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Kisspeptin Neuron Integration of Sex Steroid and Circadian Signaling

By

Jacob Scott Moeller

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Endocrinology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Lance Kriegsfeld, Chair
Professor George Bentley
Professor Daniela Kaufer

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Kisspeptin Neuron Integration of Sex Steroid and Circadian Signaling

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Abstract

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Doctor in Philosophy in Endocrinology

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Professor Lance Kriegsfeld, Chair

Fertility in women and across mammalian species requires a series of temporally coordinated neuroendocrine events by the circadian timing system, with disruptions to circadian timing having a pronounced negative impact on female reproductive function across species, including humans. For example, irregular menstrual/estrous cycles, diminished fertility, and increased miscarriages/fetal resorption are associated with disrupted circadian timing. Ovulation and subsequent fertility in across spontaneously ovulating mammals require the preovulatory luteinizing hormone (LH) surge, a hormonal signaling cascade initiated prior to the onset of the activity period that is stimulated by direct and indirect neuronal signals to the reproductive axis originating from the suprachiasmatic nucleus (SCN) of the hypothalamus. Throughout most of the ovulatory cycle, the negative feedback effects of ovarian sex steroids maintain the gonadotropins, LH and follicle-stimulating hormone (FSH), at low levels prior to ovulation. However, during a brief time window on the morning of proestrus when systemic levels of estradiol peak organizing responsiveness of circuits governing reproductive axis activity, daily SCN signals to the reproductive axis drive LH surge initiation. Specifically, SCN-derived signals drive the activity of stimulatory kisspeptin neurons, while concomitantly suppressing activity of the inhibitory RFRP-3 neurons. This dissertation focuses on understanding the cellular mechanisms underlying timed-changes in reproductive axis activity in response to temporally coordinated kisspeptin and RFRP-3 signals by the circadian system in LH surge generation. Whereas most studies to date have focused on their role in LH pulse generation and as the locus for estradiol negative feedback, the stimulatory role for arcuate kisspeptin (KNDy) neurons in LH surge generation was examined in Experiment 1. Although the role in anteroventral periventricular (AVPV) kisspeptin neurons is well established for their integration of sex steroid and circadian signals for the initiation of the LH surge, the mechanisms by which estrogen, progesterone, and vasopressin are temporally integrated individually and synergistically with circadian molecular timekeeping to drive timed AVPV kisspeptin cell responsiveness to vasopressin signals for LH surge initiation was investigated in Experiment 2. Finally, because the reproductive axis exhibits time-dependent changes to Kp signals for LH surge generation, Experiment 3 examined whether the reproductive axis demonstrated daily changes sensitivity to

RFRP-3 signals for LH surge generation in a similar manner to kisspeptin signaling. Taken together, findings from experiments discussed herein uncover novel cellular mechanisms for several critical signaling molecules integrated into the reproductive axis in temporally coordinated activity required for LH surge generation.

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Whereas my actual dissertation itself – yes, all of those scientific jargon-laden pages – is the culmination and celebration of my academic and scientific achievements, the stimulating, pedagogical review of the many studies completed and used in the preparation of this dissertation proved to be the celebration for me of those who aided me in the completion of these experiments during my graduate studies at Berkeley. With the vast majority my previous seven years succinctly wrapped up in a little over 100 pages presented in this dissertation, I think the African proverb "it takes a village to raise a child" needs to be updated to "it takes a village to complete a PhD" because it took a 'village' of wonderful and incredible people helping and supporting me throughout my completion of this work. I want to recognize and celebrate those who were integral in my completing this dissertation.

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Chapter 1: General Introduction

1.1 Introduction

The daily rotation of the earth on its axis results in predictable, 24 h cycles in daylight, temperature, and selection pressure (e.g., daily changes in predation risk, fertility, and sexual motivation), requiring organisms to markedly alter physiology and behavior across the day. Consequently, life on Earth has led to the evolution of the circadian timing system to predict recurring changes in the environment and to alter behavior and physiology in anticipation of changing demands. To accomplish this task, the circadian system coordinates the timing of physiological and behavioral events with natural changes in the rising and setting of the sun.

The circadian system confers a selective advantage by allowing organisms to anticipate rhythmic and predictable environmental changes and adjust physiology and behavior accordingly. Although circadian rhythms are endogenously generated to effectively synchronize internal timing with the external environment, exposure to sunlight during the day and darkness at night entrains (synchronizes) these rhythms to environmental time (Hughes et al., 2015). Unfortunately, a major consequence of contemporary lifestyles and technological advancements omnipresent in the modern world is increased exposure to sun-free environments during the day, artificial lighting and blue-light-emitting electronic devices at night, and the dissimilarity between one's internal time and their time-dependent daily obligations (social jet lag) (Roenneberg et al., 2019). This combination results in an incongruence between the endogenous circadian timing system and the external environment, leading to chronic and pervasive 'jet lag' (Bedrosian & Nelson, 2013; Fonken & Nelson, 2014). Such concerns have attracted the attention of the medical community, with the American Medical Association adopting a policy statement on the dangers of light at night for health and reproductive functioning (Stevens et al., 2013).

Because hormones travel through the bloodstream, they can have widespread influence in the central nervous system (CNS) and periphery (Buijs et al., 2021; Gotlieb et al., 2019; Moeller et al., 2022). Through these actions, hormones regulate a wide range of processes, such as metabolism, sexual motivation and behavior, sleep-wake cycles, and immune function on a daily schedule (Buijs et al., 2021; Gotlieb et al., 2019; Moeller et al., 2022). Coordinated timing of neuroendocrine events is also fundamental for successful female reproduction across mammalian species, including humans (Boden et al., 2013; Gamble et al., 2013; Kriegsfeld et al., 2018; Mahoney, 2010; Simonneaux et al., 2017; Williams & Kriegsfeld, 2012). Each phase of the female reproductive cycle, from ovulation, pregnancy and fetal development, to parturition, requires specifically-timed patterns of hormone secretion regulated by the circadian system (Blaustein et al., 1994; Egli et al., 2004; Kriegsfeld et al., 2002; McEwen et al., 1987; Mong & Pfaff, 2003), and disruptions to circadian timing have marked, negative consequences for female reproductive health. For example, women with irregular work schedules or frequent travel across time zones experience abnormal menstrual cycles (Lawson et al., 2011; Y. Wang et al., 2016), reduced fertility (Ahlborg et al., 1996; Fernandez et al., 2016), and increased miscarriage rates (Fernandez et al., 2016; Lawson et al., 2012; Nurminen, 1998). Analogously, in rodents, ablation of the master circadian clock in the brain, blocking relevant clock output signals, or disrupting the genes driving circadian clock function at

the cellular level, lead to pronounced deficits in ovulation and reproductive success (Chu et al., 2013; Miller et al., 2004; Nunez & Stephan, 1977; van der Horst et al., 1999; Wiegand & Terasawa, 1982).

1.2 The Circadian Timing System

The circadian timing system is comprised of a master brain clock in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Lehman et al., 1987; R. Y. Moore & Eichler, 1972; Ralph et al., 1990; Stephan & Zucker, 1972) and subordinate oscillatory systems throughout the brain and periphery (Cox & Takahashi, 2019; Patke et al., 2020). The SCN receives monosynaptic communication from retinal ganglion cells in the eye via a direct retino-hypothalamic tract (Morin & Allen, 2006) that coordinates the phase of endogenously generated SCN rhythms with environmental time. In turn, the SCN communicates broadly to subordinate oscillators throughout the CNS and periphery to coordinate system-wide timing. The SCN achieves this feat through monosynaptic communication to neuroendocrine cells in the brain that mediate hormone secretion and via multisynaptic communication to the periphery via the autonomic nervous system (Buijs et al., 2021; Gotlieb et al., 2019; Moeller et al., 2022; Tonsfeldt, Mellon, et al., 2022).

At the cellular level, circadian rhythms are generated by interlocked, autoregulatory transcription-translation feedback loops whereby protein products feed back to the cell nucleus to regulate the transcription of their own genes (Mohawk et al., 2012; Patke et al., 2020). The core feedback loop begins with a heterodimer of two clock proteins, CLOCK and BMAL1, driving transcription of the Period (Per1, Per2, and Per3) and Cryptochrome (Cry1 and Cry2) genes. During the course of the day, these genes are transcribed into their respective proteins that eventually heterodimerize and feed back to the cell nucleus to repress CLOCK:BMAL1-mediated transcription. In addition, the rate of PER and CRY protein degradation is controlled by casein kinase 1δ (CK1δ) and CK1ϵ (Gallego & Virshup, 2007; Narasimamurthy et al., 2018) and the F-box proteins, FBXL3 and FBXL21 (Hirano et al., 2013; Yoo et al., 2013). Following the discovery of the core feedback loop, it became clear that additional, interlocking feedback loops participate in the molecular clockwork. Specifically, a second well-established feedback loop begins with CLOCK/BMAL1-mediated transcription of the genes for the nuclear receptors, REV-ERBα and REV-ERBβ (Preitner et al., 2002), which, like PER/CRY heterodimers, individually feed back to the cell nucleus and compete with retinoic acid-related orphan receptors (i.e., RORα, RORβ, and RORγ) binding sites (i.e., ROR binding elements) at the Bmal1 gene, to positively (ROR) and negatively (REV-ERB) regulate Bmal1 transcription (Sato et al., 2004). Additional feedback mechanisms and regulatory elements maintain ~24 h rhythmicity but are beyond the scope of the work presented herein (see Cox and Takahashi, 2019 (Cox & Takahashi, 2019) and Patke et. al, 2020 (Patke et al., 2020) for further details).

Circadian timekeeping via a molecular clockwork is a universal property of cells throughout the brain and body, with virtually all cells exhibiting circadian timekeeping (Balsalobre et al., 1998; Nagoshi et al., 2004; Welsh et al., 2004; Yamazaki et al., 2000; Yoo et al., 2004). In rodents, the SCN is comprised of around 20,000 cells that are uniquely and strongly coupled to maintain synchrony among independent cellular oscillator with slightly different periods even in the absence of light (Evans, 2016; Herzog, 2007). By contrast, without communication from the master SCN clock or peripheral

signaling capable of system-wide oscillator coordination, synchronized rhythmicity within and among extra-SCN brain loci and peripheral organs uncouples and is lost after several cycles (Abe et al., 2002; Yamazaki et al., 2000). At the tissue level, the deterioration of rhythmicity results from a loss of coupling among cellular oscillators having slightly different periods rather than extra-SCN cellular oscillators losing rhythmic function (Welsh et al., 2004).

1.3 Circadian Timing in Female Reproduction

All stages of female reproduction in mammals, including follicular/gamete development and ovarian steroidogenesis (Chen, Zhao, Chu, et al., 2013; Chen, Zhao, Kumazawa, et al., 2013), ovulation (Karsch et al., 1997; Levine, 1997; Levine & Ramirez, 1982a; Mereness et al., 2016; Sellix et al., 2010; Tonsfeldt, Mellon, et al., 2022; Williams et al., 2011), behavioral estrus (Blaustein et al., 1994; McEwen et al., 1987; Mong & Pfaff, 2003), fertilization (M. H. Johnson et al., 2002), implantation (Greenhill, 2014; Liu et al., 2014a; J. Xu et al., 2016), and parturition (Gamble et al., 2013; Miller & Taskahashi, 2013), rely on timed activity of the HPG axis coordinated by the circadian timing system. Unlike their male counterparts, reproductive activity in female mammals is characterized by predictable ovulatory cycles (menstrual cycles in women and non-human primates and estrous cycles in rodents).

Prior to ovulation, the gonadotropins, LH and FSH, stimulate ovarian follicle growth and maturation while increasing concentrations of estradiol (E2; an estrogen) secreted from the developing ovarian follicle act to maintain these gonadotropins at low concentrations through E2 negative feedback (Caraty et al., 1989; Chongthammakun & Terasawa, 1993; Sarkar & Fink, 1980; Shupnik et al., 1988). In spontaneously ovulating rodents, elevated concentrations of E2 combine with circadian signals originating in the SCN to stimulate the preovulatory LH surge (i.e., estrogen positive feedback) (Gotlieb et al., 2019; Moeller et al., 2022; Simonneaux & Piet, 2018; Tonsfeldt, Mellon, et al., 2022). In women (Dozortsev & Diamond, 2020) and non-human primates (Kenealy et al., 2017; Xia et al., 1992), an extended estradiol peak is required for ovulation. Whereas the LH surge in women and non-human primates does not appear to require the SCN as in rodents, findings that the LH surges occurs within a restricted time window in women (Kerdelhue et al., 2002) and that circadian dysregulation negatively impacts the menstrual cycle (Lawson et al., 2012; Y. Wang et al., 2016) suggest that coordinated circadian timing is required for normal reproductive function in women. Likewise women with a single nucleotide polymorphisms in either the Bmal1 (Kovanen et al., 2010) or Clock (Hodzic et al., 2018) genes experience subfertility. Circadian disruption through shift work is also associated with subfecundity (Bisanti et al., 1996; Mills & Kuohung, 2019), with night shift or rotating shift work during pregnancy associated with an increased risk of preterm birth and low birth weights (Mamelle et al., 1984; McDonald et al., 1988; Patke et al., 2020; X. Xu et al., 1994), gestational hypertensive disorders (Cai et al., 2019; Hammer et al., 2018), and miscarriage (Begtrup et al., 2019; Cai et al., 2019). Although, some studies report no significant association between night work and adverse pregnancy outcomes (e.g.,(Specht et al., 2019)).

In rodent studies, destruction of the molecular clockwork leads to deficits in LH surge generation, ovulation, and fertility (Alvarez et al., 2003; Boden et al., 2010; Chu et al., 2013; Dolatshad et al., 2006; Kennaway et al., 2004; Liu et al., 2014a; Mereness et

al., 2016; Miller et al., 2004; Ratajczak et al., 2009; Zheng et al., 2019). Similarly, chronic phase shifts of the light:dark cycle (akin to jet lag) suppress the LH surge and reduce the number of successful matings compared to controls (Bahougne et al., 2020; Summa et al., 2012). Conditional deletion of the homeodomain transcription factors sine oculis homeobox 3 (Six3) in neurons leads to weakened SCN rhythms and, due to a reduced ovarian response to LH, a reduced number of oocytes ovulated during superovulation (Hoffmann et al., 2021). These results are consistent with findings in mice showing that the ovaries exhibit cyclic changes in the response to LH and that a loss of rhythmicity at the level of the ovary reduces fertility (Mereness et al., 2016). Mice in which food is restricted to the light phase to create misalignment between internal timing and the environment have reduced rates of mating behavior and resultant reproductive output (Swamy et al., 2018). Similarly, gestational chronodisruption results in a variety of health risks for offspring that often last well into adulthood. Specifically, in adult offspring, prior gestational chronodisruption is associated with metabolic disturbances (de Almeida Faria et al., 2016; Mendez et al., 2016; Varcoe et al., 2011), learning and memory deficits (Fujioka et al., 2011; Vilches et al., 2014), behavioral abnormalities (Smarr et al., 2017; P. Zhang et al., 2017), and disrupted circadian hormone profiles (Mendez et al., 2016; Salazar et al., 2018; Vilches et al., 2014). Taken together, these findings underscore the importance of circadian stability for optimal fertility, pregnancy success, and developmental programming of offspring health and adult behavior.

1.4 Circadian Control of the Preovulatory LH Surge

Across spontaneously ovulating mammals, the LH surge is ubiquitous and required for ovulation (Elkind-Hirsch et al., 1984; Levine & Ramirez, 1982a; Moline et al., 1981; Sarkar et al., 1976a). The LH surge occurs in the early in the morning in women (Kerdelhue et al., 2002) and in diurnal rodents (Mahoney et al., 2004), but in the early evening in nocturnal rodents (Christian & Moenter, 2010), allowing coordination of fertility with the active time of day. As indicated previously, the circadian timing system integrates with the reproductive axis to regulate the timing of the preovulatory LH surge. The following sections describe how the circadian system acts at several hypothalamic loci to regulate the LH surge and ovulation.

1.4.1 Circadian Control of the Preovulatory LH surge: GnRH Neurons

The SCN and its neural outputs are essential for the LH surge. Early studies in rodents demonstrated that SCN lesions (Brown-Grant & Raisman, 1977; Wiegand et al., 1980; Wiegand & Terasawa, 1982) and/or ablations that severed connections between the SCN and the anterior hypothalamus resulted in acyclicity (Nunez & Stephan, 1977). In rodents, two major neuropeptidergic SCN cell outputs have been implicated as key signaling molecules for the generation of the preovulatory LH surge: neurons that synthesize vasoactive intestinal polypeptide (VIP) in the retinorecipient SCN core and neurons that synthesize arginine vasopressin (AVP) in the dorsal SCN shell (R. Y. Moore et al., 2002). At the time of the LH surge, both VIP (Francl et al., 2010) and AVP (Kalsbeek et al., 1995a; Schwartz et al., 1983) are maximally released from SCN-derived neuronal projections, and antagonizing either VIP (Harney et al., 1996; Sun et al., 2012) or AVP communication (Funabashi et al., 1999; Palm et al., 1999) attenuates LH surge amplitude.

Several lines of evidence point to VIPergic SCN cells in the regulation of GnRH cell activity required for the LH surge. For example, VIPergic SCN neurons project monosynaptically to hypothalamic GnRH neurons (Horvath et al., 1998; Van der Beek et al., 1997) that express the VIP receptor, VPAC2 (M. J. Smith et al., 2000), and those GnRH neurons receiving monosynaptic SCN VIPergic input are preferentially activated (i.e., express cFOS) on the afternoon of proestrus concomitant with the LH surge (van der Beek et al., 1994; Van der Beek et al., 1997). SCN VIPergic innervation of GnRH neurons is sexually dimorphic, with greater VIPergic innervation emerging in females around puberty (Horvath et al., 1998; Kriegsfeld et al., 2002) and persisting into adulthood (Horvath et al., 1998). In vitro, VIP stimulation potentiates GnRH secretion (Ohtsuka et al., 1988; Samson et al., 1981; Zhao & Kriegsfeld, 2009) through increased GnRH neuron firing (Christian & Moenter, 2008; Piet et al., 2016).

Furthermore, functional molecular timekeeping in GnRH cells is required for GnRH stimulation of pulsatile and surge LH release. GnRH cells express clock genes (Bittman, 2019; Chappell et al., 2003; Tonsfeldt et al., 2019; Zhao & Kriegsfeld, 2009), and GnRH mRNA expression (Choe et al., 2013) and release (Chappell et al., 2003; Tonsfeldt et al., 2011) is rhythmic. Circadian timekeeping in GnRH cells may underlie temporal changes in GnRH cell responsiveness to upstream neurochemical signals, as in vitro studies using serum-synchronized GT1-7 cells demonstrate time-dependent changes in GnRH release by VIP stimulation (Zhao & Kriegsfeld, 2009). Moreover, disruptions to molecular circadian timekeeping in GnRH cells results in decreased LH pulse frequency (Chappell et al., 2003), disrupted estrous cycles (Bittman, 2019; Chappell et al., 2003; Dolatshad et al., 2006; Miller et al., 2004), and LH surge abnormalities (Bittman, 2019; Miller et al., 2004). Taken together, these results suggest that molecular timekeeping in GnRH cells gates responsiveness of the HPG axis to upstream neurochemical signals required for proper timing of the LH surge and ovulation.

Importantly, GnRH neurons do not express estrogen receptor-alpha (ERα), the ER subtype responsible for mediating the negative and positive feedback effects of E2 (Herbison & Theodosis, 1992; Radovick et al., 2012; Wintermantel et al., 2006), pointing to an E2-responsive neuron population upstream of the GnRH system that integrates circadian and E2 signals to mediate the timing of negative and positive feedback. The discovery of two neuropeptides with opposing actions on the HPG axis paved the way to identifying these upstream mediators and examining their roles in LH surge generation. As described below, the SCN acts to coordinate the timing of E2 negative feedback with the induction of E2 positive feedback via timed actions on RFamide-related peptide 3 (RFRP-3; the mammalian ortholog of avian gonadotropin-inhibitory hormone [GnIH]) and kisspeptin (Kp) neurons, respectively. These circadian-mediated, E2-responsive regulators that lie upstream of the GnRH system are discussed further below.

1.4.2 Circadian Control of the Preovulatory LH surge: Kisspeptin Neurons

The locus of integration for circadian and E2-ERα-mediated signals required for positive feedback effects of E2 on the initiation of the preovulatory LH surge remained largely unknown until the discovery of kisspeptin (Kp), a potent stimulator of the GnRH system (de Roux et al., 2003a; Seminara et al., 2003a). The role of Kp as a positive regulator of reproduction was first uncovered through investigations of individuals with idiopathic hypogonadotropic hypogonadism (IHH), a condition characterized by low or

absent circulating levels of LH, failure to advance through pubertal development, and reproductive incompetence in adulthood. In a subset of family members diagnosed with IHH and in transgenic mice, loss of function mutations to the genes that encode Kp (Kiss1) or its cognate receptor, GPR54 (Kiss1r), manifest as IHH (d'Anglemont de Tassigny et al., 2007; de Roux et al., 2003a; Lapatto et al., 2007; Seminara et al., 2003a).

Kp cell bodies are found in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei of the hypothalamus in rodents (Clarkson et al., 2008; Clarkson & Herbison, 2006; J. T. Smith, Cunningham, et al., 2005; J. T. Smith, Dungan, et al., 2005) and in the preoptic area (POA) and infundibulum in humans (Hrabovszky et al., 2010). The distribution of Kp is sexually dimorphic in both rodents and humans, with a greater number of Kp cell bodies observed in the AVPV of female rodents (Clarkson & Herbison, 2006; Kauffman et al., 2007; Smith, Cunningham, et al., 2005) and fewer Kp fibers and cell bodies are observed in men (Hrabovszky et al., 2010), a finding that may explain the sexually dimorphic LH surge. Moreover, in female rodents, the majority of Kp cells express ERα, with approximately 70% of AVPV and 99% of ARC Kp neurons expressing ERα (Clarkson & Herbison, 2006; Dubois et al., 2015; Gottsch et al., 2004; Greives et al., 2007; Revel et al., 2006; J. T. Smith, Cunningham, et al., 2005; J. T. Smith, Dungan, et al., 2005).

In vivo and in vitro, E2 differentially regulates Kp neuronal activity and Kiss1 gene and peptide expression in ARC and AVPV Kp cells, with elevated levels of E2 positively and negatively driving neuronal activity and Kp peptide/gene expression in AVPV and ARC Kp populations, respectively (de Croft et al., 2012; Ducret et al., 2010; Gottsch et al., 2009a; Kauffman et al., 2007; Robertson et al., 2009; J. T. Smith, Cunningham, et al., 2005). Both Kp cell populations project directly to GPR54-expressing GnRH neurons (d'Anglemont de Tassigny et al., 2007; Williams et al., 2011), with approximately 90% of GnRH expressing GPR54 (Han et al., 2005; Herbison et al., 2010). Kp acts directly on GnRH neurons to drive neuronal activity (Han et al., 2005; Pielecka-Fortuna et al., 2008) and GnRH and LH release (Gottsch et al., 2004; Matsui et al., 2004; Zhao & Kriegsfeld, 2009).

Nearly all studies to date have focused on the role of AVPV Kp cells as the locus for E2 positive feedback and LH surge generation by the circadian system (Gotlieb et al., 2018; Moeller et al., 2022; Simonneaux & Piet, 2018; Tonsfeldt, Mellon, et al., 2022). The AVPV has long been a site of interest in LH surge generation as AVPV-lesioned rats fail to ovulate or exhibit an LH surge (Gu & Simerly, 1997; Ronnekleiv & Kelly, 1988; Wiegand et al., 1980; Wiegand & Terasawa, 1982) and cFOS activation in the AVPV occurs concomitant with the LH surge (Le et al., 1999)l. Following the discovery of Kp, these activated AVPV cells were found to express Kp (J. T. Smith et al., 2006). It was later shown that optogenetic activation of these cells in ovariectomized-E2-treated mice induces surge levels of LH similar in amplitude to that observed during an endogenous LH surge (Piet et al., 2018a).

Although elevated E2 concentrations increase Kiss1 gene and peptide expression (Gottsch et al., 2009a; Robertson et al., 2009; J. T. Smith et al., 2006), Kp secretion (Jacobs et al., 2016), and cellular activity (de Croft et al., 2012; Ducret et al., 2010; Robertson et al., 2009; L. Wang et al., 2018, 2019) in AVPV Kp cells, E2 signaling alone is not sufficient to stimulate an LH surge and SCN signaling is required. In mice and hamsters, the SCN sends monosynaptic AVPergic projections to AVPV Kp cells that

express the AVP receptor, V1a (Hrabovszky et al., 2010; Williams et al., 2011). In hamsters, central infusion of AVP stimulates AVPV Kp cell activity (Williams et al., 2011) with analogous results observed in mouse brain slice (Piet, Fraissenon, et al., 2015). Likewise, optogenetic stimulation of AVPergic SCN fibers increases AVPV Kp cell activity (Jamieson et al., 2021).

E2 is in a position to modulate the impact of AVP signaling in AVPV Kp cells through several mechanisms; E2 treatment increases the number of SCN-derived AVP terminal appositions onto Kp neurons (Vida et al., 2010), induces rhythmic V1a expression in AVPV Kp cells (Smarr et al., 2013), and restores Kp cell responsiveness to AVP in OVX+E2-treated mice (Piet, Fraissenon, et al., 2015). Despite altering these variables, AVPV Kp cells respond indiscriminately to central AVP infusion regardless of time on the day of proestrus in hamsters and mice (Piet, Fraissenon, et al., 2015; Williams et al., 2011). In contrast to Kp cells, AVP infusions only activate GnRH cells on the afternoon of proestrus in hamsters, suggesting that the gating of the LH surge does not occur at the level of kisspeptin neurons but, instead, downstream of Kp signaling (Williams et al., 2011). In support of this notion, timed changes in the responsiveness and sensitivity to Kp are observed in serum-synchronized immortalized GnRH cells (Tonsfeldt et al., 2011; Zhao & Kriegsfeld, 2009)(Tonsfeldt et al., 2011; Zhao & Kriegsfeld, 2009). This gating likely results from a daily rhythm in Gpr54 gene and protein expression in GnRH cells, a rhythm that is dependent on E2 (Tonsfeldt et al., 2011).

It is surprising that AVPV Kp cells are not differentially responsive to AVP signaling across the day of proestrus, as V1a and Kiss1 mRNA expression exhibit daily changes in the presence of E2 (Jacobs et al., 2016; Robertson et al., 2009; Smarr et al., 2013). This finding suggests that molecular circadian timekeeping in AVPV Kp cells may underlie these changes and contribute to reproductive function, independent of daily alterations in responsiveness to SCN signaling. Indeed, autonomous timekeeping has been observed in Kp cells (Chassard et al., 2015; Jacobs et al., 2016), and several findings suggest a role for an autonomous Kp cell clockwork in the LH surge. For example, genetic deletion of Bmal1 in only in Kp cells disrupts LH surge timing and amplitude (Bittman, 2019; Tonsfeldt et al., 2019). Additionally, in serum-synchronized ARC Kp cells, E2 treatment phase delays Bmal1 and Per2 gene expression relative to untreated cells (Jacobs et al., 2016), pointing to interactions between E2 and Kp-cell clockwork timing. Finally, in mice, PER1 rhythms in AVPV Kp cells are phase advanced in proestrus relative to diestrus (Chassard et al., 2015). Further studies are needed in which inputs to AVPV Kp cells are optogenetically and/or chemogenetically manipulated in mice lacking core clock genes in this cell type to fully understand the role played by autonomous circadian timing in LH surge generation.

In addition to E2 and AVP signaling, astrocyte-derived neuroprogesterone (neuroP4) signals acting on AVPV Kp cells appear to be essential for the generation of the LH surge. Proestrous levels of E2 stimulate astrocyte neuroP4 production (P. E. Micevych & Sinchak, 2008; P. Micevych & Sinchak, 2008; Sinchak et al., 2003), and infusion of aminoglutethimide (AGT), a P450scc inhibitor (steroidogenesis inhibitor), blocks LH surge induction (P. E. Micevych et al., 2003) and arrests estrous cyclicity until AGT is metabolized (P. E. Micevych & Sinchak, 2008). This finding is consistent with observations in women, where the LH surge is attenuated by treatment with either a PGR antagonist or progesterone synthesis inhibitor (Batista et al., 1992; Croxatto et al., 1993;

Shoupe et al., 1987). Moreover, E2 not only stimulates neuroP4 production in astrocytes but also increases AVPV Kp cell sensitivity to neuroP4 signals, with E2 treatment increasing progesterone receptor (Pgr) expression in AVPV Kp cells (P. E. Micevych et al., 2003; Mittelman-Smith et al., 2015a; Mohr, Wong, et al., 2021; Shughrue et al., 1997; Simerly et al., 1996; J. Zhang et al., 2014a). NeuroP4 signaling in AVPV Kp is essential for the generation of the LH surge (Sinchak et al., 2020a); mice with specific deletion of Pgr in Kp cells lack an E2-induced LH surge (Gal et al., 2016; Stephens et al., 2015) that is restored upon re-expression of Pgr in AVPV Kp cells (Mohr, Wong, et al., 2021).

Although progesterone classically acts as a transcription factor, it appears that in AVPV Kp cells PGR is trafficked to the plasma membrane and signals through Src kinase, a non-receptor tyrosine kinase, to alter Kp neuronal activity, stimulate Kiss1 gene transcription, and induce Kp release (Boonyaratanakornkit et al., 2001, 2007; Chuon et al., 2021a; Mittelman-Smith et al., 2018; Pang et al., 1988). Likewise, activation of PGR or Src kinase in immortalized AVPV Kp cells increases intracellular Ca^{2+} levels and stimulates Kp gene expression and release, whereas antagonizing PGR or inhibiting Src kinase abolishes these effects (Mittelman-Smith et al., 2015a, 2018). Finally, treatment of OVX+ E2-treated female rats with an Src kinase inhibitor prevents LH surge induction (Chuon et al., 2021a). Whether or not there is a circadian component to neuroP4 signaling in AVPV Kp cells and the induction of the LH surge remains to be determined.

In contrast to AVPV Kp cells and their role in LH surge generation, a role for ARC Kp cells in LH surge generation is less well studied. Most studies to date have explored a role for ARC Kp cells as the GnRH pulse generator and locus for E2 negative feedback (Clarkson et al., 2017; Goodman & Lehman, 2012; Herbison, 2018; Lehman, Coolen, et al., 2010; Li et al., 2009; Millar et al., 2010; Nagae et al., 2021; Navarro et al., 2009; Yip et al., 2015). However, several reports suggest that ARC Kp cells may participate in LH surge generation. For example, rodents with specific ablation of the ARC Kp population exhibit abnormal estrous cycles and LH surge abnormalities (Hu et al., 2015; Mittelman-Smith et al., 2016a; Specht et al., 2019). Likewise, timed optogenetic activation of ARC Kp cells in OVX mice results in surge-like levels of LH release (Lin et al., 2021). Furthermore, SCN AVP/VIP circadian signals may modulate ARC Kp cell activity as ARC Kp cells express receptors for both AVP/VIP (Lukas et al., 2010; Mohr, Wong, et al., 2021; Mounien et al., 2006; Ronnekleiv et al., 2014) and AVP/VIP alter the cellular activity of ARC Kp cells (Mansano et al., 2021; Schafer et al., 2018). Tract tracing studies to date have not revealed direct SCN projections to ARC Kp cell (A. M. Moore et al., 2018; Yeo et al., 2019), suggesting that either AVP/VIP arise from another source or that AVP/VIP is released into the cerebrospinal fluid or vasculature of the brain to act at the level of the ARC (Schwartz et al., 1983; Taub et al., 2021).

1.4.3 Circadian Control of the Preovulatory LH surge: RFRP-3 Neurons

In their search for novel hypothalamic peptides implicated in reproduction, at the turn of the millennium Kazuyoshi Tsutsui and colleagues discovered a novel neuropeptide expressed in the paraventricular nucleus (PVN) of Japanese quail with axonal terminals projecting to the median eminence. Given that cells expressing this novel neuropeptide appeared communicate with the hypophyseal portal system, Tsutsui and colleagues examined the impact of this novel RFamide (i.e., R=Arginine, F=Phenylalanine) peptide on cultured anterior pituitary glands, finding that its application inhibited gonadotropin

release in a dose dependent manner without impact on other pituitary secretions. As a result, Tsutsui and colleagues named this neuropeptide gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000). In mammals, the Rfrp gene encodes a precursor molecule that produces the peptides RFRP-1, -2, and -3, with RFRP-3 considered to be the mammalian ortholog of avian GnIH (Clarke et al., 2008; Fukusumi et al., 2001; Hinuma et al., 2000; Kriegsfeld, 2006; Tsutsui et al., 2009; Ukena et al., 2002; Yoshida et al., 2003).

In most cases, RFRP-3 suppresses (c.f., further below) GnRH and gonadotropin secretion across mammalian species, with central and peripheral injections of RFRP-3 rapidly decreasing LH concentrations in most species (Clarke et al., 2008; Ducret et al., 2009; George et al., 2017; Gotlieb et al., 2019; M. A. Johnson et al., 2007; Kadokawa et al., 2009; Kriegsfeld, 2006; M. Wu et al., 2009). In most rodent species, RFRP-3 immunoreactive cell bodies are observed exclusively in the dorsomedial nucleus of the hypothalamus (DMH) with projections to numerous brain regions, including the POA, AVPV, ARC, PVN, organum vasculosum of the lamina terminalis (OVLT), and median eminence (Kriegsfeld, 2006; Ukena & Tsutsui, 2001). RFRP-3 fibers contact 20-40% of GnRH neurons in rodents and sheep (Clarke et al., 2008, 2008; Kriegsfeld et al., 2006; Rizwan et al., 2012; Ubuka et al., 2012) providing a direct mechanism for GnRH cell inhibition. RFRP-3 exerts its actions at target cells via the G-protein coupled receptor (GPR), GRP147 (also called NPFF1) (Bonini et al., 2000), which is expressed in 15-33% of GnRH neurons in mice (Poling et al., 2012; Rizwan et al., 2012) and in pituitary gonadotropes (Gibson et al., 2008; Sukhbaatar et al., 2014). RFRP-3 is also in a position to regulate Kp secretion, with 20% of AVPV and 35% of ARC Kp neurons receiving close contacts from RFRP-3 cells (Rizwan et al., 2012) and 5-16% of AVPV and 25% of ARC Kp neurons expressing Gpr147 (Rizwan et al., 2012).

GPR147 is typically coupled to an inhibitory G-protein (Gαi) that suppresses downstream intracellular signaling pathways (Hinuma et al., 2000; Shimizu & Bedecarrats, 2010), but in some cases, GPR147 may be coupled to the stimulatory G-proteins, Gαs or Gαq, to stimulate downstream intracellular signaling pathways (Gouarderes et al., 2007). RFRP-3 treatment of cultured brain slices suppresses electrical activity in GnRH cells (Ducret et al., 2009; Wu et al., 2009) and decrease c-Fos expression in GnRH cells in in vivo studies (Anderson et al., 2009). The suppressive actions of RFRP-3 on HPG axis activity do not appear to be mediated at the level of Kp neurons, as Kiss1 gene expression in both the AVPV (Kanasaki et al., 2019) and ARC Kp populations (Ancel et al., 2012; Tumurbaatar et al., 2018) are unaffected in vitro and in vivo following RFRP-3 treatment.

The effects of RFRP-3 on the reproductive axis are both sex and species dependent. In long-day breeding male Siberian hamsters, central RFRP-3 administration results in increased LH concentrations when given during short-day conditions (Ubuka et al., 2012). Analogous results are observed in Syrian hamsters maintained in long day conditions (Ancel et al., 2012). Additionally, in male mice, RFRP-3 treatment stimulates LH secretion, whereas RFRP-3 inhibits LH release during proestrus in female mice, but has no impact when given during diestrus (Ancel et al., 2017). These disparate effects of RFRP-3 on reproductive axis activity across species and sexes may arise from differential coupling of GPR147 to inhibitory and stimulatory G-proteins (Gouarderes et al., 2007).

There are several lines of evidence demonstrating that RFRP-3 neurons integrate circadian and estrogenic signals to mediate the effects of E2 negative feedback. First, RFRP-3 neurons express ERα, and E2 injections increase RFRP-3 neuronal activity (measured by cFOS) (Kriegsfeld et al., 2006). The activational state of RFRP-3 neurons changes across the estrous cycle, with decreased levels of activation (indicated by cFOS expression) observed at the time of the LH surge in Syrian hamsters and mice (Gibson et al., 2008; Poling et al., 2017), pointing to removal of E2 negative feedback at this time. Similarly, Rfrp expression is decreased in ewes during the preovulatory period (Clarke et al., 2012). Analogous to findings seen for GnRH cells following central kisspeptin injections, the HPG axis exhibits daily changes in responsiveness to RFRP-3, with injections around the time of the surge, but not prior, suppressing LH secretion in ovariectomized Syrian hamsters (Gotlieb et al., 2019).

As with Kp neurons, RFRP-3 neurons are direct targets of the SCN, with SCN-derived AVP- and VIPergic fibers forming close appositions with RFRP-3 neurons in Syrian hamsters (Russo et al., 2015). Furthermore, VIP modulates RFRP-3 neuron activation, decreasing neuronal activational state around the time of the LH surge, likely coordinating the release from E2 negative feedback; central AVP administration does not affect RFRP-3 neuron activity (Russo et al., 2015). AVP application to mouse brain slice during the afternoon of diestrus, but not proestrus, increases RFRP-3 neuron firing (Angelopoulou et al., 2021), pointing to potential species differences in the impact of AVP. It is unclear whether either AVP or VIP acts directly on RFRP-3 neurons, as there are no reports of AVP receptor expression in RFRP-3 neurons to date and few (~10%) RFRP-3 neurons express VIP receptors (Russo et al., 2015). Lastly, RFRP-3 neurons keep circadian time intrinsically, with the clock protein, PER1, peaking around the time of dark onset (Russo et al., 2015). Together, these results suggest RFRP-3 cells integrate circadian and estrogenic signals to appropriately coordinate the timing of E2 negative feedback and allow for initiation of the preovulatory LH surge through downstream disinhibition.

1.5 Goals

Temporal homeostasis is critical for normal brain and body functioning. The aim of the following studies was to investigate the role of autonomous circadian timekeeping and the integration of sex steroid and circadian neurochemical signaling in Kp cells in coordinating their responsiveness to SCN stimulation required for LH surge generation and subsequent ovulation. Specifically, these studies aimed to elucidate the role for steroidogenic and circadian signaling pathways for coordinating the timing of the switch from positive to negative feedback for LH surge generation. Within these experiments, we also sought to understand how steroidogenic and circadian signals organize circadian timekeeping in kisspeptin cells to coordinate the switch from negative to positive feedback for LH surge initiation. Lastly, we examined whether time-dependent changes were specific to Kp neurons or if there was timed changes in RFRP-3 activity as well in modulating LH release in the LH surge. Specifically, we investigated how the reproductive axis exhibits daily changes to RFRP-3 for LH surge generation.

Chapter 2: Arcuate Kisspeptin Neurons Contribution to LH Surge Generation

2.1 Introduction

Ovulation and subsequent fertility across female mammals requires a series of precisely timed neuroendocrine events that culminate in gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus stimulating a preovulatory surge in luteinizing hormone (LH) required for ovulation (Moeller et al., 2022; Simonneaux & Piet, 2018). Critical to the initiation of the LH surge are dynamic changes in estradiol (E2) concentrations over the ovarian cycle, with increasing amounts of E2 secreted from developing ovarian follicles over the cycle. Circulating E2 concentrations from developing ovarian follicles increase over the follicular phase of the cycle and peak prior to ovulation, the timepoint at which the ovarian follicles have fully matured (de la Iglesia & Schwartz, 2006; Gotlieb et al., 2018; Kriegsfeld & Silver, 2006). Although LH secreted from the anterior pituitary gland is responsible for stimulating ovarian steroidogenesis, the negative feedback effects of E2 maintain low gonadotropin concentrations prior to ovulation (Christian & Moenter, 2008). However, at the time of LH surge when E2 concentrations are highest, there is a temporary shift from E2-negative to E2-positive feedback that stimulates the LH surge (Karsch et al., 1997; Levine, 1997).

GnRH neurons do not express estrogen receptor (ER)-α, the ER subtype responsible for mediating the negative and positive feedback effects of E2 (Wintermantel et al., 2006). Instead, forebrain kisspeptin (Kp) neurons are a locus of integration for E2 negative and positive feedback. Kp signaling is critical for typical reproductive function, as individuals with loss of function mutations to the either the gene encoding Kp, Kiss1, or its cognate receptor, GPR54 (Kiss1r), experience idiopathic hypogonadotropic hypogonadism, a condition characterized by low or absent levels of circulating LH, failure to advance through puberty, and reproductive incompetence in adulthood (d'Anglemont de Tassigny et al., 2007; de Roux et al., 2003b; Lapatto et al., 2007; Seminara et al., 2003b). Kp cell bodies are found in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei of the hypothalamus in rodents, and both Kp populations project directly to GnRH neurons (d'Anglemont de Tassigny et al., 2007; Williams et al., 2011) to drive GnRH neuron activity (Han et al., 2005; Pielecka-Fortuna et al., 2008) and subsequent GnRH and LH release (Gottsch et al., 2004; Matsui et al., 2004; Zhao & Kriegsfeld, 2009). Moreover, both Kp populations express ER-α, with ER-α expression observed in ~70% of AVPV and ~99% ARC Kp cells, respectively (Clarkson & Herbison, 2006; Dubois et al., 2015; Gottsch et al., 2004; Greives et al., 2007; Revel et al., 2006; J. T. Smith, Cunningham, et al., 2005; J. T. Smith, Dungan, et al., 2005).

E2-ERα-mediated signals in Kp cells is critical for typical reproductive function (Dubois et al., 2015; Shivers et al., 1983; Wintermantel et al., 2006), and have been reported to differentially regulate Kp cell activity and Kiss1 gene and peptide expression across Kp populations, with increased E2 levels positively and negatively driving neuronal activity and Kp gene/peptide expression in AVPV and ARC Kp populations, respectively (de Croft et al., 2012; Ducret et al., 2010; Gottsch et al., 2009b; Kauffman et al., 2007; Robertson et al., 2009; J. T. Smith, Cunningham, et al., 2005). Nearly all studies to date have focused on the role of AVPV Kp cells in E2-positive feedback and LH surge generation. Conversely, ARC Kp neurons co-express neurokinin B and dynorphin (KNDy neurons) and are thought to be a major driver of pulsatile LH release and are suggested

as the locus for E2-negative feedback (Clarkson et al., 2017; Goodman & Lehman, 2012; Herbison, 2018; Lehman, Coolen, et al., 2010; Li et al., 2009; Millar et al., 2010; Nagae et al., 2021; Navarro, 2012; Yip et al., 2015). However, more recent studies suggest an additional role for ARC Kp cells in the generation of a normal LH surge. For example, knockdown of ARC Kp expression in rats results in decreased LH surge amplitude (Hu et al., 2015), rodents with selective ablation of ARC Kp cells exhibit abnormal estrous cycles and LH surge abnormalities (Helena et al., 2015; Hu et al., 2015; Mittelman-Smith et al., 2016a), and optogenetic stimulation of ARC Kp cells in OVX-E2-treated mice induces surge-like levels of LH release (Lin et al., 2021).

The ability of Kp neurons to initiate the LH surge requires sequential priming with E2 followed by progesterone stimulation of a neural origin (neuroP4) (P. E. Micevych et al., 2003; P. Micevych & Sinchak, 2008; Sinchak et al., 2020a). Proestrous levels of E2 not only induce hypothalamic astrocyte production of neuroP4 but also function to increase Kp sensitivity to neuroP4 signals by upregulating Kp cell progesterone receptor (Pgr) expression in the AVPV (Göcz, Rumpler, et al., 2022; P. E. Micevych et al., 2003; Mittelman-Smith et al., 2015b; Mohr, Wong, et al., 2021; Shughrue et al., 1997; Simerly et al., 1996; J. Zhang et al., 2014b). Mice with global or Kp cell specific deletion of Pgr expression do not exhibit LH surges (Chappell et al., 1997, 1999; Gal et al., 2016; Sleiter et al., 2009; Stephens et al., 2015). Although the selective reinstatement of Pgr expression in AVPV Kp neurons is capable of rescuing the LH surge in Kp Pgr knockout mice, the amplitude of the surge is dampened (Mohr, Esparza, et al., 2021), suggesting a potential role for PGR signaling in ARC Kp cells in augmenting the LH surge. The role of neuroP4 signaling in ARC Kp cells for the generation of the preovulatory LH surge, however, remains to be determined and represents one focus of the present study.

Although the convergence of E2 and P4 signals on Kp cells is required for LH surge generation, they are not sufficient to stimulate the LH surge individually or synergistically. Instead, these signals set the stage for stimulation of Kp cells by a circadian signal from the suprachiasmatic nucleus (SCN) of the hypothalamus, the master circadian pacemaker (Moeller et al., 2022; Piet, Fraissenon, et al., 2015; Williams et al., 2011), to Kp cells (Palm et al., 1999). AVPV Kp cells express the vasopressin (AVP) receptor, V1a, receive monosynaptic SCN-derived AVPergic communication to drive the LH surge generation (Vida et al., 2010; Williams et al., 2011), and molecular circadian timekeeping in Kp cells is required for typical LH surge timing and magnitude (Bittman, 2019; Chu et al., 2013; Dolatshad et al., 2006; Khan & Kauffman, 2012; Miller et al., 2004; Tonsfeldt, Mellon, et al., 2022). Although tract tracing studies have not uncovered monosynaptic AVP projections from the SCN to ARC Kp neurons (A. M. Moore et al., 2018; Yeo & Herbison, 2011), the SCN rhythmically releases AVP into the CSF that presumably reaches the ARC, ARC Kp neurons express V1a (Lukas et al., 2010; Mohr, Wong, et al., 2021; Mounien et al., 2006; Ronnekleiv et al., 2014), and these cells exhibit altered cellular activity following AVP treatment (Schafer et al., 2018).

The present study sought to determine the actions of ovarian E2, P4, and circadian signaling to ARC Kp neurons in the switch from E2-negative to E2-positive feedback and sensitivity to AVPergic signaling required for the generation of the preovulatory LH surge.

2.2 Materials and Methods

2.2.1 mHypoA-KISS/GFP-3 Cell Culture and Reagents

mHypoA-KISS/GFP-3 (CELLutions Biosystems, Burlington, Ontario, CLU507) (ARC Kp cells) are a clonal, immortalized Kiss1-expressing cell line originating from arcuate nucleus (ARC) of adult, female Kiss-GFP transgenic mice generated by Dr. Robert Steiner (University of Washington, Seattle, WA). Immortalization was attained by a retroviral transfer of Simian virus-40 T-antigen and neuronal proliferation was induced by ciliary neurotrophic factor treatment (Treen et al., 2016). Monolayer mHypoA-KISS/GFP-3 cultures were maintained in humidified atmosphere at 37°C with 5% CO_2 and cultured in 75 cm^2 flasks containing normal growth medium that is comprised of high-glucose Dulbeco's Modified Eagles Medium (DMEM; Gibco, Carlsbad, CA, #11965118) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO, #F2442) and 1% penicillin-streptomycin (PS; Sigma Aldrich, St. Louis, MO, #P4333). Cells were passaged at 85-90% confluency and not used after 10 passages.

Serum-free, phenol red-free DMEM (FSDMEM; Hyclone, Logan, UT, #SH30284) supplemented with 1% PS was used following serum shock. (17ß)-Estra-1,3,5(10)-triene-3,17-diol (E2; Tocris Bioscience, Minneapolis, MN, #2824) was dissolved in absolute ethanol (ETOH) to obtain a stock concentration of 10mM and stored at -20°C. Stock E2 was diluted with ETOH to 100 nM and subsequently diluted to final concentration of 100 pM with SFDMEM, as described previously (Jacobs et al., 2016). Progesterone-water soluble (P4; Sigma Aldrich, St. Louis, MO #P7556) was reconstituted in water to yield a 1mM stock concentration and was subsequently stored at -20°C prior to studies. For experiments, stock P4 was diluted in SFDEMEM supplemented with 100 pM E2 or vehicle to a final concentration of 1nM. [Arg^8]-Vasopressin acetate salt (AVP; Sigma Aldrich, St. Louis, MO #V9879) was dissolved in water for a 100 µM stock concentration and stored at -20°C prior to studies. For dose response and time course experiments, AVP was subsequently diluted with SFDMEM to a final concentration of 0.5 nM, 1 nM, and 5 nM, with water as the vehicle control.

2.2.2 Steroid and Peptide Treatments

For all experiments, mHypoA-KISS/GFP-3 cells were seeded at a density of 400,000 cells per well in a 6-well plate and incubated overnight in normal growth medium to allow for cells to attach. Medium was then changed to FSDMEM for 24 h, and cells were next transferred into FSDMEM supplemented with 100 pM E2 or vehicle and incubated for 24h. Next, 4 h prior to AVP stimulation, media was refreshed to remove accumulated secreted kisspeptin resulting from baseline release and 1 nM P4 or vehicle was added to SFDMEM supplemented with 100 pM E2 and then AVP or vehicle was added to experimental wells and incubated for 45 mins following P4 treatment. After incubation, media was collected and pooled from wells of corresponding treatment conditions and cells were harvested for either protein or RNA expression analysis.

2.2.3 Quantitative RT-PCR

Following treatment, stimulated cells and untreated controls were harvested and total mRNA was purified. Total mRNA from treated and untreated wells was isolated using the PureLink RNA Mini Kit (Ambion, Austin, TX, #12183025) with and on-column DNase I (Ambion, Austin, TX, #12185010) digestion according to manufacturers' protocols. Subsequently, total RNA concentration and purity were determined using a NanoDrop

2000c (Thermo Fisher Scientific, Waltham, MA, #ND-2000C). cDNA was synthesized from 1 µg of total mRNA using the SensiFAST cDNA Synthesis Kit (Bioline, Memphis, TN, #BIO-65054) and stored at -20°C. The reverse transcription was performed at 42°C for 20 min followed by a 5 min termination at 85°C. A total of 100 ng of cDNA template was amplified using SensiFAST SYBR No-ROX Kit (Bioline, Memphis, TN, #BIO98020). Samples were run in triplicate using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, #1855485) with an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 25 s for v1a, esr1, per2, bmal1, and 40 cycles of 95°C for 15 s and 63°C for 25 s for pgr. Primers were designed using PrimerBLAST (National Library of Medicine, Bethesda, MD) and aimed to span intro-exon junctions when possible to control for genomic DNA contamination. Efficiency for each primer pair was determined by a standard curve using 3-fold serial dilutions of cDNA, with values between 95.4-111.5% for each primer pair. Specificity of each primer pair was determined by non-template and no reverse transcriptase controls, a single-peak melt curve analysis, and gel electrophoresis of amplicons. Primer sequences, amplicon size, and annealing temperature are listed in **Table 1**. The relative expression levels for each gene of interest were determined using the $2^{-\Delta\Delta Ct}$ method. Gene expression was normalized to tbp, the gene coding for TATA-box binding protein.

2.2.4 Western Blotting

At the time of protein harvest, cells were placed on ice, washed twice with cold Dulbecco's phosphate buffered saline (PBS; Sigma Aldrich, St. Louis, MO, D8537), and lysed in radioimmunoprecipitation (RIPA) assay buffer (Cell Signaling Technology, Danvers, MA, #9806) supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, #5872). Lysates were centrifuged at 14,000 rpm for 15 min at 4°C, with the supernatant collected and stored at -80°C. Protein concentration was determined with the Pierce Rapid Gold BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, #A53226), with 25 µg of protein per sample resolved on a 4-20% SDS polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, #4568093) and electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, #1620175). Membranes were blocked for 1 h at room temperature with 5.0% nonfat milk or bovine serum albumin (BSA) (wt/vol) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and then incubated overnight with the primary antibody at 4°C with gentle rocking. Primary antibodies with associated dilutions used are listed in **Table 2**. Primary antibodies were diluted in either 2.0% BSA or 2.0% non-fat milk in 0.1% TBS-T per manufacturers' recommendations. Membranes were then washed 3 times for 10 min with TBS-T prior to incubation with species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (diluted in 2.0% BSA or 2% non-fat milk in TBS-T) sourced from EMD Millipore (Burlington, MA) for 2 h at room temperature. Blots were washed three times with 0.1% TBS-T for 10 min and proteins of interest were visualized on a ChemiDocXRS+ (Bio-Rad Laboratories, Hercules, CA, 1708265) using SuperSignal West Femto or Atto chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, #34095 or #A38556, respectively) per the manufacturer's instructions. Membranes were stripped with Re-Blot Plus Mild Antibody Stripping Solution (EMD Millipore, Burlington, MA, #2502) according to manufacturer's instructions. This process of primary and secondary antibody incubation was repeated for the housekeeping gene, vinculin. Densitometric analysis of

images were performed using Image Lab 5.2.1 (Bio-Rad, Hercules, CA, #1709690), with the optical density for both signal and background calculated by the software. Total signal intensity was then calculated by subtracting the pixel density from that of the background to that of the protein of interest. Relative expression was determined by dividing the corrected signal for the protein of interest by the corrected signal for the housekeeping gene.

2.2.5 Statistics

The impact of treatment was analyzed using t-tests and one-way analysis of variance analysis (ANOVA) with Tukey's post hoc test where appropriate. All statistical analyses were performed using PRISM (GraphPad Software Inc., San Diego, CA). All data are presented as the mean ± SEM. $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Estradiol stimulates Pgr gene expression in arcuate ARC Kp cells

Previous rodent RNA sequencing studies indicate that E2 treatment significantly increases Pgr expression in AVPV Kp cells of OVX-E2-treated animals (Göcz, Rumpler, et al., 2022; Mohr, Wong, et al., 2021). To determine the impact of E2 on Pgr expression in ARC Kp cells, we treated mHypoA-KISS/GFP-3 cells with 100pM or vehicle for 24 h and harvested mRNA for gene expression analysis. Analogous to results observed in AVPV Kp cells, E2-treatment significantly induced progesterone receptor mRNA expression in then ARC cell population (5.478 ± 1.441-fold increase from controls; $p< 0.05$) (**Figure 1A)**.

2.3.2 E2, P4, and AVP treatments stimulate Kp peptide expression in ARC Kp cells

Generation of the preovulatory LH surge is thought to require convergence of E2 (J. T. Smith, Cunningham, et al., 2005; Wintermantel et al., 2006), neuroP4 (Mohr, Esparza, et al., 2021; Stephens et al., 2015), and AVP (Funabashi et al., 1999; Palm et al., 1999; Williams et al., 2011) signals at AVPV Kp cells. Whether or not convergence of this neuropeptidergic and hormonal signaling at ARC Kp neurons participates in LH surge generation has not been explored. Here we find that Kp peptide expression was significantly increased following E2 (2.563 ± 0.3124; $p< 0.05$), P4 (1.325 ± 0.1123; $p<0.05$), and AVP (1.087 ± 0.1611; $p<0.05$) treatments relative to controls (0.3467 ± 0.06556). Conversely, E2/P4 (0.26 ± 0.09886; p=NS) and E2/P4/AVP (0.8683 ± 0.03156; NS) co-treatments did not impact Kp peptide expression (**Figure 1C**). Moreover, E2 treatment significantly increased Kp peptide expression relative to all groups ($p<0.05$), whereas Kp peptide expression was significantly decreased in E2/P4 co-treated samples relative to P4- ($p<0.05$) and AVP-treated ($p<0.05$) samples.

2.3.3 E2/P4 co-treatment potently stimulates CREB activation in ARC Kp cells

Previous studies using mHypoA-KISS/GFP-3 AVPV cells indicate that E2 modulates Kp gene expression through regulation of Creb1 via a membrane-initiated signaling pathway, as E2 modulation of Kiss1 gene expression is lost with siRNA

knockdown of Creb1 gene expression (Treen et al., 2016). In the present work, 100 pM E2 treatment (0.2017 ± 0.05275; $p<0.05$) significantly downregulated phospho-CREB (pCREB) expression relative to controls (0.6167 ± 0.06515; $p<0.05$), E2/P4 cotreatment (1.168 ± 0.1268; $p<0.05$) significantly increased pCREB expression, whereas no differences were observed following P4 (0.3133 ± 0.03051; p=NS) or AVP (0.5933 ± 0.08425; p=NS) stimulations relative to controls (**Figure 1B**). E2/P4 co-treatment significantly upregulated pCREB expression over all groups ($p<0.05$), but pCREB expression was significantly reduced following E2 treatment relative to AVP-stimulated samples ($p<0.05$).

2.3.4 E2/P4 co-treatment rescues ER-α protein expression in ARC Kp cells

Because Kp peptide expression is increased following E2, P4, and AVP treatments and E2-ER-α-mediated signaling is suggested to mediate Kiss1 gene expression in the AVPV (Gottsch et al., 2009b; J. T. Smith, Cunningham, et al., 2005), ER-α protein and gene expression were assessed to determine if increased Kp peptide expression is from a loss of E2-ER-α-mediated signaling in regulation of Kp peptide expression. ER-α protein expression following E2/P4 co-treatment (1.137 ± 0.08977; p=NS) did not differ relative to control (1.372 ± 0.0660). However, treatment with E2 (0.8833 ± 0.04372), P4 (0.7517 ± 0.05108; $p<0.05$), and AVP (0.9433 ± 0.1064; $p<0.05$), and co-treatment with E2/P4/AVP (0.8867 ± 0.06687; $p<0.05$), significantly decreased ER-α protein expression relative to controls (**Figure 2A**). Significant differences, however, were not observed in underlying ER-α (Esr1) gene expression (**Figure 2B**).

2.3.5 E2 treatment induces phosphorylation of Erk 1/2

Because E2- and neuroP4-membrane-initiated signals (Mittelman-Smith et al., 2017, 2018) are reported to stimulate Erk 1/2 phosphorylation in AVPV Kp neurons, phosphorylation of Erk 1/2 was measured to determine whether differential regulation of Erk 1/2 signaling underlies observed changes in CREB phosphorylation and Kp expression. Although Erk 1/2 phosphorylation did not differ from controls (0.3533 ± 0.1037; p=NS) following P4 (0.5233 ± 0.1020; p=NS) and AVP (0.5667 ± 0.08480; p=NS) or co-treatment with E2/P4/AVP (0.2533 ± 0.08535; p=NS), E2 treatment (1.643 ± 0.2958) significantly increased CREB phosphorylation relative to all other treatment groups ($p<0.05$). Although E2/P4 co-treatment (0.9500 ± 0.06875; p=0.07 [NS]) resulted in a nonsignificant trend towards increased Erk 1/2 phosphorylation relative to control, Erk 1/2 phosphorylation was significantly increased ($p<0.05$) relative to E2/P4/AVP co-treatment (**Figure 2C**). To determine whether changes in Erk 1/2 phosphorylation were driven by underlying differences in Erk 1/2 expression, Erk 1/2 expression was measured, and no differences were observed across groups (p=NS) (**Figure 2D**).

2.3.6 Circadian clock function is not altered by any treatment or co-treatment

Previously, ARC Kp cells were found to have a functional circadian clock (Jacobs et al., 2016), and disruption of clock function in Kp cells (Bittman, 2019; Tonsfeldt, Cui, et al., 2022) results in disrupted LH surge timing and amplitude. To determine whether circadian clock function was impacted by E2, P4, or AVP treatments or co-treatments,

expression of the core clock gene, Period2 (Per2), was measured. None of the treatments or co-treatments were found to impact Per2 expression (p=NS in all cases). (**Figure 3**).

2.4 Discussion

The identity of the neural locus for E2-negative feedback and LH pulse generation has long been thought to be ARC Kp neurons (Lehman, Coolen, et al., 2010). However, recent studies indicate that ARC Kp cells may also serve a role in LH surge generation (Helena et al., 2015; Lin et al., 2021; Mittelman-Smith et al., 2016a). Because previous in vivo studies established E2, neuroP4, and AVP signals converging on Kp cells as a requirement for stimulation of an LH surge with typical surge dynamics (e.g., timing and amplitude), the present study sought to understand how sex steroid (E2, neuroP4) and circadian (AVP) signals are integrated in ARC Kp neurons for the timed initiation of the LH surge. The present studies reveal several novel findings: 1) ARC Kp peptide and progesterone receptor gene (Pgr) expression are significantly increased following 24 h E2 treatment (**Figure 1**), 2) P4 stimulation reverses E2-stimulated increases in Kp peptide expression (**Figure 1C**), 3) P4 treatment following E2 treatment significantly increased CREB phosphorylation over all treatments and co-treatments and reversed E2-induced decreases in CREB phosphorylation (**Figure 1B**), 4) P4 stimulation following E2 pretreatment significantly reduced phosphorylated Erk 1/2 relative to E2 treatment (**Figure 2C**), 5) E2 treatment reduced ER-α protein expression, an outcome partially reversed by P4 treatment (**Figure 2A**), and 6) E2, P4, or AVP signals neither individually nor combined impact circadian clock function in ARC Kp cells (**Figure 3**).

Findings from the present study suggest that E2 treatment in ARC Kp cells increases Kp peptide expression similar to what is observed in an AVPV Kp-like immortalized hypothalamic cell line (Mittelman-Smith et al., 2015a) and suggest that E2-stimulated increases in ARC Kp peptide expression are likely occurring through upregulation of Erk 1/2 signaling and decreased activation of CREB-mediated gene expression, which are a consequence of membrane-initiated E2 signaling (P. E. Micevych et al., 2017). Conversely, findings from the present study also suggest the E2-negative feedback effects on Kp expression observed in in vivo studies are likely occurring through E2-primed, neuroP4-negative feedback actions on ARC Kp cells, as P4 treatment following E2 pretreatment reverses E2-induced increases in Kp peptide expression. The observation that Kp peptide expression and Erk 1/2 phosphorylation are correlated with increased CREB phosphorylation in ARC Kp cells, suggests that E2-stimulated P4 actions regulating Kp expression are occurring through the differential activation of CREB-mediated gene transcription and inhibition of Erk 1/2 signaling, likely occurring through membrane-initiated neuroP4 activation of protein kinase A (PKA) or protein kinase C (PKC) signaling pathways (Mani & Oyola, 2012; P. E. Micevych & Sinchak, 2018).

Because ARC Kp cells have long been thought to be the locus for direct E2-negative feedback effects, the observation that E2 treatment stimulates ARC Kp peptide expression was unexpected. Previous work using animals with genetic deletion of the gene encoding for ER-α found Kiss1 expression is increased following OVX (Gottsch et al., 2009b; J. T. Smith, Cunningham, et al., 2005), and Kiss1 mRNA is decreased in mHypoA-KISS/GFP-3 cells following treatment with membrane-impermeable E2-BSA (Treen et al., 2016). Moreover, in KT-aR1 cells, a different immortalized ARC Kp cell line, Kiss1 expression is decreased following low dose (2-10 pM) E2 treatment (Jacobs et al.,

2016). However, consistent with findings presented herein, higher doses (25 pM and above) of E2 can stimulate non-significant increases in Kiss1 expression in both mHypoA-KISS/GFP-3 and KT-aR1 cells (Jacobs et al., 2016; Treen et al., 2016). The likely explanation for treatments using lower doses (2-10 pM) of E2 and E2-BSA resulting in decreased Kiss1 mRNA expression (Jacobs et al., 2016; Treen et al., 2016) is that the genomic and membrane-initiated effects of E2 signals differentially regulate Kiss1 expression, with membrane-initiated signaling negatively and genomic positively regulating Kp gene expression, respectively. Because the K_d value for ER-α is 100 pM (Kuiper et al., 1997), lower doses (2-10 pM) of E2 used in previous studies likely only fully activate membrane-initiated signaling pathways and are incapable of fully activating ER-α-mediated genomic signaling pathways. The present study used a dose of 100 pM for E2 treatment, a dose capable of fully activating both genomic and membrane-initiated ER-α signaling pathways. This finding is also consistent with observations from studies examining the neural mechanisms responsible for mediating sexually receptive behaviors in rats, where high/low doses of E2 have differential effects on membrane ER-α protein expression, with high doses of E2 leading to ER-α removal from the membrane and targeted for proteosome degradation (Sirinathsinghji et al., 1986). This finding suggests that low E2 concentrations during early follicular phase of the cycle negatively regulate ARC Kp expression; however, at the time of proestrous when E2 concentrations peak, E2 stimulates Kp peptide expression for normal surge initiation.

Consistent with early rodent studies showing Pgr expression is increased after E2 treatment (Göcz, Rumpler, et al., 2022; Göcz, Takács, et al., 2022; Mohr, Wong, et al., 2021), ARC Kp cells demonstrated increased Pgr expression following E2 treatment. Because selective reinstatement of Pgr expression in AVPV Kp cells in animals with targeted deletion of Pgr expression from Kp cells is capable of restoring the LH surge but only with a reduced amplitude (Mohr, Esparza, et al., 2021), the finding that P4 stimulation following E2 pretreatment decreased Kp peptide expression in ARC Kp cells was unexpected. Since E2-ER-α-mediated signaling is required to induce ARC Kp Pgr expression along with P4 production, studies using Kp ER-α knockout animals are unable to dissociate E2- versus P4-dependent signaling events due to sensitivity to P4 signals being contingent upon ER-α-stimulated transcription of Pgr. This finding underscores the need further in vitro studies to substantiate findings obtained in vivo by examining the individual and combined effects of critical signaling molecules.

Whereas E2 treatment was observed to significantly downregulate CREB phosphorylation while increasing Kp peptide expression, P4 stimulation following E2 pretreatment reversed E2-induced reductions in CREB phosphorylation and in increased Kp peptide expression. Because Kp expression is low while CREB phosphorylation peaks, the present findings suggest that increased CREB phosphorylation negatively regulates Kp expression in ARC Kp cells. This observation is consistent with a previous report using E2-treated ARC Kp cells, with Creb1 gene expression significantly reduced by siRNA targeting Creb1 expression showing the ability for E2 to negatively regulate Kiss1 mRNA expression is lost following transient knockdown of Creb1 expression (Treen et al., 2016). Moreover, the differential phosphorylation of CREB observed in the present study following E2 and P4 stimulation suggest that E2 and P4 bind to membrane bound receptors that function similar to G-coupled protein receptors (GPCRs) for activation of different downstream signaling pathways (P. E. Micevych et al., 2017; Sinchak et al.,

2020a, 2020a), resulting in the differential regulation of CREB-mediated gene transcription and subsequent Kp peptide expression.

Previous observations from in vitro studies that were then verified in subsequent in vivo studies using animal models indicate that membrane-initiated P4 signaling in AVPV Kp cells following E2 conditioning increases Erk 1/2 phosphorylation via activation of Src kinase, which, in turn, stimulates Kp peptide release and increased Kp peptide and gene expression for the initiation of the LH surge (Chuon et al., 2021b; Mittelman-Smith et al., 2018). Therefore, the present study sought to determine whether differential activation of Erk 1/2 signaling by E2 and P4 is responsible for the disparate effects of E2 and P4 on CREB phosphorylation and Kp peptide expression. Paralleling findings from AVPV Kp cells exhibiting increased Erk 1/2 phosphorylation corresponding with increased Kp peptide expression (Mittelman-Smith et al., 2018), E2-treated ARC Kp cells exhibited increased Erk 1/2 activity with increased Kp peptide expression, a finding reversed by P4 stimulation. Although the observation that Kp peptide expression is stimulated along with increased Erk 1/2 activity in both ARC and AVPV Kp cells (Mittelman-Smith et al., 2018), the ligand responsible for stimulating increased Erk 1/2 activity differs across the two populations. In the present study, E2 was found to increase Erk 1/2 phosphorylation, whereas P4 signals are reported to increase AVPV Kp cell Erk 1/2 phosphorylation, suggesting E2- and P4-stimulated signaling pathways converge on the Erk 1/2 pathway in controlling Kp expression for normal LH surge generation. Findings from the present study are consistent with previous reports examining E2 regulation of ARC Kp Erk 1/2 activity in sexually receptive behaviors, with E2 stimulating increased Erk 1/2 phosphorylation in female rats without associated increases in CREB activity. Because increased CREB phosphorylation was not observed with increased Erk 1/2 phosphorylation following E2 treatment in the present study, these results suggest that activation of CREB-mediated gene transcription is not a downstream target of E2-initiated Erk 1/2 signaling. Taken together, these findings further support the notion that ARC Kp cells have stimulatory actions through E2-mediated increases in Kp peptide expression for normal LH surge generation.

Provided the impact E2 signaling has on Pgr is conserved across AVPV and ARC Kp cells, with E2 treatment resulting in increased Pgr expression (Göcz, Rumpler, et al., 2022; Göcz, Takács, et al., 2022; Mittelman-Smith et al., 2015a; Mohr, Wong, et al., 2021), the mechanism by which P4 signals stimulate Kp peptide expression differs across populations. Previous studies established P4 signaling in AVPV Kp cells stimulates Kp expression via increased Erk 1/2 activity, whereas findings presented herein indicate P4 reverses E2-stimulated increases in P4 peptide expression through induction of CREB-mediated gene expression. Because CREB phosphorylation was not observed with increased Erk 1/2 phosphorylation, CREB is an unlikely target of Erk signaling and is likely activated by protein kinase A (PKA) or protein kinase C (PKC) signaling. The activation of PKA and PKC and subsequent increased CREB phosphorylation depends upon increases in the effector molecule, cyclic adenosine monophosphate (cAMP) (reviewed in (Stork & Schmitt, 2002)) and has been suggested to regulate female rodent receptive behaviors in the ARC (reviewed in (Boulware et al., 2005)). Because P4 treatment resulted in decreased and increased Erk 1/2 and CREB phosphorylation, respectively, and cAMP activation of PKA and PKC signaling pathways have been demonstrated to decrease Erk 1/2 phosphorylation (reviewed in (G.-Y. Wu et al., 2001)), the finding that

P4 treatment results in decreased Erk 1/2 phosphorylation supports the notion P4 stimulates a PKA/PKC-mediated signaling pathway for CREB-mediated decreases in Kp peptide expression while also reducing Erk 1/2-mediated signaling events for reduced Kp expression.

Interestingly, observations from the present study suggests that E2 mediates ARC Kp sensitivity, in part, through regulation of ER-α protein expression. Whereas E2 treatment was found to significantly decrease protein expression, E2 was not observed to negatively regulate Esr1 mRNA expression. Prolonged E2 stimulation results in the removal and degradation of ER-α protein from the membrane in the ARC nucleus (P. Micevych & Dominguez, 2009) and observed decreases in ER-α protein expression in the present study are consistent with this finding. Although Esr1 expression was not impacted by E2 treatment in the present study, E2 stimulation was found to decrease Esr1 mRNA expression in ARC Kp cells (Treen et al., 2016). The discrepancy between the previous study and the observations presented herein are likely due to the difference in concentration of E2 used. Whereas 10 and 100 nM E2 treatment was found to lead to decreased Esr1 expression in the previous study, 100 pM E2 treatment was used in the present study.

Taken together, findings from the present study indicate several parallels in the physiology across AVPV and ARC Kp cell populations. Early and more recent work has established that both populations are required for generation of a normal LH surge (reviewed in (Moeller et al., 2022)). Furthermore, physiology in both populations is dependent upon and regulated by E2 and P4 membrane-initiated signaling mechanisms (reviewed in (P. E. Micevych & Sinchak, 2018; Mittelman-Smith et al., 2017)). Moreover, the mechanisms utilized for regulating Kp cell physiology are consistent across populations. For instance, high doses of E2 mediate Kp peptide and Pgr mRNA expression in a similar manner across the two populations. Likewise, although the ligand stimulating increases in Erk 1/2 activity differ across the two populations, the impact of increased Erk 1/2 activity is consistent. Because timed Kp cellular activity is required for initiation of the LH surge, future studies examining the temporal regulation of AVPV and ARC Kp cell physiology will shed light on conserved mechanisms regulating the physiology of both populations.

2.5 Tables

Gene	Primer Name	Primer Sequence	Amplicon Length (BP)
V1a	V1A FWD	TCTTCATCGTCCAGATGTGGTC	90
	V1A REV	CCAGTAACGCCGTGATCGT	
Esr1	ESR1 FWD	ATGAAAGGCGGCATACGGAAAG	94
	ESR1 REV	CACCCATTTCATTTCGGCCTTC	
Pgr	PGR FWD	AGGTCTACCCGCCATACCTT	193
	PGR REV	TTATGCTGCCCTTCCATTGC	
Per2	PER2 FWD	CCAAGCATCCAGCCCTGTTT	170
	PER2 REV	CGTTTGGTTTGCGCATGAAC	
Bmal 1	BMAL 1 FWD	GACCTACTCTCCGGTTCCCT	89
	BMAL 1 REV	GCATATTCTAACTGGTAGTCAGTGG	
Tbp	TBP FWD	TTGGCTAGGTTTCTGCGGTC	179
	TBP REV	TGGAAGGCTGTTGTTCTGGT	

Table 1. Primers used for qPCR gene expression assays.

Peptide/Protein Target	**Name of Antibody**	**Manufacturer, Catalog Number**	**Host Species; Monoclonal or Polyclonal**	**Dilution Used**
KISS-1	Anti-KiSS-1 Antibody, clone 8H4.1	EMD Millipore, #MABC60	Mouse; Monoclonal	1:1000
phospho-MAPK	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb	Cell Signaling, #4370	Rabbit; Monoclonal	1:2000
Total MAPK	p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell Signaling, #4695	Rabbit; Monoclonal	1:1000
Estrogen Receptor-alpha	Estrogen Receptor alpha Monoclonal Antibody (1D5)	Invitrogen, #MA5-13191	Mouse; Monoclonal	1:1000
phospho-CREB	Phospho-CREB (Ser133) (1B6) Mouse mAb	Cell Signaling, #9196	Mouse; Monoclonal	1:1000
Vinculin	Vinculin (E1E9V) XP Rabbit mAb	Cell Signaling, #13901	Rabbit; Monoclonal	1:1000

Table 2. Antibodies used for peptide/protein content analysis.

2.6 Figures

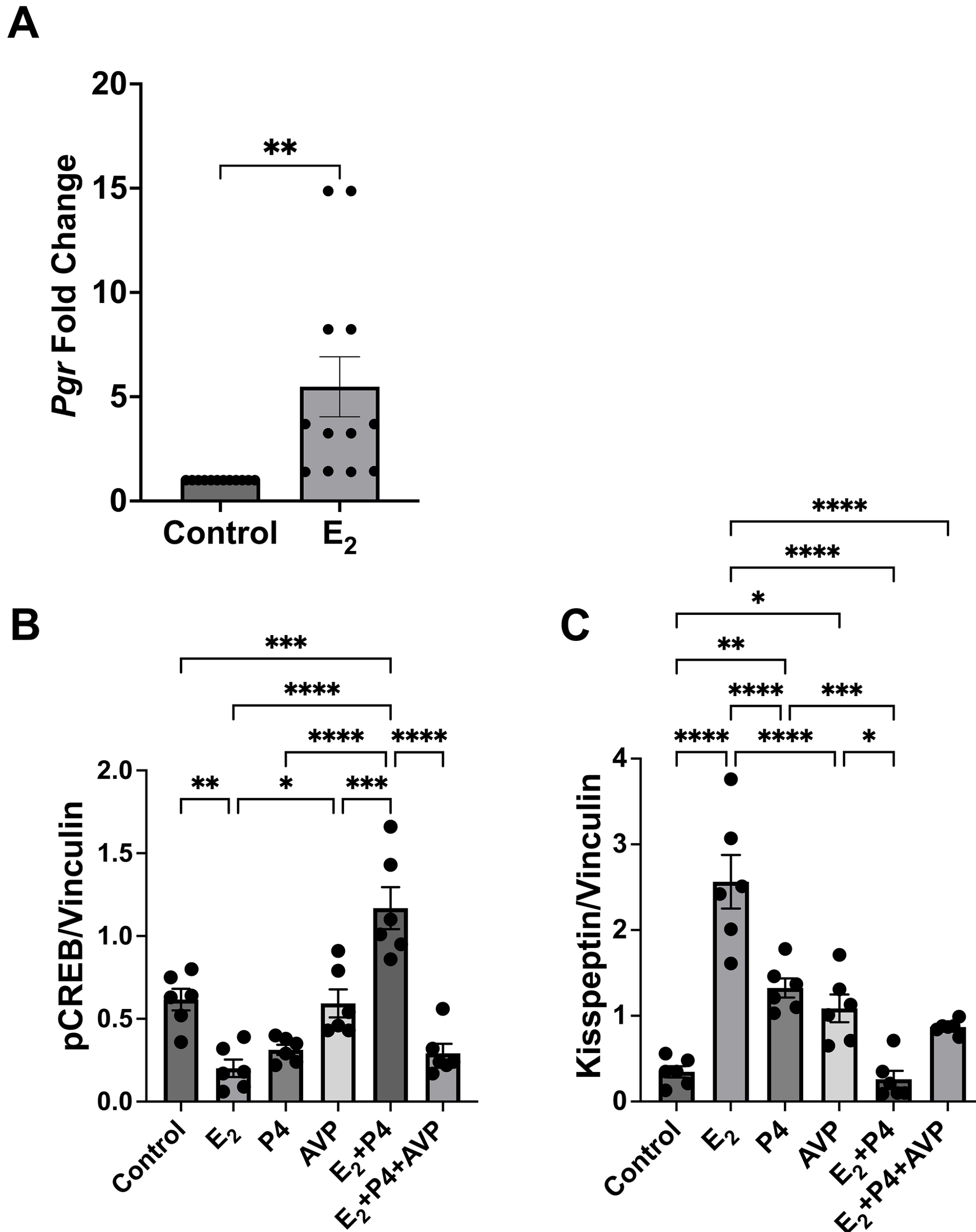


Figure 1. Regulation of kisspeptin expression through modulating CREB activity. **A.** Estradiol stimulates progesterone receptor (*Pgr*) mRNA expression. **B.** CREB phosphorylation is reduced in the presence of estradiol and is increased by progesterone stimulation following estradiol pretreatment. **C.** Estradiol, progesterone, and vasopressin stimulate kisspeptin peptide expression. Data are represented as mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, and ****, $P < 0.0001$.

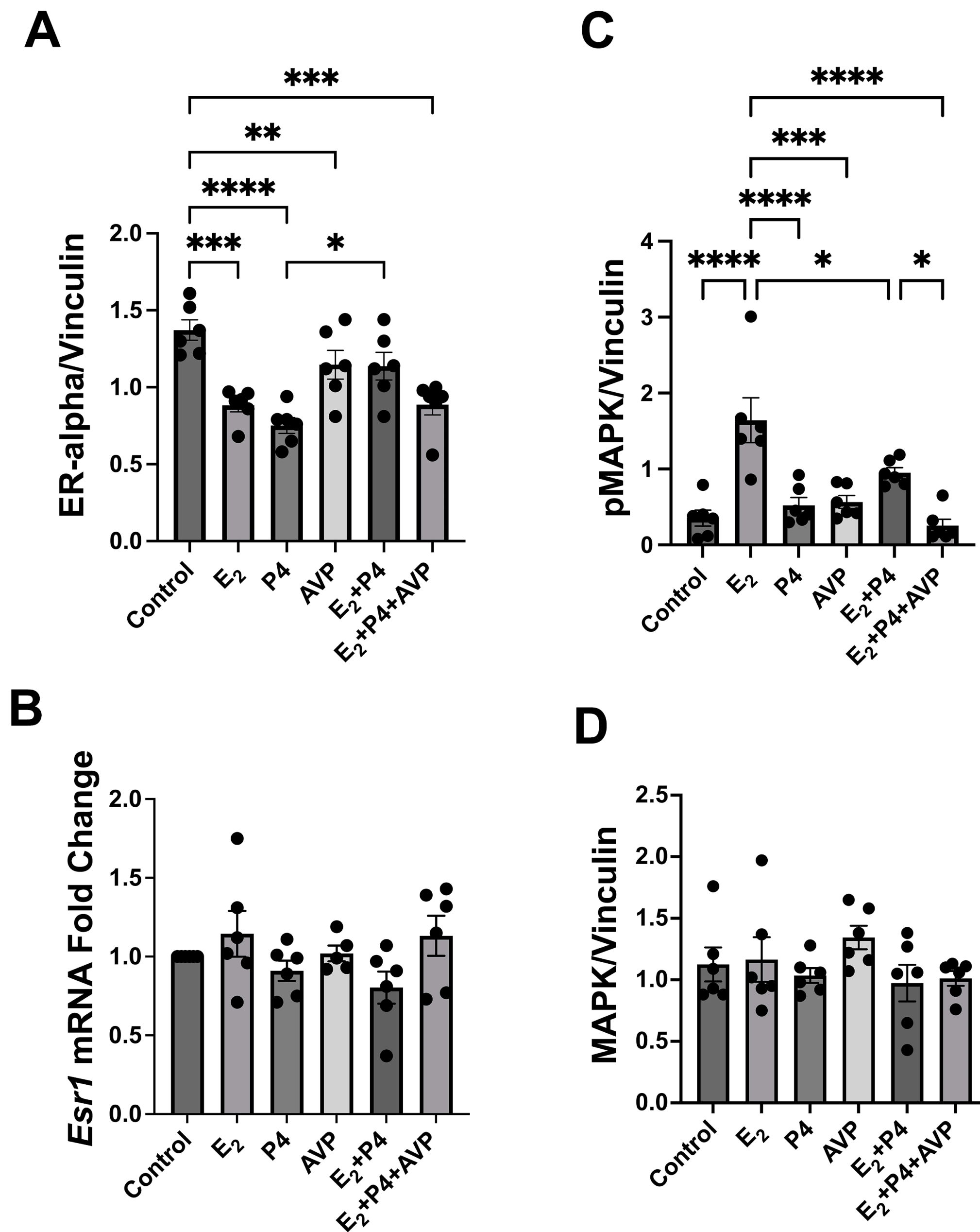


Figure 2. Signaling pathways involved in mediating kisspeptin peptide expression and CREB phosphorylation. **A.** Estrogen, progesterone, and vasopressin stimulation reduce estrogen receptor-α protein expression. **B.** Estrogen receptor-α (*Esr1*) mRNA expression is not impacted by estradiol, progesterone, or vasopressin treatments or co-treatment. **C.** MAPK activity is induced in ARC kisspeptin cells following estradiol treatment. **D.** Mitogen expression in ARC kisspeptin cells is not impacted by estradiol, progesterone, or vasopressin treatments or co-treatment. Data are represented as mean ± SEM. Data are represented as mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, and ****, $P < 0.0001$.

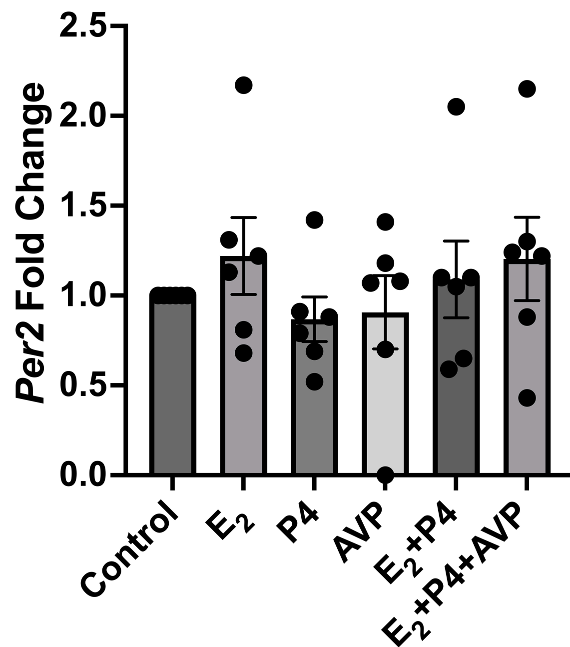


Figure 3. The circadian clock gene, *Period 2*, mRNA expression in ARC kisspeptin cells is not impacted by estradiol, progesterone, or vasopressin treatments or co-treatments. Data are represented as mean ± SEM. P = NS.

3.1 Introduction

The circadian control of ovulation is conserved across spontaneously-ovulating mammalian species (Boden et al., 2013; Cahill et al., 1998; Levine & Ramirez, 1982b; Mahoney et al., 2004; Moenter et al., 1992; Moline et al., 1981; Sarkar et al., 1976b) achieving temporal coordination of fertility and sexual behavior to maximize reproductive fitness (Blaustein et al., 1994; Egli et al., 2004; Kriegsfeld et al., 2002; McEwen et al., 1987; Mong & Pfaff, 2003). Women experiencing chronic circadian disruption exhibit irregular menstrual cycles (Lawson et al., 2011; Y. Wang et al., 2016), reduced fertility (Ahlborg et al., 1996; Fernandez et al., 2016), and increased miscarriage rates (Fernandez et al., 2016; Lawson et al., 2012; Nurminen, 1998). Analogously, findings from rodent studies reveal that temporal homeostasis is required for normal reproductive function, as destruction of the circadian timing system impairs luteinizing hormone (LH) surge generation, ovulation, and subsequent fertility (Bahougne et al., 2020; Boden et al., 2010; Chu et al., 2013; Dolatshad et al., 2006; Kennaway et al., 2004; Liu et al., 2014b; Mereness et al., 2016; Miller et al., 2004; Ratajczak et al., 2009; Summa et al., 2012; Zheng et al., 2019).

Ovulation in females is temporally confined to the onset of the activity period in spontaneously ovulating mammals (Kerdelhue et al., 2002; Levine & Ramirez, 1982b; Mahoney et al., 2004) and is stimulated by a surge of the gonadotropin, LH, that is released into systemic circulation from the anterior pituitary gland in response to gonadotropin-releasing hormone (GnRH) stimulation (Gotlieb et al., 2018; Moeller et al., 2022). Prior to ovulation, ovarian follicle growth and maturation is stimulated by the gonadotropins, LH and follicle stimulating hormone (FSH) and, in turn, increasing amounts of estradiol (E2) secreted from the developing ovarian follicle maintain low gonadotropin levels low through E2-negative feedback (Caraty et al., 1989; Chongthammakun & Terasawa, 1993; Sarkar et al., 1976b; Sarkar & Fink, 1980). At the time of ovulation, however, when elevated E2 levels are combined with daily circadian signals, there is brief transition from E2-negative to E2-positive feedback stimulated by the suprachiasmatic nucleus (SCN) of the hypothalamus, resulting in the initiation of the preovulatory LH surge (Moeller et al., 2022; Simonneaux & Piet, 2018; Tonsfeldt, Mellon, et al., 2022). The negative and positive feedback effects of E2 on gonadotropin secretion are mediated by estrogen receptor (ER)-α (Wintermantel et al., 2006). Because GnRH neurons do not express ER-α (Herbison & Theodosis, 1992), the hypothalamic neuron population that integrates both circadian and sex steroid signals remained largely unknown until the discovery of kisspeptin (Kp) (de Roux et al., 2003a; Seminara et al., 2003a).

Kp signaling is essential for initiating the LH surge and normal reproductive function, as individuals with loss of function mutations to either the Kp (*Kiss1*) or to *Kiss1r* genes experience idiopathic hypogonadotropin hypogonadism (d'Anglemont de Tassigny et al., 2007; de Roux et al., 2003b; Lapatto et al., 2007; Seminara et al., 2003a). Neurons expressing Kp critical to reproductive function are located in the rodent arcuate (ARC) and anteroventral periventricular nuclei (AVPV) of the hypothalamus, with ~90% of all Kp neurons expressing ER-α (J. T. Smith, Cunningham, et al., 2005). Whereas ARC Kp neurons mediate GnRH pulsatility, basal LH levels, and E2-negative feedback (Goodman

& Lehman, 2012; Herbison, 2018; Lehman, Merkley, et al., 2010; Millar et al., 2010; Yip et al., 2015), AVPV Kp cells positively drive LH surge initiation and are the locus for E2-positive feedback (Moeller et al., 2022; Piet, 2023). Early studies established that AVPV Kp cells have a direct stimulatory role in LH surge generation with these cells projecting directly to GnRH neurons expressing the cognate receptor for Kp (Clarkson & Herbison, 2006; Williams et al., 2011; Yip et al., 2015), GPR54 (also called *Kiss1r*). This population of Kp cells exhibits peak activation concomitant with the LH surge (Williams et al., 2011), and Kp infusion stimulates GnRH cell activation and LH release (Han et al., 2005). Although E2 stimulates AVPV *Kiss1* expression (Mittelman-Smith et al., 2015a; J. T. Smith, Cunningham, et al., 2005) and E2-ER-α-mediated signals are critical for LH surge generation (Wintermantel et al., 2006), the exact role that E2 signaling has in conferring timed changes in AVPV Kp cell physiology for LH surge generation is unknown.

The induction of the LH surge also requires neuroprogesterone (neuroP4) signals synthesized and secreted from hypothalamic astrocytes, as blockade of neuroP4 synthesis on the morning of proestrous in rats prevents the LH surge (Chaban et al., 2007; P. E. Micevych et al., 2003; P. Micevych & Sinchak, 2008). As E2 concentrations rise over the cycle from ovarian follicle development, E2 not only stimulates hypothalamic neuroP4 synthesis but also functions to increase AVPV Kp cell sensitivity to neuroP4 by stimulating AVPV Kp cell *Pgr* expression (Mittelman-Smith et al., 2015a, 2018; J. Zhang et al., 2014a). Kp cells are a critical locus for PGR signaling in LH surge induction, as the LH surge is disrupted in mice with Kp cell specific genetic deletion of *Pgr* (Gal et al., 2016; Stephens et al., 2015), and rescued upon reinstatement of *Pgr* expression in AVPV Kp cells (Mohr, Esparza, et al., 2021). Although PGR expression is observed in the nucleus and plasma membrane of AVPV Kp cells (Mittelman-Smith et al., 2015a), membrane-initiated signaling involving Src and mitogen-activated protein kinase (MAPK) is suggested to augment AVPV Kp cell activity. This proposed pathway is based on the observation that neuroP4 treatment results in increased intracellular calcium (Mittelman-Smith et al., 2015a), increases *Kiss1* gene expression (Mittelman-Smith et al., 2018), and stimulates Kp peptide release (Mittelman-Smith et al., 2018) in AVPV Kp cells pretreated with E2 via PGR-Src mediated increases in MAPK activity (Chuon et al., 2021a; Mittelman-Smith et al., 2018). The observation that infusion of the Src inhibitor, PP2, into the AVPV of rats on the morning of proestrous prevents LH surge initiation (Chuon et al., 2021a), suggests that timed stimulation of AVPV Kp cells with neuroP4 is needed, however it is unknown whether the production of, or sensitivity to neuroP4, exhibits temporally confined windows of sensitivity.

Although E2 and neuroP4 signals are required for AVPV Kp cells initiation of the LH surge, these signals, alone or in concert, are not sufficient to stimulate the LH surge. Instead, I hypothesize that the LH surge is triggered by a daily arginine vasopressin (AVP) signal from the suprachiasmatic nucleus (SCN) of the hypothalamus, the master circadian pacemaker, to AVPV Kp cells that express the AVP receptor, *V1a*, when threshold levels of E2 and neuroP4 signals converge at AVPV Kp cells (Williams et al., 2011). In rodents SCN AVP afferent projections to AVPV Kp neurons stimulate the LH surge (Vida et al., 2010; Williams et al., 2011), AVP is maximally released at the time of the LH surge (Francl et al., 2010; Kalsbeek et al., 1995b; Schwartz et al., 1983), V1A antagonists attenuate LH surge amplitude (Funabashi et al., 1999; Palm et al., 1999), and AVP stimulation increases AVPV Kp cell activity (Piet, de Croft, et al., 2015; Piet, Fraissenon, et al., 2015).

Although AVPV Kp neurons respond indiscriminately to AVP stimulation regardless of hormone status and time of day (Williams et al., 2011), the ability for AVP to stimulate AVPV Kp cells to trigger an LH surge is temporally confined (Kerdelhue et al., 2002; Levine & Ramirez, 1982b; Mahoney et al., 2004), suggesting that time-dependent responsiveness of AVPV Kp cells to stimulate the LH surge is organized by the molecular circadian clock. The molecular clock is a ubiquitous property throughout cells in the brain and periphery (Miller et al., 2007; Panda et al., 2002), including Kp cells (Chassard et al., 2015; Jacobs et al., 2016). An intact molecular clock is needed for normal reproductive function systemically and locally in AVPV Kp cells, as women with a single nucleotide polymorphism in the clock gene, *Bmal1,* have disrupted fertility (Kovanen et al., 2010) and rodent studies demonstrated global (Chu et al., 2013; Dolatshad et al., 2006; Khan & Kauffman, 2012; Miller et al., 2004) and Kp cell specific (Bittman, 2019; Tonsfeldt, Mellon, et al., 2022) impairments to the molecular clock in mice lead to disruptions to the LH surge generation and overall reproductive function.

Because the ability for AVPV Kp cells to stimulate the LH surge following timed, daily SCN-derived AVP stimulation requires both E2 and neuroP4 priming and is temporally restricted, we sought to determine how each of these signals acts individually and synergistically on an immortalized, serum-synchronized AVPV Kp cell line to organize timed changes in physiology. Furthermore, we sought to determine how E2, neuroP4, and AVP signaling in AVPV Kp cells impacts molecular clock timing to coordinate timed changes in AVPV Kp cell physiology required for LH surge generation.

3.2 Materials and Methods

3.2.1 mHypoA-KISS/GFP-4 Cell Culture and Reagents

mHypoA-KISS/GFP-4 (CELLutions Biosystems, Burlington, Ontario, CLU508) are clonal, an immortalized *Kiss1*-expressing cell line originating from anteroventricular periventricular nucleus (AVPV) of adult, female Kiss-GFP transgenic mice generated by Dr. Robert Steiner (University of Washington, Seattle, WA). Immortalization was attained by a retroviral transfer of Simian virus-40 T-antigen and neuronal proliferation was induced by ciliary neurotrophic factor treatment (Treen et al., 2016). Monolayer mHypoA-KISS/GFP-4 cultures were maintained in humidified atmosphere at 37°C with 5% CO_2 and cultured in 75 cm^2 flasks containing normal growth medium that is comprised of high-glucose Dulbeco's Modified Eagles Medium (DMEM; Gibco, Carlsbad, CA, #11965118) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO, #F2442) and 1% penicillin-streptomycin (PS; Sigma Aldrich, St. Louis, MO, #P4333). Cells were passaged at 85-90% confluency and not used after 10 passages.

Serum-free, phenol red-free DMEM (FSDMEM; Hyclone, Logan, UT, #SH30284) supplemented with 1% PS was used following serum shock. (17ß)-Estra-1,3,5(10)-triene-3,17-diol (E2; Tocris Bioscience, Minneapolis, MN, #2824) was dissolved in absolute ethanol (ETOH) to obtain a stock concentration of 10mM and stored at -20°C. Stock E2 was diluted with ETOH to 100 nM and subsequently diluted to final concentration of 100 pM with SFDMEM, as described previously (Jacobs et al., 2016). Progesterone-water soluble (P4; Sigma Aldrich, St. Louis, MO #P7556) was reconstituted in water to yield a 1mM stock concentration and was subsequently stored at -20°C prior to studies. For experiments, stock P4 was diluted in SFDEMEM supplemented with 100 pM E2 or vehicle

to a final concentration of 1nM. [Arg^8]-Vasopressin acetate salt (AVP; Sigma Aldrich, St. Louis, MO #V9879) was dissolved in water for a 100 μM stock concentration and stored at -20°C prior to studies. For dose response and time course experiments, AVP was subsequently diluted with SFDMEM to a final concentration of 0.5 nM, 1 nM, and 5 nM, with water as the vehicle control.

3.2.2 Serum Shock, Steroid, and Peptide Treatments

For all experiments, mHypoA-KISS/GFP-4 cells were seeded at a density of 400,000 cells per well in a 6-well plate and incubated overnight in normal growth medium to allow for cells to attach. For dose response experiments, medium was then changed to FSDMEM for 24 h, and cells were next transferred into FSDMEM supplemented with 100 pM E2 or vehicle and incubated for 24 or 48 h prior to sample collection. Next, 4 h prior to AVP stimulation, media was refreshed to remove accumulated secreted kisspeptin resulting from baseline release and 1 nM P4 or vehicle was added to SFDMEM supplemented with 100 pM E2 or vehicle. Treatment with 1 nM P4 has been previously well established (Mittelman-Smith et al., 2015a, 2018). At each time point, AVP or vehicle was added to experimental wells and incubated for 45 mins. After incubation, media was collected and pooled from wells of corresponding treatment conditions and cells were harvested for either protein or RNA expression analysis.

For time course experiments, cells were serum shocked with DMEM supplemented with 50% FBS and 1% PS for 2h to synchronize oscillations in core circadian clock genes (Balsalobre et al., 1998). Next, media was changed to FSDMEM or FSDMEM supplemented with 100 pM E2 and cells were incubated for 12h for clear rhythmicity in core circadian clock genes to be established. Four hours prior to AVP stimulation, 1 nM P4 or vehicle was added to SFDMEM and at the time of AVP stimulation, cells were stimulated with 50 nM or vehicle for 45 mins. After incubation, media was collected and pooled from wells of corresponding treatment conditions and cells were harvested for either protein or RNA expression analysis. At each time point, treated samples were compared with untreated controls from the 0h time point for determination of fold change in expression.

3.2.3 Quantitative RT-PCR (qRCR)

Following treatment, stimulated cells and untreated controls were harvested and total mRNA was purified. Total mRNA from treated and untreated wells was isolated using the PureLink RNA Mini Kit (Ambion, Austin, TX, #12183025) with and on-column DNase I (Ambion, Austin, TX, #12185010) digestion according to manufacturers' protocols. Subsequently, total RNA concentration and purity were determined using a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, #ND-2000C). cDNA was synthesized from 1 μg of total mRNA using the SensiFAST cDNA Synthesis Kit (Bioline, Memphis, TN, #BIO-65054) and stored at -20°C. The reverse transcription was performed at 42°C for 20 min followed by a 5 min termination at 85°C. A total of 100 ng of cDNA template was amplified using SensiFAST SYBR No-ROX Kit (Bioline, Memphis, TN, #BIO98020). Samples were run in triplicate using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, #1855485) with an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 25 s for *V1a, Esr1, Per2, Bmal1,* and 40 cycles of 95°C for 15 s and 63°C for 25 s for *Pgr*. Primers were designed

using PrimerBLAST (National Library of Medicine, Bethesda, MD) and aimed to span intro-exon junctions when possible to control for genomic DNA contamination. Efficiency for each primer pair was determined by a standard curve using 3-fold serial dilutions of cDNA, with values between 95.4-111.5% for each primer pair. Specificity of each primer pair was determined by non-template and no reverse transcriptase controls, a single-peak melt curve analysis, and gel electrophoresis of amplicons. Primer sequences, amplicon size, and annealing temperature are listed in **Table 1**. The relative expression levels for each gene of interest were determined using the $2^{-\Delta\Delta Ct}$ method. Gene expression was normalized to *Tbp*, the gene coding for TATA-box binding protein.

3.2.4 Western Blotting

At the time of protein harvest, cells were placed on ice, washed twice with cold Dulbecco's phosphate buffered saline (PBS; Sigma Aldrich, St. Louis, MO, D8537), and lysed in radioimmunoprecipitation (RIPA) assay buffer (Cell Signaling Technology, Danvers, MA, #9806) supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, #5872). Lysates were centrifuged at 14,000 rpm for 15 min at 4°C, with the supernatant collected and stored at -80°C. Protein concentration was determined with the Pierce Rapid Gold BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, #A53226), with 25 µg of protein per sample resolved on a 4-20% SDS polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, #4568093) and electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, #1620175). Membranes were blocked for 1 h at room temperature with 5.0% nonfat milk or bovine serum albumin (BSA) (wt/vol) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and then incubated overnight with the primary antibody at 4°C with gentle rocking. Primary antibodies with associated dilutions used are listed in **Table 2**. Primary antibodies were diluted in either 2.0% BSA or 2.0% non-fat milk in 0.1% TBS-T per manufacturers' recommendations. Membranes were then washed 3 times for 10 min with TBS-T prior to incubation with species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (diluted in 2.0% BSA or 2% non-fat milk in TBS-T) sourced from EMD Millipore (Burlington, MA) for 2 h at room temperature. Blots were washed three times with 0.1% TBS-T for 10 min and proteins of interest were visualized on a ChemiDocXRS+ (Bio-Rad Laboratories, Hercules, CA, 1708265) using SuperSignal West Femto or Atto chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, #34095 or #A38556, respectively) per the manufacturer's instructions. Membranes were stripped with Re-Blot Plus Mild Antibody Stripping Solution (EMD Millipore, Burlington, MA, #2502) according to manufacturer's instructions. This process of primary and secondary antibody incubation was repeated for the housekeeping gene, vinculin. Densitometric analysis of images were performed using Image Lab 5.2.1 (Bio-Rad, Hercules, CA, #1709690), with the optical density for both signal and background calculated by the software. Total signal intensity was then calculated by subtracting the pixel density from that of the background to that of the protein of interest. The final signal was calculated by dividing the corrected signal for the protein of interest by the corrected signal for the housekeeping gene.

3.2.5 Kisspeptin Enzyme-Linked Immunosorbent Assay (ELISA)

Media was pooled from corresponding wells of the same treatment (two wells in total) designated for gene and peptide/protein expression and immediately stored at -

80°C at the time of sample collection. Samples containing 2 mL of media were desiccated overnight at 30°C using a Vacufuge (Eppendorf, Enfield, CT, #022820168) and reconstituted in 225 µL of PBS. Quantification of released Kp was determined using a mouse enzyme-linked immunosorbent assay (ELISA; Biomatik, Wilmington, DE, #EKU05505) per manufactures instructions. Samples and standards were run in duplicate.

3.2.6 Statistics

The impact of treatment was analyzed using t-tests or one- or two-way analyses of variance analysis (ANOVA) with Tukey's post hoc tests where appropriate. All statistical analyses were performed using PRISM (GraphPad Software Inc., San Diego, CA). Circadian analysis for clock gene expression was conducted using NiteCap (Brooks et al., 2022). All data are presented as the mean ± SEM. $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Estrogen Dose Response

Because previous studies indicated E2 to be stimulatory to Kp expression in AVPV cells (Mittelman-Smith et al., 2015a; J. T. Smith, Cunningham, et al., 2005; Treen et al., 2016), Kp expression was measured following treatment with increasing doses of E2 to determine the optimal dose of E2 for culture experiments. Although neither 1 pM E2 (0.426 ± 0.06091; p=NS), 25 pM E2 (0.2127 ± 0.02267; p=NS), 100 pM E2 (0.4043 ± 0.1157; p=NS), 1 nM E2 (0.5737 ± 0.1158; p=NS), 10 nM E2 (0.4191 ± 0.06072; p=NS) or 100 nM E2 (0.4074 ± 0.05978; p=NS) were statistically different from control (0.3695 ± 0.03055), 1 nM E2 was found to be most stimulatory to Kp expression (**Figure 1A**). Additional studies also suggested E2 modulates *Pgr* (Mittelman-Smith et al., 2015a, 2018; Mohr, Wong, et al., 2021) therefore, Pgr expression was assessed following increasing doses of E2 treatment. E2 was found to potently stimulate *Pgr* expression, with 100 pM E2 (4.885 ± 1.185 fold change; $p<0.05$), 1 nM E2 (4.908 ± 0.5140 fold change; $p<0.05$), and 100 nM E2 (4.467 ± 0.7051 fold change; $p<0.05$) was found to significantly upregulate *Pgr* expression relative to control (**Figure1B**. Moreover, ER-α signaling is imperative to Kp cell ability to stimulate the LH surge, therefore ER-α (*Esr1*) gene expression was measured following increasing doses of E2. 1 pM (0.8831 ± 0.02608 fold change; $p<0.05$), 25 pM (0.8247 ± 0.02874 fold change; $p<0.05$), 100 pM (0.8219 ± 0.01990 fold change; $p<0.05$), 1nM (0.8140 ± 0.03378 fold change; $p<0.05$), 10 nM (0.6533 ± 0.01733 fold change; $p<0.05$), and 100 nM (0.7011 ± 0.01588 fold change; $p<0.05$) of E2 were found to downregulate *Esr1* expression relative to control, with 10 nM being most inhibitory to *Esr1* expression (**Figure 1C**). Furthermore, because AVP receptor expression was suggested to be mediated by E2 (Smarr et al., 2013), *V1a* expression was also measured following increasing doses of E2 treatment. *V1a* expression was found to significantly decrease ($p<0.05$) following increasing E2 treatment, as 25 pM (0.7255 ± 0.03308 fold change; $p<0.05$), 10 nM (0.5988 ± 0.04064 fold change; $p<0.05$), and 100nM (0.5468 ± 0.1197 fold change; $p<0.05$) relative to control (**Figure 1D**). Additionally, 10 nM E2 treatment significantly decreased *V1a* expression relative to 1 pM E2 (0.8482 ± 0.03915

fold change; $p<0.05$). Lastly, because E2 has been suggested to modulate circadian clock function in AVPV Kp cells in rats, the circadian clock gene, *Period (Per) 2*, was assessed. Increasing E2 treatments were not found to affect *Per2* expression (p=NS) (**Figure 1E**).

3.3.2 Vasopressin Dose Response

The LH surge has been indicated to be triggered by a daily AVP signal from the SCN to AVPV Kp cells (Palm et al., 1999; Williams et al., 2011). To determine the optimal dose of AVP treatment for stimulating maximum Kp release, cells were stimulated with increasing doses of AVP following 24 h pretreatment with 100 pM E2. Cells were pretreated with E2 to stimulate Kp production. Indicating a stimulatory effect on Kp release, increasing doses of AVP were found to significantly decrease cellular Kp content. Whereas 100 pM E2 (0.8902 ± 0.09977; p=NS) treatment resulted in a non-significant increase in cellular Kp content relative to control (0.6969 ± 0.03997), 10 nM (0.4857 ± 0.07930; $p<0.05$), 50 nM (0.4242 ± 0.03156; $p<0.05$), and 100 nM (0.4953 ± 0.02279; $p<0.05$) AVP stimulation following 24 h 100 pM E2 pretreatment resulted in significant decreases in cellular Kp content relative to 100 pM E2 treatment. Furthermore, 50 nM AVP stimulation following 100 pM E2 pretreatment resulted in significant decreases in cellular Kp content relative to 0.5 nM AVP (0.8727 ± 0.06250; $p<0.05$), 1 nM AVP (0.7281 ± 0.03859; $p<0.05$), and 5 nM AVP (0.7568 ± 0.05146; $p<0.05$) and 100 nM AVP treatment significantly reduced cellular Kp content relative to 0.5 nM AVP (**Figure 2**).

3.3.3 Progesterone Dose Response

Because P4 has been shown to regulate Kp gene (*Kiss1*) expression, receptors for key signaling molecules were assessed following increasing doses of P4 treatment (Mittelman-Smith et al., 2018). To determine whether P4 treatment altered sensitivity to E2 signaling, *Esr1* expression was measured following increasing doses of P4 treatment. No significant (p=NS) impact of P4 treatment was observed on *Esr1* following increasing doses of P4 treatment (**Figure 3A**). E2 treatment (3.410 ± 0.05033 fold change; $p<0.05$) was found to significantly upregulate *Pgr* expression relative to control and to 10 pM (0.7667 ± 0.05175 fold change; $p<0.05$), 100 pM (0.8500 ± 0.1453 fold change; $p<0.05$), 1 nM (0.6700 ± 0.05508 fold change; $p<0.05$), 10 nM (0.6633 ± 0.1041 fold change; $p<0.05$), or 100 nM P4-treated (0.4767 ± 0.04380 fold change; $p<0.05$), however, increasing doses of P4 treatment do not impact *Pgr* expression relative to control except in 100 nM P4-treated cells. 100 nM P4 treatment significantly decreased *Pgr* expression relative to control and 100 pM P4-treated cells ($p<0.05$) (**Figure 3B**). *V1a* expression, on the other hand, was indicated to be positively regulated by P4, with increasing doses stimulating *V1a* expression. Whereas 100 pM E2 treatment led to non-significant decreases in *V1a* expression (0.7900 ± 0.05859 fold change; p= NS) relative to control, 10 pM (1.447 ± 0.1162 fold change; $p<0.05$), 100 pM (1.357 ± 0.168 fold change; $p<0.05$), 1 nM (1.383 ± 0.09387 fold change; $p<0.05$), and 10 nM P4 (1.120 ± 0.1193 fold change; $p<0.05$) treatment significantly increased *V1a* expression relative to 100 pM E2-treated samples. Moreover, 10 nM pM P4 treatment significantly increased *V1a* expression relative to control (**Figure 3C**). Lastly, when examining the impact of increasing P4 treatment on *Per2* expression, there were no significant differences of treatment relative to control, however 10 nM P4 (1.443 ± 0.08686 fold change; $p<0.05$) treatment

significantly increased *Per2* expression relative to 100 pM P4 treatment (0.8900 ± 0.02517 fold change; p<0.05) (**Figure 3D**).

3.3.4 Impact of Estrogen, Progesterone, and Vasopressin Co-Treatment

Because the convergence of E2, P4, and AVP signals on AVPV Kp cells is required to stimulate the LH surge, the impact of the combined treatment was examined (Moeller et al., 2022). Whereas E2, P4, and AVP co-treatment did not have an impact on *Esr1* expression (p=NS) (**Figure 4A**), there was a significant stimulatory effect of co-treatment on *Pgr* expression. 50 nM AVP (1.080 ± 0.01528 fold change; p= NS) and 1 nM P4 (0.6700 ± 0.05508 fold change; p= NS) treatment did not affect *Pgr* expression relative to control, however treatment with 50 nM AVP (p<0.05), 1 nM P4 (p<0.05), 100 pM E2 (3.410 ± 0.05033 fold change; p<0.05) and 1 nM P4/50 nM AVP (2.883 ± 0.03844 fold change; p<0.05) co-treatment significantly increased *Pgr* expression following 24 h 100 pM E2 pretreatment relative to control, 50 nM AVP, and 1 nM P4 treated samples. Furthermore, co-treatment with 1 nM P4/50 nM AVP following 100 pM E2 pretreatment significantly decreased *Pgr* expression relative to 100 pM E2 and 50 nM AVP and 1 nM P4-treated samples following 100 pM E2 pretreatment (**Figure 4B**). Whereas no significant differences in *V1a* expression were observed for any treatment relative to control (1.00 ± 0.000), 1 nM P4 (1.383 ± 0.09387 fold change; p= NS) treatment significantly increased *V1a* expression relative to 100 pM E2 (0.7900 ± 0.05859 fold change; p<0.05), 50 nm AVP (1.057 ± 0.1040 fold change; p<0.05), and 50 nM AVP, 1 nM P4, and 1 nM P4/50 nM AVP (0.6567 ± 0.05364 fold change; p<0.05) co-treatment following 24 h 100 pM E2 pretreatment (**Figure 4C**). Lastly, clock gene function appears to be impacted by co-treatment with E2, P4, and AVP, with 1 nM P4/50 nM AVP co-treatment (1.830 ± 0.06351 fold change; p<0.05) following 24 h 100 pM E2 pretreatment significantly increasing *Per2* expression relative to control (1.000 ± 0.000), 50 nM AVP (1.150 ± 0.02000 fold change; p<0.05), 100 pM E2 (1.287 ± 0.1499 fold change; p<0.05), 1 nM P4 (1.157 ± 0.03180 fold change; p<0.05), and 50 nM AVP treatment following 24 h 100 pM E2 pretreatment. Furthermore, 50 nM AVP treatment and 1 nM P4 treatment following 24 h 100 pM E2 pretreatment significantly upregulated *Per2* expression relative to control (**Figure 4D**).

Next, because all of these are molecules are stimulatory to AVPV Kp cell ability to stimulate the LH surge, the effects of each individual treatment and co-treatment were assessed in stimulating Kp release. Co-treatment with 1nM P4/50 nM AVP following 24 h 100 pM E2 (47.20 ± 1.838 pg/ml; p<0.05) pretreatment significantly increased Kp release relative to control (36.08 ± 3.094 pg/ml), whereas no other treatment or co-treatment had a significant impact on Kp release (**Figure 4E**).

Because *Per2* expression was affected by E2, P4, and AVP co-treatment, circadian clock function was assessed using serum shocked mHypoA-KISS/GFP-4 cells. *Per2* expression was rhythmic across all treatments, however the timing of the peak for the rhythm and period length was affected by treatment. The peak of *Per2* expression was observed to occur at 4 h for control and E2-treated samples, whereas the peak was advanced following AVP, P4, and co-treatment with E2, P4, and AVP. The peak of the rhythm was advanced to 2 h following P4 and E2, P4, and AVP co-treatment, whereas the rhythm peaked at 0 h following stimulation with AVP. The length of the *Per2* rhythm was observed to be 24 h in control and E2-treated samples, whereas the length of the

period for the *Per2* rhythm was extended to 28 h following P4, AVP, and E2, P4, and AVP co-treatment (**Figure 4F**).

To further assess circadian clock function in AVPV Kp cells, *Bmal1* expression was measured. Across all treatments, *Bmal1* expression was found to be rhythmic by co-signor analysis, however the period length and the timing for peak *Bmal1* expression were impacted by treatment. The period for rhythmic *Bmal1* expression was found to be 28 h in control, E2 and E2, P4, and AVP co-treated samples, whereas AVP or P4 treatment shortened the period length 24 h. Lastly, the timing for the peak of *Bmal1* expression was also impacted by treatment, with peak *Bmal1* expression observed to occur at 16 h in control, AVP, P4, and E2, P4, and AVP-co-treated groups and at 14 h following E2 treatment (**Figure 4G**).

3.3.5 Time-Dependent Induction of Key Signaling Pathways and Gene Expression Changes in Response to Steroidogenic and Neuropeptidergic Signals Across 48 h

For visibility, all time course data are presented as both line and bar graphs (**Figure 5**). Bar graphs indicate group differences at each time point. To examine whether cell-synchronized circadian rhythms persist in culture conditions, expression of two, core clock genes, *Per2* and *Bmal1,* was examined over 48 h. *Per2* expression showed a main effect of time ($F (3.707, 36.76) = 361.4$, $P<0.05$) and group ($F (4, 10) = 4.502$, $P<0.05$) as well as an interaction between time and treatment ($F (48, 119) = 5.249$, $P<0.05$) . AVP reduced *Per2* expression at 32 h relative to controls and P4-treated cells ($P<0.05$ in each case) (**Figure 5A**). *Bmal1* expression uncovered a main effect of time ($F (4.098, 40.64) = 290.8$, $P<0.05$) along with an interaction between time and treatment ($F (48, 119) = 3.639$, $P<0.05$). At 20 h, P4, AVP, and E2/P4/AVP co-treatment reduced *Bmal1* expression ($P<0.05$ in each case). Similar findings were seen at 44 and 48 h, although E2 suppression of *Bmal1* reached significance at 48 h ($P<0.05$) (**Figure 5B**).

Next, expression of key receptors impacted in LH surge generation were examined. *Pgr* expression exhibited a main effect of time ($F (4.538, 45.00) = 28.27$, $P<0.05$) and group ($F (4, 10) = 10.07$, $P<0.05$) and an interaction between time and treatment ($F (48, 119) = 2.854$, $P<0.05$). E2 and P4 were generally suppressive to *Pgr* expression as E2 treatment suppressed *Pgr* expression at 0 and 44 h relative to controls and P4 suppressed expression at 0 and 4 h relative to controls ($P<0.05$ in each case). Likewise, and E2/P4/AVP co-treatment reduced *Pgr* expression at 0, 12, and 44 h ($P<0.05$ in each case) (**Figure 5C**). For *Esr1* expression there was a significant main effect of time ($F (5.566, 55.19) = 25.54$, $P<0.05$) and an interaction between time and treatment ($F (48, 119) = 5.308$, $P<0.05$). At 24 h, E2/P4/AVP co-treatment increased *Esr1* relative to controls, while exhibiting a reduction at 28h ($P<0.05$ in each case). At 32 and 36 h, E2 and P4 were suppressive to *Esr1*, with P4 showing suppression at 48 h relative to E2-treated cells ($P<0.05$ in each case) (**Figure 5E**). For *V1a* expression, a main effect of time ($F (3.925, 38.92) = 62.11$, $P<0.05$) and group ($F (4, 10) = 10.07$, $P<0.05$) as well as an interaction between time and treatment ($F (48, 119) = 2.854$, $P<0.05$) was observed. In contrast to *Pgr* expression, E2 was stimulatory to *Esr1* at some time points, with E2 stimulating *Esr1* expression at 12 and 36 h relative to controls and AVP-treated cells ($P<0.05$ in each case) (**Figure 5D**).

3.3.6 Time-Dependent Induction of MAPK Signaling Across 48 h

Lastly, because Src-mediated increases in MAPK activity have been observed in response to P4 signaling in AVPV Kp cells for LH surge initiation (Chuon et al., 2021b; Mittelman-Smith et al., 2018), timed changes in MAPK phosphorylation were measured as a ratio of phosphorylated MAPK to total MAPK to account for any underlying changes in total MAPK expression (**Figure 6**). There was a main effect of time ($F (2.366, 25.64) = 12.21$, $P<0.05$) on pMAPK/MAPK content. Relative to control, AVP and P4 treatment at 40 h was found to decrease pMAPK/MAPK activity. Although the variability is high across samples, MAPK activity is generally increased across all treatments and co-treatments relative to other time points.

3.4 Discussion

Generation of the preovulatory LH surge required for ovulation requires the convergence of E2 (J. T. Smith, Cunningham, et al., 2005), centrally-produced P4 (Mohr, Esparza, et al., 2021; Stephens et al., 2015), and AVP (Palm et al., 1999; Williams et al., 2011) onto AVPV Kp neurons. The present study aimed to determine how the sensitivity to each of these signals is regulated by endogenous circadian timing in AVPV Kp cells. Key findings from the present study include: 1) increasing concentrations of E2 downregulate *Esr1* expression in AVPV Kp cells, 2) *Pgr* expression is potently stimulated by increasing doses of E2, 3) *V1a* expression is suppressed by E2 and stimulated by increasing doses of P4, 4) neither E2 nor P4 stimulates *Per2* expression, but *Per2* expression was increased following co-treatment with E2, P4, and AVP, 5) increasing doses of AVP lead to decreases in Kp cellular content, 6) co-treatment with E2, P4, and AVP stimulated maximum Kp peptide release, and 7) despite having clear rhythms in clock gene expression over 48 h, AVPV Kp cells do not exhibit consistent time-dependent changes in responsiveness to upstream signaling molecules. These findings suggest that each of these key signaling molecules are integrated into AVPV Kp cells to orchestrate timing of responsiveness required for generation of the preovulatory LH surge.

Previous experiments using AVPV Kp cells established that *Esr1* expression is downregulated following increasing doses of E2 treatment (Treen et al., 2016), and results from the present study are consistent with this observation. Furthermore, *Esr1* expression was not impacted by either AVP or P4 treatment, suggesting that E2 alone is responsible for mediating AVPV Kp cell sensitivity to E2 signals required for stimulating the preovulatory LH surge. Because P4 signaling is required for stimulation of the LH surge, sensitivity to P4 was examined in in AVPV Kp cells. Consistent with findings from both *in vivo* (Göcz, Takács, et al., 2022; Mohr, Wong, et al., 2021) and *in vitro* (Mittelman-Smith et al., 2015a, 2018) experiments, increasing doses of E2 was found to potently stimulate *Pgr* expression in mHypoA-KISS/GFP-4 cells. However, *Pgr* expression was not found to be impacted by either AVP or P4 treatments.

Findings by our lab and others established that AVP acts to stimulate the LH surge (Palm et al., 1999; Williams et al., 2011). Findings from the experiments presented herein indicate AVPV Kp cells express the principal central receptor for AVP and are responsive to AVP stimulation, with increasing doses of AVP leading to significant decreases in cellular Kp content, suggesting increased Kp release. Provided AVP triggers Kp release to stimulate the LH surge, this observation is consistent with the notion that AVP acts as the trigger for the LH surge. Although the responsiveness of AVPV Kp cells to AVP was

suggested to be mediated by E2 (Smarr et al., 2013), findings from the study presented did not find *V1a* expression to be impacted by E2 treatment. Instead, *V1a* expression increased following P4 treatment. Thus, increasing concentrations of ovarian-derived E2 likely stimulate hypothalamic astrocytes to synthesize P4 of neural origin as previously shown (Sinchak et al., 2020b) and, in turn, P4 increases sensitivity to AVP signaling.

Generation of the LH surge requires intact circadian signaling in Kp cells, as destruction of the molecular clock leads to LH surge disruptions (Bittman, 2019; Tonsfeldt et al., 2019). Because E2, P4, and AVP all have the capacity to impact molecular clock function, *Per2* expression was assessed. Neither E2 or P4 were found to individually impact *Per2* expression. However, co-treatment with P4 and AVP following E2 pretreatment resulted in significant increases in *Per2* expression. These results suggest that the convergence of these three signals is required to appropriately set the time for the circadian molecular clock. When assessing how E2, P4, and AVP impact the clock function in serum synchronized AVPV Kp cells, all treatments resulted in rhythmic clock function. Results from the present study indicated E2, P4, and AVP co-treatment impacted both the timing for the period length and peak timing for *Per2* expression, with E2, P4, and AVP co-treatment found to increase the period length as well as advance the timing of the peak for *Per2* expression. Substantial alterations in *Per2, V1a, Esr1* in the 32-26 h time point followed by subsequent increases in pMAPK/MAPK, point to potential time-dependent sensitivity of AVPV Kp cells in response to signals initiating the LH surge.

Although E2 increases the firing rate of AVPV Kp cells, it was unknown whether increased activity led to increases in Kp release responsible for stimulating GnRH cells to release a bolus of GnRH required for stimulating the LH surge (Piet, de Croft, et al., 2015; Piet, Fraissenon, et al., 2015). To determine whether there are increases in Kp release following E2, P4, and AVP treatment or co-treatment with E2, P4, or AVP, Kp release was measured. Although each treatment was found to stimulate Kp release, only co-treatment with E2, P4, and AVP was found to maximally stimulate Kp release, consistent with the notion that the convergence of hormonal and SCN signaling is required for AVPV Kp-cell stimulation of ovulation.

Taken together, findings from the experiments presented herein indicate that the convergence of E2, P4, and AVP signals onto AVPV Kp cells is likely needed to appropriately stimulate appropriate receptor expression and subsequent sensitivity to the combination of these molecules. Furthermore, results suggest that each of these signals is integrated at the level of AVPV Kp to impact the timing of the molecular circadian clock to appropriately time the initiation of the surge. Lastly, results from the experiments presented herein suggest that the integration of the each of these signals is required to maximally stimulate Kp peptide release for typical LH surge generation.

3.5 Tables

Primer Pair Name	Primer Name	Primer Sequence	Amplicon Length (BP)
V1a	FWD	TCTTCATCGTCCAGATGTGGTC	90
	REV	CCAGTAACGCCGTGATCGT	
Esr1	FWD	ATGAAAGGCGGCATACGGAAAG	94
	REV	CACCCATTTCATTTCGGCCTTC	
Pgr	FWD	AGGTCTACCCGCCATACCTT	193
	REV	TTATGCTGCCCTTCCATTGC	
Per2	FWD	CCAAGCATCCAGCCCTGTTT	170
	REV	CGTTTGGTTTGCGCATGAAC	
Bmal1	FWD	GACCTACTCTCCGGTTCCCT	89
	REV	GCATATTCTAACTGGTAGTCAGTGG	
Tbp	FWD	TTGGCTAGGTTTCTGCGGTC	179
	REV	TGGAAGGCTGTTGTTCTGGT	

Table 1. Primers used for qPCR gene expression assays.

Peptide/Protein Target	Name of Antibody	Manufacturer, Catalog Number	Host Species; Monoclonal or Polyclonal	Dilution Used
KISS-1	Anti-KiSS-1 Antibody, clone 8H4.1	EMD Millipore, #MABC60	Mouse; Monoclonal	1:1000
phospho-MAPK	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb	Cell Signaling, #4370	Rabbit; Monoclonal	1:2000
Total MAPK	p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell Signaling, #4695	Rabbit; Monoclonal	1:1000
Estrogen Receptor-alpha	Estrogen Receptor alpha Monoclonal Antibody (1D5)	Invitrogen, #MA5-13191	Mouse; Monoclonal	1:1000
phospho-CREB	Phospho-CREB (Ser133) (1B6) Mouse mAb	Cell Signaling, #9196	Mouse; Monoclonal	1:1000
GAPDH	GAPDH (14C10) Rabbit mAb	Cell Signaling, #2118	Rabbit; Monoclonal	1:1000
Vinculin	Vinculin (E1E9V) XP Rabbit mAb	Cell Signaling, #13901	Rabbit; Monoclonal	1:1000

Table 2. Antibodies used for peptide/protein content analysis.

3.6 Figures

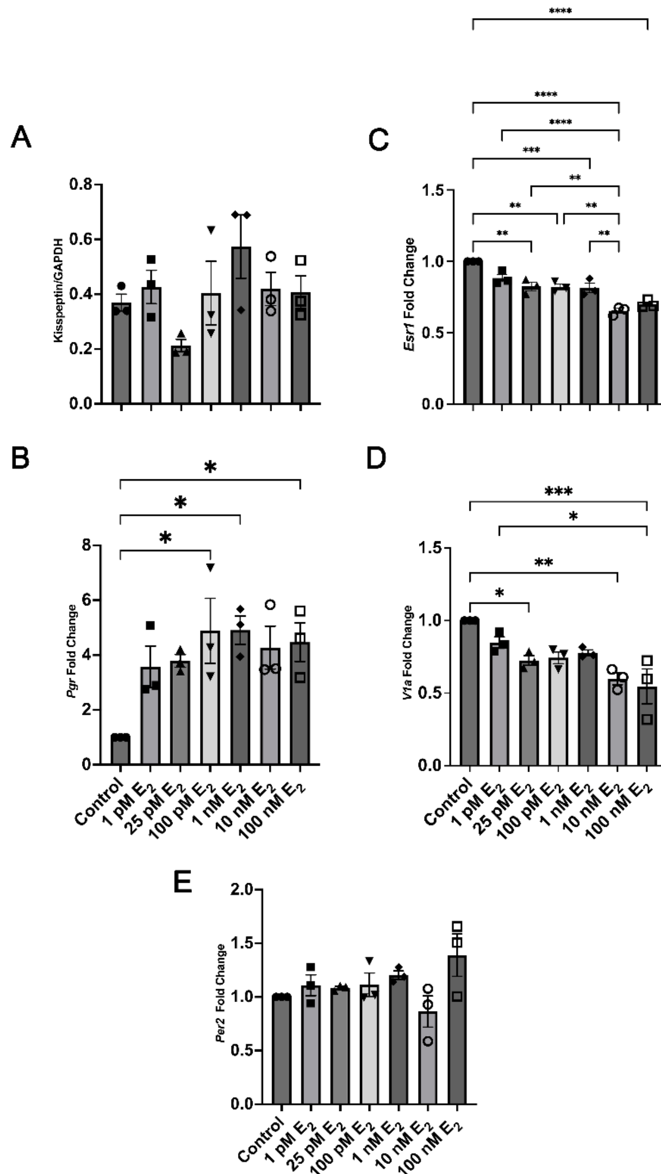


Figure 1. The effects of increasing doses of estradiol (E2) treatment on cellular kisspeptin peptide content and *Esr1*, *Pgr*, *V1a,* and *Per2* mRNA expression. **A.** Although not statistically significant, kisspeptin cellular peptide content is increased following 24 h treatment with 100 pM E2 **B.** Progesterone receptor (*Pgr*) mRNA expression is significantly increased following increasing doses of estradiol treatment. **C.** Estrogen receptor-α (*Esr1*) mRNA expression is decreased following increasing doses of 24 h E2 treatment. **D.** Vasopressin receptor (*V1a*) mRNA expression is decreased following increasing doses of E2 **E.** Expression of the clock gene, *Period 2* (*Per2*), is not impacted by increasing concentrations of E2 treatment. Data are represented as mean ± SEM. *, P < 0.05, **, P < 0.01, ***, P < 0.001, and ****, P < 0.0001.

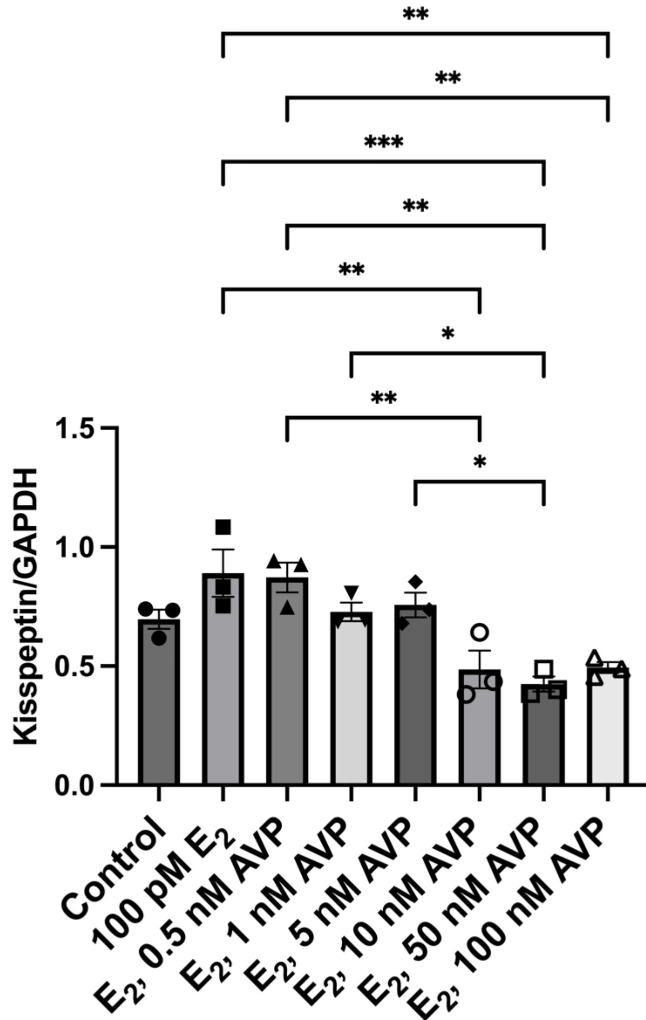


Figure 2. The impact of increasing doses of vasopressin treatment on kisspeptin cellular content. Cellular kisspeptin content was found to decrease following increasing doses of vasopressin treatment in the presence of 100 pM estradiol. Data are represented as mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.

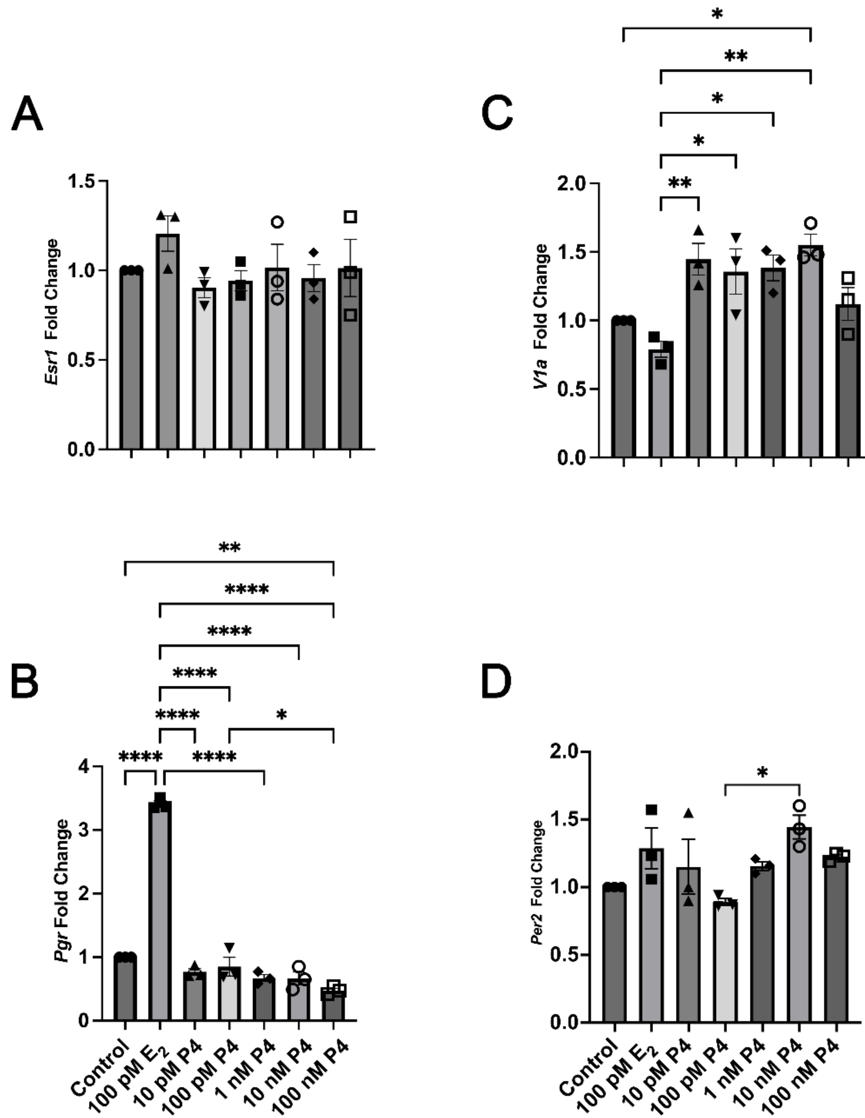


Figure 3. The impact of increasing doses of progesterone (P4) on *Esr1*, *Pgr*, *V1a*, and *Per2* mRNA expression. **A.** Estrogen receptor-α (*Esr1*) mRNA expression is not impacted by increasing concentrations of P4 stimulation. **B.** Progesterone receptor (*Pgr*) mRNA expression is increased following 24 h 100 pM estradiol treatment but is not impacted by exposure to increasing doses of P4. **C.** Vasopressin receptor (*V1a*) mRNA expression is increased following increasing doses of P4 stimulation. **D.** Expression of the core clock gene, *Period 2* (*Per2*) is increased following increasing doses of P4 treatment. Data are represented as mean ± SEM. *, P < 0.05, **, P < 0.01, ***, P < 0.001, and ****, P < 0.0001.

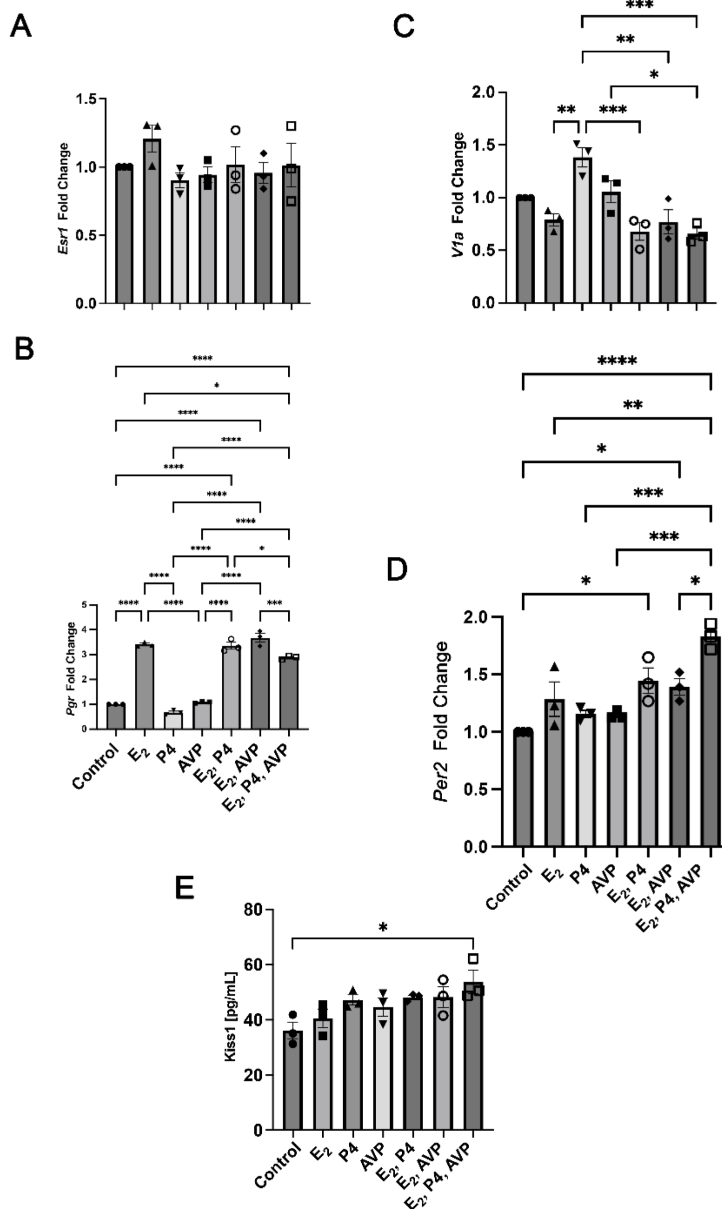


Figure 4. The impacts of estradiol (E2), progesterone (P4), and vasopressin (AVP) treatments and co-treatment on *Esr1, Pgr,* V1a, and *Per2* mRNA expression and *in vitro* kisspeptin release into culture media. **A.** Estrogen receptor-α (*Esr1*) mRNA expression is not impacted by E2, P4, or AVP treatment or co-treatment. **B.** Progesterone receptor (*Pgr*) mRNA expression is significantly increased following E2 treatment and co-treatment with either E2, P4 or E2, AVP or E2, P4, AVP. **C.** Vasopressin receptor (*V1a*) mRNA expression is increased by 1 nM P4 treatment but not impacted by any co-treatment. **D.** Expression of the core clock gene, *Per2*, is significantly increased following E2, P4 and E2, P4, and AVP co-treatments. **E.** Released kisspeptin peptide into culture media is significantly increased following E2, P4, and AVP co-treatment. Data are represented as mean $\pm$ SEM. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, and ****, $P < 0.0001$.

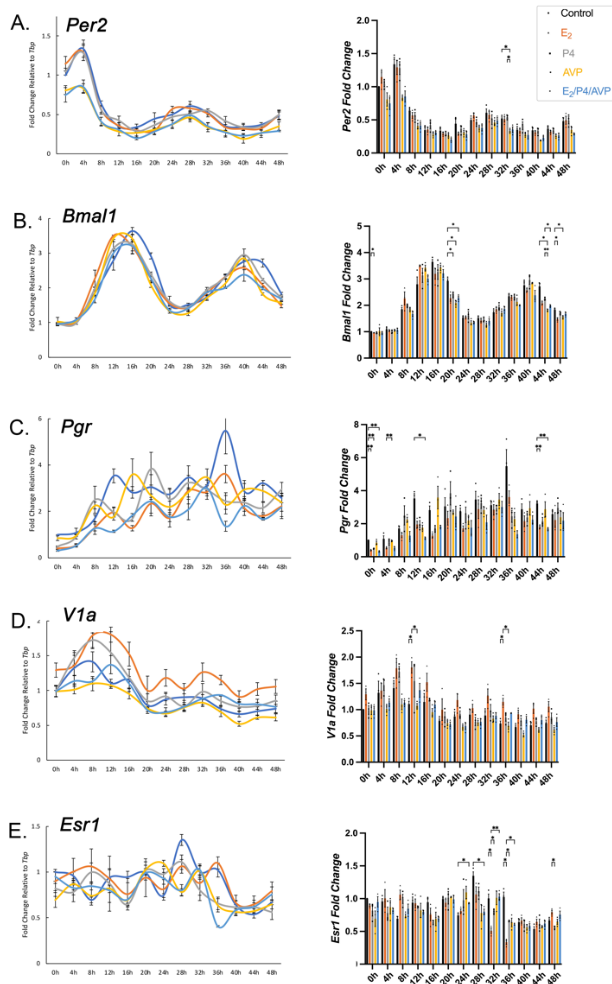


Figure 5. The time-dependent impacts of estradiol (E2), progesterone (P4), and vasopressin (AVP) treatment and co-treatment on *Per2*, *Bmal1*, *Pgr*, *V1a*, and *Esr1* mRNA expression over 48 h sampling period were measured. **A.** Treatment or co-treatment with E2, P4, and AVP over 48 h were found to have a main effect of time and treatment along with an interaction between time and treatment on the mRNA expression of the core clock gene, *Per2*. **B.** A main effect of time and an interaction between time and treatment were found to impact mRNA expression of the core clock gene, *Bmal1*, following E2, P4, and AVP treatments and co-treatment **C.** Although E2 and P4 treatments were generally suppressive to progesterone receptor (*Pgr*) mRNA expression, a main effect of time and an interaction between time and treatment were observed to significantly impact *Pgr* expression following E2 treatment. **D.** A main effect of time and an interaction between time and treatment were found to significantly impact vasopressin receptor (*V1a*) mRNA expression. **E.** A main effect of time and an interaction between time and treatment or co-treatment with E2, P4, or AVP were observed to significantly impact estrogen receptor-α (*Esr1*) mRNA expression. The same data set is represented as both a line and bar graph for ease of visibility. Data are represented as mean $\pm$ SEM. *, $P < 0.05$ and **, $P < 0.01$.

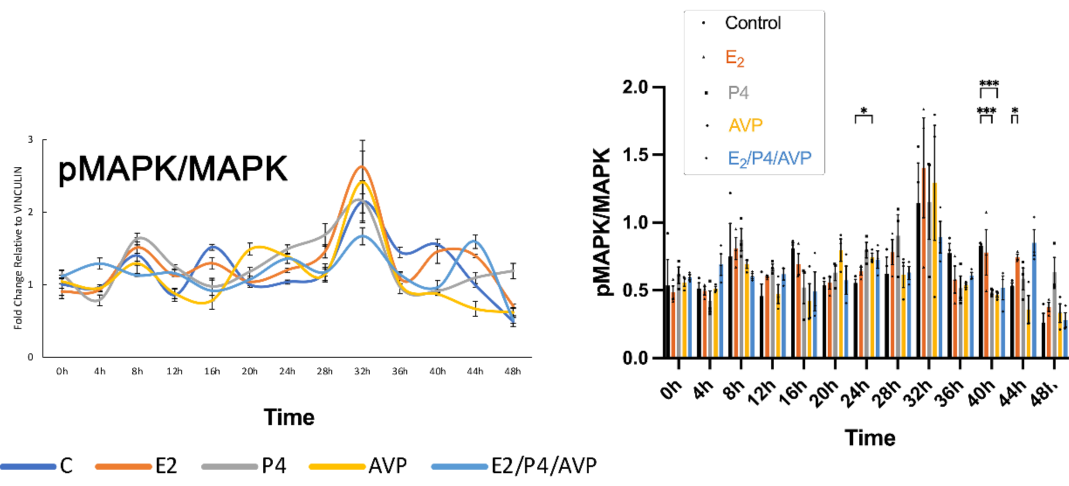


Figure 6. The time-dependent impacts on activation of mitogen-activated protein kinase (MAPK) signaling following timed stimulation with estradiol (E2), progesterone (P4), and vasopressin (AVP) treatment and co-treatment over 48 h. A main effect of time was observed on cellular pMAPK/MAPK content. The same data set is represented as both a line and bar graph for ease of visibility. Data are represented as mean ± SEM. *, $P < 0.05$ and ***, $P < 0.001$.

Chapter 4: Time-of-day-dependent sensitivity of the reproductive axis to RFamide-related peptide-3 inhibition in female Syrian hamsters

4.1 Introduction

Circadian timing is critical for female reproduction with disruptions to circadian timing leading to pronounced deficits in female reproductive health. For example, women with irregular sleep or work cycles have decreased fertility and increased rates of miscarriages (Gamble et al., 2013; Gotlieb et al., 2018; Mahoney, 2010; Simonneaux et al., 2017). In spontaneously ovulating species, the timing of the luteinizing hormone (LH) surge required for ovulation is under strict circadian regulation by the suprachiasmatic nucleus (SCN) of the hypothalamus, the master mammalian brain clock (Everett & Sawyer, 1950; Kriegsfeld, 2013; Legan & Karsch, 1975; Simonneaux et al., 2017). The dependence of ovulation on circadian timing coordinates a limited time window of fertility with sexual motivation and activity to maximize reproductive success, with the preovulatory LH surge occurring during early mornings in women and diurnal rodents (Cahill et al., 1998; Mahoney et al., 2004; McElhinny et al., 1999) and in late afternoon in nocturnal rodents (Gibson et al., 2008; Robertson et al., 2009; Turek et al., 1984). To ensure appropriate oocyte maturation at the time of ovulation, the neuroendocrine circuit initiating ovulation has an additional reliance on estradiol (E2) signaling from developing follicles. As the follicles develop during the follicular phase of the ovulatory cycle, increasing concentrations of E2 are secreted to maintain LH at low concentrations via negative feedback. However, just prior to ovulation, peak E2 concentrations act via positive-feedback to initiate the LH surge that triggers ovulation (Christian & Moenter, 2010; de la Iglesia & Schwartz, 2006; Gotlieb et al., 2018; Kriegsfeld, 2013; Legan & Karsch, 1975; Piet et al., 2013; Simonneaux & Piet, 2018). Previous findings by our group and others suggest that the temporary shift from negative- to positive-feedback is coordinated by the SCN (Gibson et al., 2008; Khan & Kauffman, 2012; Russo et al., 2015; Simonneaux & Piet, 2018; Williams & Kriegsfeld, 2012).

The SCN modulates reproductive axis function via direct and indirect communication to the hypothalamic-pituitary-gonadal (HPG) axis. At the time of the LH surge, monosynaptic vasoactive intestinal peptide (VIP) projections from the SCN directly stimulate gonadotrophin-releasing hormone (GnRH) neurons, with FOS ex- pression increased in GnRH neurons receiving VIP input around this time (Piet et al., 2018b; van der Beek et al., 1994). GnRH neurons do not express estrogen receptor-α (ERα), the receptor subtype that mediates E2-positive and E2-negative-feedback (Wintermantel et al., 2006). To modulate the balance of negative and positive E2 feedback, the SCN coordinates the activity of two opposing ERα-expressing neuropeptidergic systems that lie upstream of the GnRH system: the stimulatory neuropeptide, kisspeptin, and the inhibitory neuropeptide, RFamide-related peptide-3 (RFRP-3; the mammalian orthologue of avian gonadotrophin-inhibitory hormone [GnIH]) (Gibson et al., 2008; Henningsen et al., 2017; Kriegsfeld et al., 2006, 2018; Piet, Fraissenon, et al., 2015; Rizwan et al., 2012; Russo et al., 2015; Schafer et al., 2018; Simonneaux et al., 2017; Vida et al., 2010; Williams et al., 2011). The SCN coordinates cellular activity of RFRP-3 neurons to suppress the reproductive axis outside the time window of the LH surge and to allow for the transient suppression of E2-negative-feedback around the time of the LH surge (Gibson et al., 2008; Henningsen et al., 2017; Russo et al., 2015). RFRP-3 neurons are

concentrated in the dorsomedial hypothalamus (DMH) and project broadly to hypothalamic loci that contain GnRH neurons and fibers (i.e., medial septum, diagonal band of Broca, preoptic area, anterior hypothalamus and arcuate nucleus [ARC]) in addition to the ventromedial nucleus of the hypothalamus and brainstem (Kriegsfeld et al., 2006). RFRP-3 cell projections form direct contacts with GnRH neurons expressing the RFRP-3 receptor, GPR147 (Kriegsfeld et al., 2006; Rizwan et al., 2012; Ubuka et al., 2013), permitting direct suppression of GnRH neuron activity and consequent LH release (Ducret et al., 2009; Kriegsfeld et al., 2006; Russo et al., 2015; Ubuka et al., 2009, 2012; Williams et al., 2011; M. Wu et al., 2009). In some species, RFRP-3 neurons may also act on the anterior pituitary to mediate LH release because RFRP-3 neurons directly project to the median eminence and GPR147 is expressed in the pituitary (Bentley et al., 2009; Clarke et al., 2008; Gibson et al., 2008; J. T. Smith, 2012; Ubuka et al., 2009). Finally, RFRP-3 may modulate the HPG axis via a subpopulation of ARC kisspeptin neurons that express GPR147 (Poling et al., 2013).

Concomitant with RFRP-3 suppression at the time of the LH surge, the SCN stimulates kisspeptin neurons located in antero- ventral periventricular nucleus (AVPV) that, in turn, stimulate the GnRH system and the LH surge (Chassard et al., 2015; Piet et al., 2018b; Williams et al., 2011; Zhao & Kriegsfeld, 2009). Although we have previously shown that kisspeptin neurons are indiscriminately sensitive to SCN signaling across the day in Syrian hamsters, GnRH neurons exhibit time-dependent sensitivity to kisspeptin stimulation, responding more robustly in the afternoon than in the morning (Williams et al., 2011). This additional mechanism of temporal control further ensures precision in the timing of the LH surge and ovulation. The present study examined whether this time-dependent sensitivity of the GnRH system is unique to kisspeptin or whether daily changes in reproductive system sensitivity also occur in response to RFRP-3 inhibition to further coordinate the precise timing of the LH surge.

Because reproductive axis inhibition is essential prior to ovulation, we hypothesized that the GnRH system is maximally responsive to RFRP-3 in the morning, prior to the LH surge. If true, then LH concentrations should be inhibited by RFRP-3 in the morning but not (or to a greater degree than) in the afternoon. However, it is also possible that the GnRH system is maximally responsive to RFRP-3 in the afternoon, because this is a time during which RFRP-3 neurons are transiently inactive (Ancel et al., 2017; Gibson et al., 2008; Russo et al., 2015). If it is the case that maximal responsiveness of the GnRH system occurs in the afternoon, then LH concentrations should be inhibited by RFRP-3 in the afternoon but not (or to a greater degree than) in the morning. RFRP-3 inhibition of LH occurs via changes in LH peptide secretion, which may or may not reflect changes in mRNA expression. Likewise, inhibition of LH is possibly accompanied by changes in GnRH peptide release which may or may not be reflected in changes in Gnrh mRNA expression. Finally, RFRP-3 may modify LH production and/or release via direct impact on pituitary gonadotrophs or indirectly via kisspeptin neurons in the ARC that control GnRH pulsatility (Navarro, 2012; Tsutsumi & Webster, 2009; Yip et al., 2015). To select among these possibilities, we examined daily changes in HPG axis sensitivity to RFRP-3 inhibition in ovariectomized (OVX) female hamsters administered RFRP-3 or saline in the morning (prior to the LH surge) or late afternoon (around the time of the LH surge).

4.2 Materials and Methods

4.2.1 Animals

Thirty-four (>8 weeks old) female Syrian hamsters (Mesocricetus auratus) were purchased from Charles River (Wilmington, MA, USA) and maintained under a 14:10 hour light/dark cycle (lights on 6.00 am) at 23 ± 1°C with food and water available ad libitum. A 14:10 hour light/dark cycle was employed to create a “long day” light regimen because Syrian hamsters are seasonal breeders that breed under long day conditions. All procedures were approved by the Animal Care and Use Committee at the University of California, Berkeley and conformed to principles enumerated in the NIH guide for the use and care of laboratory animals.

4.2.2 Surgical Procedures

After a 2-week acclimation period, all hamsters were OVX to eliminate E_2-negative-feedback. Surgeries were conducted under isoflurane anesthesia with buprenorphine (s.c., 0.1 mg kg^{-1}) provided for analgesia. After a 2-week recovery, a guide cannula (22GA, 6 mm; Plastics One Inc., Roanoke, VA, USA) was stereotaxically implanted under deep anesthesia (ketamine-xylazine cocktail (i.p., 60/5 mg kg^{-1}) directed at the lateral ventricle. For cannular implantation, the head was shaved and prepared for surgery and then animals were placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Guide cannulae were placed at specific coordinates relative to bregma: 1.3 mm mediolateral, 1.1 mm posterior and 3 mm ventral from the surface of the dura mater. Following surgery, a dummy cannula (6.5 mm; Plastics One) was inserted into each guide cannula to prevent obstruction. Buprenorphine was administered before and after the surgeries for analgesia (s.c., 0.1 mg kg^{-1}). Following the procedure, hamsters were singly housed for the remainder of the study. Animals were given 1 week to recover before assessing cannula placement via injections of angiotensin-II (5 ng angiotensin-II in 2 µL sterile 0.9% saline) and examination of subsequent drinking behavior. Immediate drinking exhibited by hamsters confirmed the location of the cannula in the lateral ventricle.

4.2.3 Pharmacological Manipulations and Sample Collection

Saline (5 µL) or RFRP-3 (100 or 500 ng in saline) (Syrian hamster RFRP: ILSRVPSLPQRF-NH2; Phoenix Pharmaceuticals Inc., Belmont, CA, USA) was injected (i.c.v.) in the morning (3 h after lights on, n = 6 per group) or in the afternoon (3 h before lights off, n = 6-7 per group), at a rate of 0.5 µL per 30s, while the animals were freely moving about their home cage. Blood samples were collected from the retro-orbital sinus 20 minutes following injection and centrifuged at 1400 g for 15 minutes. Serum was collected and stored at −20˚C until assayed. Two weeks later, animals were injected again with RFRP-3 (100 ng per 5 µL saline) or saline (5 µL) in the morning or afternoon (n = 6-9 per group) and animals were euthanized 2 hours later. Brains and pituitaries were flash-frozen, and brains were sectioned at 300 µm and transferred to RNAlater (AM7021; Ambion, Austin, TX, USA) for one night at 4˚C and −20˚C thereafter until further processing. A 3-mm biopsy punch was used to microdissect the DMH and ARC in a single punch and a 2-mm biopsy punch was used to microdissect the medial preoptic area (mPOA) and AVPV bilaterally (**Figure 1**). RNA was extracted using ISOLATE II RNA Mini Kit (BIO-52073; Bioline, Memphis, TN, USA) and reverse transcribed to cDNA following

all manufacturer's instructions (iScript RT Supermix, 170-8841; Bio-Rad, Hercules, CA, USA). A random, representative sample of RNA (for each tissue n = 8) was assessed for RNA quality on an Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and yielded an average RNA integrity number of 7.3 or higher. To confirm LH suppression and further validate the detection levels of the LH enzyme-linked immunosorbent assay (ELISA) at low concentrations, five hamsters were injected with E_2 benzoate (EB) (100 µg in 200 µL of sesame oil) and retro-orbital blood samples were collected 90 minutes later.

4.2.4 qRT-PCR

Analysis of relative gene expression via qRT-PCR was performed using SSOAdvanced SYBR Green Supermix (1725272; Bio-Rad, Hercules, CA, USA). Samples were analyzed on a CFX384 machine (Bio-Rad) using 10-µL reaction volumes with a two-step amplification for 40 cycles followed by a melt curve. Primers were designed from published sequences for Syrian hamsters using NCBI Primer BLAST software (https://www.ncbi.nlm.nih. gov/BLAST) (**Table 1**). Primer sets were validated for specificity using positive, negative, no reverse transcriptase and no template controls and were confirmed with a single-peak melt curve and DNA electrophoresis for correct product length. Efficiency of each primer set was determined by standard curve; primers were 94.7%-105.4% efficient with R^2 values above 0.99. All samples were run in triplicate. Replicate sets in which Cq values varied beyond 0.5 cycles were excluded from analysis and the resulting data were analyzed in Excel (Microsoft Corp., Redmond, WA, USA) using the delta Cq method (Pfaffl, 2001). The geometric mean of the expression of two housekeeping genes was used for reference. Because the expression of housekeeping genes was found to vary with time of day or treatment between brain regions, samples from different brain regions were analyzed with different reference genes. Gapdh and Actb were used as reference genes for pituitary samples (Cq ranges were 20.3-24.26 and 20.6-24.64, respectively), whereas Hmbs and Tbcc were used as reference genes for DMH and ARC samples (Cq ranges were 23.9-26.8 and 21.35-24.24, respectively) and B2m and Rplp16 were used as reference genes for AVPV and POA samples (Cq ranges were 20.78-23.88 and 18.77-21.53, respectively). Housekeeping genes were not significantly different across groups, and in all gene replicate groups, the Cq SD was lower than 0.2. Although Kiss1 mRNA expression was measured in the DMH and ARC, it was not assessed in the AVPV and POA as a result of late and unstable amplification, indicating low mRNA expression, possibly as a result of the OVX. All data are expressed as fold-change over morning saline hamsters. Some samples did not have sufficient cDNA to quantify the expression of all genes; thus, the sample sizes vary for different genes measured.

4.2.5 Assessment of LH Levels

LH concentrations were quantified in duplicate with an ELISA, using a modified protocol that was kindly provided by Jens D. Mikkelsen (Copenhagen University Hospital, Denmark) (Ancel et al., 2012). Briefly, 96-well micro- titer plates were coated with 50 µL of bovine LHβ 518B7 monoclonal antibody (kindly provided by Lillian E. Sibley, UC Davis, CA, USA) and incubated overnight at 4˚C. Excess antibody was removed, and plates were washed three times with 200 µL of 10 mmol L^{-1} phosphate- buffered saline with 0.05% Tween 20 (PBS-T). Plates were blocked for 1 hour at room temperature using 5% skim

milk powder in PBS-T. Following washes, 50 µL of each sample and standards (mouse RIA kit; AF Parlow, National Hormone and Pituitary Program, University of California, Harbor Medical Center, Los Angeles, CA, USA) diluted in assay buffer was added to each well and incubated for 2 hours at room temperature. Plates were then washed and 50 µL of rabbit polyclonal LH antibody (AFP240580Rb; AF Parlow, National Hormone and Pituitary Program) were added into each well and incubated for 90 minutes at room temperature. After washing, a 1:2000 dilution of polyclonal goat anti-rabbit immunoglobulin G conjugated to horse- radish peroxidase (P0448; Dako, Glostrup, Denmark) was added to each well and incubated for 1 hour at room temperature. After washing, O-phenylenediamine (00-2003; Invitrogen, Carlsbad, CA, USA) in citrate buffer was added to each well and the reaction was allowed to proceed for 30 minutes at room temperature in darkness before being stopped by the addition of 3 mol L^{-1} HCl to each well. Light absorbance was immediately read at 490 nm with a reference of 655 nm. Representative random serum samples were assessed by the Center for Research in Reproduction at the University of Virginia (Charlottesville, VA, USA) (UVA) and Pearson's r = 0.97 correlation was found between the LH values obtained at UVA and the values generated by the 'in-house' LH ELISA. The assay was also validated by assessing parallelism with the standard curve as well as blood samples collected 90 minutes following EB administration used to suppress LH concentrations. Assay sensitivity was 0.002 ng mL^{-1} and intra- and inter-assay variability were 1.1% and 3.4%, respectively.

4.2.6 Statistical Analysis

Group comparisons were examined using a two-way analysis of variance (ANOVA). In instances where assumptions of normality and/or equal group variance were violated, data were analyzed by planned contrasts based on a priori hypotheses and corrected for multiple comparison with Bonferroni's inequality test. Statistical analyses were performed in SPSS (IBM Corp., Armonk, NY, USA) and PRISM (GraphPad Software Inc., San Diego, CA, USA). All data are reported as the mean ± SEM. $P < .05$ was considered statistically significant. The data that support the findings of this study are available from the corresponding author upon reasonable request.

4.3 Results

4.3.1 Circulating LH

LH concentrations were measured to examine whether the GnRH system exhibits daily changes in sensitivity to RFRP-3 inhibition. Consistent with the timing of the LH surge in the afternoon, baseline LH concentrations (saline groups) were significantly different across the day, increasing from 18.65 ± 3.8 ng mL^{-1} in the morning to 29.6 ± 4.3 ng mL^{-1} in the afternoon ($P < .044$; 95% confidence interval (CI) = −1.998 to 23.87) (n = 6-7 per group) (**Figure 2**). In addition, 100 ng RFRP-3 significantly decreased circulating LH concentrations in the afternoon 20 minutes after administration (from 29.6 ± 4.3 ng mL^{-1} to 14.7 ± 2.8 ng mL^{-1} [$P < .006$; 95% CI = −25.89 to −3.883]). At this same dose, no differences were found between saline and RFRP-3 administration in the morning ($P > .05$). No effects were observed with the 500 ng dose of RFRP-3 ($P > .05$ in all cases; data not shown). Finally, EB markedly suppressed

LH concentrations 90 minutes post-administration (decreasing to 5.47 ± 0.9 ng mL^{-1}; t = 2.808, P < .01; data not shown), further validating the LH assay.

4.3.2 Pituitary Gene Expression

Pituitary gene expression was measured to examine whether changes in LH concentrations are accompanied by changes at the mRNA level and whether pituitary cells exhibit the potential for direct inhibition by RFRP-3 (i.e., changes at the level of the pituitary independent of changes in the GnRH or kisspeptin systems) (n = 6-9 per group) (**Figure 3**). Within each treatment (saline or RFRP-3), no differences in Lhβ mRNA expression were found across time of day. However, RFRP-3 significantly decreased pituitary Lhβ subunit mRNA expression in the afternoon (P < .04; 95% CI = −1.092 to 0.07406) but not in the morning (P > .05) compared to saline, consistent with the impact of this peptide on circulating LH. By contrast, pituitary Gnrh-r mRNA expression did not differ at any timepoint regardless of treatment. However, a significant time X treatment interaction was found for pituitary Gpr147 mRNA expression ($F_{1,24}$ = 2.427, P < .019), with RFRP-3 significantly decreasing pituitary Gpr147 mRNA expression in the afternoon (P < .016; 95% CI = −1.702 to −0.08974) but not in the morning (P > .05).

4.3.3 mPOA Gene Expression

To examine whether changes in LH concentrations are mediated via the classic GnRH-LH pathway, mPOA Gnrh expression and Gpr147 mRNA expression were assessed (n = 5-9 per group) (**Figure 4**). Baseline mPOA Gnrh expression (i.e., saline groups) was significantly reduced in the afternoon relative to morning (P < .01; 95% CI = −0.5464 to −0.05362). Additionally, RFRP-3 significantly decreased mPOA Gnrh mRNA expression in the morning (P < .024; 95% CI = −0.5581 to −0.002333) but not in the afternoon (P > .05). mPOA Gpr147 mRNA expression did not differ at any timepoint for either treatment (P > .05 in all cases).

4.3.4 ARC Gene Expression

RFRP-3 may modify LH production and/or release indirectly via kisspeptin neurons in the ARC that control GnRH pulsatility (A. M. Moore et al., 2018). Thus, we examined the expression of Kiss1 and Gpr147 mRNA in the ARC following RFRP-3 and saline treatments (n = 7-9 per group) (**Figure 5**). Within each treatment (saline or RFRP-3), no difference in mRNA expression was found across time of day. However, RFRP-3 significantly decreased ARC Kiss1 mRNA expression in the afternoon (P < .022; 95% CI = −1.229 to −0.01701) but not in the morning (P > .05) compared to saline controls. In the ARC, Gpr147 mRNA baseline expression (saline groups) exhibited a non-significant trend in which afternoon levels were reduced compared to morning (P < .054; 95% CI = −0.2739 to 2.418). No effect of RFRP-3 was observed for ARC Gpr147 mRNA expression at either timepoint (P > .05 in each case).

4.4 Discussion

The results of the present study indicate that the reproductive axis responds to RFRP-3 in a time-dependent manner, with central RFRP-3 administration in the afternoon, but not the morning, reducing circulating LH and down-regulating pituitary Lhβ subunit mRNA expression. These findings support the notion that the reproductive axis is

most sensitive to RFRP-3 inhibition around the time of the LH surge (i.e., late afternoon). Because previous studies have established that administration of RFRP-3 around the time of ovulation suppresses the GnRH/LH surge (Anderson et al., 2009) and sexual motivation (Piekarski et al., 2013), this finding further highlights the importance of RFRP-3 cellular inhibition at this time, as we and others have previously shown (Gibson et al., 2008; Henningsen et al., 2017; Russo et al., 2015). Additionally, consistent with previous findings in this species (Williams et al., 2011), the present findings further establish daily changes in the reproductive axis that are coordinated with the timing of the LH surge, even in the absence of estrogen. Taken together, these outcomes highlight the importance of circadian-controlled RFRP-3 system inhibition with respect to permitting the LH surge and coordinating maximal fertility with sexual motivation (**Figure 6**).

To explore where daily changes in sensitivity to RFRP-3 are mediated, we examined the expression of Gpr147, the cognate receptor for RFRP-3. In the brain, Gpr147 is expressed in GnRH cells (Ducret et al., 2009; Poling et al., 2012; Rizwan et al., 2012; Ubuka et al., 2012), in the pituitary (Clarke et al., 2008; Gibson et al., 2008; J. T. Smith, 2012; Ubuka et al., 2009), and in kisspeptin neurons (Poling et al., 2013; Rizwan et al., 2012), providing three potential loci at which such changes may occur. Specifically for kisspeptin neurons, 12%-15% of AVPV kisspeptin cells express Gpr147 and 25% of ARC kisspeptin neurons express Gpr147 in both male and female mice (Poling et al., 2013; Rizwan et al., 2012). Likewise, ~35% of ARC kisspeptin neurons receive RFRP-3 immunoreactive fiber contacts (Poling et al., 2013). In the present study, RFRP-3 had no effect on the expression of Gpr147 in the mPOA or AVPV, suggesting enhanced RFRP-3 signaling via Gpr147 in these regions is not responsible for increased responsiveness to RFRP-3 inhibition in the afternoon. Likewise, hypothalamic Gnrh mRNA levels were not reduced in the afternoon by infusion of RFRP-3. Furthermore, RFRP-3 did not influence Gnrh-r mRNA expression in the pituitary. These findings suggest that changes in the sensitivity of hypothalamic GnRH neurons, or reduced pituitary sensitivity to GnRH, do not underlie the enhanced suppression of LH by RFRP-3 in the afternoon. Whether or not the enhanced suppression of LH in the afternoon by RFRP-3 is a result of inhibition of GnRH peptide release, post-transcriptional/translational events regarding GPR147 (e.g., more GRP147 receptors are available/translated in the afternoon) or the specific time intervals between RFRP-3 administration and sampling represents an important area for future inquiry.

Although the present findings do not support a role for altered GnRH cell sensitivity to RFRP-3 signaling or changes in pituitary sensitivity to GnRH across the day, the findings suggest that daily changes in the suppressive actions of RFRP-3 might occur at the level of ARC kisspeptin cells. Specifically, we observed a substantial reduction in Kiss1 mRNA expression in the ARC following afternoon, but not morning, RFRP-3 administration. These findings point to the possibility that ARC kisspeptin cells may act on GnRH terminals to modulate their output across the day in response to upstream mediators. GnRH neurons possess unique axonal projections to the median eminence that also exhibit dendritic functions (Herde et al., 2013; Iremonger & Herbison, 2015; Yip et al., 2015). These so-called ‘dendrons’ allow for synaptic input and the integration of information to control the release of GnRH. In several species, ARC kisspeptin neurons exhibit axo-axonal contacts with GnRH neurons (Matsuyama et

al., 2011; Uenoyama et al., 2011), as well as projections to the internal and external layer of the median eminence (Yip et al., 2015). Our results show that the expression of ARC Kiss1 mRNA co-varies with circulating LH levels, with RFRP-3 acting to reduce both Kiss1 mRNA expression and circulating concentrations of LH in the afternoon but not in the morning, consistent with this pathway of control. In support of this possibility, ablation of ARC kisspeptin neurons leads to atypical LH surge amplitude (Helena et al., 2015; Mittelman-Smith et al., 2016b). The present findings are also in agreement with a recent study demonstrating RFRP-3 suppression of ARC kisspeptin expression in free-cycling Syrian hamsters maintained in long photoperiods (Henningsen et al., 2017). This same previous study found that hamsters injected with RFRP-3 in the afternoon, but not in the morning, exhibit suppression of LH concentrations when in proestrus. Furthermore, ARC kisspeptin neurons receive monosynaptic input from RFRP-3 neurons and express the RFRP-3 receptors (Poling et al., 2013). Taken together, the present and previous findings support the notion that RFRP-3 cells are in a position to modify LH secretion via actions on ARC kisspeptin cells and these cells differ in their response to RFRP-3 across the day.

In addition to actions on the ARC kisspeptin cell population, daily changes in RFRP-3 sensitivity may also be mediated at the level of the pituitary because pituitary Gpr147 and Lhβ mRNA expression are reduced following RFRP-3 treatment in the afternoon but not in the morning. Future studies in which RFRP-3 are administered peripherally in the morning and afternoon are necessary to examine this possibility because it is unclear whether injections of RFRP-3 in the present study enter the hypophyseal portal system. Across species (e.g., sheep, mice, hamsters, macaques, and humans), RFRP-3 projections to the median eminence and RFRP-3 receptor expression in the pituitary have been reported (Clarke et al., 2008; Gibson et al., 2008; Glanowska et al., 2014; Sukhbaatar et al., 2014; Ubuka et al., 2009). By contrast, neither RFRP-3 projections to the median eminence, nor its receptor, are found in some species (Harbid et al., 2013; Rizwan et al., 2009; J. T. Smith et al., 2010; Yano et al., 2003). In cultured pituitaries across species, RFRP-3 administration inhibits gonadotrophin production and release (Kadokawa et al., 2009; Pineda et al., 2010; Sari et al., 2009), suggesting the potential for inhibition in vivo. Although the present study did not assess this pathway directly, the expression of pituitary Gpr147 mRNA exhibited a similar pattern to that of LH and Lhβ, with RFRP-3 inhibition of Grp147 mRNA expression in the afternoon but not in the morning. These findings suggest potential actions of RFRP-3 that ultimately affect pituitary level responsiveness to this neuropeptide.

In the present study, hamsters were ovariectomized to eliminate E_2-negative-feedback. In the absence of E_2, the pattern of LH in the saline (control) groups resembled the expected pattern, with LH concentrations being higher in the afternoon than in the morning (Gibson et al., 2008). However, this daily change is not reflected in Gnrh mRNA expression. Also, in contrast to expectation, RFRP-3 suppressed Gnrh expression in the mPOA in the morning but not in the afternoon, contrasting with patterns of circulating LH that are not inhibited by morning RFRP-3 treatment. These unexpected relationships between the pattern of Gnrh mRNA and daily change in LH are possibly a result of the disparity between the time at which blood and brain samples were collected (i.e., blood samples were collected 20 min post- treatment, whereas brains were collected 2 h post-treatment). It is also possible that post-transcription/translation

modifications lead to differential GnRH peptide release (Kim et al., 2014). Finally, we cannot exclude the possibility that the removal of endogenous ovarian hormones alters the typical hypothalamic response to RFRP-3 communication. Future studies examining the time course of gene transcription/ translation and the association with peptide release will help to select among these possibilities.

The results of the present study suggest that the mechanisms driving LH secretion differ depending on the time of day and neurochemical environment. Specifically, in the absence of RFRP-3 administration (i.e., saline conditions) or during times that RFRP-3 is administered when it is typically released (i.e., the morning), the mechanisms driving LH secretion converge at the level of GnRH neurons. However, when RFRP-3 is administered in the afternoon, a time during which it is not typically released, it appears to bypass direct communication with the GnRH system, and instead, acting via ARC kisspeptin cells and/or directly on the pituitary. This latter circumstance might result in the case of circadian disrupted individuals, including women who are jetlagged, have irregular shift work hours or are exposed to light at night-time (e.g., from electronic devices), which are conditions associated with marked deficits in ovulatory cycling (Gamble et al., 2013; Mahoney, 2010).

In conclusion, the present findings indicate that time-dependent sensitivity to regulators of the HPG axis is not unique to kisspeptin stimulation of the GnRH system, at least in Syrian hamsters. The re- productive axis is maximally responsive to RFRP-3 administration in the afternoon, with no effect in the morning, even in the absence of estrogen. During the afternoon, RFRP-3 appears to inhibit LH secretion via actions on ARC kisspeptin cells and the pituitary rather than the GnRH system. These findings further highlight the importance of timed suppression of the RFRP-3 system at the appropriate time of day to allow for the LH surge and ovulation. These findings raise the possibility that, in cases of circadian disruption (e.g., irregular sleep patterns, night-time exposure to light-emitting devices, shift work), mistimed RFRP-3 release may be responsible for the compromised fertility seen across species, including humans.

4.5 Tables

Primer	Primer Name	Primer Sequence	Amplicon Length (BP)
Lhβ	FWD	CGGCTACTGTCCTAGCATGG	102
	REV	AGGCGGACAGATGTGAAGTG	
Gnrh-r	FWD	TCATCTTCACCCTCACACG	121
	REV	GTGGCAAATGCGACTGTCAT	
Gnrh	FWD	AGGGACCTTCGAGGAGTTCT	88
	REV	TGTGGATCCTTTGGTGCTGAT	
Kiss1	FWD	TGGTTATCTTTGACCTCCGGC	105
	REV	TGCCAAGAAGCCAATGTGGT	
Gpr147	FWD	CCGGTTGGCCTTTTGACAAT	140
	REV	CAGCTTCTCACGGAAAGGGT	
Gapdh	FWD	ACAGTCAAGGCTGAGAACGG	116
	REV	TCCACAACATACTCGGCACC	
Actb	FWD	GACCCAGATCATGTTTGAGACCT	112
	REV	TCCGGAGTCCATCACAATGC	
B2m	FWD	TGGCCGTGGTCTTTCTGATG	139
	REV	TGGAACTGCGACACATAGCA	
Rplp16	FWD	ATCTACTCCGCCCTCATCCT	159
	REV	GCAGATGAGGCTTCCAATGT	
Hmbs	FWD	TATCCTGGATGTTGCACGGC	165
	REV	TCTCAACACCCAGTGGTTCA	
Tbcc	FWD	CAGTGGGACTGAGCACTAGC	156
	REV	TAGCAAAAGCCCCGGGTTAG	

Table 1. Primers used for qPCR gene expression assays.

4.6 Figures

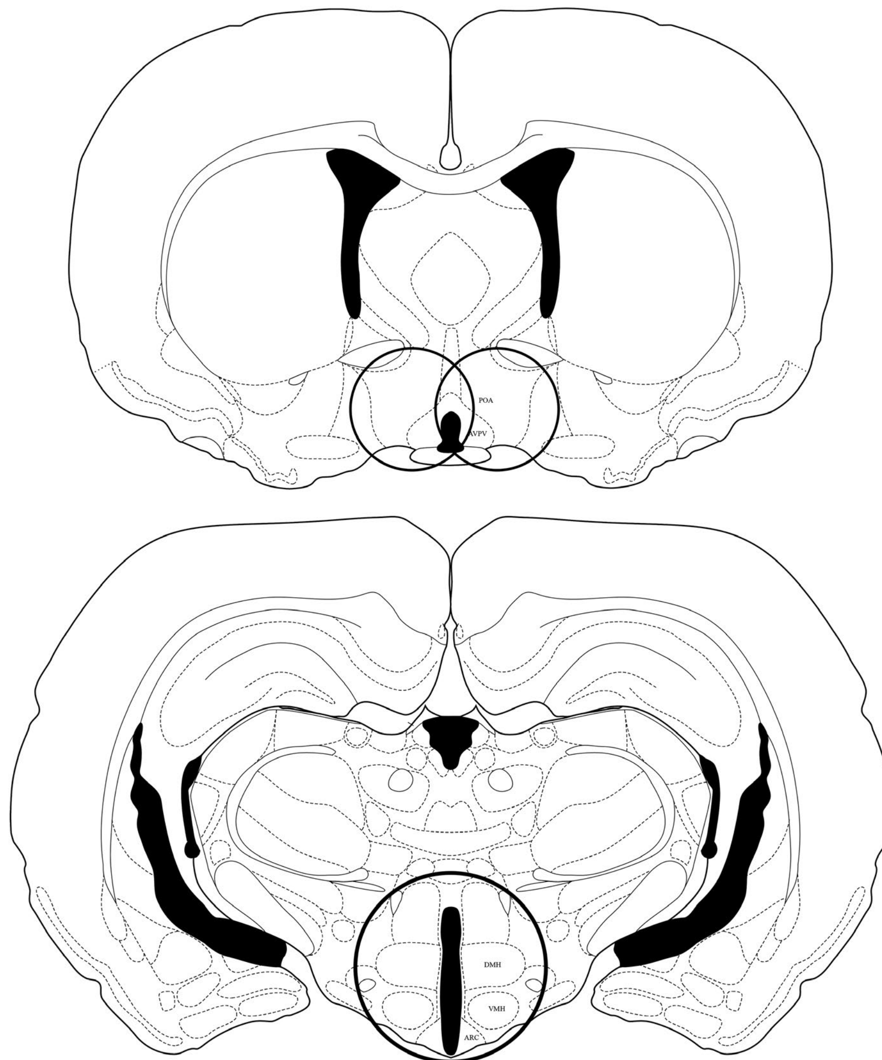


Figure 1. An illustration of the location of samples punched for reverse transcriptase-polymerase chain reaction analysis. Brains were flash-frozen and cut at 300 μm and then transferred to RNAlater for one night. A 2-mm biopsy punch was used to microdissect the medial preoptic area and anteroventral periventricular nucleus bilaterally (top) and a 3-mm biopsy punch was used to microdissect the dorsomedial hypothalamus and arcuate nucleus in a single punch (bottom). Illustrations are adapted and modified from the Stereotaxic Atlas of the Golden Hamster Brain (Morin, 2001). ARC = arcuate nucleus, AVPV = anteroventral periventricular nucleus, DMH = dorsomedial hypothalamus, POA = preoptic area, VMH = ventromedial nucleus of the hypothalamus.

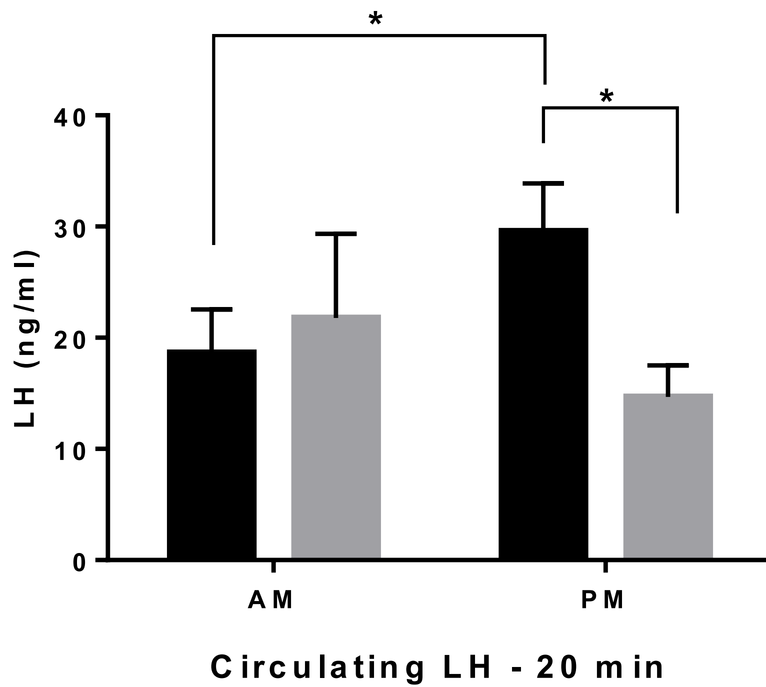


Figure 2. Central administration of RFamide-related peptide-3 (RFRP-3) (100 ng) inhibits circulating luteinizing hormone (LH) in the afternoon 20 minutes post-administration (n = 6, 6, 6 and 7 for am saline, am RFRP-3, pm saline, and pm RFRP-3, respectively). Data are presented as the mean ±SEM. *P < .05.

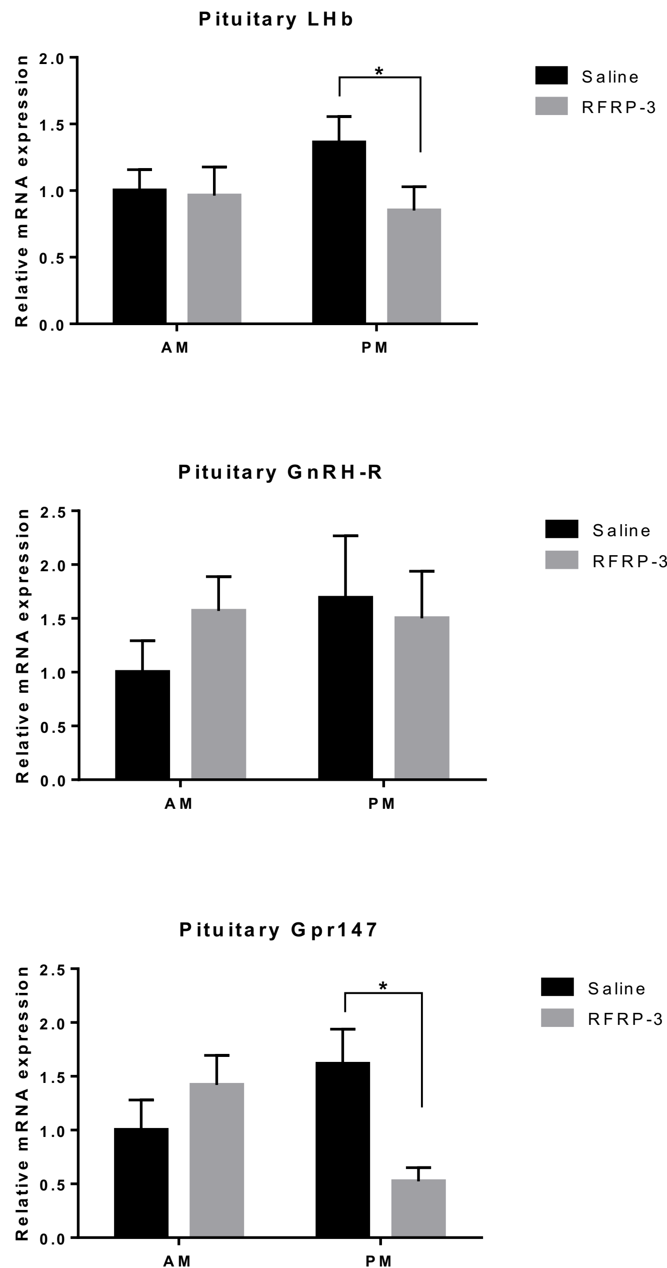
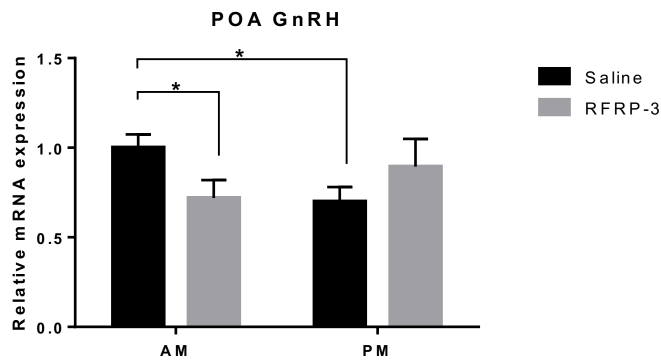


Figure 3. Central administration of RFamide-related peptide-3 (RFRP-3) suppresses pituitary Lhβ subunit mRNA expression in the afternoon but not the morning (top; n = 6, 6, 6 and 7 for am saline, am RFRP-3, pm saline, and pm RFRP-3, respectively). Pituitary Gnrh-r mRNA expression is not affected by either RFRP-3 or time of day (middle; n = 6, 8, 7 and 7 for am saline, am RFRP-3, pm saline and pm RFRP-r, respectively). RFRP-3 suppresses pituitary Gpr147 mRNA expression in the afternoon but not the morning (bottom; n = 6, 8, 9 and 5 for am saline, am RFRP-3, pm saline, and pm RFRP-3, respectively). Data are presented as the mean ± SEM. *P < .05.



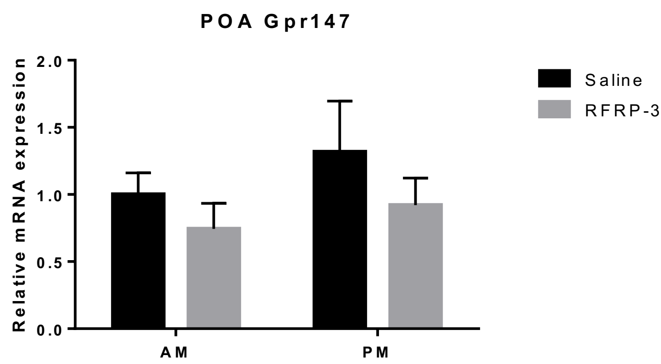
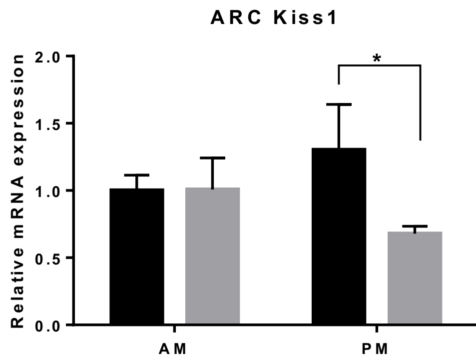


Figure 4. mPOA Gnrh mRNA expression is suppressed by central administration of RFamide-related peptide-3 (RFRP-3) in the morning but not in the afternoon (top; n = 6, 6, 7 and 9 for am saline, am RFRP-3, pm saline, and pm RFRP-3, respectively). RFRP-3 does not alter mPOA Gpr147 mRNA expression (bottom; n = 7, 5, 7 and 8 for am saline, am RFRP-3, pm saline, and pm RFRP-r, respectively). Data are presented as the mean ± SEM. *P< .05.



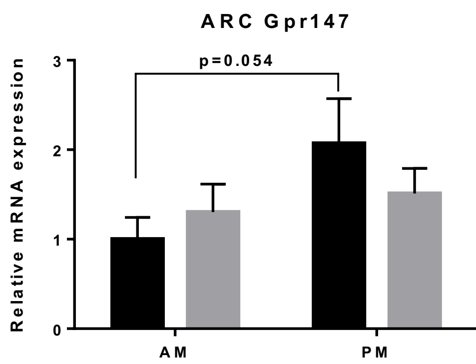


Figure 5. Central administration of RFamide-related peptide-3 (RFRP-3) suppresses arcuate nucleus (ARC) Kiss1 mRNA expression in the afternoon but not in the morning (top; n = 7, 8, 7 and 9 for am saline, am RFRP-3, pm saline, and pm RFRP-3, respectively). No effect of RFRP-3 on ARC Gpr147 mRNA expression was observed (bottom; n = 6, 8, 7 and 9 for am saline, am RFRP-3, pm saline, and pm RFRP-3, respectively). Data are presented as the mean ± SEM. *P < .05.

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