucleus that is effected by menopause in women.

Future experiments need to determine how 17β-estradiol influences the SCN and core-clock mechanisms in females at different stages of the menstrual cycle and in response to menopausal changes in sex-steroid levels. First, we need to determine the impact of menstrual cycle status (early follicular, late follicular, and mid-luteal phases) on ER-expressing projections from the mPOA and BNST to key output signals in the SCN, such as VIP and AVP neurons (De La Iglesia 1999). Second, how these ER-expressing inputs are altered by the changes in sex-steroids during transition to menopause, using animals at differences stages on the perimenopause, needs to be assessed. Finally, the impact of cycle status and the transition to menopause on peripheral brain and organs canonical clock mechanism needs to studies in order to understand how reproductive changes during aging impact the function of clock-regulated pathways locally, in the brain and rest of the body.

It comes as little surprise that there are differences between men and women in their response to hormones is the SCN. First, the SCN of females has not been consistently shown to express ERs (Kruijver & Swaab 2002, Vida et al. 2008). On the other hand, there is unambiguous evidenced that AR is expressed in the SCN of males

and responds to androgens (Wu et al. 1995, Rees & Michael 1982, Zhou et al. 1994, Fernandez-Guasti et al. 2000). Evolutionarily this makes sense; women have cyclic hormone patterns where 17β-estradiol and progesterone levels increase at varying intervals over the ~28-day menstrual cycle and it would not be beneficial if every time the women shows an LH surge that the master oscillator in the SCN that controls all of the rhythms throughout the body, is resynchronized. However, males have mostly constant sex-steroid levels and so it may be beneficial to have sex-steroids act directly on the SCN in males to help synchronize core-clock rhythms in this nucleus.

One of the studies on the cohort from the Massachusetts Male Aging Study examined hormone levels in healthy men and found much different results from the whole data set. Feldman et al. reported a 10-15% increase in T levels was correlated with a healthier lifestyle characterized by the absence of excess drinking, obesity, chronic illness, or prescription medication (Feldman et al. 2002). This is an important point, because the rhesus macaques used in the current study represent the “healthy lifestyle” group, which did not have as drastic of a change in hormone levels with age and may not show as severe changes in the HPG axis. Future studies in animal models may want to test the impact of obesity and excessive drinking on age-related changes hormone levels and the HPG axis, as the results may be more translatable to our current human demographic

The HPG-axis controls a number of circadian activities including rhythmic release of GnRH, ACTH, LH, and GH (Griffin & Ojeda 2000). Even reproductive hormones, like T, have a 24-hour pattern of release (Bremner et al. 1983). Affymetrix Gene Profiling revealed diurnal changes in primary core-clock genes, *NPAS2,* in the ARC and SCN.

Many more clock genes has a diurnal rhythm in the ARC. This is an important finding because aging is associated with changes in hormonal rhythms, sleep-wake behavior, and other body rhythms. It is possible that clock genes show changes in rhythmicity with age because many of the modifiers were rhythmic in the aged animals, whereas core-clock genes were not. This suggests that there was a compensatory mechanism in the ARC working to maintain the core-clock rhythm. NR1D1 was inhibiting BMAL1 expression to keep the positive limb in sync. CSNK1 was phosphorylating the PERs and CRYs. Finally, NPAS2 was heterodimerizing with CLOCK to compensate for changes in BMAL1 rhythms. Future experiments should compare young and old animals necropsies during the day and night to further characterize changes in core-clock rhythms change with age.

In Chapters I and III, subdissected hypothalamic tissue was collected based on anatomical reference points, such as the third ventricle, optic chiasm, and median eminence. In the ARC nucleus of females, the subdissections were precise because of the major changes in KISS1 and NKB expression were identical to other histological based experiments (Rance et al. 2009). The subdissection of the male ARC was the same as the females and supports previous observations that there are minimal changes in the HPG axis of aging males (Gore & Hayes 2015). However, it is important to mention that subtle changes in the expression of low-expressing genes may have been masked by noise from neighboring nuclei that may have been included in the dissection, like the ventromedial hypothalamus, or periventricular hypothalamus or from glial cells. Similarly, the subdissected SCN of males in Chapter III may have included some median preoptic area (MPOA) preoptic periventricular (POP) nucleus gene

expression. However, there was very low standard error in the gene expression values and the key signals known to be expressed in the SCN and not the mPOA or POP were present on the array, so I believe these subdissections of the SCN were also highly specific (see Appendix K).

ERs are expressed in many brain areas outside of ARC nucleus and 17β-estradiol is involved in a diverse number of functions in the brain, including learning and memory, neuroprotection, olfaction, vision, coordination and balance, muscle movement, and seasonal rhythms. On the other hand, T is associated with vestibular and auditory processes and visceral sensory responses and ARs have a different pattern of expression in the male brain. The future of hormone therapy lies in spatially targeted treatments, via SERMS or Selective Androgen Receptor Modulators (SARMS), and carefully controlled temporal delivery, i.e., T at night and E near the time of the final menstrual period.

Taken as a whole, these data suggest that neurons in the ARC of females and SCN of males retain the capacity to respond to hormone therapy despite aging. Moreover, the gene expression changes in the ARC of aging female monkeys may be important for understanding the causes and consequences of menopause and for the development of therapies for menopausal disorders. The characterization of the non-human primate SCN fills a gap in our knowledge between the rodent and human circadian clock mechanism and shows that core-clock modifiers like NR1D1 and CSNK1E may play an important role in the SCN during aging. Finally, understanding the central effects of hormone therapy is important, given that both testosterone and DHEA are being widely promoted for use in elderly men.

Appendix A: Protocol for Probe Cleaning (if disposable homogenizers are not available)

1. Prepare 0.5 M sodium hydroxide (NaOH) Probe Cleaning Solution

   Calculation: 0.5 mole NaOH · 40 grams NaOH = 20 grams NaOH L 1 mole NaOH L Procedure: Weigh 20 grams of NaOH and place into a glass bottle containing 1 L Diethylpyrocarbonate (DEPC) water.

   Place the glass bottle in a rubber bucket of water and put it in the autoclave for a programmed sterilization cycle. After the sterilization cycle is complete, remove the rubber bucket of water and the bottle of NaOH, and allow to cool 5-10 minutes.

   Tube Preparation: Pour 10 ml of NaOH into a 15 ml Corning Centrifuge Tube. One tube should be prepared per original source tissue sample.

2. DEPC Water Probe Cleaning Solution:

   Tube Preparation: pour 10 ml of DEPC water into 15 ml Corning Centrifuge Tube. Prepare one tube per original source tissue sample.

3. Prepare 50%, 70%, and 100% Ethanol (ETOH) Probe Dehydrating Solutions

   Tube preparation: pour 5 ml ETOH to 5 ml DEPC water into 15 ml Corning Centrifuge Tube for 50% ETOH, pour 7 ml ETOH to 3 ml DEPC water into 15 ml Corning Centrifuge Tube for 70% ETOH, and pour 10 ml of ETOH in 15 ml Corning Centrifuge Tube for 100% ETOH wash. One tube should be prepared per original source tissue sample.

4. Prepare Chloroform Dehydration Tubes: Pour 10 ml of Chloroform into a 15 ml Corning Centrifuge Tube right before isolation experiment. The Chloroform will deform the plastic and may not fit around the homogenizer if it is left out too long. One tube should be prepared per original source tissue sample.
5. Probe Cleaning and Dehydration: Wash probe with 0.5 M NaOH for 2 minutes. Wash probe with DEPC water for 1 minute. Dehydrate the probe: Wash with 50%, 70% and 100% ETOH for 1 minute each. Throw tubes away after. Dehydrate the probe with Chloroform for 1 minute. Save Chloroform tube for proper disposal, do not throw away or put down the sink. Finally, allow the probe to air dry, by turning the probe on and off until no liquid is present on the probe.

Appendix B: Tissue Disruption, Homogenization, and RNA Extraction using Qiagen's RNeasy Mini Kit

1. Personal protective equipment (PPE) must be worn when homogenizing primate tissue and the following experiments must be performed in a biosafety hood.
2. Weigh the tissue prior to extraction. The maximum capacity is 30 mg for for each spin column. Note that the Qiagen RNeasy Micro Kit should be used for samples < 10 mg. For samples with a high lipid content, like the adrenal gland, you may use a larger tissue sample per column (~40 mg), homogenize the tissue for longer (Step 2), elute off the lipid layer (at Step 5) to get the RNA to bind to the column.
3. Place the tissue sample in a 1-ml round bottom microcentrifuge tube. Add 600 μl of Buffer RLT. Conversely, if your samples size is larger you may use a 15-ml corning conical tube and add 600 μl for every 30 mg of tissue. Bring the tube up underneath the probe and homogenize for 1 minute on high speed. After 1 minute, lower the speed and get all of homogenate out of the probe onto the sides of the tube. This is especially important when working with smaller tissue samples. Then place the sample in a rack until you have finished extracting. Sample integrity is maintained at room temperature.
4. Rinse the probe with DEPC water and then follow the steps in the probe cleaning protocol in **Appendix A** or use a fresh disposable homogenizer.
5. RNA Binding and Adsorption Step: Pipette all of the lysate into a 1.5 ml microcentrifuge tube (pointed bottom). If you have a larger sample then you

will need multiple tubes and you must transfer 600 μl to each microcentrifuge tube.

6. Place the tube into a centrifuge for 3 minutes at maximum speed. Carefully pipette 180 μl of the lysate one at a time into a new 1-ml microcentrifuge tube, careful to not disrupt the pellet at the bottom that contains lipids that will clog the column at a later step.
7. Add 600 μl of 70% Ethanol Solution to each microcentrifuge tube and vortex each tube for 30 seconds. Do not centrifuge.
8. Contaminant Removal Step: Pipette 700 μl of the sample in the spin column provided in the Qiagen RNeasy Kit. Centrifuge for 30 seconds at > 10,000 rpm. Throw away collection tube and replace with new collection tube.
9. Transfer remaining 700 μl and repeat Step 7. Throw away tube and replace the collection tube. Add 500 μl of Buffer RPE to spin column. Centrifuge for 30 seconds at > 10,000 rpm to wash the spin column membrane. Throw away tube and replace the collection tube. Add another 500 μl of Buffer RPE and Centrifuge for 2 minutes at >10,000 rpm to wash the spin column membrane.
10. Throw away collection tube and replace with new collection tube. Dry spin the column for 1 minute at > 10,000 rpm.
11. Finally, place the Spin Column on top of the 1.5-ml microcentrifuge tube provided in the Qiagen RNeasy Kit (with a snap lid). Add 50 μl RNase-Free Water directly to the spin column membrane.

12. Total RNA Elution: Centrifuge for 1 minute at > 10,000 rpm to elute the RNA. Pipette the eluted RNA back onto the column. Centrifuge for 1 minute at >10,000 rpm to elute the total RNA. Store at -80º C degrees.
13. RNA concentration can be measure using a 2100 Agilent Bioanalyzer (microcapillary electrophoresis). High quality RNA will have two ribosomal peaks at 18S and 28S.
14. RNA concentrations can also be measure using the NanoDrop-1000. Open the ND-1000 V3.3.0, found on the desktop. Choose the nucleic acid option. Clean the top and bottom of the measurement pedestal with a Kim Wipe. Load 2 μl of RNase free water and click “Ok” to initialize the instrument. Clean the top and bottom of the measurement pedestals with a Kim Wipe. Choose RNA-40 from the “Sample Type” option, found on the right of the screen. Load 2 μl of the liquid your samples was re-hydrated in, press F3. Clean top and bottom measurement pedestals with a Kim Wipe. Load 2 μl of sample, type in sample number in provided box, press F1. Clean top and bottom measurement pedestals with a Kim Wipe, after each new sample. A260/A280 should be > 2.0. If it A260/A280 is < 2.0 then it is possible that there is protein contamination, presence of degraded RNA, or excess free nucleotides and the sample must be re-extracted.

Appendix C: Disposal homogenizer probe cleaning

1. Once you are finished isolating RNA, disassemble the disposable homogenizer and place it in a beaker filled with deionized water (DI) water.
2. Carefully clean both pieces of the homogenizer using hot water and use the brush to thoroughly clean the outer surface of the probe.
3. Next, put both pieces of the homogenizer in 10% bleach for 15 minutes.
4. Then rinse the homogenizer with DI water.
5. The homogenizer should then soak in RNase Zap or RNase Away for one hour.
6. Rinse with DI water.
7. Dry in fume hood.
8. Re-assemble the probe parts and wrap in foil.
9. Autoclave the wrapped probes on a short and dry cycle.

Appendix D: cDNA synthesis protocol using SuperScript III First-strand Synthesis Kit

1. Denature RNA: Add DEPC water, random hexamers, dNTPs, RNA sample according to RNA concentration. Ex. for RNA sample with a concentration of 200 ng/μl combine 4.16 μl of RNA sample to 3.82 μl of DEPC water, 1 μl of random hexamers, and 1 μl of dNTPs in 0.2 or 0.5-ml tube. Vortex and place on ice.
2. Put in thermocycler for Step 1 at 65° C for 5 minutes and Step 2 at 4° C for 5 minutes.
3. Annealing RNA: Prepare master mix of 10x RT Buffer, 25 mM MgCl2, 0.1 M DTT, RNase OUT, and SuperScript III. Combine 2 μl of 10x RT Buffer, 4 μl 25 mM MgCl2, 2 μl 0.1M DTT, 1 μl RNase OUT, and 1 μl Superscript III with the RNA mixture. Vortex and place on ice.
4. Put in thermocycler for Step 1 at 25° C for 10 minutes, Step 2 at 50° C for 50 minutes, Step 3 at 85° C for 5 minutes, and Step 4 for 4° C for 30 minutes.
5. Remove RNA: Add 1 μl RNase H to RNA mixture.
6. Put in thermocycler for Step 1 at 37° C for 10 minutes and Step 2 at 4° C for 30 minutes.

Appendix E: Reverse-transcriptase PCR

5x	TBE Buffer (1L)
54 g	Tris Base
27.5 g	Boric Acid
0.5M EDTA (pH 8.0)	20 ml

DI water up to 1L

1. Combine Super Mix, DEPC water, cDNA, forward-primer, and reverse-primer. For example, combine 22.5 μl of SuperMix, 3 μl DEPC water, 1 μl cDNA, 0.5 μl F-primer, and 0.5 μl R-primer in 0.2 or 0.5-ml tube.
2. Determine appropriate thermocycler program on NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or directly on thermocycler.
3. Run 2% agarose gel: combine 1 g of agarose in 50 ml of TBE and heat in microwave, swirl solution until agarose dissolves.
4. Add 1 μl of ethidium bromide (50 μg/ ml) for a concentration of 0.5 μg/ ml.
5. Pour solution into electrophoresis mold and insert combs.
6. Mix 1 μl of DNA loading buffer (dark blue dye) with 10 μl of sample on piece parafilm.
7. Once the agarose gel has cooled, you may load the samples into the gel wells along with a DNA Ladder in the first column.
8. Run electrophoresis at 70 volts until dye has crossed the gel.
9. Visualize the gel on Gel Doc and save image for pixel intensity analysis.

Appendix F: Real-time PCR (for example, Using the Standard Curve Methods)

1. Standard curve calculations: Determine the number of cDNA samples and genes you plan to assess. Calculate a serial dilution to use on your cDNA samples. For example, using the combined cDNA from multiple arcuate (ARC) nucleus samples serial dilutions are made at for example, 1:1, 1:3, 1:9, 1:27. 1:3 dilutions have typically been selected to assess expression of all the genes in an experiment at the same time, including genes expressed at lower levels, like steroidogenic enzymes. For confirming high throughput methods, like microarray and RNAseq, a higher dilution can be used. Each cDNA dilution, for each gene of interest, is pipetted in triplicate for each dilution to improve accuracy of results. A standard curve plate will contain a cDNA mixture of all the mRNA samples at the four dilutions. If you have 7 rows (for 6 genes, include extra row for margin of error) multiplies by 3 columns, multiplied by 2 μl samples = 42 μl. Round up to 50 μl of cDNA needed for each concentration. The 1:1 dilution will contain 50 μl of combined cDNA from all your samples in a 0.5-ml tube. You must transfer 25 μl of the 1:1 to the next 0.5-ml tube for 1:3 dilution, which you will add 50 μl of RNase-free water. You will then transfer 25 μl of the 1:3 dilution to the third 0.5 ml tube and add 50 μl of RNase-free water for the 1:9 dilution. Transfer 25 μl of the 1:9 dilution to a new 0.5 ml tube and add 50 μl of RNase free water. In the end, you should have 50 μl of the first three dilutions (1:1, 1:3, and 1:9) and 75 μl of the final dilution (1:27).

Serial dilution calculations:

$\frac{\text{Volume}_{\text{post-transfer}}}{\text{Volume}_{\text{pre-transfer}}} = 1 - \text{Dilution Factor} \rightarrow \frac{50\ \mu l}{\text{Volume}_{\text{pre-transfer}}} = 1 - (1/3)$ $\text{Volume}_{\text{pre-transfer}} = \frac{50\ \mu l}{(2/3)} = 75\ \mu l$

$\text{Volume}_{\text{transfer}} - \text{Volume}_{\text{pre-transfer}} - \text{Volume}_{\text{post-transfer}} = 75\ \mu l - 50\ \mu l = 25\ \mu l$

$\text{Volume}_{\text{water}} - \text{Volume}_{\text{pre-transfer}} - \text{Volume}_{\text{transfer}} = 75\ \mu l - 25\ \mu l = 50\ \mu l$

2. Standard curve solution preparation: Prepare the serial dilutions calculated in Step 1. Make up each individual probe sets Gene Master Mix. For example, if you have 6 samples then for each samples you will need to make a volume for 15 wells (12 wells per row and 2 empty wells and 1 well for margin of error).

	Standard Formula per well (μl)	Per 15 wells μl
Master mix	5	5 x 15 = 75
TaqMan Probe (10 μm)	0.5	0.25 x 15 = 3.75
Primer F (10 or 25 μm)	0.3	0.3 x 15 = 4.5
Primer R (10 or 25 μm)	0.3	0.3 x 15 = 4.5
RNase-free water	2.15	2.15 = 32.25
Total Volume	8.0	8 x 15 = 120

3. Prepare the master mix for each probe set and add 8 μl to each well and 2 μl of each dilution in triplicate to wells on a 384-well plate. In the NTC well, pipet 2 μl RNase-free water instead of cDNA. Once you are finished pipetting you must

carefully apply the MicroAmp Optical Adhesive Film and using MicroAmp Optical Adhesive Film Sealing Instrument to seal the plate.

4. Experimental curve calculation: determine the appropriate volume for the cDNA dilution of your sample, from your Standard Curve, you will need for each of your Real-time qPCR plates.
5. For example, if you have 46 cDNA samples that will analyzed in triplicate for 6 genes of interest then you will multiply 6 by 8 μl cDNA sample (for each plate) = 48 → 50 μl of 1:3 cDNA concentration with a margin of error included.

   μl cDNA sample to add = (Dilution Factor) (Total Volume)

   μl cDNA sample to add = (1/3) (50 μl)

   μl cDNA sample to add = 16.67 μl

   Total Volume = μl of RNase-free water to add + μl cDNA sample to add

   50 μl = 16.67 μl + μl of RNase-free water to add

   μl of RNase free water to add = 50 μl – 16.67 μl = 33.33 μl

6. Experimental solution preparation: the next step is the same as Step 2.

	Standard Formula per well (μl)	Per x wells μl
Master mix	5	5 (x)
TaqMan Probe (10 μm)	0.5	0.25 (x)
Primer F (10 or 25 μm)	0.3	0.3 (x)
Primer R (10 or 25 μm)	0.3	0.3 (x)
RNase-free water	2.15	2.15 (x)

Total Volume	8.0	8 (x)

7. Experimental Real-Time qRT-PCR Run: Prepare the master mix for each probe set and add 8 μl to each well and 2 μl of each dilution in triplicate to wells on a 384-well plate. In the NTC well, pipet 2 μl RNase-free water instead of cDNA. Once you are finished pipetting you must carefully apply the MicroAmp Optical Adhesive Film and using MicroAmp Optical Adhesive Film Sealing Instrument to seal the plate.
8. Centrifuge the Standard Curve and Experimental plate in an Eppendorf Centrifuge 5810R for 1 minute at 2000 rpm at room temperature. Plates can be store at 4° C until they are to be run on thermocycler.

Appendix G: Primer and Probe Design

1. Determine the sequence of interest from high throughput gene expression method, like microarray or RNAseq, or from a previously published manuscript.
2. Blast the sequence on Blastn NCBI to find the full NCBI reference sequence and copy the sequence into a new document and highlight the nucleotides of interest.
3. Upload the sequence in Primer Express and determine primer and probe set that covers the sequence of interest with the lowest penalty score.
4. Blast just the primers in Primer Blast to make sure they do not bind to another gene.

Appendix H: Immunohistochemistry protocol

Solutions:

Tris Buffer (pH 7.6) (4 L)

4 L DI water

24.24 g Trizma HCl

5.56 g

Trizma base

36 g NaCl

Calibrate with HCl

Citrate Buffer (pH 6.0)

2 L DI water

3.84 g Citric Acid Anhydrous to 1.8 L of DI water

Calibrate with NaOH and fill to 2L with DI water

Day 1:

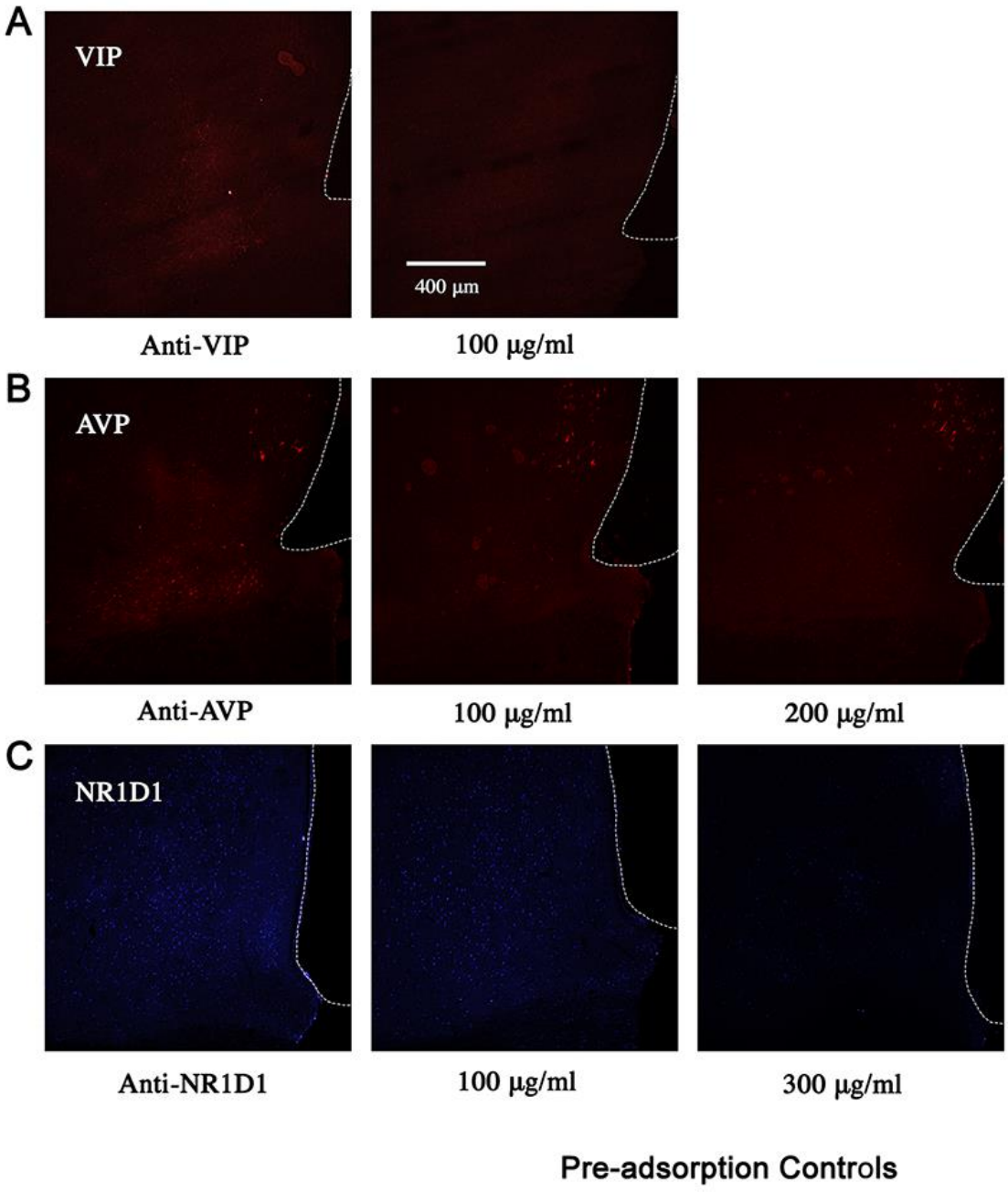
1. Hot water bath antigen retrieval: Once hot water bath reaches 80° C you may transfer the 1 ml microcentrifuge tubes, containing 800 μl of citrate buffer, to the hot water bath. One tube per brain section.
2. Wash tissue in Tris Buffer for 5 minutes in 6, 12, or 24-well plate. Depending on the availability/cost of the primary antibody and the size of the tissue sections.

3. Then transfer sections to the individual microcentrifuge tubes and place in the hot water bath for 30 minutes.
4. Allow samples to cool for 15 minutes and then transfer tissue from microcentrifuge tube back to fresh Tris Buffer and wash for 5 minutes.
5. Block tissue for 30 minutes with 3% of the appropriate normal serum (usually goat or horse).
6. Incubate the samples for 24-48 hours at room temperature on shaker. Combine primary antibody with 0.001% Triton-X in Tris Buffer. Note that a dilution curve experiment should be conducted at the beginning of each new experiment to determine the appropriate concentrations to use on the tissue, which may vary depending on preservation. Finally, expensive or rare antibodies must be frozen at -20° C immediately after this step for reuse. Mold and the the Triton-X will ruin the antibody if left at higher temperatures. Note, repeat thawing and freezing of antibodies damage them and is not recommended.

Day 2:

7. Wash tissue in Tris Buffer for 3x 5 minutes.
8. Incubate samples in secondary antibody for 1 hour. Secondary antibody should be prepared in Tris Buffer and because the fluorescence intensity can vary it is optimal to test the secondary antibody that will be used in the experiment. Usually, concentrations of 1:200-1:400 are sufficient.
9. Wash tissue in Tris Buffer for 3x 5 minutes.
10. Mount section on slide precoated with poly-L-lysine and dry for 1 hour.
11. Coverslip with appropriate glycerol based media and store at -20° C.

Appendix I: Pre-adsorption of VIP, AVP, and NR1D1



To confirm antibody specificity, the immunohistochemistry in Appendix G was used, with the minimum concentration of primary antibody to obtain visible staining, VIP (1:2000), AVP (1:3000), and NR1D1 (1:200), without and with preadsorption with 100-300 μg/ml

of VIP, AVP, or NR1D1 for 1 h, which was sufficient to abolish visible staining the SCN. Finally, the Sudan Black protocol (Appendix I) was used to block lipofuscin autofluorescent. Together, these controls provide evidence that the antibodies were specific VIP, AVP, and NR1D1 proteins.

Appendix J: Sudan Black B Protocol

Solutions:

Tris Buffer (pH 7.6) (4 L)

4 L	DI water
24.24 g	Trizma HCl
5.56 g	Trizma base
36 g	NaCl

Calibrate with HCl

70% ETOH (1 L*)*

300 ml	DI water
700 ml	ETOH

1. Mount sections on polylysine-L-lysine coated slides.
2. Once sections are lightly dried you may transfer them to Tris Buffer for ~5 minutes.
3. Immerse sections in 70% EtOH for 5 minutes.
4. Lay sections out and cover the slides with filtered Sudan Black B (Autofluorescence Eliminator Reagent, Millipore).

   5-7 minutes depending on lipofuscin intensity.

   Watch carefully that sections do not dry out during this step.
5. Immerse sections in 70% EtOH washes until EtOH is clear.

6. Cover slip (Prolong Diamond Antifade Mountant, Invitrogen) and store below 4°C.

Note, there will still be some background Autofluorescence, especially in the oldest animals. In the future, other lipid soluble dyes, like Sudan Red, should be tested to determine if other lipids that accumulate during aging can be blocked by preadsorption with lipid soluble dyes. This would be especially important in analyses used to measure co-expression intensity in aged animals.

Appendix K: Western Blot Protocol (Hayley GE, Eghlidi DH, Kohama SG, Urbanski HF, Raber J, Behav Brain Research 2012)

Buffers:

10X Transfer Buffer (1L)

24.2g	Tris Base
112.6g	Glycine
Fill to 1L	Mill-Q water

1X Transfer Buffer (1L)

100ml	10X Transfer buffer
200ml	Methanol
700ml	Milli-water

SDS Running Buffer (1L)

50 ml	Nupage Running buffer (20x)
950 ml	Cold DI water

10X TBS (1L)

80g	NaCl
24g	Tris Base

Adjust pH to 7.6 with HCl

1X TBS-T (1L)

100ml 10X TBS

1ml Tween-20

899ml Milli-Q water

5% Milk TBS-T

2.5g Nonfat dry milk

50ml 1X TBS-T

Homogenization

1. Weigh fresh frozen or RNAlater preserved tissue.
2. Add one 100 μl aliquot of 100x HALT protease inhibitor

   100 μl per 10 ml of lysate

   Add 1ml of cold RIPA buffer per 0.5 g of tissue
3. Homogenize tissue using dounce homogenizer, using dounce A to break of the tissue and then dounce B to lyse the cells.
4. Leave on ice for 30 minutes.
5. Spin for 30 minutes at 10,000 rpm at 4° C in centrifuge located in cold room.
6. Transfer supernatant to new tube.

NanoDrop-100 for Protein

1. Click ND-100 V3.3.0.
2. Open Protein A280.
3. Clean top and bottom pedestals with Kim Wipe.

4. Load 2 μl Milli-Q water and click okay.
5. Clean top and bottom pedestals with Kim Wipe (repeat in between each sample load).
6. Load 2 μl sample buffer press blank or F3.

   Extra sample buffer located in cold room.
7. Enter name in box on right side and load 2 μl of sample, press collect or F1.

   Check to make sure 280 concentration is < 2.0

   Print report

Dilution

1. Dilute samples to 2mg/1ml Ex. 28.85 mg/ml of protein, 13.425 ml of RIPA buffer per 1ml of sample.

Sample Buffer

1. Get protein samples and ladder from -20 freezer, add ½ vol Laemmli/BME Buffer to lysate and boil (95º C) in beaker with water on hotplate for 5 minutes. Allow samples to cool to room temperature.

   Only boil samples once, otherwise thaw the pre-boiled samples

   The protein ladder should not be boiled

   For a 19:1 dilution:

   Ex. 95μL Laemmli Sample Buffer

   5μL Beta mercaptoethanol (BME)
2. Prepare 1x SDS Running Buffer, in 1-L graduated cylinder:
3. Fill plastic tub with ice and set up gel apparatus submerged in the ice.

4. Open pre-cast gels and remove white tape-strip and comb, rinse with DI water and place in gel apparatus. Wells should face the center of the chamber.
5. Fill both chambers of apparatus with 1x Running Buffer so liquid covers above the wells. Remove bubbles with a 1-ml pipette.
6. Add 200 μl of Nupage antioxidant to center chamber.
7. Clean wells, using 200 μl micropipette triturating 1-3x in each well.
8. Add 5 μl ladder and 25 μl samples to each well, avoid air bubbles and be careful not to poke bottom of well. Load from left to right on front gel and right to left on back gel (since back gel is actually facing the opposing direction).

    Load 25 μl of protein ladder and sample to each well. If protein is low in expression try loading 40-80 μl.

    Load buffer on into the last well because lanes will travel more evenly.
9. Turn on power-supply and fit electrode/cover on top of gel apparatus.
10. Set power supply to 200V, 60 minutes, press run.

    If set up is correct, bubbles will appear.
11. Check gel after 5 minutes to make sure samples are running through gel.
12. Rinse plastic box and wedge, place on drying rack.

For 18-well gels follow instructions above except:

4. After opening precast gel, remove green comb and white strip on bottom. Place well tank toward center of chamber (numbers should read L – R).

5. Use steps 6-8 above.

6. Fill chamber to line with 1X Running Buffer.

7. Use steps 9-12 above.

Transfer Protocol

1. Get 4 trays, add a little methanol (MeOH) into one and 1x Transfer Buffer into other three.
2. With gloves, remove PVDF membrane from blue safety papers, let soak fully in MeOH.

   Cut lower left hand corner off to keep oriented of where the base of the gel is.
3. Cut 2x pieces of Whatman paper just larger than gel or PVDF membrane and let soak in 1x Transfer Buffer.
4. Soak 6x sponges in 1x transfer buffer carefully removing all air bubbles from each sponge.
5. Crack open gel with sushi knife (cut off wells and bottom extra gel), transfer to piece of Whatman paper (peel off to avoid tearing) flip onto another piece of Whatman paper, so ladder appears on your right. Place gel and paper in 1x Running Buffer and lay PVDF membrane on top.

   Cut corner should touch bottom of protein ladder.
6. Let soak in Transfer Buffer, smooth out air bubbles with sushi knife.
7. Set up transfer box, bottom to top:

   Larger half of transfer box

   3x sponges (air bubbles pressed out)

   Whatman paper

Gel

PVDF membrane (smooth out air bubbles)

Whatman paper (again, smooth out air bubbles)

3x sponges (no air bubbles) add extra if necessary for a tight fit

Top half of transfer box

8. Place transfer box in plastic box, close with wedge.
9. Fill center chamber with 1x Transfer Buffer (covering above sponges and gel).
10. Add 200 μl of Antioxidant to center chamber.
11. Fill outer chamber with cold DI water.
12. Attach electrodes and set power supply to 30V for 90 min, press run.

For 18-well gels doing above except (might need more than 1 L of Transfer Buffer:

1. Get special transfer tray and fill with transfer buffer, with the cassette open, red side up (on lip of tray). Soak 2 filter pads/gel in Transfer Buffer.
2. Soak PVDF membrane in MeOH (see 2 above).
3. Soak extra-long blotting paper (Bio-Rad brand) in Transfer Buffer.
4. Crack open gel with sushi knife, cut off wells, and cut off bottom extra gel and transfer to blotting paper.
5. Set up cassette (bottom to top):

    Black bottom

    Fiber pad

    Blot absorbing paper

    Gel

    Membrane

Blot absorbing paper

Fiber pad

Red top with hinge

6. Close cassette and secure with hinge.
7. Place in transfer box with red facing red and black facing black.
8. Add ice pack to icepack space in the back.
9. Fill chamber with 1X Transfer Buffer.
10. Attach electrodes and set power supply to 30V for 90 minutes.
11. When removing, pull and it will eventually come out.

Blocking Protocol

1. Take apart apparatus and remove PVDF membrane.

   Ladder and protein should have transferred to membrane
2. Place PVDF membrane in 5% milk/TBST in container for 1 hour on rocker.
3. Discard Whatman paper and gel, rinse boxes and sponges, place on drying rack.

   If membrane dries out for any reason re-soak in methanol until hydrated

Staining Protocol:

1. Discard milk/TBST.
2. Replace with primary antibody solution.

   10 ml 5% milk/TBST + 1:1000 dilution of antibody mixed in conical tube
3. Place membrane on rocker in deli fridge at 4º C for it to incubate overnight.
4. Carefully pour primary anti-body solution back into conical tube the next morning.
5. Rinse membrane 3x5 minutes each, in TBST.

Sign up for camera

1. Prepare secondary antibody solution:

   10 ml 5% milk/TBST

   Make (1 μl/ml; or 1:1000 dilution) anti-rabbit/mouse/chicken HRP conjugated antibody

2. Add secondary antibody solution to membrane, shake for 1 hour.
3. Discard secondary antibody solution.
4. Wash membrane 3x with TBST, 5 minutes each.
5. Mix ECL solutions, which are located in the cold room.

   Pipet 0.5 ml of each bottle, brown and white in 14 ml conical tube vortex

6. With tweezers/gloves, remove membrane from TBST and place on clear plastic.

   Evenly spread ECL solution over it using micro-pipette covering it completely, place another piece of clear plastic over it. If no cellophane is necessary, add ECL solution to membrane and rotate for 2 minutes. Avoid air bubbles.

7. Take blot over to camera.

Stripping Protocol:

1. Put membrane back into plastic container, wash 4x in DI water.
2. Add Stripping Buffer to membrane, shake for 20 minutes.
3. Discard stripping buffer, wash 4x in DI water, wash 1x in TBST, re-block for 30 minutes in in 5% milk/TBST and repeat staining steps.
4. Membranes can be stripped and re-probed 3 times, keep track.

Appendix L: RNA sequencing in the SCN of an intact adult female rhesus macaque.

To verify that subdissected macaque SCN's would express transcripts that are in the SCN I used a high-throughput method for analyzing gene expression, RNAseq. High-quality RNA was extracted from the SCN of an intact adult female rhesus (13 years old) and was processed by the OHSU Massively Parallel Sequencing Shared Resource (MPSSR). Core-clock genes and primary output signals (i.e., VIP and AVP) were expressed in the female rhesus monkey SCN. Estrogen receptor alpha (ESR1) and GPR30 (GPER) were more highly expressed than ERβ (ESR1) in the SCN (See Table 1 below). Post-mortem tissue was collected from the ONPRC Aging Resource archive. The hypothalamus was preserved in RNA later, and the SCN was subdissected. The adrenal glands, used in **Appendix L**, were flash frozen in liquid nitrogen and stored, along with the SCN, at -80° C. The samples were extracted using a Qiagen RNeasy Micro or Mini Kit (Norgen Biotek Corp., Thorold, Ontario, CA). The RNA was tested for purity, and the concentration determined using an Agilent 2100 Bioanalyzer and Nanodrop. Library preparation and subsequent deep sequencing of Rhesus macaque RNA was completed by the Integrated Genomics Laboratory (IGL) at Oregon Health and Sciences University (OHSU). Single-end read sequencing of samples was performed on lanes of an Illumina HiSeq 2000 flowcell. Reads were evaluated for elevated duplication levels (indicating possible adapter contamination or PCR bias) and sequence quality using FastQC (v0.10.1) software (Babraham Bioinformatics). Adaptive quality trimming was performed using a Trimmomatic (v0.30) algorithm favoring

sequence quality over read length (Lohse et al 2012). All other settings were left at default for single-end read data. The remaining reads were aligned with STAR (v.2.3.0), a commonly used RNA-seq aligner. Alignment utilized the most current Rhesus macaque genome build and gene annotation (BioProject accession: PRJNA214746). Aligner parameters were similar to the default parameters, with the exception of allowing 3 mismatched per 100-bp read. HTSeq (v0.5.4p3) was used to count unique reads from the set of uniquely aligned STAR reads (Anders and Huber 2010). Count data was based on exon boundaries within the latest gene annotation containing 16052 annotated genes. Counts were additionally normalized using an upper quartile normalization (Bullard et al. 2010).

Target Gene Counts		
Clock genes		SCN counts
BMAL1	ARNTL (aryl hydrocarbon receptor nuclear translocator like)	13.85
CLOCK	Clock circadian regulator	9.40
CRY1	Cryptochrome circadian clock 1	12.09
CRY2	Cryptochrome circadian clock 2	24.21
NPAS2	Neuronal PAS domain protein 2	14.00
NR1D1	Nuclear receptor subfamility 1 group D member 1	44.62
PER1	Period circadian clock 1	15.96
PER2	Period circadian clock 2	6.78
RORA	RAR related orphan receptor A	6.22
RORB	RAR related orphan receptor B	4.06
RORC	RAR related orphan receptor C	2.39
SCN output signals		
AVP	Arginine vasopressin	788.99
AVPR1A	Arginine vasopressin receptor 1A	0.59
AVPR1B	Arginine vasopressin receptor 2B	0.06
VIP	Vasoactive intestinal peptide	0.31
VIPR1	Vasoactive intestinal peptide receptor 1	0.32
Estrogen receptors		
ESR1	Estrogen receptor 1	4.17
ESR2	Estrogen receptor 2	0.85

Table 1. Data were aligned to the rhesus macaque genome (v7.6) using STAR (v.2.3.0) and gene expression measurements were calculated using HTseq (v0.5.4p3) with counts normalized to the FPKM (fragments per kilobase million). Shown above are the normalized counts for core-clock, output signal, estrogen receptor, and age-related factor genes in the SCN of an intact adult female rhesus monkey.

Appendix M: RNA sequencing in the adrenal gland of adult female rhesus macaque

To test how sex-steroids influenced core-clock and estrogen receptor gene expression in peripheral tissues I evaluated gene expression the adrenal gland of OVX adult (12 years old) and OVX and 1-month of estrogen supplemented female rhesus monkey. High quality RNA from pulverized whole adrenal glands was processed using RNAseq. An upper quartile normalization was performed using EdgeR and fold change was manually calculated in R. Multiple genes were differentially expression between OVX and OVX and estrogen treated animals including core-clock genes and estrogen receptors. *CLOCK*, *BMAL1* (ARNTL), *PER1*, *PER2*, and NR1D1 were all greater than 1-fold change. Estrogen treatment suppressed the expression of both positive and negative components of the core-clock feedback loop. The present results also suggest that short-term manipulation of the sex-steroid environment down-regulated two of the estrogen receptors in the adrenal gland.

Target Gene Counts				
Clock genes		Adrenal OVX	Adrenal OVX + E	Fold change
BMAL1	ARNTL (aryl hydrocarbon receptor nuclear translocator like)	1542.00	1222	**1.26**
CLOCK	Clock circadian regulator	1901.00	1788	**1.06**
CRY1	Cryptochrome circadian clock 1	918.00	1027	0.89
CRY2	Cryptochrome circadian clock 2	1563.00	1870	0.84
NPAS2	Neuronal PAS domain protein 2	1031.00	1034	1
NR1D1	Nuclear receptor subfamility 1 group D member 1	1191.00	1029	**1.16**
PER1	Period circadian clock 1	3999.00	2081	**1.92**
PER2	Period circadian clock 2	799.00	693	**1.15**
RORA	RAR related orphan receptor A	3929.00	5049	**1.29**
RORB	RAR related orphan receptor B	392.00	357	0.91
RORC	RAR related orphan receptor C	384.00	327	0.85
Estrogen receptors				
ESR1	Estrogen receptor 1	224.00	241	0.93
ESR2	Estrogen receptor 2	559.00	290	**1.93**

Table 2. Shows the upper quartile normalized counts in columns 2 and 3, and the fold change (OVX + E / OVX) in the last column. No filtering was performed and so very large and very small fold changes with low (<20) counts were not included.

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