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Neural Circuits and Hormonal Mechanisms Underlying Female Reproduction

Author
Gotlieb, Neta

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Neural Circuits and Hormonal Mechanisms Underlying Female Reproduction

By

Neta A Gotlieb

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Committee in charge:

Professor Lance J. Kriegsfeld, Chair
Professor George E. Bentley
Professor Linda Wilbrecht
Professor Daniela Kaufer

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Abstract

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Doctor of Philosophy in Psychology

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Professor Lance J. Kriegsfeld, Chair

'Homeostasis' is the physiological process of maintaining a stable equilibrium, operating within optimal limits to promote health and prevent illness. Deviations from homeostasis can have adverse consequences for one's health. However, physiological and behavioral needs vary markedly over the course of the day, necessitating that biological systems are adjusted correspondingly. Likewise, physiological needs change over the female reproductive cycle, with ovulation, pregnancy and fetal development, and parturition requiring specifically-timed patterns of hormone secretion regulated by the circadian system. Disruption to homeostasis, either by circadian misalignment or by exposure to stress, has marked, negative consequences for female reproductive health, including ovulation, pregnancy success and maintenance, and offspring development. The overarching goal of this dissertation research is to understand the neural and hormonal mechanisms underlying reproductive health and how these mechanisms are affected by disruption to homeostasis through circadian misalignment and stress. This dissertation considers two aspects of the female reproductive cycle, the ovulatory cycle and pregnancy. Despite a significant volume of knowledge detailing the circadian regulation of ovulation and the negative impact of stress of reproductive health, the mechanisms underlying these events remain poorly understood. Chapter 2 investigates the time-dependent sensitivity of the reproductive axis to the inhibitory neuropeptide RFamide-related peptide-3 (RFRP-3) in the control of the neuroendocrine events required for ovulation. Chapters 3-5 explore how stress hormones act on the brain, ovaries, and placenta to compromise pregnancy success and fetal development. These studies inform our understanding of the complex neural and endocrine networks regulating environmental and physiological processes involved in promoting female reproductive health.

Dedication

For Lia and Maya, who expand my heart with love every single day.

May your curiosity lead you to pursue adventures.

May your laughter leave a trail of light wherever you go.

May your imagination and creativity bring dreams to life.

Acknowledgments

I see science as an ever-growing jigsaw puzzle with scientists working diligently to contribute one piece at a time. I am fortunate to be able to contribute my own, and I am only able to accomplish this because I am standing on the shoulders of giants.

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1. General Introduction

1.1 Introduction

Walter Cannon coined the term homeostasis (standing the same) to describe the remarkable precision with which brain and bodily processes are maintained within stable operating limits to promote optimal health and prevent illness [1]. However, physiological and behavioral needs vary markedly and predictably over the course of the day, necessitating that biological systems are adjusted correspondingly. The circadian timing system synergizes with homeostatic drive to anticipate changing daily requirements and adjust central and peripheral physiology accordingly. Hormones are chief regulators of biological and behavioral events including, sexual motivation and reproduction, feeding and metabolism, sleep and vigilance, and immune function. Given the broad functional significance of hormones and their ability to travel long distances to alter physiology throughout the brain and periphery, the endocrine system is under strict circadian control [2-4].

Coordinated timing of neuroendocrine events is fundamental for successful female reproduction across mammalian species, including humans [5-10]. 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 [11-15]. 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, [16, 17], reduced fertility [18, 19], and increased miscarriage rates [19-21]. 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 [22-26].

The circadian system confers a selective advantage by allowing organisms to anticipate rhythmic and predictable environmental change 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 [27]. 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 and artificial lighting at night. This combination results in an incongruence between the endogenous circadian timing system and the external environment, leading to chronic and pervasive 'jet lag' in the modern world [28, 29]. 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 [30].

Because the majority of studies to date have concentrated on the role of circadian rhythms in female reproductive function, the present chapter focuses on this sex. Although, it is worth noting that circadian disruption negatively affects semen quality and sperm numbers [31, 32] and compromises fertility in men [33] with analogous results seen in mouse models in which the genes regulating the circadian clockwork are knocked out [34, 35]. Herein, we consider all phases of the female reproductive cycle, including ovulation, mating, pregnancy and fetal development, and

parturition and describe how the circadian system integrates with the reproductive axis to mediate reproductive success. Likewise, we consider development in relevant systems and circuits where applicable and the negative consequences of circadian disruption during each stage of reproduction.

1.2 The Circadian Timing System

The circadian system consists of a master brain clock in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus that is synchronized to environmental time via a direct retinal pathway. As indicated previously, although circadian rhythms are endogenously generated, to be adaptive for an organism and allow anticipation of environmental changes across the day, these endogenous cycles are entrained to environmental time. Light entrains the SCN via retinal communication from rods and cones that, in turn, target specialized, intrinsically photosensitive retinal ganglion cells containing the photopigment, melanopsin [36-39]. Successively, the SCN uses neural, diffusible and autonomic communication to convey timing information to the whole organism [2, 40].

Circadian rhythms are a cell-autonomous property with circadian rhythms being generated by an autoregulatory transcription-translation feedback loop consisting of clock genes and their protein products [41, 42]. The core feedback loop begins in the morning with the clock protein, CLOCK, binding to BMAL1 to drive the transcription of the Period (*Per1* and *Per2*) and Cryptochrome (*Cry1* and *Cry*2) genes. Over the course of the day, *Per* and *Cry* transcripts are translated into their respective proteins that inevitably feed back to the cell nucleus to repress CLOCK:BMAL1-mediated transcription until the next morning when transcription resumes. Whereas the *Clock* gene is constitutively expressed, an additional feedback loop driven by the CLOCK:BMAL1 complex regulates *Bmal1* transcription through repression by *Rev-erb*$\propto$ and transcriptional activation via retinoic acid receptors (RORs)(**Figure 1**). Circadian timekeeping is a ubiquitous property of cells throughout the brain and body, with virtually all cells exhibiting circadian timekeeping [42]. Even when the SCN is isolated in culture, the master clock maintains indefinite circadian rhythms at the tissue level due to unique coupling among independent oscillators. In contrast, in the absence of master clock communication or other entraining stimuli, extra-SCN brain loci and peripheral organs exhibit loss of rhythmicity after several cycles [43, 44]. This loss of rhythmicity in extra-SCN systems results from loss of coupling among cellular oscillators having slightly different periods [45].

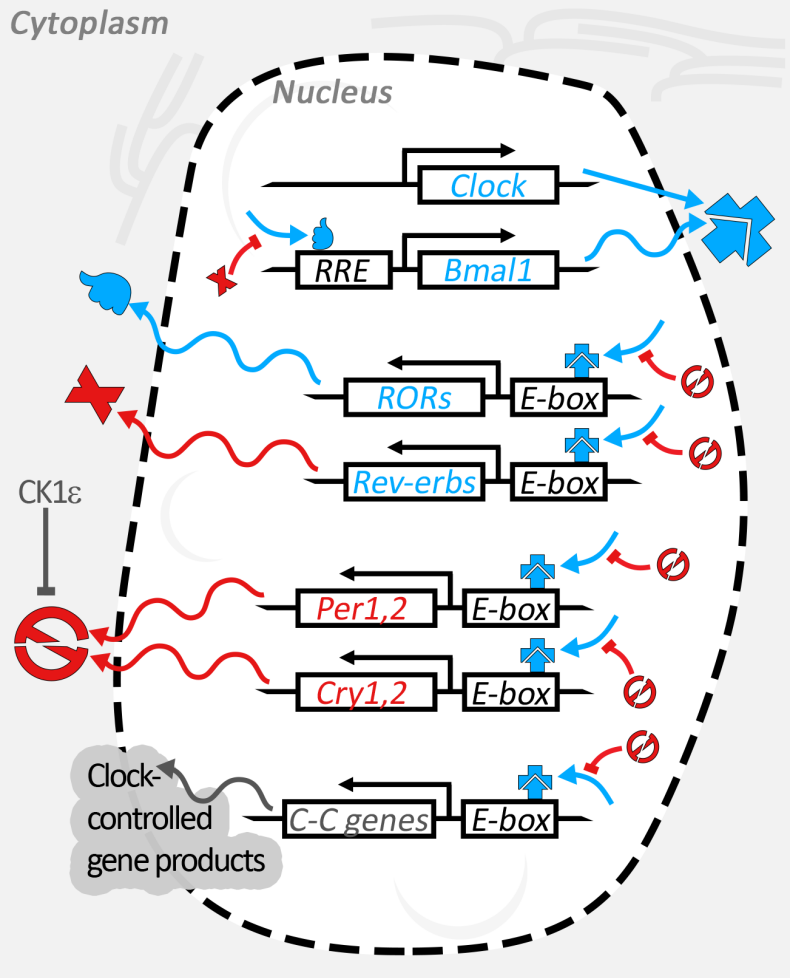

Figure 1. The molecular clockwork. A simplified model of the intracellular mechanisms responsible for mammalian circadian rhythm generation. The process begins when CLOCK and BMAL1 proteins dimerize to drive the transcription of the Per (Per1 and Per2) and Cry (Cry1 and Cry2) genes. In turn, Per and Cry are translocated to the cytoplasm and translated into their respective proteins. Throughout the day, PER and CRY proteins rise within the cell cytoplasm. When levels of PER and CRY reach a threshold, they form heterodimers, feed back to the cell nucleus, and negatively regulate CLOCK: BMAL1-mediated transcription of their own genes. Levels of Per are regulated by casein kinase 1 epsilon (CK1ε) which phosphorylates these proteins and marks them for degradation, thereby appropriately delaying negative feedback. Whereas *Clock* is constitutively expressed, a secondary feedback loop drives the transcription of ROR and Rev-Ervα that, in turn, induce rhythms in *Bmal1* transcription through stimulatory and inhibitory actions on ROR response elements (RRE) in the *Bmal1* promotor, respectively. Clock-controlled genes are tissue-specific genes that are produced rhythmically by the CLOCK:BMAL1 complex but are not part of the clockwork mechanism (i.e., do not feed back onto the clockwork).

1.3 The Hypothalamo-pituitary gonadal axis (HPG)

At the pinnacle of the HPG axis, hypothalamic gonadotropin-releasing hormone (GnRH) neurons send axonal projections to the median eminence. Here, GnRH is released from nerve terminals into the portal vasculature to trigger the secretion of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary. In turn, LH and FSH act on the gonads to stimulate sex steroid (e.g., progesterone, estradiol, and testosterone) synthesis and secretion and gametogenesis, respectively. Sex steroids and gonadotropins feed back to the hypothalamus and anterior pituitary to regulate HPG axis activity. Upstream of the GnRH system, two key neuropeptides were identified around the turn of the millennium that have pronounced inhibitory and stimulatory actions on GnRH neurons, namely RFamide-related peptide 3 and kisspeptin, respectively. The discovery and significance of these neuropeptides are described below.

A. RFamide-Related Peptide 3

In a search for novel peptides in the Arg-Phe-NH_2 (i.e., R=Arginine, F=Phenylalanine; termed RFamide peptides), Tsutsui and colleagues discovered a novel RFamide peptide that inhibited gonadotropin release from cultured quail pituitaries [46]. Because this hypothalamic neuropeptide specifically inhibited the gonadotropins without affecting other pituitary hormones, they named this peptide gonadotropin inhibitory hormone (GnIH) [46]. In birds, the GnIH precursor encodes one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) [47, 48]. In mammals, the neuropeptide precursor cDNA encodes three peptides (known as RFamide-related peptides [RFRPs]), RFRP-1, -2 and -3), with RFRP-3 thought to be the ortholog of avian GnIH [48]. Studies across mammalian species have found pronounced roles for this neuropeptide in regulating reproductive function.

The receptor for GnIH/RFRP-3 is a G-protein coupled receptor (GPR), the formerly-orphaned GPR147 (also called NPFF1 [49]). GPR147 most-commonly couples to an inhibitory G protein (Gαi), with GnIH/RFRP3 suppressing adenylate cyclase activity [50, 51]. However, in some instances, GPR147 is coupled to Gαs or Gαq proteins, where this differential coupling may account for disparity in the effects of RFRP3 based on sex or reproductive status (described further below [52]).

In most rodent species, RFRP3- cell bodies are localized exclusively to the dorsomedial hypothalamus (DMH ; reviewed in [7, 53, 54]. In mammals, RFRP-3-immunoreactive (-ir) neuronal fibers are widely distributed in the diencephalon, mesencephalon and limbic structures [55-57], providing a direct synaptic mechanism for broadly affecting neurophysiology and behavior. Across mammalian species, including humans, RFRP-3 generally suppresses GnRH and gonadotropin secretion via direct actions on GnRH cells and potentially at the level of the pituitary [56, 58-62]; reviewed in [54].

Under some circumstances, RFRP-3 stimulates the reproductive axis. For example, male Siberian hamsters (*Phodopus sungorus*) that typically breed during long, summer-like days exhibit an increase in LH concentrations following central administration of RFRP-3 when maintained in short day conditions [63]. Additionally, male Syrian hamsters maintained in long days exhibit

elevated LH and FSH in response to RFRP-3 administration [64]. Likewise, in striped hamsters (*Cricetulus barabensis*) the relationship between RFRP-3 and GnRH expression depends on sex and developmental/reproductive status [65]. Finally, RFRP3 stimulates LH secretion in male mice and inhibits LH release in female mice when estradiol concentrations are high at the time of the LH surge, but has no effect during diestrus or in ovariectomized females with low estradiol concentrations provided exogenously [66]. These findings suggest that the effects of RFRP-3 on gonadotropins may depend on the species, season, sex, and developmental status. Likewise, these findings suggest that GPR147 couples differentially with Gαi, Gαs, or Gαq based on these same variables [52].

B. Kisspeptin

Kisspeptin is also a member of the RFamide family of peptides. Kisspeptin was initially known as metastin, a tumor metastasis suppressor gene in human melanoma and breast carcinoma, discovered in 1996 in Hershey, PA [67-69]. The gene for metastin was named *Kiss1*, with the 'SS' representing suppressor sequence and 'Ki' added as an homage for the home town's famous Hershey kisses [67]. The role of kisspeptin as a positive regulator of the HPG axis was discovered in 2003 in individuals exhibiting hypophysiotropic hypogonadism were found to have a mutation in G-protein-couples receptor (GPR54), the cognate receptor for *Kiss1* gene protein products [70, 71]. These individuals exhibit low or absent circulating LH, fail to undergo puberty and are reproductively incompetent as adults. When the authors created GPR54-deficient mice, these animals exhibited an analogous phenotype [70, 71]. The protein product of the *Kiss1* gene is now commonly referred to as kisspeptin.

The Kiss1 gene encodes a family of kisspeptin peptides, beginning with a precursor polypeptide of 145 amino acids, that is cleaved into a 54 amino acid protein (named kisspeptin-54) that can be further cleaved into 10, 13, and 14 amino acid proteins, all of which share an RFamide sequence on their C-terminus, are biologically active, and share a similar affinity to GPR54 [69, 72, 73].

Kisspeptin directly stimulates the secretion of GnRH and LH across mammalian species, including humans, via direct actions on GnRH neurons [74-76]. In women with hypothalamic amenorrhea, acute administration of kisspeptin stimulates gonadotropin release [77, 78], indicating that failure of kisspeptin signaling contributes to reproductive cessation is these women. Both pituitary cells and GnRH neurons express GPR54; however, whether kisspeptin directly stimulates gonadotropin release from the pituitary requires further investigation. For example, although prominent expression of GPR54 is detected in human pituitary [72, 73], and peripheral administration of kisspeptin increases plasma LH concentrations in some cases [79], the effects of kisspeptin of cultured pituitary cells are equivocal [79-82].

Kisspeptin cell bodies are concentrated in the anteroventroperiventricular (AVPV) and the arcuate (Arc) nuclei of the hypothalamus in rodents [83-86] and in the preoptic area (POA) and infundibulum in humans [87]. A large percentage of kisspeptin cells express estrogen receptor (ER)-α [83], mediating positive (AVPV) and negative (Arc) feedback effects of estradiol in females [83, 88-91].

A number of studies suggest that Arc kisspeptin cells serve as critical components of estradiol negative feedback and the GnRH pulse generator. Arc kisspeptin neurons co-express two additional peptides, neurokinin B (NKB) and dynorphin [92-98]. Together, this population is referred to as KNDy (pronounced "candy") neurons. These triple phenotype cells communicate in an autosynaptic feedback manner in which KNDy cells project to other KNDy cells [92, 99, 100]. Local secretion of NKB stimulates kisspeptin release from KNDy cells onto GnRH neurons [101]. Mutations in NKB gene (i.e., Tac3) or its receptor (Tacr3) lead to hypogonadotropic hypogonadism [102-104]. In contrast to NKB, dynorphin inhibits Arc kisspeptin secretion and is thought to participate in the termination of each pulse [105, 106].

1.4 Circadian Control of Ovulation

In species that ovulate spontaneously, the preovulatory LH surge that initiates ovulation is ubiquitous (rodents [107-110] sheep [111], rhesus macaques [112], and women [113-115]. The LH surge occurs in early morning in women [116] and diurnal rodents [108], but in early evening in nocturnal rodents [117], coordinating the time of maximal fertility with activity. During most of the rodent estrous cycle, gonadotropin concentrations are low due to the negative feedback effects of gonadal steroids (reviewed in [117]. At the time of ovulation, however, positive feedback effects of estradiol serve a permissive role in initiating the LH surge by a timed, neural signal from the SCN (i.e., positive feedback) [6, 107, 118, 119]. This 'switch' in estradiol action results from coordinated timing of positive and negative regulators of the reproductive axis by the circadian timing system. The requirement for both high estradiol concentrations and a circadian timing signal to initiate positive feedback ensures appropriate oocyte maturation and maximal sexual motivation coincide with the time of ovulation.

As indicated previously, lesions of the SCN or the severing of connections between the SCN and the preoptic area result in acyclicity in rodents [24, 120, 121]. Furthermore, genetic impairments of the molecular clockwork also disrupt estrous cycles and the LH surge in mice [22, 26, 122, 123]. Although the SCN can support behavioral rhythms via a diffusible signal, the SCN-derived signal that generates the LH surge is neural; SCN-lesioned female hamsters receiving fetal SCN transplants that do not form neural connections with the host brain exhibit rhythmic behavior but not an LH surge [124-126]. As described further below, both direct SCN-GnRH neuronal communication, and indirect connections from the SCN to estrogen receptor-α (ERα)-expressing cell phenotypes that positively and negatively regulate the GnRH system, integrate circadian and estradiol signaling to initiate the preovulatory LH surge and ovulation (**Figure 2**).

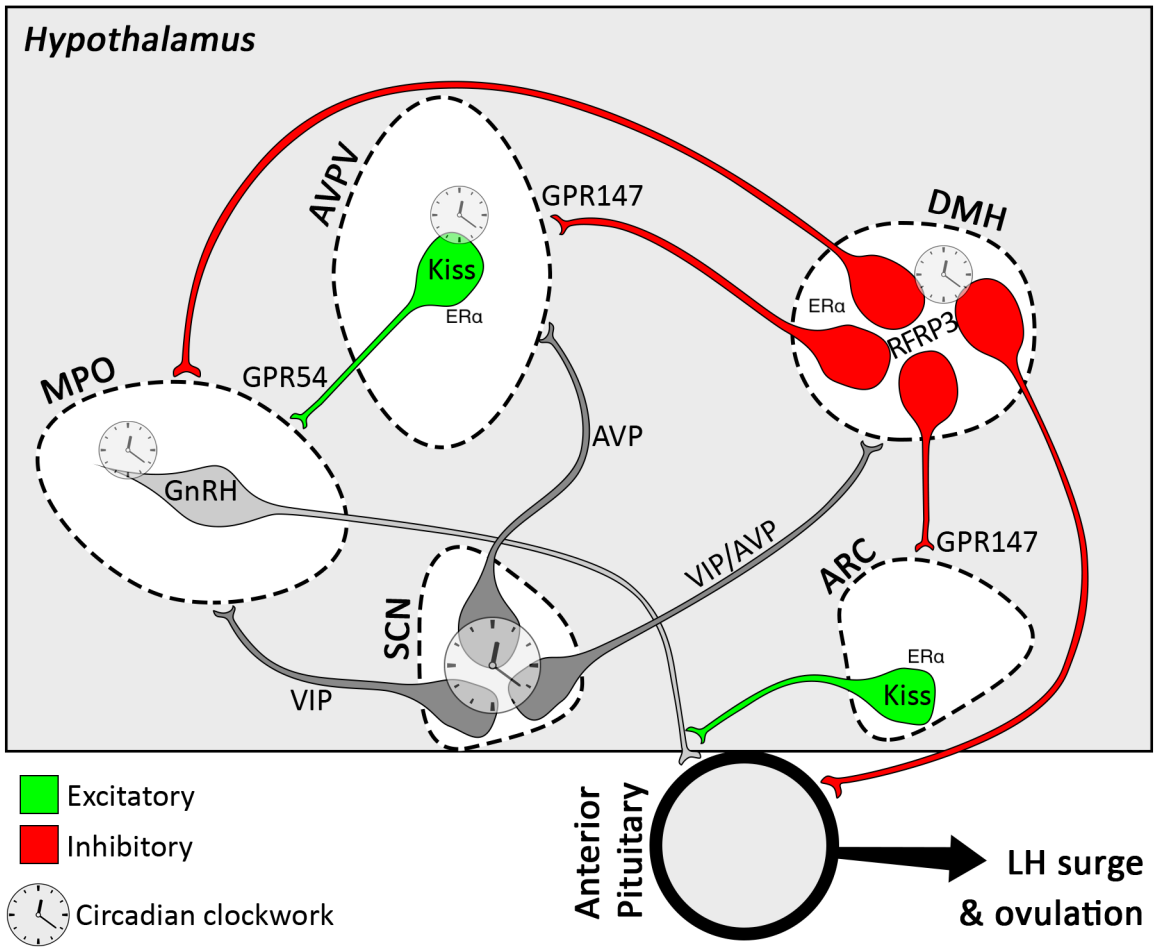

Figure 2. Circadian control of the preovulatory LH surge and ovulation. Model of interactions among the circadian, RFRP-3 and kisspeptin systems in control of the preovulatory LH surge and ovulation. At the time of the LH surge, the SCN coordinates estradiol positive feedback (through kisspeptin cell activation) with removal of estradiol negative feedback (through suppression of RFRP-3 cells in the DMH). GnRH, RFRP-3, and AVPV kisspeptin cells all exhibit rhythms in clock protein or gene expression that serve to signaling events and further temporal precision in this circuit. See text for further details.

A. Monosynaptic SCN regulation of the GnRH system in LH surge generation

Two major neuropeptidergic SCN cell phenotypes have been implicated in the generation of the preovulatory LH surge and ovulation in rodents, neurons synthesizing the neuropeptide vasoactive intestinal peptide (VIP) in the retinorecipient SCN core and vasopressin (AVP) in the dorsal SCN [127]. Both VIP and AVP exhibit peak release around the time of the LH surge [128-130] and central injections of either VIP or AVP receptor antagonists attenuate LH surge amplitude [131-134]. SCN VIPergic neurons project monosynaptically to GnRH neurons in the POA [135, 136]. Several lines of evidence suggest a role for this direct pathway in LH surge generation. First, concomitant with the LH surge, FOS, is preferentially expressed in GnRH neurons receiving VIPergic input on the afternoon of proestrous [137]. Furthermore, *in vitro*, VIP stimulates GnRH secretion [138-140], neural firing [141, 142], and intracellular calcium [142]. Finally, VIPergic

projections from the SCN to GnRH cells are sexually dimorphic, emerging during the pubertal transition into adulthood [13], with female rats demonstrating higher VIPergic innervation than males [136]. Notably, GnRH neurons do not express ERα, the estrogen receptor mediating positive and negative feedback effects of estradiol. This led researchers to search for estradiol-responsive systems upstream of the GnRH system that integrate circadian and estradiol signaling [143-145]. As described below, RFRP-3 and kisspeptin, have emerged as key, estrogen-responsive cell phenotypes upstream of the GnRH system that mediate circadian-controlled, estrogen negative and positive feedback, respectively.

B. Integration of circadian and estrogenic signaling upstream of the GnRH system

Early findings pointed to the AVPV as a likely neural locus regulating estradiol positive feedback and the LH surge. ERα expressing neurons the AVPV send monosynaptic projections to GnRH neurons, express FOS at the time of the LH surge, and AVPV lesions result in the loss of estrous cyclicity in intact and ovariectomized/estradiol-treated rats [24, 121, 146-148]. However, until relatively recently, the cell phenotype participating in estradiol positive feedback was unknown.

Following the discovery of kisspeptin, it was soon shown that ERα is expressed in the majority of AVPV and Arc kisspeptin cells [83, 149], with the AVPV kisspeptin cells mediating estradiol positive feedback and the Arc population negative feedback and GnRH pulsatility [150, 151]. The SCN sends monosynaptic AVPergic projections to AVPV estrogen-responsive kisspeptin cells (Figure 2) that, in turn, project to kisspeptin-receptor-expressing GnRH neurons in the POA to positively drive the LH surge [88, 90, 149, 152-157]. Significantly, the increased neural firing of kisspeptin cells in response to AVP is estrogen dependent, consistent with a role of kisspeptin cells in integrating estrogenic and circadian signaling [154].

In SCN lesioned rats and *Clock* mutant mice, central administration of AVP produces surge-like levels of LH [132, 158]. Importantly, exogenous AVP only stimulates LH when administered in the afternoon, the timepoint when the preovulatory LH surge occurs in rodents [159]. Given this time-dependent sensitivity, we asked if time-dependence of the surge was due to daily changes in sensitivity of kisspeptin cells to AVP stimulation, daily changes in GnRH cell sensitivity to kisspeptin stimulation, or a combination of both processes. In Syrian hamsters, AVPV kisspeptin cells do not demonstrate time-dependent changes in response to AVP stimulation, whereas GnRH neurons exhibit daily changes in their responsiveness to kisspeptin, suggestive of autonomous circadian timekeeping in cells downstream of the SCN [90]. Indeed, circadian oscillators of core clock genes that drive circadian rhythms at the cellular level are found both *in vitro* and *in vivo* in GnRH cells [138, 160, 161]. Immortalized GnRH neurons exhibit circadian rhythms in responsiveness to VIP and kisspeptin stimulation, further indicating that the GnRH system maintains circadian timing potentially as a mechanism mediating daily changes in responsiveness to upstream signaling [138]. Despite not exhibiting daily changes to AVP stimulation, kisspeptin cells express the clock gene PER1 and the AVPV exhibits sustained circadian rhythms in clock gene expression in cultured AVPV explants [153]. Whether these sustained rhythms in AVPV kisspeptin cells confer daily changes in responsiveness to upstream neurochemicals other than AVP remains to be determined.

A specific role for ARC kisspeptin cells in the generation of the preovulatory LH surge has not been established. However, several findings suggest a role for Arc kisspeptin cells in this process. For example, ablation of ARC kisspeptin cells results in abnormal estrous cycles and LH surges, indicating a potential role in generating the LH surge [162, 163]. Furthermore, this population expresses receptors for both AVP and VIP [164-166], and AVP and VIP increase intracellular calcium in subsets of Arc kisspeptin neurons in sexually dimorphic manner [167].

As mentioned previously, prior to the LH surge and ovulation, estradiol acts via negative feedback to maintain gonadotropins at low concentrations. Several lines of evidence indicate a role for RFRP-3 cells in integrating circadian and estrogenic signaling to mediate estradiol negative feedback. First, RFRP-3 cells exhibit a high activational state (as measured by FOS expression) during diestrus, reduced activity around the time of the LH surge, and increased activity soon thereafter [168]. This pattern of timing is mediated by the SCN, with both AVP/VIPergic SCN cell terminals forming close appositions with RFRP-3 cells in Syrian hamsters [168]. Likewise, RFRP-3 neurons express ERα and increase their activity in response to estradiol injections at the time of the LH surge [56, 169-171]. Additionally, analogous to effects seen for GnRH cells, VIP suppresses RFRP-3 cellular activity around the time of the LH surge, but not prior to the surge, suggesting that removal of RFPR-3-mediated estradiol negative feedback is accomplished via time-dependent sensitivity to VIP signaling [172]. This time-dependent sensitivity is associated with rhythmic clock protein expression in RFRP-3 cells [172]. Additionally, RFRP-3 cell terminals form close appositions with GnRH cell soma and terminals at median eminence, both of which express Gpr147 [56, 59, 173, 174]. The same impact of RFRP-3 is observed in both an estradiol surge implant model [168] and during the afternoon of proestrous [53]. Finally, in mice, RFRP-3 similarly inhibits LH secretion when administered at the time of the preovulatory LH surge, but not during diestrus [66]. Together, these findings suggest that RFRP-3 cells integrate estrogen and circadian signaling to time the removal of estradiol negative feedback with stimulation of the LH surge.

1.5 Implications for Circadian Rhythms in Pregnancy Success and Fetal Development

A. Circadian timing and pregnancy maintenance

The reproductive system is exposed to an array of rhythmic hormonal secretions during pregnancy [175, 176] and disruptions to maternal timing negatively impact pregnancy success and fetal development. In one early study using a chronic jet lag model in which mice were repeatedly subjected to a 6 h phase advance or delay of the light:dark cycle, jet-lagged animals exhibited a dramatic reduction in full-term pregnancy success [177]. Likewise, female mice maintained in 22 or 26 h light:dark cycles, cycles to which they cannot entrain, exhibit decreased mating behavior and experience higher rates of fetal resorption, reduced embryo weights, and delayed development [178].

Prolactin, a key hormone regulating pregnancy maintenance, is under strict circadian control in rodents. In mice and rats, for example, prolactin exhibits twice daily surges following pregnancy (a diurnal and nocturnal surge) that maintains the viability of the corpora lutea (CL) and the secretion of progesterone in the first half of pregnancy. Around mid-pregnancy, these surges cease and placental lactogens maintain progesterone secretion for the remainder of gestation

[179-183]. The SCN regulates prolactin release through the pacing of inhibiting and stimulating factors for this hormone, principally dopamine (DA) and oxytocin, respectively [180, 184, 185]. This regulation likely occurs via VIPergic projections from the SCN, as both arcuate tuberoinfundibular DA (TIDA) neurons and paraventricular nucleus (PVN) oxytocin neurons are innervated by VIPergic fibers originating in the SCN [14, 186-189]. In addition, VIP antisense oligonucleotides aimed at the SCN abolish prolactin surges in rats [133]. As would be expected given this mechanism of control, SCN lesions abolish prolactin surges [190, 191]. Likewise, prolactin surges entrain to light:dark cycles and free-run in constant darkness [192, 193]. Finally, knockdown of essential clock genes (*Per1, Per2*, and *Clock*) in the SCN abolish prolactin surges [194], and mice lacking a functional *Clock* gene exhibit reduced concentrations of progesterone and marked pregnancy failure, suggestive of disrupted prolactin timing [22]. These findings point to the critical role of precisely-timed prolactin secretion for the maintenance of pregnancy.

In addition to SCN regulation of TIDA and oxytocin neurons, kisspeptin cells project to TIDA neurons and kisspeptin administration increases prolactin release via dopaminergic cell inhibition [75, 195-197]. Furthermore, kisspeptin neurons express prolactin receptors, and hyperprolactinemia causes reduced activity of hypothalamic kisspeptin, indicating reciprocal communication between kisspeptin and TIDA neurons [75, 198]. As with TIDA and oxytocin regulation by the SCN, arcuate kisspeptin cells express VIP receptor and VIP administration alters their cellular activity [167], suggesting that the SCN likely regulates TIDA neurons directly and indirectly, via kisspeptin cells, through VIPergic signaling. Whether or not disruptions to kisspeptin cell timing affect prolactin release and pregnancy success remains to be determined.

In contrast to rodents, the pituitary gland (or pituitary hormones) is not required for the initiation and maintenance of pregnancy in humans. In the first 8 weeks of pregnancy the CL is maintained by human chorionic gonadotropin (hCG) produced by the trophoblast (a layer of tissue that later forms part of the placenta), with placental progesterone sufficient to maintain pregnancy thereafter [199]. Although humans do not exhibit a twice daily prolactin surge and progesterone secretion is maintained via the placenta, circadian disruptions profoundly reduce pregnancy success as seen in rodents [9, 200-202]. Given that the placenta is responsible for fetal-maternal nutrient exchange and maintains rhythmic clock gene and hormonal expression, it is likely that circadian disruption negatively influences pregnancy success in humans via disruptions to this structure, a hypothesis that has not yet been explored.

B. Molecular clockwork and pregnancy success

Molecular clockwork plays a key role in regulating pregnancy success, with anomalies in core clock genes resulting in marked female reproductive deficits. For example, particular single nucleotide polymorphisms in the *Bmal1* or *Clock* genes are associated with a higher rate of miscarriage in women [203, 204]. In animal models, mice with mutations or genetic ablation of clock genes (*Clock*, *Bmal1*, *Per1*, *Per2*) exhibit deficiencies in implantation, pregnancy maintenance, parturition, and have higher rates of fetal resorptions (i.e., miscarriage) [34, 205-208]. These mouse models also exhibit shorter duration prolactin surges or failure to initiate prolactin release following mating and pregnancy [189]. Likewise, *Clock* mutant mice exhibit reduced concentrations of progesterone, higher rates of fetal resorptions, and fewer full-term pregnancies [22, 208, 209], all likely do to altered prolactin secretion.

Notably, although clock gene knockout and mutant mice exhibit reduced fertility, these animals lack the gene-of-interest systemically and permanently (i.e., clock gene expression is absent in all tissues throughout development). Hence, it is not possible to determine whether the reproductive deficits originate through circadian disruption in specific reproductive tissues and/or specific time points. For example, it is possible that lacking essential clock genes throughout development leads to abnormal establishment of circuits required for mating and pregnancy maintenance (i.e., so-called organizational effects). It is equally possible that a dysfunctional cellular clockwork results in reproductive deficits through abnormal timing in these same circuits in adulthood (i.e., so-called activational effects). Moreover, even when reproductive deficits do not manifest in knockout mice, it is possible that compensatory mechanisms permit typical mating and pregnancy success. Studies applying approaches that allow for temporal and spatial specificity of genetic manipulations would help to disambiguate developmental and post pubertal roles of the circadian clockwork (e.g., *Bmal1* knockdown specifically in the SCN following puberty).

C. Circadian timing in early in fetal development

The developing embryo is exposed to a circadian environment as early as its initial migration from the oviduct into the uterus. The oviduct provides essential nutrients and growth factors to the developing embryo and, although studies examining oviduct rhythmicity are sparse, findings suggest that the oviducts regulate the embryonic environment via the rhythmic expression of clock genes as well as *plasminogen activator inhibitor 1* [210-212], a clock-controlled gene that is involved in oviduct activity during peri-implantation and is thought to play a role in embryo protection [213]. The uterus also exhibits rhythmic expression of clock genes, in both non-pregnant and pregnant females, throughout different stages of pregnancy and under constant conditions [211, 212, 214-218]. Finally, another key structure in pregnancy maintenance and regulation, the placenta, exhibits rhythmic expression of clock genes as well as glucocorticoid receptor and glucocorticoid metabolic enzyme expression [218-220]. The placenta is responsible for maternal-fetal exchanges, secreting hormones to maintain gestation and promote the health of the fetus. Taken together, the developing conceptus is exposed to a rhythmic environment from its first day, long before developing its own autonomous clock. As suggested below, this rhythmic environment is likely critical for normal fetal and postnatal development (**Figure 3**).

D. Maternal-fetal rhythm synchronization

Circadian disruptions that alter maternal endocrine timing signals can impair maternal-fetal synchronization and fetal development (**Figure 3**). A major maternal signal providing photoperiodic information to the embryo is melatonin, secreted from the maternal pineal gland at darkness. Melatonin crosses the placenta and influences the embryo's physiological rhythms and development [221-223]. Melatonin receptors are widespread in the fetal nervous system (including the SCN), as well as in peripheral organs, beginning early in fetal development [224-228]. Likewise, the placenta expresses melatonin receptors, and melatonin plays a role in placenta development [176]. Night-time melatonin secretion increases throughout pregnancy [229], and melatonin rhythms in maternal blood are mirrored in fetal circulation [223, 230]. In addition to melatonin, fetal rhythms are also entrained by maternal cortisol and body temperature rhythms [231], and possibly feeding times [232] (**Figure 3**).

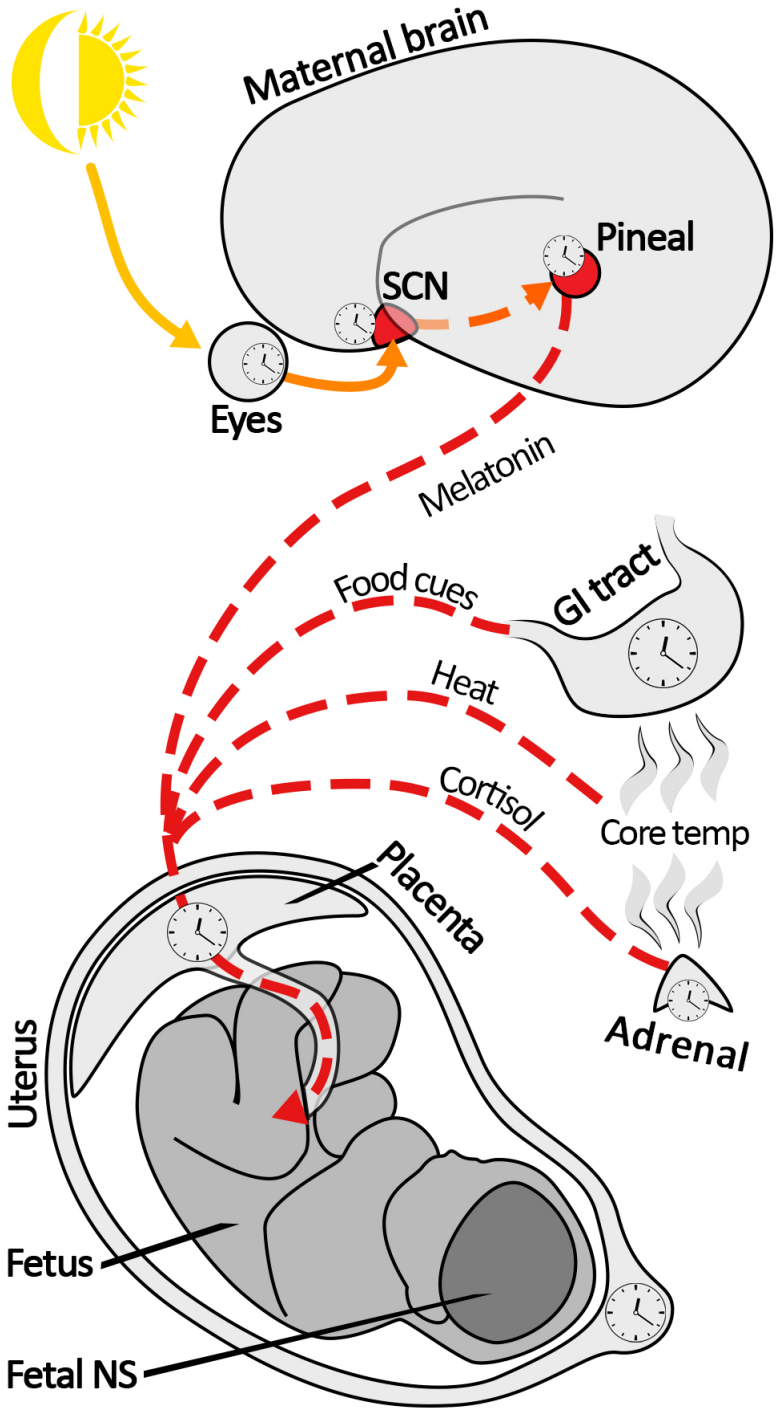

Figure 3. Maternal-fetal rhythm synchronization. The developing fetus is exposed to an array of time cues from its mother. The main signal providing rhythmic information is melatonin, secreted from the mother's pineal gland at darkness and crossing the placenta to the embryo. Additional entraining signals include maternal cortisol, body temperature rhythms and feeding times. In addition, the developing embryo is exposed to a rhythmic environment via clock genes that are rhythmically expressed in maternal reproductive tissues, including the oviduct, the uterus, and the placenta. This rhythmic environment is likely critical for normal fetal and postnatal development. Circadian disruptions that alter maternal endocrine timing signals can impair maternal-fetal synchronization and fetal development. Clocks indicate rhythmic expression of clock genes.

Because the pineal gland matures only after birth and the developing fetus and newborn do not produce their own melatonin, maternal melatonin signaling is conveyed to offspring through the placenta (*in utero*) and milk (after birth) and is required for fetal/newborn rhythms. Thus, it is not surprising that maternal circadian disruptions are reflected in fetal rhythms. In non-human primates, for example, maternal light exposure disrupts rhythmic expression of fetal clock genes that can be rescued by maternal melatonin administration [233]. Likewise, suppression of maternal melatonin by constant light exposure during pregnancy is associated with intrauterine growth restriction (IUGR), lower concentrations and altered rhythms of cortisol, modified mRNA expression of clock genes and clock-controlled genes in the fetal adrenal gland, and aberrant adrenal response to adrenocorticotropic hormone (ACTH) in rats. Melatonin administration during the subjective night rescues all of the above [234]. In *in vitro* fertilization (IVF) treatments, melatonin promotes embryo development *in vitro* [235-237]. Likewise, melatonin administration prior to IVF treatments and throughout pregnancy is associated with improved pregnancy outcomes; fertility rates are 50% higher in melatonin treated IVF cycles [238, 239]. These findings raise questions regarding the static nature of the environment that the conceptus is typically exposed to in cultured IVF conditions and whether a rhythmic environment that better mimics the *in vivo* milieu would increase IVF success rates. Likewise, the timing of IVF embryo implantation may be important to treatment success, but this remains to be determined. Nonetheless, the dependence of fetal/early postnatal rhythms on maternal environment further underscores the importance of maintaining circadian health during pregnancy and lactation.

E. Circadian disruption and fetal/offspring development

Because almost every aspect of female reproduction is regulated by the circadian system, it is not surprising that circadian disruption is associated with a host of negative reproductive outcomes. For example, shift work during pregnancy is associated with preterm birth, low birth weight, small for gestational age births, and increased risk of miscarriages [9, 200-202]. Taken together with findings indicating the prominence of circadian rhythms during pregnancy, these findings indicate that environmental temporal disruption can perturb endogenous timing and have marked negative consequences for developing progeny.

In animal models, the impact of chronic jetlag has been shown to negatively impact a host of mental and physical health parameters in both adults and developing offspring. For example, circadian disruption through twice weekly, 6 h advances of the light:dark cycle, decreases hippocampal cell proliferation and neurogenesis by >50% and leads to pronounced deficits in learning and memory in adult hamsters [240]. Although not examined, it is likely that animals can recover from these deficits after a period of time. However, in developing offspring, the impact of circadian disruption appears to be permanent, with the impact of *in utero* circadian disruption lasting until adulthood likely by modifying neural development. For example, fetal hippocampal clock and clock-controlled gene rhythms are suppressed by maternal exposure to constant light, leading to deficits in spatial memory in these offspring as adults [241]. These adult deficits are associated with dampened circadian rhythms of hippocampal clock and clock-controlled genes. Intriguingly, these deficits can be rescued by providing pregnant dams with rhythmic, exogenous melatonin [241], pointing to a potential for clinical interventions for women unable to escape circadian disruption during pregnancy. Additionally, *in utero* or early life circadian disruption impairs the ability of mice to elicit maternal care, even when crossed-fostered to non-disrupted

dams, and leads to adult deficits in social behavior and anxiety [242]. Taken together, these findings indicate that circadian disruption can have long lasting, likely permanent, impact on offspring neurobehavioral development.

1.6 Circadian control of birth

The length of gestation is determined by multiple clocks, including a principal clock monitoring fetal development and additional clock-regulated mechanisms governing labor onset and parturition timing. Fetal membrane senescence is thought to initiate the signaling cascades leading to parturition via inflammatory processes that increase uterine sensitivity to uterotonins, including prostaglandins and oxytocin (reviewed in [243]. For the sake of the present review, we focus on the circadian regulation of parturition via maternal neuroendocrine pathways, although parturition is a process regulated by multiple clocks at multiple levels (i.e., mother, fetus, and placenta) (**Figure 3**).

As different species adapted to specific temporal niches over the course of evolution, a selective advantage was gained by initiating parturition during the daytime or nighttime dependent of selective pressures. Because delivering offspring in the home den is safer than in the open areas for prey species, many diurnal species have evolved to initiate parturition at nighttime and nocturnal species at daytime. Circadian timing of labor onset or parturition has been reported in mice, rats, hamsters, sheep, pigs, horses, and primates, including humans [244-256]. In rats, the length of pregnancy is influenced by the light regimen in which animals are maintained, with longer day lengths leading to later parturition (i.e., gestational day 23 instead of 22) [257]. Clock genes are likely involved in timing parturition, as mice lacking a functional molecular clockwork either fail to enter labor or have prolonged and non-productive parturition, resulting in resorption of the fully developed embryos [22]. Furthermore, as described previously, *Clock* mutant mice experience higher rates of fetal resorptions and fewer pregnancies reaching term.

In humans, labor onset and parturition tend to cluster between the late night and early morning, exhibiting a trough in the late afternoon [9, 253, 254, 258]. A similar circadian pattern has been reported in the timing of parturition among indigenous populations living in rural areas [255] and in the onset of preterm labors [259]. Underlying the circadian rhythm of labor and birth may be a rhythm in uterine myometrial activity, with contractions peaking at nighttime [249-251]. Moreover, the nocturnal surge in uterine activity in the last trimester of gestation can predict preterm deliveries, with women who deliver prematurely losing these nocturnal surges weeks before birth [250]. Together, these findings suggest that the mechanisms underlying parturition are under circadian regulation across species.

The species-specific difference in circadian phase of parturition likely results from antiphase uterine cell responses to endocrine signals. In humans, uterine contractions exhibit a peak at night and a trough in the morning [249, 250]. This pattern may result from motility-enhancing factors, such as oxytocin, estrogens and prostaglandins, being higher at nighttime [249, 260], and from the actions of nighttime melatonin secretion from the pineal gland [176, 261]. In a proof of principle study, late-term pregnant women exposed to bright light at night exhibited suppression in plasma melatonin concentrations that were associated with a reduction in contraction intensity [254], likely resulting in the potentiation of oxytocin's actions on the uterus

[262]. Whether night-time melatonin potentiates the actions of Pitocin (synthetic oxytocin) in women induced into labor, potentially achieving a faster and safer process, has not been explored.

In contrast to humans, melatonin exhibits a negative (tocolytic) effect on oxytocin-induced uterine contractility in nocturnal rodents [252, 253, 256, 263]. Thus, although oxytocin stimulates uterine contractions in both diurnal and nocturnal species, and melatonin peaks at night in both cases, this hormone exhibits opposite effects on the myometrium. Rats in which the source of melatonin is eliminated via pinealectomy deliver their pups independent of time of day. However, when melatonin is administered to pinealectomized females at the onset of the dark phase, the circadian rhythm of parturition is rescued. If melatonin is administered at the onset of the light phase or in a constant release manner (i.e., via capsules), the circadian pattern of deliveries is not regained [252]. This finding suggests that melatonin signaling is required at the appropriate time to initiate birth, likely due to interactions with other regulators of this complex process (e.g., oxytocin).

1.7 Stress and the Reproductive System

The circadian timing system participates in all aspects of female reproduction, from ovulation to childbirth. Disruptions to temporal homeostasis, either by circadian disruption or by stress-exposure, have marked negative consequences for ovulation, pregnancy success and maintenance, and offspring development.

Hypothalamo-Pituitary Adrenal (HPA) Axis

The HPA axis regulates arousal and energy mobilization under typical conditions and rapidly appropriates energy from stored sources in response to a stressor to facilitate the fight or flight response. Analogous to the HPG axis, the hypothalamic peptide, corticotropin-releasing hormone (CRH), released into the anterior pituitary blood supply stimulates the release of adrenocorticotropic hormone (ACTH). ACTH released into systemic circulation, in turn, acts on the adrenal cortex to stimulate glucocorticoid (CORT) release. CORT acts broadly within the brain and body through negative feedback to inhibit its own production. Humans, non-human primates and rodents exhibit pronounced rhythms in CORT that persist in constant conditions, rising prior to waking and falling in anticipation of sleep [2, 264, 265]. Like the ovaries, the adrenal glands exhibit rhythms in clock gene expression that likely drive daily changes in responsiveness to ACTH stimulation and stress [266-269]. Rhythms in adrenal CORT secretion and clock gene expression are eliminated by SCN lesions [270, 271], suggesting that circadian rhythms in individual cells of the adrenal become uncoupled in the absence of SCN input.

The SCN drives rhythmic secretion of CORT through two apparent pathways. The first pathway indirectly targets CRH neurons in the paraventricular nucleus of the hypothalamus (PVN) through vasopressin-ergic SCN projections to an area just below the PVN (the subPVN) and the dorsomedial hypothalamus (DMH) [272-274]. In turn, the subPVN and DMH regulate CRH production. Secondly, the SCN continues through this PVN pathway, sending autonomic outflow to the adrenals through a multisynaptic projection [275]. As removal of the pituitary (and resulting abolition of ACTH secretion) does not alter clock gene rhythmicity in the adrenals [268], it is

likely that SCN control of autonomic input to the adrenal is responsible for the maintenance of coordinated adrenal cellular clocks.

Glucocorticoids can act throughout the periphery to set the phase of oscillators in individual peripheral systems without impacting the SCN and facilitate re-entrainment following jet lag [276-279]. As a result, disruptions to CORT rhythms have far-reaching, negative impact on typical functioning. Travel in humans and experimental jet lag in rodents increases CORT [240, 280], contributing to negative health consequences of circadian disruption.

Reproduction and Fecundity

Disruptions to endocrine rhythms are deviations from temporal homeostasis, which, in turn, provoke a stress response. A myriad number of human and animal studies have demonstrated the negative impact of stress on reproduction and fecundity. In many cases, decreased conception and increased miscarriage rates are associated with psychosocial or physiological stress. In humans, maternal stress is associated with increased risk for miscarriages, preterm birth, and low birth weight, particularly if stress occurrs in the first trimester of pregnancy [281-284]. Socioeconomic disadvantaged women are especially vulnerable to adverse pregnancy outcomes. Likewise, psychological stress in the form of depression and anxiety is associated with increased risk for preeclampsia, lower birth weight, and reduced head circumference [282, 283, 285, 286]. In animal models, stress exposure or administration of glucocorticoids result in higher rates of embryo resorption (the rodent equivalent of a miscarriage), reduced litter size, and intrauterine growth restriction (IUGR) [287-289]. The impact of stress on offspring development persists beyond pregnancy and birth; maternal stress is associated with increased risk for adverse health outcomes later in life, affecting offspring's metabolism, cardiovascular system, and immunity [290-292], as well as increasing the risk for neurodevelopmental disorders [293-296].

Prenatal effects of stress are thought to be mediated by increased maternal HPA activity and the consequential rise of circulating glucocorticoids [297, 298]. One potential pathway by which stress can affect the HPG axis and pregnancy is via neurons expressing the inhibitory neuropeptide RFamide-related peptide-3 (RFRP-3; the mammalian ortholog of avian gonadotropin-inhibitory hormone (GnIH) [46, 48]. RFRP-3 neurons are concentrated in the dorsomedial hypothalamus (DMH) and project to brain regions that contain GnRH neurons and fibers as well as non-GnRH loci [56]. Across mammalian species, including humans, RFRP-3 generally suppresses the reproductive axis via direct actions on GnRH cells and potentially at the level of the pituitary [56, 58-61]. RFRP-3 neurons are markedly regulated by environmental and psychosocial factors, including stress [7, 299-301]. In male mice and rats, acute and chronic immobilization stress increases RFRP-3 mRNA and protein expression [300, 301]. Likewise, restraint stress increases RFRP-3 cell activation and expression in female mice [302] and rats [299]. RFRP-3 neurons express glucocorticoid receptor (GR) [300, 303], suggesting direct impact of glucocorticoids on these cells. In a study of chronic stress when stress exposure concluded prior to mating and pregnancy, knocking down RFRP-3 during stress prevented stress-induced reproductive dysfunction [299], suggesting the involvement of these cells in mediating the negative impact of stress on reproductive functions. The means by which RFRP-3 negatively affects subsequent pregnancy remains unknown.

1.8 Goals

This dissertation is focused on the neuropeptide, RFRP-3, that inhibits GnRH neurons and is also regulated by environmental and psychosocial factors, providing a mechanistic link between circadian disruption, stress, and fertility outcomes. In chapter 2, I explored the role of RFRP-3 in daily timing of ovulation. Ovulation occurs within a discrete time window across mammals, and there is complex regulation of GnRH neurons that allows for precise timing of this event. I hypothesized that there are changes in sensitivity of the GnRH system to RFRP-3 across the day and these changes are controlled by the circadian timing system. In chapters 3-5, I investigated how stress acts on the brain and periphery to impair pregnancy success. In chapter 3 I focus on the regulation of ovarian progesterone production during pregnancy in response to stress. In chapter 4 I investigate whether stress decreases pregnancy success through activating RFRP-3 neurons and whether these neurons communicate with TIDA neurons that regulate prolactin secretion. Chapter 5 asks whether stress-related changes occurring in early pregnancy are maintained across pregnancy past the cessation of stress, what are the morphological and functional effects on the placenta, and how these affect fetal development. Finally, I conclude with a broad consideration of my work as a whole and propose avenues for future research.

1.9 Acknowledgements

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2. Time-of-day-dependent Sensitivity of the Reproductive Axis to RFamide-related Peptide-3 Inhibition in Female Syrian Hamsters

2.1 Abstract

In spontaneously ovulating rodent species, the timing of the luteinizing hormone (LH) surge is controlled by the master circadian pacemaker in the suprachiasmatic nucleus (SCN). The SCN initiates the LH surge through the coordinated control of two, opposing neuropeptidergic systems that lie upstream of the gonadotropin-releasing hormone (GnRH) neuronal system, the stimulatory peptide, kisspeptin, and the inhibitory peptide, RFamide-related peptide-3 (RFRP-3; the mammalian ortholog of avian gonadotropin-inhibitory hormone (GnIH)). We have previously shown that the GnRH system exhibits time-dependent sensitivity to kisspeptin stimulation, further contributing to the precise timing of the LH surge. To examine whether this time-dependent sensitivity of the GnRH system is unique to kisspeptin, or a more common mechanism of regulatory control, we explored daily changes in the response of the GnRH system to RFRP-3 inhibition. Female hamsters were ovariectomized to eliminate estradiol (E2) negative feedback and RFRP-3 or saline were centrally administered in the morning or late afternoon. LH concentrations and *Lhβ* mRNA expression did not differ between morning RFRP-3-and saline-treated groups but were markedly suppressed by RFRP-3 administration in the afternoon. However, RFRP-3 inhibition of circulating LH at the time of the surge does not appear to act via the GnRH system as no differences in mPOA *Gnrh* or RFRP-3 receptor *Gpr147* mRNA expression were observed. Rather, RFRP-3 suppressed arcuate nucleus *Kiss1* mRNA expression and potentially impacted pituitary gonadotropes directly. Together, these findings reveal time-dependent responsiveness of the reproductive axis to RFRP-3 inhibition, potentially via variation in the sensitivity of arcuate nucleus kisspeptin neurons to this neuropeptide.

2.2 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 [5, 9, 10, 304]. 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 [10, 305-307]. 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 [108, 113, 308] and in late afternoon in nocturnal rodents [168, 309, 310]. 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 maturing follicles develop during the follicular phase of the ovulatory cycle, increasing concentrations of E2 are secreted to maintain LH at low concentrations through negative feedback. However, just prior to ovulation, peak E2 concentrations act through positive feedback to initiate the LH surge that triggers ovulation [117, 304, 306, 307, 311-313]. Previous findings by our group and others suggest that the temporary shift from negative to positive feedback is coordinated by the SCN [6, 122, 168, 172, 313].

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 GnRH neurons, with FOS expression increased in GnRH neurons receiving VIP input around the time of the LH surge [137, 314]. GnRH neurons do not express estrogen receptor α (ERα), the receptor subtype that mediates E2 positive and negative feedback. 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 ortholog of avian gonadotropin-inhibitory hormone (GnIH)) [7, 10, 53, 56, 90, 152, 154, 168, 172, 315, 316]. The SCN coordinates cellular activity of RFRP-3 neurons to suppress the reproductive axis outside the time window of the LH surge and allow for the transient suppression of E2 negative feedback around the time of the surge [53, 168, 172]. 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) in addition to the ventromedial nucleus of the hypothalamus and brainstem [56]. RFRP-3 cell projections form direct contacts with GnRH neurons expressing the RFRP-3 receptor, GPR147 [56, 315, 317]. Furthermore, RFRP-3 directly suppresses GnRH neuron activity and consequent LH release [56, 61-63, 90, 172, 318]. In some species, RFRP-3 neurons may also act on the anterior pituitary to mediate LH release as RFRP-3 neurons directly project to the median eminence and GPR147 is expressed in the pituitary [58, 168, 318-320]. Finally, RFRP-3 may modulate the HPG axis via a subpopulation of arcuate nucleus (ARC) kisspeptin neurons that express GPR147 [321].

Concomitant with RFRP-3 suppression at the time of the LH surge, the SCN stimulates kisspeptin neurons located in anteroventral periventricular nucleus (AVPV) that, in turn, stimulate

the GnRH system and the LH surge [90, 138, 153, 314]. Whereas 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 [90]. This additional mechanism of temporal control likely 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 if daily changes in reproductive system sensitivity also occur in response to RFRP-3 inhibition to enhance the precision of the 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 typically transiently inactive [66, 168, 172]. 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 through 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 gonadotropes, or indirectly via kisspeptin neurons in the ARC that control GnRH pulsatility [157, 322, 323]. 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).

2.3 Material and Methods

Animals

Thirty-four >8-week-old female Syrian hamsters (*Mesocricetus auratus*) were purchased from Charles River (Wilmington, MA) and maintained on a 14:10 light:dark cycle (lights on at 06:00, lights off at 20:00) at 23 ± 1°C with food and water available *ad libitum*. A 14:10 light:dark cycle was employed to create a "long day" light regimen, as 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.

Experimental Procedure

Surgical Procedures

After a 2 wk acclimation period, all hamsters were ovariectomized (OVX) to eliminate E2 negative feedback. Surgeries were conducted under isoflurane anesthesia with buprenorphine (s.c., 0.1 mg/kg) provided for analgesia. After a 2 wk recovery, a guide cannula (22GA, 6 mm; PlasticsOne, San Diego, CA, USA) was stereotaxically implanted under deep anesthesia

(ketamine-xylazine cocktail (i.p., 60/5 mg/kg) directed at the lateral ventricle. For cannular implantation, the head was shaved, prepared for surgery, and animals were placed in a stereotaxic apparatus (Kopf, Tujunga, CA). Guide cannulae were placed at the following 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; PlasticsOne, San Diego, CA, USA) 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). Following the procedure, hamsters were singly housed for the remainder of the study. Animals were given 1 wk 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.

Pharmacological manipulations and sample collection

Five µl of saline or RFRP-3 (100 or 500 ng in saline) (Syrian hamster RFRP: ILSRVPSLPQRF-NH2, purchased from Phoenix Pharmaceuticals, CA, USA) were injected (i.c.v.) in the morning (3 h after lights on, n=6/group) or in the afternoon (3h before lights off, n=6-7/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 mins following injection and centrifuged at 1400 RCF for 15 mins. Serum was collected and stored at -20˚C until assayed. 2 weeks later, animals were injected again with RFRP-3 (100 ng/5 µl saline) or saline (5 µl) in the morning or afternoon (n=6-9/group) and animals were sacrificed 2h later. Brains and pituitaries were flash frozen and brains were sectioned at 300 µm and transferred to RNAlater (AMBION, AM7021, Grand Island, NY, USA) for one night at 4˚C and -20˚C thereafter until further processed. 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 mPOA and AVPV bilaterally (**Figure 1**). RNA was extracted using ISOLATEII RNA mini kit (Bioline, BIO-52073, Memphis, TN, USA) and reversed transcribed for RT-PCR (iScript RT supermix, BIO-RAD, 170-8841, Hercules, CA, USA). A random, representative sample of RNA (for each tissue n=8) was assessed for RNA quality on an Agilent Technologies Bioanalyzer and yielded an average RNA integrity number (RIN) of 7.3 or higher. To confirm LH suppression and further validate the detection levels of the LH ELISA at low concentrations, 5 hamsters were injected with estradiol benzoate (EB, 100 µg in 200 µl sesame oil) and retro-orbital blood samples were collected 90 min later as described above.

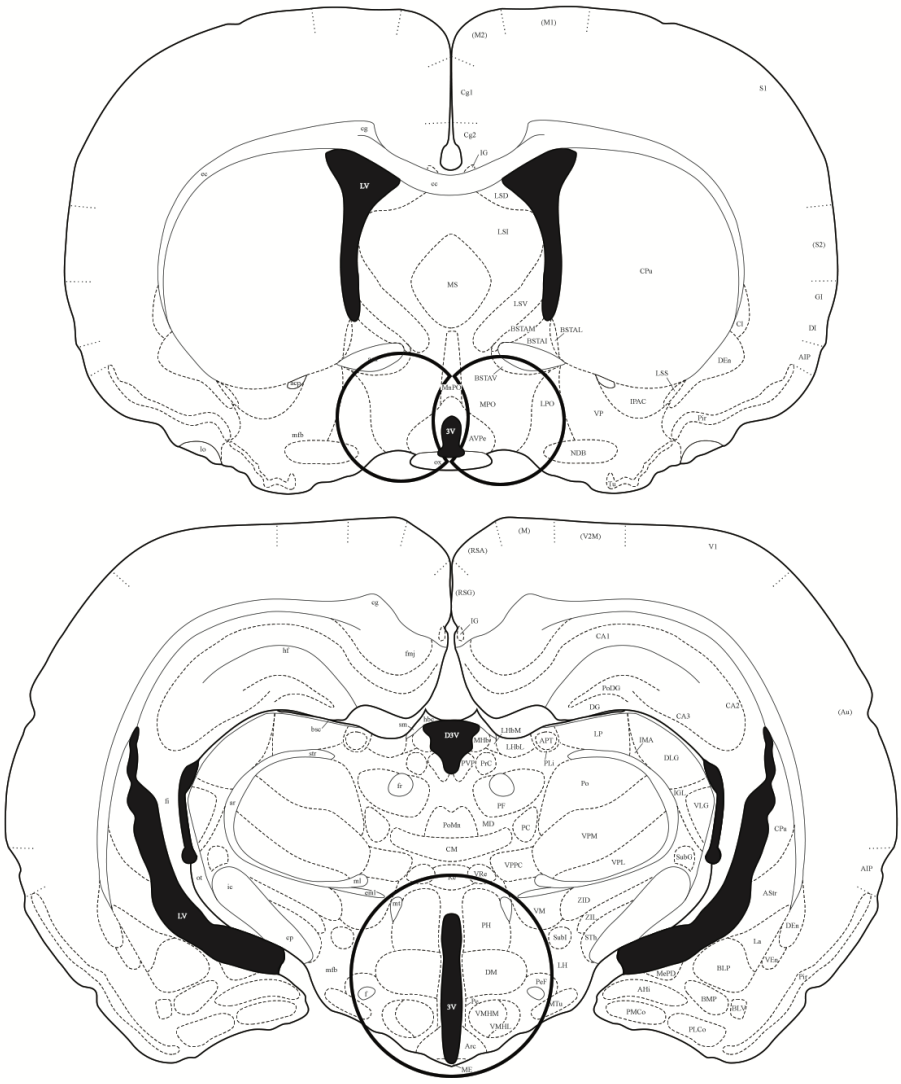

Figure 1. An illustration of the location of samples punched for RT-PCR analysis. Brains were flash frozen and cut at 300µm and then transferred to RNAlater for one night. A 2mm biopsy punch was used to microdissect the mPOA and AVPV bilaterally (left), and a 3mm biopsy punch was used to microdissect the DMH and ARC in a single punch (right). Illustrations adapted and modified from the Stereotaxic Atlas of the Golden Hamster Brain by L.P Morin and R.I Wood (2000) [341].

qRT-PCR

Analysis of relative gene expression via qRT-PCR was performed using SSOAdvanced SYBR Green supermix (BIO-RAD, 1725272, Hercules, CA, USA). Samples were run on a BIO-RAD CFX384 machine with 10µl reaction volumes with a 2-step amplification for 40 cycles followed by a melt curve. Primers were designed from published sequences for Syrian hamsters using NCBI Primer BLAST software (**Table 1**). Primer sets were validated for specificity using positive, negative, no reverse transcriptase, and no template controls, and confirmed with a single-peak melt curve and 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 resulting data were analyzed in Microsoft Excel following the delta delta Cq method [324]. The geometric mean of 2 housekeeping genes' expression 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 the pituitary (Cq ranges were 20.3-24.26 and 20.6-24.64, respectively), whereas *Hmbs* and *Tbcc* were used as reference genes for the DMH and ARC (Cq ranges were 23.9-26.8 and 21.35-24.24, respectively), and *B2m* and *Rplp16* were used as their 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 between all groups, and in all gene replicate groups Cq Standard deviation was smaller than 0.2. Whereas *Kiss1* mRNA expression was measured in the DMH and ARC, it was not assessed in the AVPV and POA due to late and unstable amplification, indicating low mRNA expression, possibly as a result of the OVX. All data are expressed as a fold-change over morning, saline hamsters. Some samples did not have sufficient cDNA to quantify the expression of all genes, thus sample sizes vary for different genes measured.

Primer	Forward	Reverse	Product Size
Lhβ	CGGCTACTGTCCTAGCATGG	AGGCGGACAGATGTGAAGTG	102
Gnrh-r	TCATCTTCACCCTCACACG	GTGGCAAATGCGACTGTCAT	121
Gnrh	AGGGACCTTCGAGGAGTTCT	TGTGGATCCTTTGGTGCTGAT	88
Kiss1	TGGTTATCTTTGACCTCCGGC	TGCCAAGAAGCCAATGTGGT	105
Gpr147	CCGGTTGGCCTTTTGACAAT	CAGCTTCTCACGGAAAGGGT	140
Gapdh	ACAGTCAAGGCTGAGAACGG	TCCACAACATACTCGGCACC	116
Actb	GACCCAGATCATGTTTGAGACC T	TCCGGAGTCCATCACAATGC	112
B2m	TGGCCGTGGTCTTTCTGATG	TGGAACTGCGACACATAGCA	139
Rplp16	ATCTACTCCGCCCTCATCCT	GCAGATGAGGCTTCCAATGT	159
Hmbs	TATCCTGGATGTTGCACGGC	TCTCAACACCCAGTGGTTCA	165
Tbcc	CAGTGGGACTGAGCACTAGC	TAGCAAAAGCCCCGGGTTAG	156

Table 1. Primers used for qRT-PCR.

Assessment of LH levels

LH concentrations were quantified with an ELISA, using a modified protocol that was kindly provided by Jens D. Mikkelsen (Copenhagen University Hospital, Denmark) [64], and all samples were run in duplicate. Briefly, 96-well microtiter 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 3 times with 200 µl of 10mM PBS with 0.05% Tween 20 (PBS-T). Plates were blocked for 1h 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 h at room temperature. Plates were then washed and 50µl of rabbit polyclonal LH antibody (AFP240580Rb, AF Parlow, National Hormone and Pituitary Program, University of California, Harbor Medical Center, Los Angeles, CA, USA) were added into each well and incubated for 90 mins at room temperature. After washing, a 1:2000 dilution of polyclonal goat anti-rabbit IgG conjugated to horseradish peroxidase (DAKO Cytomation, catalog # P0448, Santa Clara, CA, USA) was added to each well and incubated for 1 h at room temperature. After washing, o-Phenylenediamine (OPD, Invitrogen, catalog # 00-2003, Camarillo, CA, USA) in citrate buffer was added to each well and the reaction was allowed to proceed for 30 mins at room temperature in darkness before being stopped by the addition of 3M 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 (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 min following E2 benzoate (EB) administration used to suppress LH concentrations. Assay sensitivity was 0.002 ng/ml and intra- and inter-assay variability were 1.1% and 3.4%, respectively.

Statistical analysis

Group comparisons were examined using a two-way analysis of variance (ANOVA). In instances which assumptions of normality and/or equal group variance were violated, data was analyzed by planned contrasts for comparisons between specific groups was made on the basis on *a priori* hypotheses and corrected for multiple comparison with Bonferroni's inequality test. Statistical analyses were performed in SPSS (Armonk, New York, USA) and Prism (San Diego, CA, USA). All data are reported as mean ± standard error of the mean (SEM) with $p < 0.05$ considered statistically significant. The data that support the findings of this study are available from the corresponding author upon reasonable request.

2.4 Results

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 to the afternoon, baseline LH concentrations (saline groups) were significantly different across the day, increasing from 18.65 ng/ml ± 3.8 in the morning to 29.6 ng/ml ± 4.3 in the afternoon (p<0.044,

95% confidence interval: -1.998 to 23.87) (**Figure 2**, n=6-7/group). 100 ng RFRP-3 significantly decreased circulating LH concentrations in the afternoon 20 mins after administration (from 29.6 ng/ml ± 4.3 to 14.7 ng/ml ± 2.8 (p<0.006, 95% confidence interval: -25.89 to -3.883). At this same dose, no differences were found between saline and RFRP-3 administration in the morning (p>0.05). No effects were observed with the 500 ng dose of RFRP-3 (p>0.05 in all cases; data not shown). Finally, EB markedly suppressed LH concentrations 90 mins post administration (decreasing to 5.47 ng/ml ± 0.9, t=2.808, p<0.01, data not shown), further validating the LH assay.

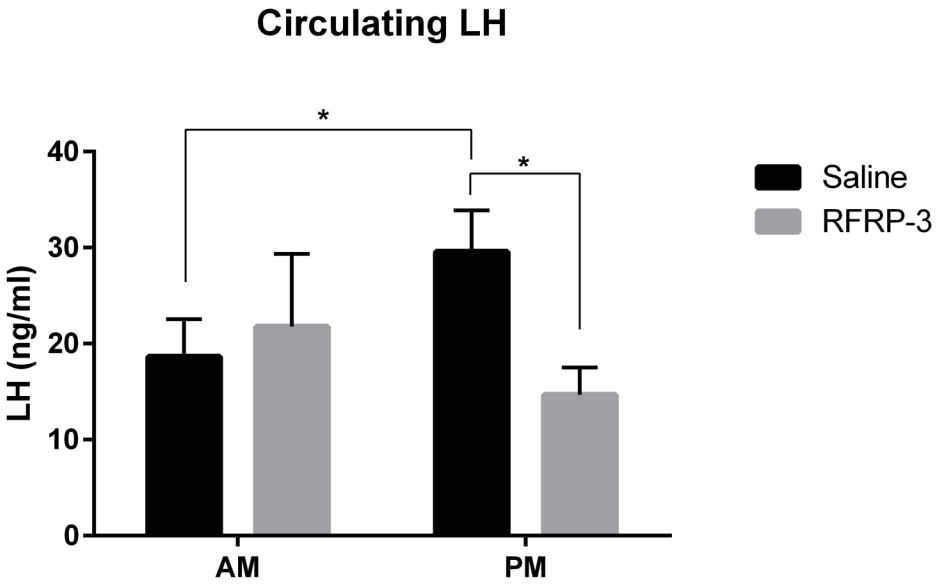

Figure 2. Central administration of RFRP-3 (100 ng) inhibits circulating LH in the afternoon 20 min post administration. n = 6, 6, 6, 7, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively. Data are presented as mean ± standard error of the mean. * p < 0.05.

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) (**Figure 3**, n=6-9/group). 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<0.04, 95% confidence interval: -1.092 to 0.07406) but not in the morning (p>0.05), compared to saline, consistent with the impact of this peptide on circulating LH. In contrast, pituitary *Gnrh-r* mRNA expression did not differ at any time point regardless of treatment. However, a significant time X treatment interaction was found for pituitary *Gpr147* mRNA expression ($F(1,24)=2.427$, p<0.019), with RFRP-3 significantly decreasing pituitary *Gpr147* mRNA expression in the afternoon (p<0.016, 95% confidence interval: -1.702 to -0.08974) but not in the morning (p>0.05).

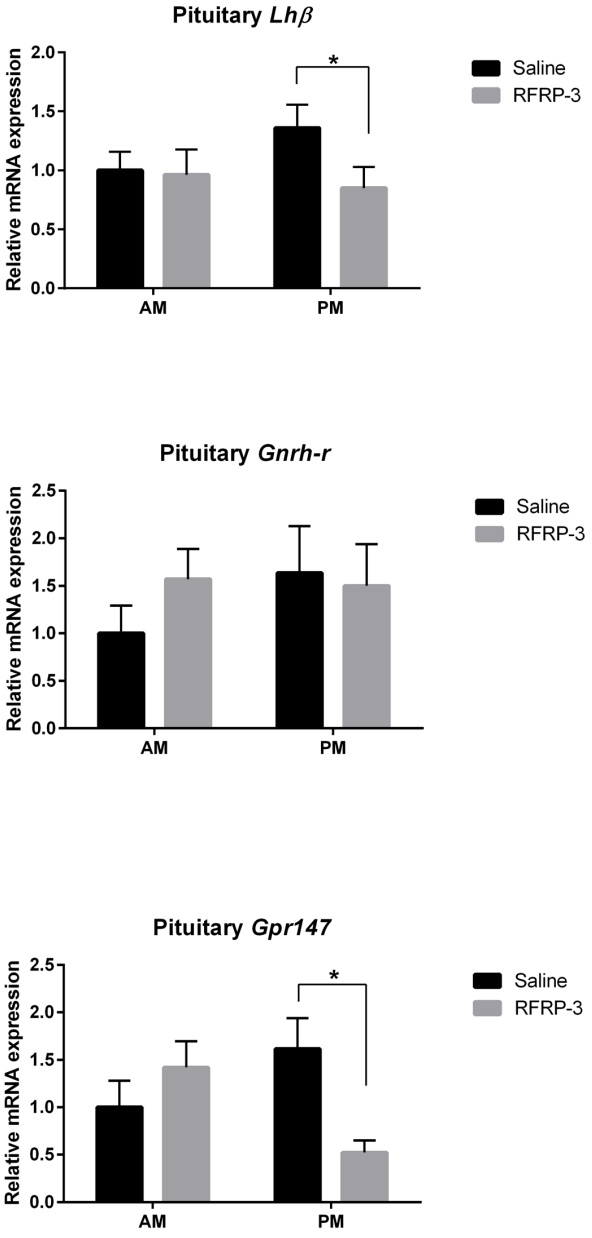

Figure 3. Central administration of RFRP-3 suppresses pituitary *Lhβ* subunit mRNA expression in the afternoon but not the morning (top; n = 6, 6, 6, 7, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). Pituitary *Gnrh-r* mRNA expression is not affected by either RFRP-3 or time of day (middle; n = 6, 8, 7, 7, for AM saline, AM RFRP-3, PM saline, PM RFRP-r, respectively). RFRP-3 suppresses pituitary *Gpr147* mRNA expression in the afternoon but not the morning (bottom; n = 6, 8, 9, 5, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). Data are presented as mean ± standard error of the mean. * $p < 0.05$.

mPOA gene expression

To examine whether changes in LH concentrations are mediated via the classic GnRH-LH pathway, mPOA *Gnrh* and *Gpr147* mRNA expression were assessed (**Figure 4**, n=5-9/group). Baseline mPOA *Gnrh* expression (i.e., saline groups) was significantly reduced in the afternoon relative to morning ($p<0.01$, 95% confidence interval: -0.5464 to -0.05362). Additionally, RFRP-3 significantly decreased mPOA *Gnrh* mRNA expression in the morning ($p<0.024$, 95% confidence interval: -0.5581 to -0.002333) but not in the afternoon ($p>0.05$). mPOA *Gpr147* mRNA expression did not differ at any time point for either treatment ($p>0.05$ in all cases).

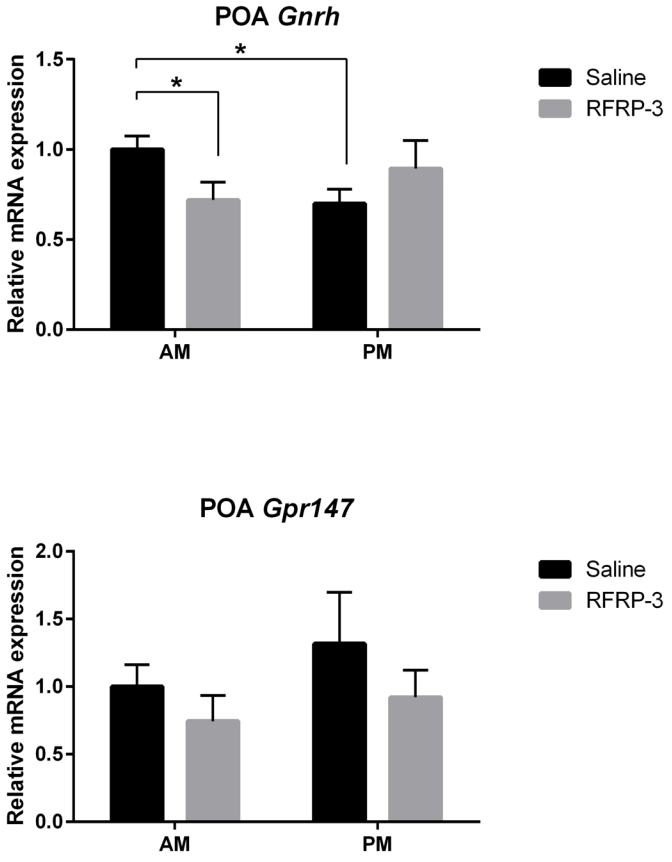

Figure 4. mPOA *Gnrh* mRNA expression is suppressed by central administration of RFRP-3 in the morning but not in the afternoon (top; n = 6, 6, 7, 9, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). RFRP-3 does not alter mPOA *Gpr147* mRNA expression (bottom; n = 7, 5, 7, 8, for AM saline, AM RFRP-3, PM saline, PM RFRP-r, respectively). Data are presented as mean ± standard error of the mean. * $p < 0.05$.

ARC gene expression

RFRP-3 may modify LH production and/or release indirectly via kisspeptin neurons in the ARC that control GnRH pulsatility [325]. Thus, we examined the expression of *Kiss1* and *Gpr147* mRNA in the ARC under RFRP-3 and saline treatments (**Figure 5**, n=7-9/group). 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 ($p<0.022$, 95% confidence interval: -1.229 to -0.01701) but not in the morning ($p>0.05$), 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 ($p<0.054$, 95% confidence interval: -0.2739 to 2.418). No effect of RFRP-3 was observed for ARC *Gpr147* mRNA expression at either time point ($p>0.05$ in each case).

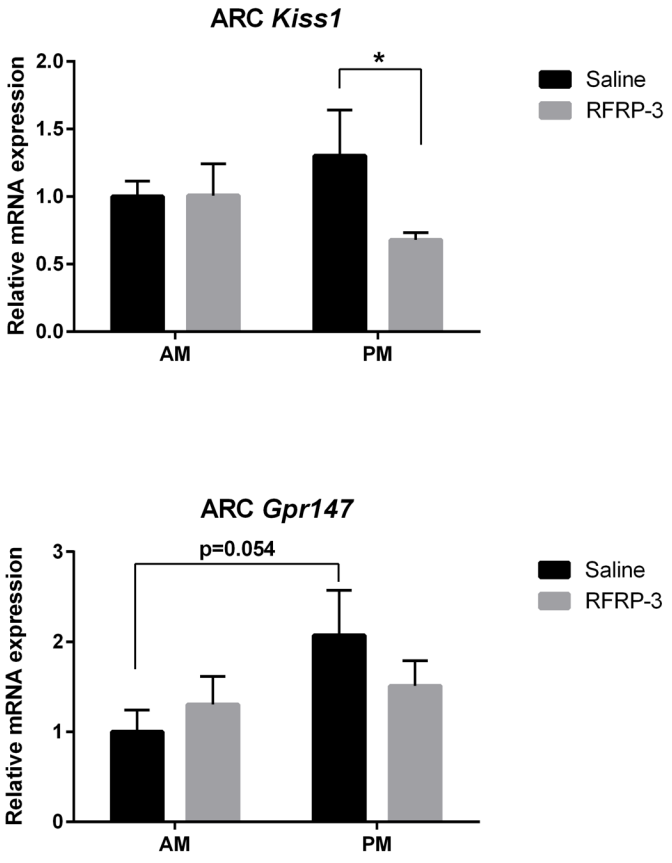

Figure 5. Central administration of RFRP-3 suppresses ARC *Kiss1* mRNA expression in the afternoon but not in the morning (top; n = 7, 8, 7, 9, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). No effect of RFRP-3 on ARC *Gpr147* mRNA expression was observed (bottom; n = 6, 8, 7, 9, for AM saline, AM RFRP-3, PM saline, PM RFRP-3, respectively). Data are presented as mean ± standard error of the mean. * $p < 0.05$.

2.5 Discussion

The present findings 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 downregulating 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). As previous studies have established that administration of RFRP-3 around the time of ovulation suppresses the GnRH/LH surge [326] and sexual motivation [327], this finding further underscores the importance of RFRP-3 cellular inhibition at this time as we and others have previously shown [53, 168, 172]. Additionally, consistent with previous findings in this species [90], 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. Together, these outcomes underscore the importance of circadian-controlled RFRP-3 system inhibition to permit the LH surge and coordinate maximal fertility with sexual motivation.

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 [61, 63, 171, 315], in the pituitary [58, 168, 318, 320], and in kisspeptin neurons [315, 321], 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 KNDy neurons express *Gpr147* in both male and female mice [315, 321]. Likewise, ~35% of ARC kisspeptin neurons receive RFRP-3 immunoreactive fiber contacts [321]. In the present study, RFRP-3 had no effect on the expression of *Gpr147* in the mPOA and AVPV, suggesting that 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 [157, 328, 329]. 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 [330, 331] as well as projections to the internal and external layer of the median eminence

[157]. 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 KNDy neurons leads to atypical LH surge amplitude [162, 163]. 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 [53]. This same 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 [321]. Together, the present and previous findings support the working model that RFRP-3 cells are in a position to modify LH secretion through 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, as 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 as it is unclear whether or not 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 [58, 168, 318, 332, 333]. In contrast, neither RFRP-3 projections to the median eminence nor its receptor are found in some species [57, 334-336]. In cultured pituitaries across species, RFRP-3 administration inhibits gonadotropin production and release [337-339], suggesting the potential for inhibition *in vivo*. Although our 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 current study, hamsters were ovariectomized to eliminate E2 negative feedback. In the absence of E2, 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 [168]. However, this daily change is not reflected in *Gnrh* mRNA expression. Also contrary 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 is possibly due to 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 [340]. Finally, we cannot exclude the possibility that the removal of 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 present findings 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 mechanism 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, instead acting through ARC kisspeptin cells and/or directly on the pituitary (**Figure 6**). 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 nighttime (e.g., from electronic devices), conditions associated with marked deficits in ovulatory cycling [5, 9].

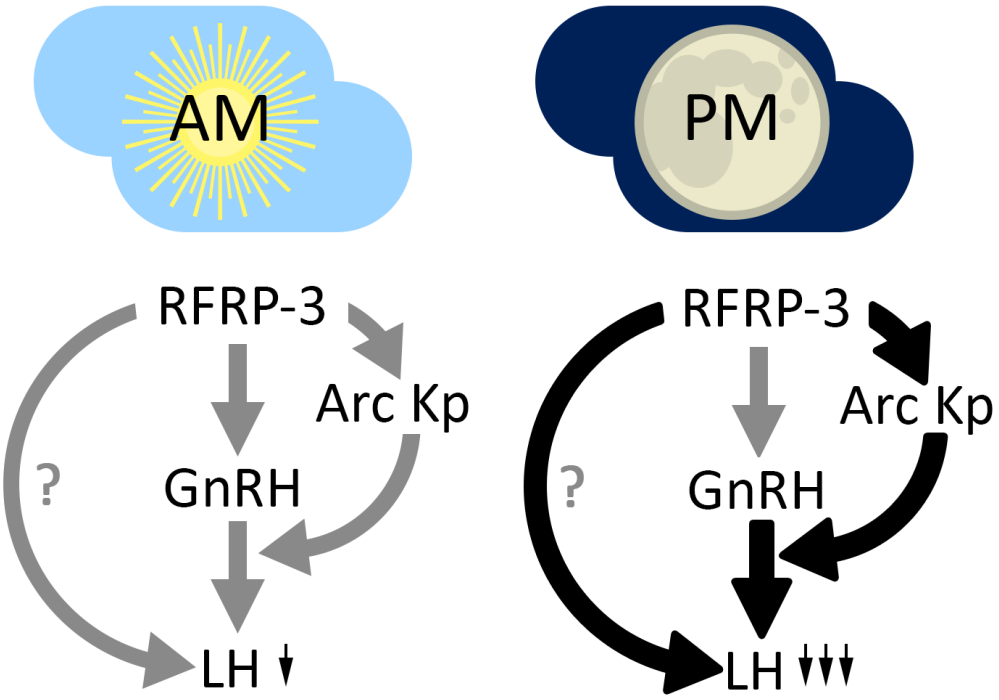

Figure 6. Proposed model by which RFRP-3 leads to greater suppression of LH in the afternoon relative to morning injections. In the morning, RFRP-3 is proposed to have actions via GnRH cells, and potentially pituitary gonadotropes, to suppress LH. In the afternoon, based on the present findings, it is suggested that RFRP-3 acts to more potently suppress LH via additional suppression of ARC kisspeptin cells that mediate GnRH release, potentially in combination with actions on GnRH soma and/or pituitary gonadotropes. Black and thicker lines indicate points of proposed increased RFRP-3 suppression of the reproductive axis in the PM relative to AM conditions.

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 reproductive 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 through 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, nighttime exposure to light-emitting devices, shift work), mistimed RFRP-3 release may be responsible for compromised fertility seen across species, including humans.

2.6 Acknowledgements

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3. Pregnancy stage determines the effect of chronic stress on ovarian progesterone synthesis.

3.1 Abstract

Stress-induced glucocorticoid release is thought to be a primary driver by which maternal stress negatively impacts pregnancy outcomes, but the neuroendocrine targets mediating these adverse outcomes are less well understood. We hypothesized that stress-induced glucocorticoid secretion influences pituitary hormone secretion, resulting in altered ovarian progesterone synthesis. Using a chronic restraint model of stress in mice, we quantified pituitary hormones, steroid hormone production, and expression of ovarian genes that support progesterone production at both early- (day 5) and mid-pregnancy (day 10). Females subjected to daily restraint had elevated baseline corticosterone during both early- and mid-pregnancy. However, lower circulating progesterone was observed only during early pregnancy. Lower progesterone production was associated with lower expression of steroidogenic enzymes in the ovary of restrained females during early pregnancy. There were no stress-related changes to luteinizing hormone (LH) or prolactin (PRL). By mid-pregnancy, circulating LH decreased regardless of treatment, and this was associated with down-regulation of ovarian steroidogenic gene expression. Our results are consistent with a role for LH in maintaining steroidogenic enzyme expression in the ovary, but neither circulating PRL nor LH were associated with the stress-induced inhibition of ovarian progesterone production during early pregnancy. We conclude that chronic stress impacts endocrine networks differently not only in pregnant and non-pregnant mammals, but also in different stages of pregnancy.

3.2 Introduction

Maternal stress increases the likelihood of adverse pregnancy outcomes in many mammals [342], including humans [343, 344]. Adverse outcomes include total failure (miscarriage, or resorption) as well as a range of sub-lethal effects, including lower birth weight of offspring, slower growth rates, and altered social and anxiety behaviors [345-347]. One mechanism by which stress can produce these adverse outcomes is by increasing activity of the hypothalamic-pituitary-adrenal (HPA) axis: when animals experience stress, the HPA axis increases glucocorticoid release from the adrenal gland, and this release of glucocorticoids (above homeostatic levels) impacts pregnancy progression and fetal development [297, 298]. Some of these effects result from the inhibition of the primary pregnancy maintenance hormone, progesterone. If progesterone is too low during early pregnancy, embryo implantation and/or the pregnancy will fail [209, 348, 349], and more broadly, low progesterone throughout pregnancy can adversely affect placental growth and development [350].

Circulating progesterone during early pregnancy in humans and other mammals is inversely correlated with circulating glucocorticoids [351, 352], and stress exposure during pregnancy is associated with lower circulating progesterone concentrations [352-354]. Despite clear evidence of these associations, the pathway by which glucocorticoids inhibit progesterone production during pregnancy is unknown [297, 352].

In non-pregnant female mammals, glucocorticoids regulate ovarian function primarily through action on the hypothalamus and pituitary. For example, in non-pregnant females,

glucocorticoids alter hypothalamic and pituitary hormone release (including luteinizing hormone [LH] and prolactin [PRL]), and these changes can result in lower sex steroid production (estrogens and progestogens) from the ovary [350]. The association between glucocorticoids and progesterone release during pregnancy could potentially reflect action through these same circuits. However, as described below, pregnancy requires substantial changes to regulatory networks and activity of endocrine axes (the reproductive axis being only one of many), and it is therefore possible that the association between glucocorticoid and progesterone production during pregnancy results from novel interactions among endocrine organs or from interactions that are less important in non-pregnant females. Furthermore, pregnancy is dynamic and the effects of chronic stress on endocrine outcome measures (including glucocorticoid and progesterone production) are likely to change across pregnancy progression.

In rodents, the amount of progesterone produced during pregnancy depends on steroidogenic activity in the corpora lutea (CL) in the ovary. Increased steroidogenic activity by the CL is a function of activity across two pathways [355, 356]. First, inhibition of the enzyme *20αHSD*, which usually metabolizes progesterone; and second, increased expression of steroidogenic enzymes, especially P450 cholesterol side-chain cleavage enzyme (*P450SCC*). The pituitary hormones prolactin (PRL) and luteinizing hormone (LH) control these pathways, respectively [355-358]. Failure or decreased function of any of these signaling pathways within the ovary during early pregnancy can increase the likelihood of adverse pregnancy outcomes [359-361]. Though the placenta begins to contribute progesterone to circulation by mid-pregnancy [356, 362], the ovary is thought to be required for pregnancy maintenance throughout gestation in mice [363].

We hypothesized that chronic stress affects ovarian steroidogenesis across the first half of pregnancy (early- to mid-pregnancy) by modulating the pituitary hormones (LH, PRL) that mediate these responses in non-pregnant animals. To test this possibility, we used chronic restraint to model chronic stress in mice, and we measured pituitary and ovarian hormone production, and gene expression in candidate ovarian steroidogenic pathways during early- and mid-pregnancy. We predicted that restrained females would have elevated circulating concentrations of glucocorticoids, specifically corticosterone, which would be associated with lower circulating progesterone. Furthermore, we predicted that the pituitary hormone signaling (circulating concentrations of PRL or LH, and receptor expression in the ovary) would be concomitantly lower in restrained females.

3.3 Materials and Methods

Animals

C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA) and housed in ventilated cages on a 14:10 light/dark cycle (lights on at 06:00, lights off at 20:00) with *ad libitum.* access to food and water. Experimental animals were pair-housed with the male throughout the experiment. All animals were allowed to acclimate for at least 1 week. Females used in these experiments were 8-10 weeks old. All protocols were approved by the UC Berkeley Office of Laboratory Animal Care and were consistent with NIH guidelines for the care and use of laboratory animals.

Experimental procedures

Successful mating was determined either through observation of at least two intromissions during timed mating trials or by the identification of a vaginal plug the morning following pairing. The morning after mating or on which a vaginal plug was found was considered Day 1 of pregnancy. Females were then pseudo-randomly assigned to restraint stress or control (unrestrained) groups such that assignment between groups was balanced across the length of the experiment (see Table 1 for total sample sizes). All females were weighed each morning prior to treatment. Animals assigned to the chronic restraint stress group were moved each morning, beginning on Day 1, to a separate room where they were restrained in a modified 50 mL plastic tube. Animals were also exposed to predator odor during restraint: each day, 15 uL of predator odor (undiluted fox urine, Minnesota Trapline, Inc; Pannock, MN) was freshly soaked into a new cotton ball and placed in the cage with each mouse during restraint. Daily restraint lasted four hours from 08:00 to 12:00 (relative to lights-on). Restraint was repeated daily until tissue collection. Unrestrained females remained in their home cages.

Treatment	Day of Pregnancy Collected		Total
	Day 5	Day 10	
Control	10	12	22
Restrained	9	15	24
		Total	46

Table 1. Summary sample sizes used in experiment.

Females were euthanized on either day 5 (early-) or day 10 (mid-) pregnancy. All animals were euthanized via intraperitoneal injection of sodium pentobarbital (200 mg/kg) followed by rapid decapitation or perfusion. In animals euthanized via decapitation, trunk blood was collected into 1.5 mL Eppendorf tubes and the ovaries were rapidly dissected from the body, cleaned of fat, and flash frozen in isopentane on dry-ice. The number of developing fetuses for each side of the uterus was counted in females collected at mid-pregnancy, and fetal developmental abnormalities or resorption sites were recorded by an observer unaware of the individual's treatment. In animals euthanized via perfusion, blood was collected via the retro-orbital sinus immediately prior to perfusion; the uterus and ovaries were clamped and removed prior to perfusion, and these tissues were immediately dissected and frozen as previously described. Tissues were stored at -80°C until extraction and analysis. Blood was centrifuged at 1300 rcf for 10 min. and plasma removed. Plasma was centrifuged a second time for 1 min. and then aliquoted and stored at -80°C. Blood samples were collected an average of 2:41 ± 0:30 minutes from lifting the cage (Average ± St.Dev.), with a median time to collection of 2:37 (N = 46). Samples collected more than 4 minutes after lifting the cage were excluded from analyses.

Hormone analyses

Progesterone was quantified using Cayman Chemical Progesterone ELISA (Item No. 582601, Ann Arbor, MI). Intra-and inter-assay variations for progesterone were 3.9% and 5.1%, respectively. Baseline corticosterone was quantified using Enzo corticosterone ELISA kit (ADI-900-097; Enzo Life Sciences, Inc., Farmingdale, NY) using the manufacturer's protocol for small sample volumes. Intra-and inter-assay variations were 4.8% and 7.9%, respectively. LH levels were quantified using an LH ELISA, modified from [64]. The protocol was kindly provided by Jens D Mikkelsen (Copenhagen University Hospital, Denmark). Briefly, 96-well microtiter plates were coated with 50µl of bovine LHβ 518B7 monoclonal antibody (kindly provided by Lillian E Sibley, UC Davis) and incubated overnight at 4 C. Excess antibody was removed, and the plates were washed with 200µl/well of 10mM PBS with 0.1% Tween 20. The plates were blocked using 5% skim milk powder in PBS-T and incubated for 1 h at room temperature. Following washes, 50µl of sample or standards of mouse LH (mouse RIA kit, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA), diluted in assay buffer, were added per well in duplicates and incubated for 2 h at room temperature. The plates were washed and 50µl of Rabbit polyclonal LH antibody (AFP240580Rb, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA) were added into each well, then incubated at room temperature for 90 min. After washing, 50µL Polyclonal Goat Anti-Rabbit IgG conjugated to horseradish peroxidase (DAKO Cytomation, catalog # P0448) was added at 1:2000 dilution and incubated for 1 h at room temperature. After washing, 100µl of o-Phenylenediamine (OPD (Invitrogen, catalog # 00-2003)) in citrate buffer were added to all the wells. The color reaction was allowed to develop for 30 min in the dark. The enzyme was stopped by adding 50µl of 3M HCl per well and the OD of each well was immediately read at 490 nm with a reference of 655 nm.

Samples which did not reach the limit for detection for the LH assay were assigned the lowest measurable value (0.078 ng/mL; N = 7, all females from mid-pregnancy). Intra- and inter-assay variations were 5.9% and 3.59%, respectively.

Three samples (all in the mid-pregnancy group) gave values that were nearly 10 times greater than the average of all other samples (1.19, 1.71, and 2.12 ng/mL compared to the average of 0.18 ng/mL [range: 0.078-0.53 ng/mL]). Such values are comparable to LH values measured in ovariectomized mice that were run in the same assay as internal controls. However, we could not determine any reason to suspect that the values measured were inaccurate. Accordingly, we include these data points in the figure, and present analyses with and without these samples included.

Prolactin was assayed using the mouse prolactin ELISA kit from Abcam (ab100736, Cambridge, MA). Intra-assay variation for prolactin was 2.8%.

Some samples did not have sufficient plasma to quantify all hormones, thus sample sizes vary for different hormone measures.

Gene expression analysis

Total RNA was extracted from whole ovaries (ISOLATE II RNA Mini-kit, BIO-52073, Bioline USA Inc., Taunton, MA). The RNA quality of a random subset of samples (N = 10) were analyzed on an Agilent Technologies Bioanalyzer and yielded an average RNA integrity number (RIN) of 9.5 (Range: 8.8 to 10). We reverse transcribed 1.0 ug of RNA (iScript Advanced cDNA synthesis Kit for RT-qPCR, Bio-Rad Laboratories Inc., Hercules, CA). cDNA was diluted 1:25 in nuclease-free water immediately prior to performing quantitative PCR. Quantitative PCR was performed using duplicate 10 uL reactions with a 2-step amplification for 40 cycles followed by a melt curve. All primers used were validated prior to analyses by confirming single-peak melt curves, correct product length, and acceptable efficiency (all primer pairs between 85 and 101% efficiency). Primer sequences and annealing temp. are provided in Table 2. Any wells with aberrant melt-curves were excluded from expression analysis. C_T values were corrected for efficiency; relative expression was calculated using methods by Pfaffl and colleagues [73]. All data are expressed as fold-change over mid-pregnancy, restrained individuals.

Target	Forward Primer	Reverse Primer	T_A
TBP	GGGAGAATCATGGACCAG	GGCTGTGGAGTAAGTC CTGT	55
PRLRL	ATAAAAGGATTTGATACTCA TCTGCTAGAG	TGTCATCCACTTCCAA GAACTCC	60
StAR	CTTGGCTGCTCAGTATTGAC	TGGTGGACAGTCCTTA ACAC	55
P450SCC	CGATACTCTTCTCATGCGAG	CTTTCTTCCAGGCATCT GAAC	55
LHR	CTCCAGAGTTGTCAGGGTCG	AGGTGAGAGATAGTCG GGCG	60
20αHSD	ATGAGCTTTTGCCTAAAGAT GAG	GTTAGACACCCCGATG GAC	55

Table 2 Primers used for quantitative PCR analyses of gene expression in C57BL/6J mice. Primers for *PRLRL* were taken from (5). T_A: Annealing Temperature, °C.

Statistical analyses

All analyses were run in RStudio 0.98.1091 with the nlme and multcomp packages.

We evaluated the change in mass across pregnancy in the restrained and unrestrained groups by calculating percent change in mass per day (of initial body mass) from days 1-6 and days 6-9. We identified day 6 as the point at which chronically stressed females began to gain mass by visually inspecting mass across pregnancy (Fig. 1). Slope was statistically evaluated using a one-way ANOVA with post-hoc comparisons among means using a Holms-Sidak correction for multiple comparisons.

Progesterone, corticosterone, and LH were log-transformed for analyses to meet assumptions of residual normality. Blood collection method (retro-orbital vs. trunk blood) significantly affected progesterone measurements, and because samples collected from animals at day 5 were all collected via retro-orbital bleeds, we only included progesterone measurements from day 10 animals that were collected via the retro-orbital sinus in the analysis. We ran a one-way ANOVA with planned contrasts to test for differences among groups based on our *a priori* predictions. We tested for differences between restrained and unrestrained individuals during early- and mid-pregnancy (planned contrasts 1 & 2), and we tested for differences between early and mid pregnancy (planned contrast 3). The correlations between baseline corticosterone and progesterone were assessed using Pearson's product-moment correlation. A difference in circulating prolactin between restrained and unrestrained animals during early pregnancy was analyzed using Welch's two sample t-test.

Gene expression analyses were carried out using a repeated-measures linear regression model including a random effect of individual to account for use of both ovaries. All genes were log-transformed to fulfill assumptions of normality of residuals. Again, we used planned contrasts to test for *a priori* differences. We used Pearson's product-moment correlation to examine correlations between expressed genes. All tests were considered statistically significant at $P < 0.05$. Because we used planned comparisons, we did not correct p-values for multiple comparisons. Figures show untransformed data and use mean ± S.E., except where noted.

3.4 Results

Maternal mass and litter size

Females exposed to chronic restraint stress lost body mass during early pregnancy, in contrast to unrestrained females, which gained mass (Fig 1A). Once chronically stressed females began gaining mass (after day 6), they gained mass at the same rate as unrestrained animals (One-way ANOVA: $F_{3,42} = 44.07$, $P < 5e^{-13}$; Fig. 1B). Regardless of treatment, pregnant mice gained mass between successful mating and mid-pregnancy (CON: 2.80±0.13 g; STR: 0.63±0.12 g), however unrestrained females gained more mass (Welch's T-test, $t_{17.5} = 12.03$, $P < 7e^{-10}$; Fig 2A).

There were no overall differences in total number of fetuses per female at mid-pregnancy (data not shown, $P > 0.3$). However, evidence of early resorption and underdeveloped fetuses was apparent in 2 of 15 females (13%) exposed to daily restraint stress, whereas 0 of 12 (0%) unrestrained females showed signs of resorption or underdeveloped fetuses.

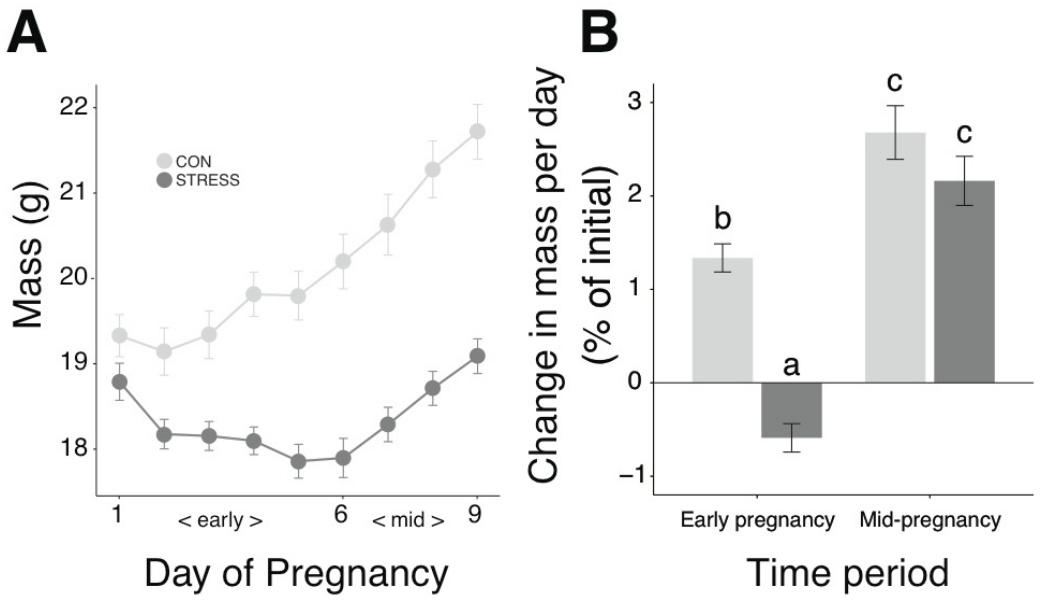

Figure 1 (A) Patterns of weight gain across pregnancy in unrestrained mice (CON, light line; N = 20) and mice exposed to chronic restraint stress (STRESS, dark line; N = 23). (B) When exposed to chronic restraint stress, pregnant female mice (dark bar) lost mass between days 1-6 relative to pregnant unrestrained mice, which gained weight (light bar). However, between day 6 and day 9, chronically stressed females gained weight at rates comparable to unrestrained females (though absolute mass is still less than unrestrained females; see 2A). Letters above bars indicate significantly different post-hoc comparison ($P < 0.03$ for all).

Steroid hormones

Baseline corticosterone (CORT) increased as pregnancy progressed (Fig. 2A; AOV: $F_{3,28} = 10.65$, $P < 8e^{-5}$; Pregnancy: $t = 2.573$, $P < 0.015$). Chronic restraint stress elevated baseline CORT during both early and mid-pregnancy (Fig. 2A; AOV: $F_{3,28} = 10.65$, $P < 8e^{-5}$; planned contrasts: Early: $t = 2.554$, $P < 0.016$; Mid: $t = 4.11$, $P < 0.0003$). Chronic stress also resulted in lower circulating progesterone, but only during early pregnancy (Fig. 2B; AOV: $F_{3,26} = 16.26$, $P < 4e^{-6}$; planned contrasts: Early: $t = -5.776$, $P < 5e^{-6}$; Mid: $t = 0.492$, $P < 0.63$). Baseline CORT was correlated with circulating progesterone during early pregnancy (Fig. 2C; Pearson-R = -0.78, $t_7 = -3.32$, $P < 0.013$), but not during mid-pregnancy (Pearson-R = 0.16, $t_9 = 0.48$, $P > 0.60$).

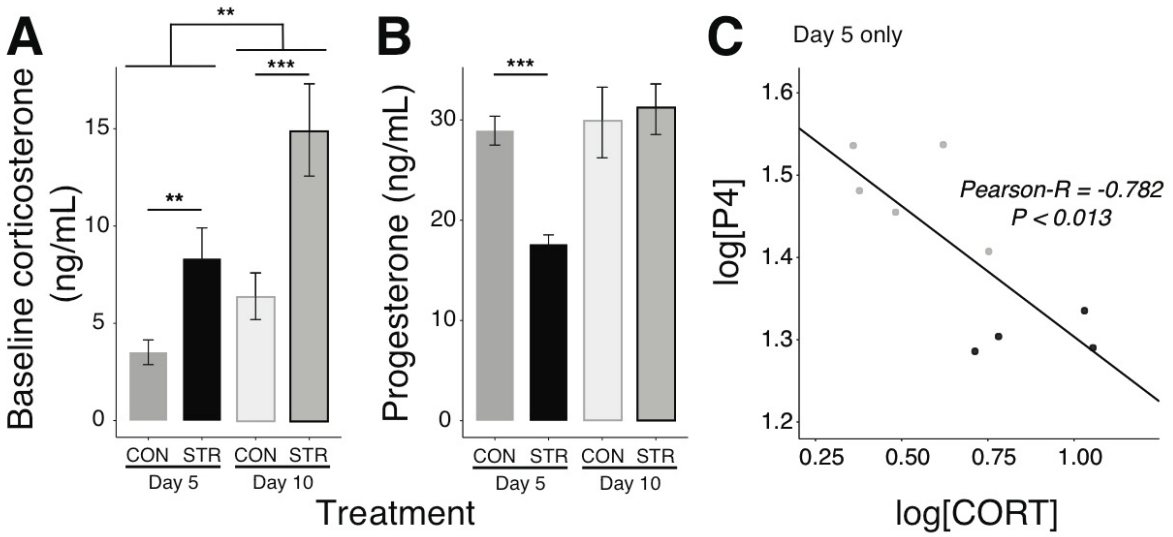

Figure 2 (A) Baseline corticosterone was elevated in chronically-stressed (STR), pregnant female mice relative to unrestrained (CON) females at both day 5 and day 10 of pregnancy. Baseline corticosterone increased from early to mid-pregnancy (day 5 to day 10), and was elevated in restrained females relative to unrestrained at both time points. Day 5: CON, N = 5; STR, N = 4; Day 10: CON, N = 11; STR, N = 12. (B) Baseline progesterone was lower in chronically-stressed (STR), pregnant female mice on day 5 relative to unrestrained (CON) females, but not on day 10. (C) On day 5, baseline corticosterone was inversely correlated with circulating progesterone. Day 5: CON, N = 10; STR, N = 9; Day 10: CON, N = 5; STR, N = 6. ** P < 0.02; *** P < 0.001, planned comparisons.

Pituitary hormones

Circulating prolactin did not differ between unrestrained and chronically restrained females during early pregnancy (Fig. 3A; Welch's t-test $t_{7.984} = -1.27$, P = 0.24). When all LH measures are included the analyses, circulating LH did not vary across pregnancy or with treatment (P > 0.15 for all). However, these three points in the mid-pregnancy group (see Fig. 3B) are all at least two times greater than any other measured value and, when included, they are responsible for a 5-fold increase in standard deviation within the mid-pregnancy group. When these points are excluded, circulating LH was lower during mid-pregnancy relative to early pregnancy (AOV: $F_{3,26} = 4.711$, P < 0.009; planned contrasts: Pregnancy: t = -3.72, P < 0.0009), though it still did not differ between unrestrained and chronically restrained females (Fig. 3B; planned contrasts: Early: t = 0.091, P < 0.93; Mid: t = 0.773, P < 0.45).

Ovarian gene expression

During early pregnancy, the expression of two steroidogenic enzymes (Steroidogenic acute regulatory protein [*StAR*] and p450 cholesterol side-chain cleavage enzyme [*SCC*]) were lower in chronically-stressed animals compared to unrestrained females (*StAR*, Early: $t_{34,32} = -3.46$, P < 0.0015; *SCC*, Early: $t_{34,32} = -2.41$, P < 0.0220; Fig. 4). Expression of these genes in the ovary during mid-pregnancy was lower relative to early pregnancy, and there was no difference in expression between chronically stressed and unrestrained individuals during mid-pregnancy

(*StAR*, Pregnancy: $t_{34,32} = -7.21$, $P < 0.0001$, Mid: $t_{34,32} = -0.043$, $P < 0.96$; *SCC*, Pregnancy: $t_{34,32} = -8.30$, $P < 0.0001$, Mid: $t_{34,32} = -0.414$, $P < 0.68$; Fig. 4).

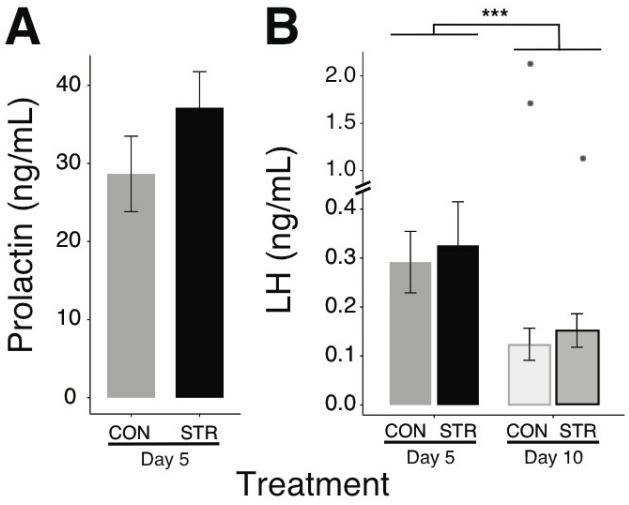

Figure 3 (A) Circulating concentration of prolactin did not differ between unrestrained (CON) and restraint-stressed (STR), pregnant mice on day 5 of pregnancy ($P > 0.2$) (B) Circulating concentration of LH varied between early and mid-pregnancy, but not with stress. PRL - Day 5: CON, N = 5; STR, N = 5. LH - Day 5: CON, N = 5; STR, N = 4; Day 10: CON, N = 9; STR, N = 12. *** $P < 0.001$, planned comparisons.

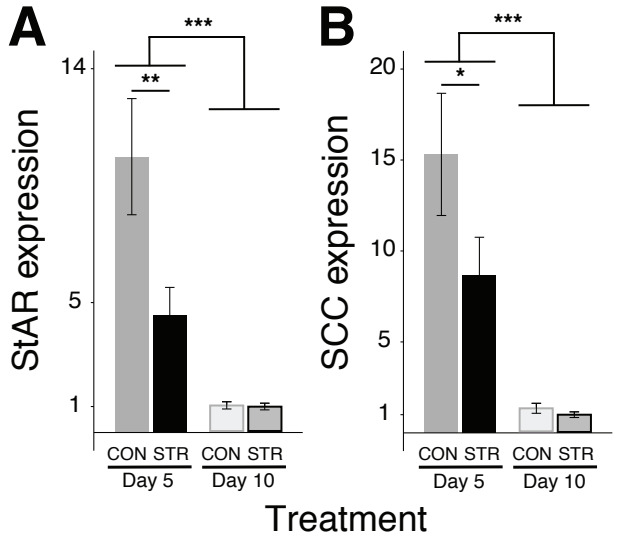

Figure 4 Expression of two steroidogenic enzymes in the ovary is modulated by stress and pregnancy progression. (A) Fold-change expression of steroidogenic acute regulatory protein (*StAR*) was decreased in restraint-stressed (STR) animals during early pregnancy related to unrestrained females (CON), but down-regulated in both groups during mid-pregnancy (B) Fold-change expression of cholesterol side chain cleavage enzyme (*SCC*) was also lower in restraint-stressed animals during early pregnancy, but down-regulated in both stressed and unrestrained animals during mid-pregnancy. Plot shows untransformed data. Day 5: CON, N = 20; STR, N = 16; Day 10: CON, N = 18; STR, N = 17. Samples sizes report number of ovaries. Both ovaries were used from most individuals within a repeated measures analysis (see section 3.3.4). * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$, planned comparisons.

Expression of the long prolactin receptor isoform (*PRLRL*) was lower in restrained females during early pregnancy (Early: $t = -2.28$, $P < 0.029$; Fig. 5A), but not during mid-pregnancy (Mid: $t = -0.084$, $P < 0.93$), and there was no overall difference in expression between early and mid-pregnancy (Pregnancy: $t = -0.772$, $P < 0.45$). Expression of the receptor for LH (*LH*R) and the enzyme *20αHSD* decreased during mid-pregnancy relative to early pregnancy (*LHR*, Pregnancy: $t = -4.62$, $P < 0.0001$; *20α*, Pregnancy $t = -5.22$, $P < 0.0001$), but there was no difference related to stress treatment (*LHR*: Early: $t = -0.59$, $P < 0.56$; Mid: $t = 0.43$, $P < 0.67$; *20α*: Early: $t = -0.077$, $P < 0.94$; Mid: $t = 0.62$, $P < 0.54$; Fig 5B,C).

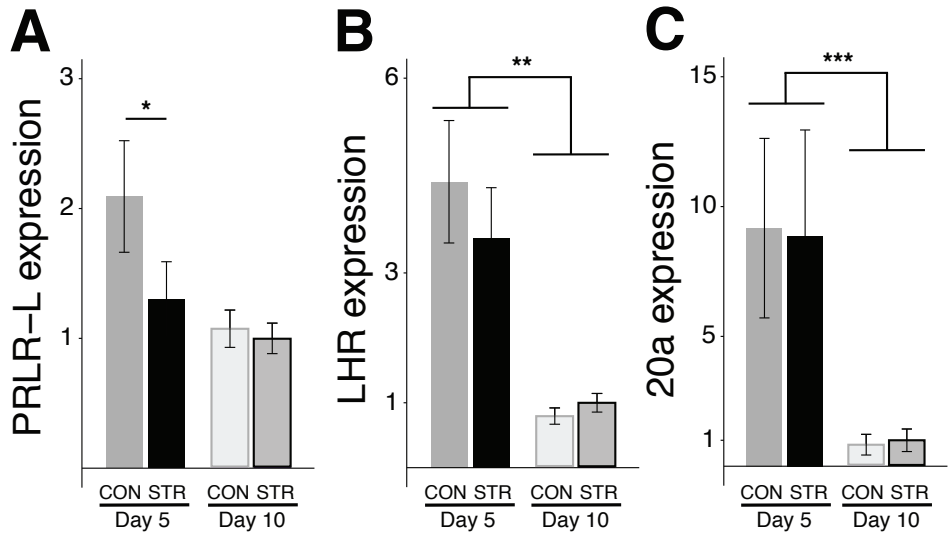

Figure 5 Expression of other candidate genes know to be important for ovarian progesterone production during early pregnancy are unaffected by restraint stress. (A) The long-form prolactin receptor (*PRLRL*) was not inhibited by chronic restraint during early pregnancy, but not during mid-pregnancy. (B) Luteinizing hormone receptor (*LHR*) was not affected by restraint stress during early pregnancy, but showed substantial down-regulation by mid-pregnancy in both groups. (C) *20αHSD* (*20α*) was also not affected by restraint stress during early pregnancy, but was down regulated in the ovary by mid-pregnancy in both groups. Plot shows untransformed data. Day 5: CON, N = 20; STR, N = 16; Day 10: CON, N = 18; STR, N = 17. Samples sizes report number of ovaries. Both ovaries were used from most individuals within a repeated measures analysis (see section 3.3.4). * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$, planned comparisons.

We found a strong correlation between the expression of *PRLRL* and expression of the two steroidogenic enzymes (*StAR* and *SCC*; Fig. 6). The relationships between the steroidogenic enzymes and *PRLRL* were consistent between early pregnancy (Day 5; *SCC*: Pearson-R = 0.98, $t_{34} = 29.60$, $P < 2.2e^{-16}$; *StAR*: Pearson-R = 0.97, $t_{34} = 26.82$, $P < 2.2e^{-16}$), and mid-pregnancy (Day 10; *SCC*: Pearson-R = 0.83, $t_{32} = 8.37$, $P < 1.47e^{-9}$; *StAR*: Pearson-R = 0.89, $t_{32} = 11.25$, $P < 1.18e^{-12}$). The relationships between these gene transcripts shift in late pregnancy; the steroidogenic enzymes are down regulated, while there is no longer a difference between restrained and unrestrained females in *PRLRL* (see Fig 4A,B and Fig 5A). However the slope of the line that explains the correlation between steroidogenic enzymes and PRLR-L appears to be similar (Fig. 6A,B).

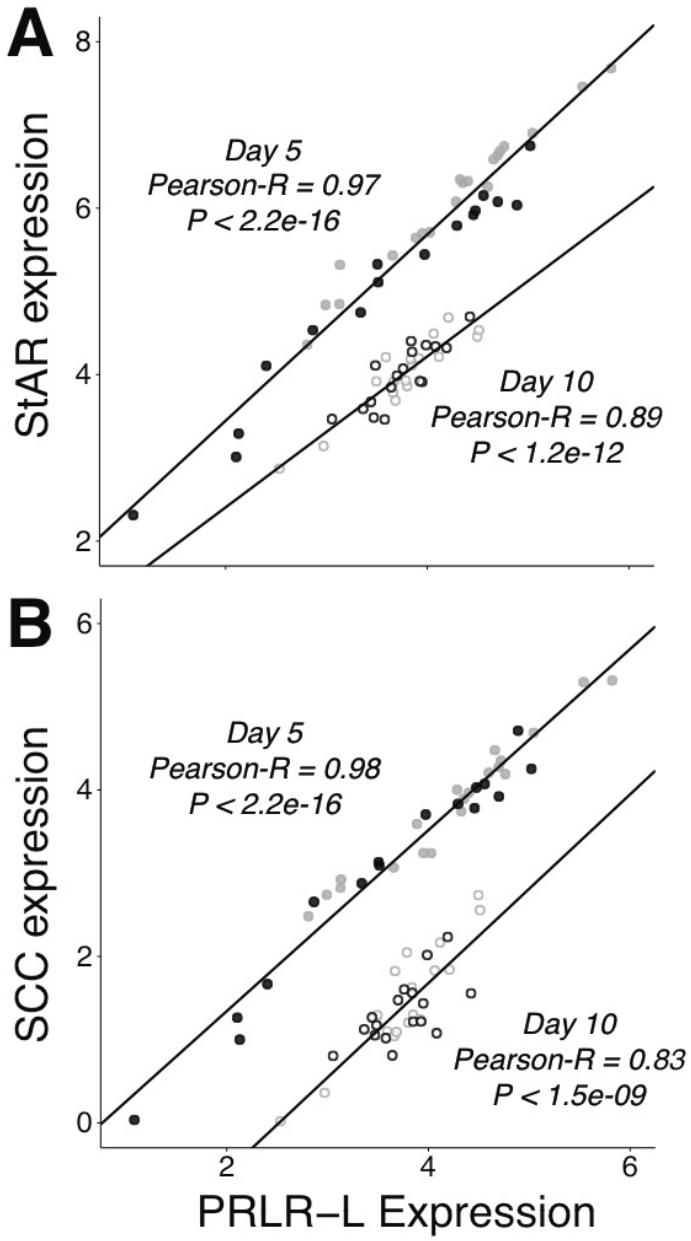

Figure 6 Expression of the long-form of the prolactin receptor (*PRLRL*) was correlated with expression of steroidogenic enzymes in the ovaries (Light orange: Day 5, Unrestrained; Dark orange: Day 5, restraint stress; Dark purple: Day 10, Unrestrained; Light purple: Day 10, restrained). (A) *PRLRL* covaries with expression of steroidogenic acute regulatory protein (*StAR*) across pregnancy. (B) *PRLRL* covaries with expression of *SCC* across pregnancy. Plot shows log-transformed data.

3.5 Discussion

General conclusions

We found that ovarian progesterone production is sensitive to restraint stress. Our results suggest that glucocorticoids do not inhibit progesterone release during pregnancy via a top-down (hypothalamic-pituitary) mechanism within the HPG axis, because basal pituitary LH and PRL secretion were unaffected by stress. The down-regulated expression of steroidogenic enzymes in the ovary by mid-pregnancy suggests that the majority of circulating progesterone at this time point may no longer be from the ovary. In contrast to the ovary during early pregnancy, mid-pregnancy progesterone synthesis appears resilient to restraint stress and elevated baseline glucocorticoids. Though there have been concerted efforts to understand the extent to which chronic stress alters reproductive outcomes, our results make it clear that there is substantial work still needed to describe and test the basic interactions between the HPA and reproductive axes across different stages of pregnancy. Understanding the functional network between these and other endocrine axes during pregnancy will help to establish the mechanisms connecting maternal stress to reproductive failure.

Effects of stress-induced corticosterone (CORT) release on progesterone during early pregnancy

The inverse correlation we found between baseline CORT and progesterone production during early pregnancy is consistent with other rodent studies [364]. Direct action of CORT on ovarian progesterone synthesis is unlikely to explain the relationship between circulating CORT and progesterone (for more, see [297, 355, 365, 366]), and we found no evidence to support the hypothesis that chronic stress alters the basal release of pituitary hormones (LH and PRL) during early pregnancy. Instead, placental factors that regulate corticosterone metabolism (e.g., 11β-HSD) and/or ovarian progesterone synthesis (e.g., placental lactogens) are promising areas for further study. Careful attention to placental endocrine activity and sensitivity *in vivo* during early pregnancy, especially related to glucocorticoid receptor isoform expression [367], may facilitate the identification of new functional mechanisms by which CORT impacts progesterone synthesis.

In addition, progesterone production and CORT secretion during pregnancy could be connected through other shared upstream regulators. Because restraint stress resulted in initial loss of body mass, suggesting that restrained females entered a negative energy balance, endocrine or metabolic signals associated with changes in energy balance could be responsible for changes to baseline CORT and progesterone production. For example, the adipose hormone leptin promotes ovarian progesterone production [359], is inhibited by chronic stress [368, 369], and is inversely related to CORT during negative energy balance in mice [370]. Mapping the interactions between energy balance circuits and reproductive function specifically in early pregnancy is likely to identify new connections between stress and adverse pregnancy outcomes.

Progesterone production during mid-pregnancy

In mid-pregnancy, *StAR* and *SCC* were dramatically down-regulated relative to early pregnancy, and there were no longer any differences in gene expression between restrained and

unrestrained females. The decrease in expression of *StAR* and *SCC* suggests that ovarian steroidogenic activity is lower in mid-pregnancy. While these results counter the classic suggestion that the ovary is required for progesterone production throughout pregnancy in mice [363], they are consistent with the idea that decreasing pituitary LH release by mid-pregnancy causes a decline in ovarian progesterone production [356]. In further support of the latter idea, circulating LH concentrations were lower during mid-pregnancy relative to early-pregnancy in this study. We also found a novel correlation between *PRLRL* and *StAR* and *SCC*. The strong co-regulation between these genes and differential sensitivity to stress across pregnancy underscores the need to better understand the regulatory networks that control ovarian progesterone production.

Even though restrained females continued to exhibit elevated baseline CORT during mid-pregnancy, circulating progesterone no longer differed between restrained and unrestrained females. Moreover, even though progesterone remained elevated, steroidogenic genes in the ovary were considerably down-regulated, suggesting the ovary is much less steroidogenically active by mid-pregnancy. Circulating progesterone during mid-pregnancy may instead reflect placental steroidogenesis. Interestingly, circulating progesterone appears to be insensitive to chronic stress (elevated CORT) during mid-pregnancy. Further work to establish the source of mid-pregnancy progesterone is needed in order to determine how the apparent insensitivity to CORT develops across pregnancy.

Caveats

Though our results present a relatively clear picture of how chronic stress affects reproductive function during the first half of pregnancy, there are some important caveats. First, only ovarian mRNA, not protein, was measured, raising the possibility that protein expression and activity may differ meaningfully. Second, both prolactin and luteinizing hormones are released in a pulsatile fashion such that single-time point measurement may miss dynamic changes in the pulse rate or peak size for either hormone resulting from chronic stress exposure. The three LH samples showing exceptionally high values likely reflect the pulsatile nature of this hormone, whereas the majority (31/33) measures reflect basal levels as expected. Evaluating upstream changes in protein and gene expression within the pituitary and hypothalamus and/or serial blood samples would be required to conclusively determine whether temporal changes in pituitary hormone production and release could explain the relationship between glucocorticoids and progesterone during early pregnancy. However, most studies evaluating the effects of stress on pituitary hormone release (LH in particular) find differences using single time point measures (e.g. [300, 371]), and we were able detect a change in LH across pregnancy.

More broadly, it is worth considering that animals or people that experience chronic stress during pregnancy are likely to experience stress before pregnancy as well. Geraghty et al. [299] showed that chronic stress prior to pregnancy in rats was associated with lower reproductive success, but that these effects could be ameliorated by inhibiting production of a stress-induced neuropeptide, gonadotropin-inhibitory hormone (GnIH), in the hypothalamus leading up to pregnancy. Thus, their results demonstrate that effects of chronic stress on central (hypothalamic) processes can explain some stress-related reproductive failures that occur during pregnancy. Whether we can differentiate between mechanisms that come into play prior to pregnancy versus

during pregnancy, and furthermore whether this difference is functionally meaningful, will be important moving forward.

Conclusions

Taken together, our results present a first step towards identifying the endocrine network that connects psychological stress to reproductive function during early pregnancy. Importantly, the effects of chronic stress on the reproductive axis during pregnancy do not seem to be acting through the well-known circuits that play a role in non-pregnant females. Combining hormone production measures and ovarian gene expression across pregnancy progression offers a new perspective for understanding the endocrine networks through which stress impacts pregnancy.

3.6 Acknowledgements

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4 Neural Circuits and Endocrine Mechanisms Underlying Risks of Prenatal Stress in a Mouse Model

4.1 Introduction

Reproduction, including ovulation and pregnancy, is controlled by a neural pathway from the hypothalamus to the pituitary gland and the gonads called the hypothalamo-pituitary-gonadal (HPG) axis. At the pinnacle of the HPG axis, neurons in the hypothalamus manufacture and release gonadotropin-releasing hormone (GnRH), a key regulator of reproductive functions. In turn, GnRH stimulates the release of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), two neuropeptides that act on the gonads and are critical for regulating ovulation and pregnancy. FSH and LH stimulate gametogenesis and hormone synthesis and secretion (e.g., progesterone, estradiol, testosterone), respectively. Whereas progesterone is typically considered critical for pregnancy maintenance, it is also involved in endometrium growth, oocyte implantation, and improving blood flow and oxygen supply [372]. Low progesterone concentrations can lead poor pregnancy outcomes, including implantation failure and adverse effects on placental and fetal development [209, 348-350].

A crucial progesterone regulator is the hormone prolactin, also required for successful reproductive functions. Prolactin is typically associated with its role in promoting lactation; however, this hormone has myriad effects on physiology and reproduction, including immune function, osmoregulation, and reproductive behavior (reviewed in [184]). Prolactin enhances progesterone secretion by potentiating the steroidogenic effects of LH in granulosa-luteal cells in the ovary and inhibiting the 20α-hydroxysteroid dehydrogenase enzyme that inactivates progesterone [373, 374]. In pregnant mice and rats, prolactin exhibits twice daily surges (a diurnal and nocturnal surge) that maintain the viability of the corpora lutea (CL) and the secretion of progesterone in the first half of gestation [375-377]. Around mid-pregnancy, these surges cease and placental lactogens maintain progesterone secretion for the remainder of gestation [179-183]. Prolactin release is primarily negatively and positively regulated by dopamine (DA) and oxytocin, respectively [180, 184, 185]. Under most circumstances prolactin is maintained via hypothalamic inhibition by tuberoinfundibular dopaminergic (TIDA) neurons located in the arcuate nucleus and projecting to the anterior pituitary blood portal system [378-381]. Dopamine has a direct effect on pituitary lactotrophs by binding to D2 receptors expressed on the cell membrane of lacotrophs [380, 382].

In many cases, adverse pregnancy outcomes (i.e., decreased conception and increased miscarriage rates, low birth weight, developmental delays) are associated with psychosocial or physiological stress [342-344]. Stress exposure activates the hypothalamic-pituitary-adrenal (HPA) axis, leading to elevation in glucocorticoids secreted from the adrenal gland. High concentrations of circulating glucocorticoids suppress the HPG axis primarily via direct and indirect actions on the hypothalamus and pituitary [297, 298]. During pregnancy, high glucocorticoid concentrations are associated with lower progesterone and adverse pregnancy outcomes [351-354, 383]. Notably, even when stress exposure concludes prior to mating and pregnancy, it leads to reproductive dysfunction [299]. Similar to progesterone, altered prolactin secretion is predictive of poor pregnancy outcomes [22, 194]. In humans, stress stimulates prolactin release [384]. In animal studies, prolactin secretion is influenced by stress in a bi-modal

manner; when pre-stress levels are low prolactin secretion is stimulated, when pre-stress levels are high prolactin secretion is inhibited [381, 385-388]. Numerous findings detail the negative impact of stress on prolactin secretion and pregnancy in general, however, the means by which stress acts on the brain and periphery to compromise reproductive success remains poorly understood.

One potential pathway by which stress can affect the HPG axis and pregnancy is via neurons expressing the inhibitory neuropeptide RFamide-related peptide-3 (RFRP-3; the mammalian ortholog of avian gonadotropin-inhibitory hormone (GnIH) [46, 48]. RFRP-3 neurons are concentrated in the dorsomedial hypothalamus (DMH) and project to brain regions that contain GnRH neurons and fibers as well as non-GnRH loci [56]. Across mammalian species, including humans, RFRP-3 generally suppresses the reproductive axis via direct actions on GnRH cells and potentially at the level of the pituitary [56, 58-61]. RFRP-3 neurons are markedly regulated by environmental and psychosocial factors, including stress [7, 299-301]. In male mice and rats, acute and chronic immobilization stress increases RFRP-3 mRNA and protein expression [300, 301]. Likewise, restraint stress increases RFRP-3 cell activation and expression in female mice [302] and rats [299]. RFRP-3 neurons express glucocorticoid receptor (GR) [300, 303], suggesting direct impact of glucocorticoids on these cells. In a study of chronic stress when stress exposure concluded prior to mating and pregnancy, knocking down RFRP-3 during stress prevented stress-induced reproductive dysfunction [299], suggesting the involvement of these cells in mediating the negative impact of stress on reproductive functions. The means by which RFRP-3 negatively affects subsequent pregnancy remains unknown. Moreover, as pregnancy requires substantial changes in regulatory networks and activity of endocrine axes, it is unclear whether RFRP-3 activity is modified during pregnancy and whether stress during pregnancy affects RFRP-3 activity similarly to that of non-pregnant animals.

Because RFRP-3 neurons express glucocorticoid receptors, we hypothesized that when stress occurs during pregnancy, it impairs pregnancy outcomes through the activation of RFRP-3 neurons, similar to non-pregnant animals. Additionally, because prolactin secretion is critical for the maintenance of pregnancy, and prolactin levels predict miscarriages in humans, we asked whether RFRP-3 projections to the arcuate nucleus affect dopaminergic signaling regulating prolactin release and maintenance of progesterone levels throughout pregnancy. To answer these questions, we subjected pregnant female mice to chronic stress and compared their hypothalamic neuroendocrine profiles to that of non-stressed pregnant (control) animals.

4.2 Materials and Methods

Animals

C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA) and housed in ventilated cages on a 14:10 light/dark cycle (lights on at 07:00, lights off at 21:00) with food and water available ad libitum. Experimental animals were pair-housed with an age-matched male throughout the experiment. All animals were allowed to acclimate for at least 1 week prior to commencing the experiment. Females used in these experiments were 10-12 weeks old. All protocols were approved by the UC Berkeley Office of Laboratory Animal Care and were consistent with NIH guidelines for the care and use of laboratory animals.

Experimental procedures

Virgin, female mice were paired with sexually-experienced males and examined for vaginal copulatory plugs every morning. The morning on which a plug was found was considered gestational day 1. Females were then pseudo-randomly assigned to chronic stress or control (non-stressed) groups such that assignment between groups was balanced across the length of the experiment. All females were weighed each morning prior to treatment. Animals assigned to the chronic psychological stress group were moved each morning, beginning on day 1, to a separate room where they were restrained in a modified 50 mL centrifuge tube (Corning, Corning, NY, USA. See Figure 1 for illustrates the modified tube). Animals were also exposed to predator odor during restraint: each day, 15 uL of predator odor (undiluted fox urine, Minnesota Trapline, Inc; Pannock, MN) was freshly soaked into a new cotton ball and placed in the cage with each mouse during restraint. Daily stress exposure lasted four hours from 09:00 to 13:00 (relative to lights-on). Stress exposure was repeated daily for 4 or 9 days, depending on group. Non-stressed females remained in their home cages.

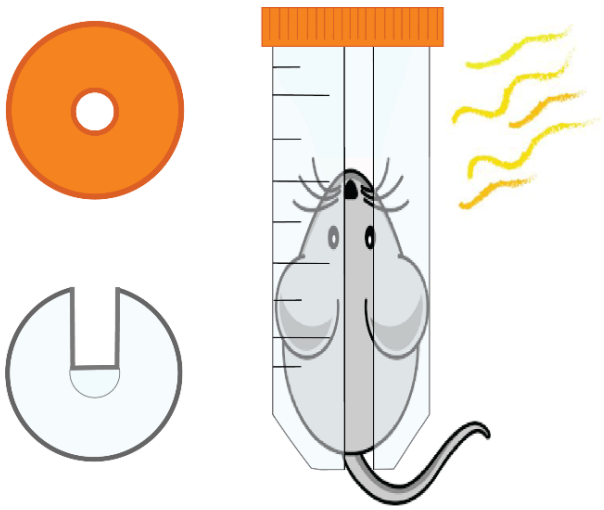

Figure 1. Stress treatment. Animals were exposed to chronic stress beginning on day 1 of gestation. Stress treatment included an immobilization paradigm combined with predator odor. Mice were restrained in a modified 50 mL centrifuge tube in which a hole was created at the center of the cap and a ridge was cut open along the tube, which allowed placing the mice in the tubes without anesthesia. In addition, each day, 15 uL of fox urine was freshly soaked into a new cotton ball and placed in the cage with each mouse during restraint. Daily restraint lasted four hours. Restraint was repeated daily for 4 or 9 days, depending on group. Illustration modified from a creation by Kathryn Wilsterman (University of Montana, Missoula, MT).

Females were euthanized on either day 5 (early-) or day 10 (mid-) of pregnancy. All animals were euthanized via intraperitoneal injection of sodium pentobarbital (200 mg/kg) followed by rapid decapitation or perfusion. In animals euthanized via decapitation (n=9-11/group), trunk blood was collected into 1.5 mL Eppendorf (Fisher Scientific) tubes. The number of developing embryos for each side of the uterus was counted. Pituitaries were collected and flash

frozen in isopentane on dry-ice. Brains were collected and hypothalami dissected and divided in half along the midline before flash freezing. One half of each hypothalami was used for gene expression analysis whereas the other half was used for RFRP-3 peptide quantification using an ELISA. In animals euthanized via perfusion (n=6/group), blood was collected via the retro-orbital sinus immediately prior to perfusion. Tissues were stored at -80°C until extraction and analysis. Blood was centrifuged at 1300 g for 15 min at 4°C and plasma removed. Plasma was centrifuged a second time for 1 min. and then aliquoted and stored at -80°C. Blood samples were collected in less than 3 min from lifting the cage.

Hormone analyses

Progesterone was quantified using the Cayman Chemical Progesterone ELISA (Item No. 582601, Ann Arbor, MI). Intra-and inter-assay variations for progesterone were 3.6% and 6.8%, respectively. Baseline corticosterone was quantified using the Enzo corticosterone ELISA kit (ADI-900-097; Enzo Life Sciences, Inc., Farmingdale, NY) according to the manufacturer's protocol for small sample volumes. Intra-and inter-assay variations were 6.19% and 5.08%, respectively. Prolactin was assayed using the mouse prolactin ELISA kit from Abcam (ab100736, Cambridge, MA). Intra-and inter-assay variations were 3.6% and 4.2%, respectively. LH levels were quantified using an LH ELISA, modified from [64]. The protocol was kindly provided by Jens D Mikkelsen (Copenhagen University Hospital, Denmark). Briefly, 96-well microtiter plates were coated with 50µl of bovine LHβ 518B7 monoclonal antibody (kindly provided by Lillian E Sibley, UC Davis) and incubated overnight at 4°C. Excess antibody was removed, and the plates were washed with 200µl/well of 10mM PBS with 0.1% Tween 20. The plates were blocked using 5% skim milk powder in PBS-T and incubated for 1 h at room temperature. Following washes, 50µl of sample or standards of mouse LH (mouse RIA kit, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA), diluted in assay buffer, were added per well in duplicates and incubated for 2 h at room temperature. The plates were washed and 50µl of Rabbit polyclonal LH antibody (AFP240580Rb, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA) were added into each well, then incubated at room temperature for 90 min. After washing, 50µL Polyclonal Goat Anti-Rabbit IgG conjugated to horseradish peroxidase (DAKO Cytomation, catalog # P0448) was added at 1:2000 dilution and incubated for 1 h at room temperature. After washing, 100µl of o-Phenylenediamine (OPD (Invitrogen, catalog # 00-2003)) in citrate buffer were added to all the wells. The color reaction was allowed to develop for 30 min in the dark. The enzyme was stopped by adding 50µl of 3M HCl per well and the OD of each well was immediately read at 490 nm with a reference of 655 nm. Samples which did not reach the limit for detection for the LH assay were assigned the lowest measurable value. Intra- and inter-assay variations were 5.3% and 4.9%, respectively. Some samples did not have sufficient plasma to quantify all hormones, thus sample sizes vary for different hormone measures.

Gene expression analysis

Total RNA was extracted from pituitary samples using RNAqueous micro kit (AM1931, Ambion, Life Technologies, Carlsbad, CA). RNA concentration and purity were assessed by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific). The RNA quality of a random subset of samples were analyzed on an Agilent Technologies Bioanalyzer and yielded an average

RNA integrity number (RIN) of 7.6 or higher. Reverse transcription was performed using Takara Bio PrimeScript RT Reagent Kit with gDNA Eraser (cat. no RR047A, Mountain View, CA) and then frozen at -20°C until RT-qPCR was performed.

Analysis of relative gene expression via qRT-PCR was performed using SSOAdvanced SYBR Green supermix (BIO-RAD, 1725272, Hercules, CA, USA). Samples were run on a BIO-RAD CFX384 machine with 10µl reaction volumes with a 2-step amplification for 40 cycles followed by a melt curve. Primers were designed from published sequences for mus musculus using NCBI Primer BLAST software (Table 1). Primer sets were validated for specificity using positive, negative, no reverse transcriptase, and no template controls, and confirmed with a single-peak melt curve and correct product length. Efficiency of each primer set was determined by standard curve; primers were 95.3-106.2% 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 resulting data were analyzed in Microsoft Excel following the delta delta Cq method [324].

The geometric mean of 2 housekeeping genes' expression was used for reference. Because the expression of housekeeping genes was found to vary with treatment between tissues, samples from different tissue types were analyzed with different reference genes. *Rplp* and *Tubb* were used as reference genes for the. Housekeeping genes were confirmed to not be significantly different between all groups, and in all genes replicate group Cq Standard deviation was smaller than 0.2. All data are expressed as a fold-change over early-pregnancy, control individuals. Some samples did not have sufficient cDNA to quantify the expression of all genes, thus sample sizes vary for different genes measured.

Primer	Forward	Reverse
Prl	GTGGTTCTCTCAGGCCATCTT	GAGGAGTGTCCCTGCTTTCT
D2	GACACCACTCAAGGGCAACT	TCCATTCTCCGCCTGTTCAC
Lhβ	ACTGTGCCGGCCTGTCAACG	AGCAGCCGGCAGTACTCGGA
Rplp	ATCTACTCCGCCCTCATCCT	GCAGATGAGGCTTCCAATGT
Tubb	GGACAGTGTGGCAACCAGAT	CCCCAGACTGACCGAAAACG

Table 1. Primers used for qRT-PCR.

Histology

Immunohistochemistry

Brains were sectioned in the coronal plane at 40 μm on a Leica 3050S cryostat and stored at − 20°C in antifreeze solution until immunohistochemistry (IHC) was performed. To visualize RFRP-3 cell bodies and projections as well as TIDA neurons, double-label immunofluorescence was performed on separate sets of every fourth 40 μm brain slice, modified from a protocol by Jennings et al [389]. To localize TIDA neurons an anti-tyrosine hydroxylase (TH) antibody was used, as TH is the rate limiting enzyme in the conversion of tyrosine to dopamine. Briefly, free floating sections were washed in PBS, incubated for 10 min in 0.5% hydrogen peroxide, washed in PBS again, and then blocked for 1 h in 3% normal goat serum suspended in 0.1% Triton X-100 (PBT). Sections were then incubated for 48 h at 4 °C in a rabbit polyclonal anti-GnIH antibody (1:120,000; PAC 123/124, a generous gift from Dr. George Bentley) with 2% normal goat serum in PBT. After incubation in the primary antibody, sections were washed in PBT, incubated for 1 h in biotinylated goat anti-rabbit IgG (1:300, Vector Laboratories, Burlingame, CA), washed in PBT, and incubated for 1 h in avidin-biotin-horseradish peroxidase complex (ABC Elite Kit, Vector Laboratories). Sections were then washed with PBT followed by 0.6% biotinylated tyramide solution for 30 min. After washing with PBS, cells were fluorescently labeled with the fluorophore CY-2 strepdavidin conjugate (1:150, Jackson ImmunoResearch Laboratories, West Grove, PA). Next, sections were washed with PBS and incubated for 1 h with 3% normal donkey serum suspended in PBT. Sections were then incubated for 48 h at 4 °C in a mouse polyclonal anti-TH antibody (1:10,000; T2928, Sigma-Aldrich, St. Louis, MO) with 2% normal goat serum in PBT. Following incubation, sections were washed and labeled with the fluorophore CY-3 donkey-anti-mouse (1:150, Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were then incubated with DAPI (1:20,000, Invitrogen, catalog # D1306) for 10 min. Finally, sections then washed with PBS and mounted on gelatin-coated slides, dehydrated and cleared with xylene, and coverslipped.

Microscopy and quantification

To determine the number of RFRP cells and the percentage of TIDA neurons in close apposition with RFRP projections, sections were examined at the conventional light microcopy level using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm) with a Zeiss Z1 microscope (Thornwood, NY). Every fourth section through the dorsomedial hypothalamus was examined for RFRP. Every fourth section through the arcuate nucleus of the hypothalamus was examined for TH and RFRP-3. Photographs were taken at ×400. Each label was captured as automated Z-stack photomicrographs at 0.5-μm increments. Investigators “blind” to the treatment conditions to which the animals were exposed identified the cell bodies and projections using Fiji. Each photo was evaluated by 2 “blind” investigators and the average assessments were used. To ensure accurate detection of TIDA neurons, additional photographs of TH cells were taken at ×10. For each animal, a brain atlas was created by aligning all photos from rostral to caudal and 3 photos containing TIDA neurons were chosen based on TH expression location in the arcuate nucleus (Figure 2).

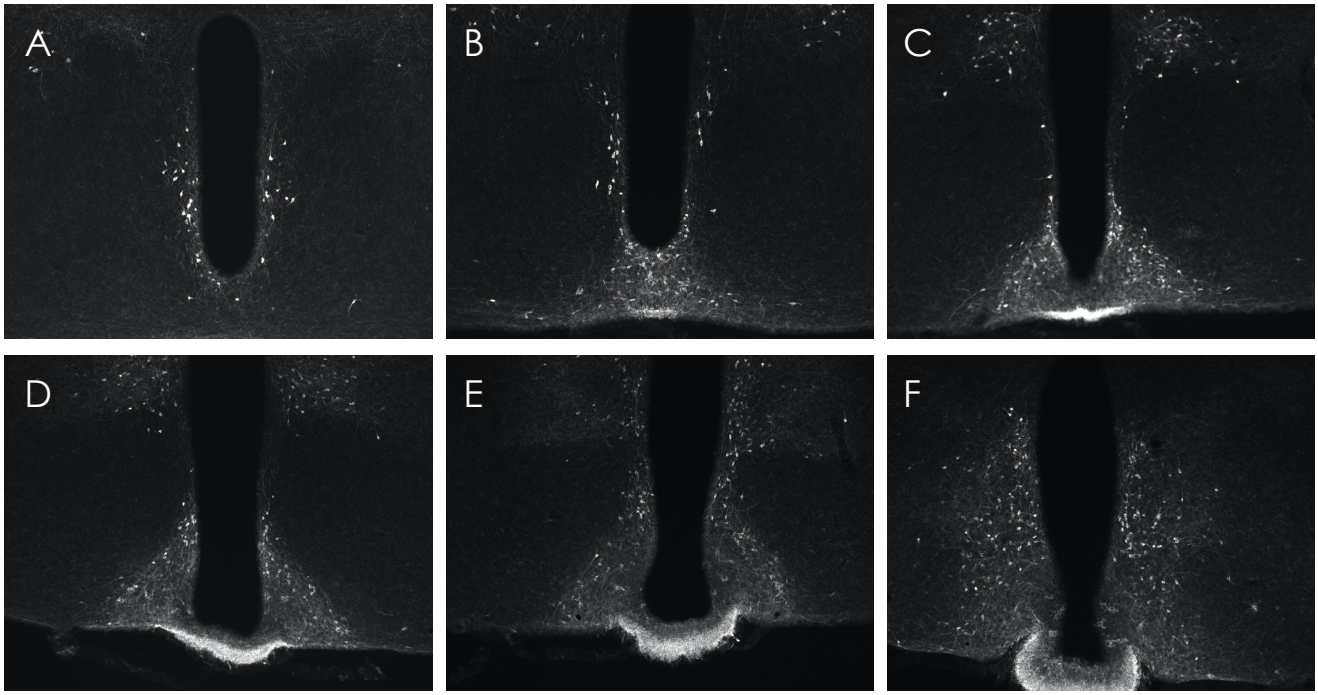

Figure 2. To determine the location of TIDA neurons every fourth section through the arcuate nucleus was examined for TH. For each animal, a brain atlas was created by aligning all photos from rostral to caudal and 3 photos containing TIDA neurons were chosen based on TH expression location in the arcuate nucleus. In this representative example, sections containing TIDA neurons are C, D, and E.

Statistical analysis

Main effects and interactions were examined using a two-way analysis of variance (ANOVA). Unpaired, one-tailed t-test, were used for planned comparisons with a hypothesized direction of effect based on previous literature. Significant main effects were examined via post hoc contrasts by Tukey's multiple comparisons test. Statistical analysis was performed in Prism (San Diego, CA, USA). All data are reported as mean ± standard error of the mean (SEM) with $P < 0.05$ considered statistically significant.

4.3 Results

RFRP-3-immunoreactive (RFRP-3-ir) cells

To test whether RFRP-3 cells are affected by stress during pregnancy, the number of hypothalamic RFRP-3-ir cells was examined (Figure 3A). The number of RFRP-3-ir cells decreased as pregnancy progressed (main effect of Days Post Conception ($F(1, 16) = 22.71$, $P < 0.0002$). This main effect was the result of a significant reduction in the number of RFRP-3-ir cells from early- to mid-pregnancy in control and stress groups: $t = 4.048$, $P < 0.001$). There was no significant main effect of stress on the number of RFRP-3-ir cells ($P > 0.05$).

The mean intensity (optic density) of RFRP-3-ir cells decreased as pregnancy progressed (main effect for Days Post Conception ($F(1, 16) = 12.93$, $P < 0.0024$; Figure 3B)). This effect was driven by a significant reduction in the mean intensity of RFRP-3-ir cells from early- to mid-pregnancy in control and stress groups: $t = 1.876$, $P < 0.04$). No significant main effect of stress on RFRP-3 cell density was found ($P > 0.05$).

The mean size of RFRP-3-ir cells did not differ among groups ($P > 0.05$; Figure 3C).

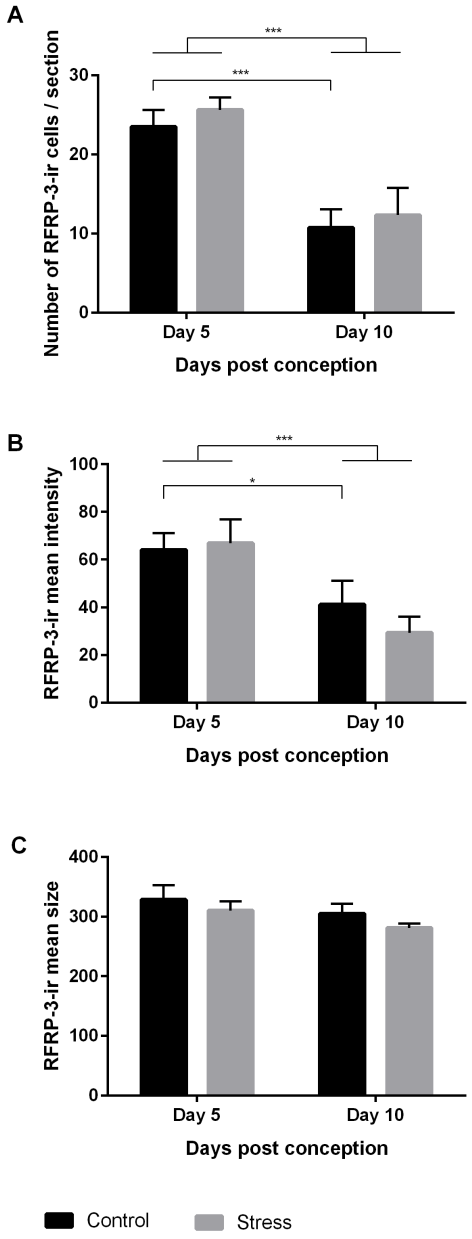

Figure 3. Number and intensity of are modulated by pregnancy progression but unaffected by stress, whereas the size of RFRP-3-ir neurons remains constant. A: the number of RFRP-3-ir cells decreased as pregnancy progressed. B: the mean intensity (optic density) of RFRP-3-ir cells decreased as pregnancy progressed. C: the mean size of RFRP-3-ir cells did not differ among groups. Data are presented as mean ± standard error of the mean. * $p < 0.05$; *** $p < 0.01$.

TH-ir cells

To examine whether the effects of stress in pregnancy are mediated by TIDA neurons, the rate limiting enzyme in dopamine synthesis, TH, was examined in hypothalamic cells (Figure 4A). The number of TH-ir cells did not differ across groups ($P > 0.05$ for both main effects and interaction).

To test whether RFRP-3 neurons potentially regulate TIDA neurons, the percentage of hypothalamic TH-ir neurons receiving close contacts from RFRP-3-ir axons was examined (Figure 4B). A small percentage of hypothalamic TH-ir neurons (TIDA) receive close contacts from RFRP-3-ir axons (a representative image at Figure 4C). The percentage of contacts exhibited an overall non-significant trend, with a decrease as pregnancy progressed (trend for Days Post Conception (F (1, 14) = 4.046, $P < 0.06$)). In early pregnancy, chronic stress increased the percentage of RFRP-3 input to TH-ir neurons in early-pregnancy ($t = 3.53$, $P < 0.006$; Figure 4B).

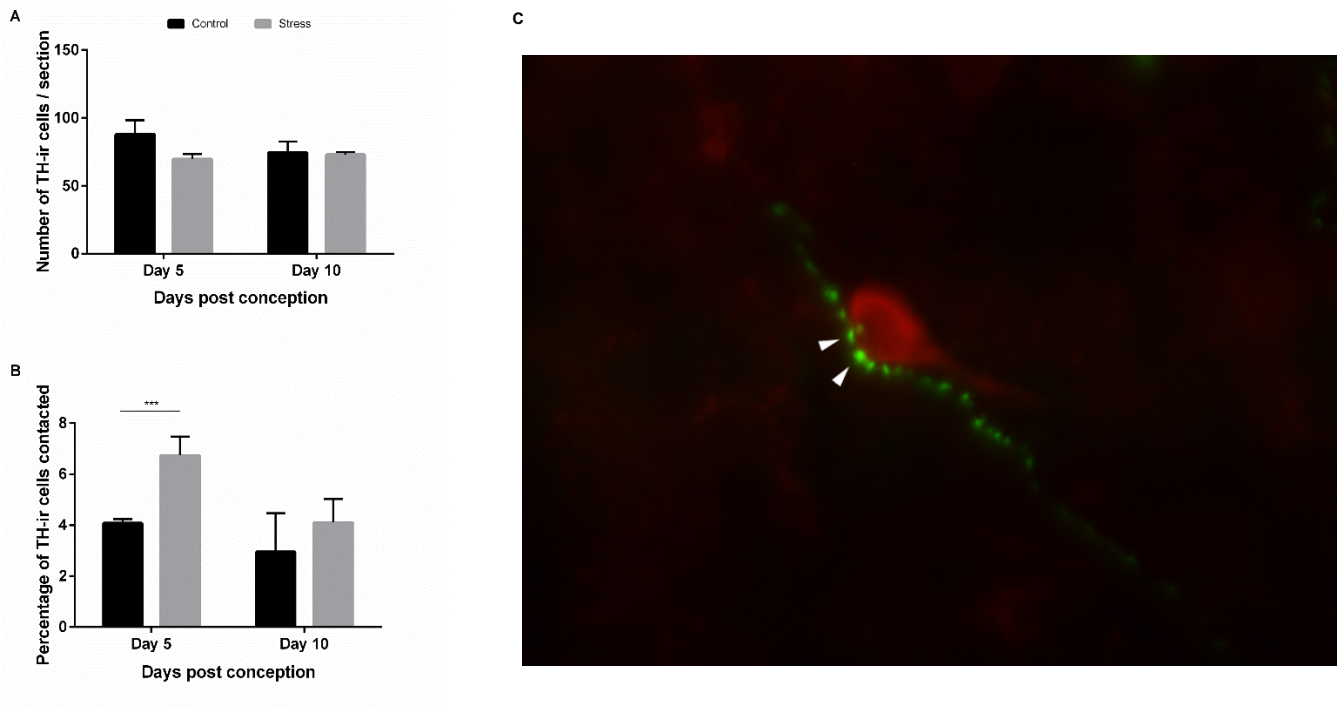

Figure 4. Percentage of TH-ir cells in close apposition with RFRP-3 fibers are modulated by stress in early- but not mid- pregnancy, whereas the number of TH+ cells are unaffected. A: the number of TH+ cells did not differ among groups. B: the percentage of TH+ neurons in close apposition with RFRP-3 projections was increased in the stress group in early pregnancy compared to non-stressed females (control) but was unaffected by stress in mid-pregnancy. C: a representative image of TH+ neuron receiving close contacts from RFRP-3-ir axon. Data are presented as mean ± standard error of the mean. *** $p < 0.01$.

Pituitary Gene Expression

The expression of pituitary prolactin (*Prl*) exhibited a significant interaction of Treatment by Days Post Conception (F (1, 31) = 4.25, $P < 0.04$), indicating that the magnitude of the difference between stress and control animals was reduced over stage of pregnancy (i.e., *Prl* expression was modulated by stress in early- but not mid-pregnancy; Figure 5A). Main effect for Treatment (F (1, 31) = 12.63, P < 0.001) was largely due to the impact of stress on *Prl* expression in early pregnancy (planned contrasts: early: $t = 4.09$, $P < 0.0003$; mid: $P > 0.05$).

Chronic stress elevated *D2 receptor* mRNA expression at all pregnancy stages (main effect for Treatment (F (1, 30) = 8.33, $P < 0.007$); planned contrasts: early: $t = 1.9$, $P < 0.04$; mid: $t = 2.209$, $P < 0.02$; Figure 5B).

The expression of *LHβ* subunit decreased as pregnancy progressed (main effect for Days Post Conception (F (1, 28) = 15.47, $P < 0.0005$); significant reduction from early- to mid-pregnancy in non-stressed animals: $t = 3.4$, $P < 0.002$), but was unaffected by stress ($P > 0.05$; Figure 5C).

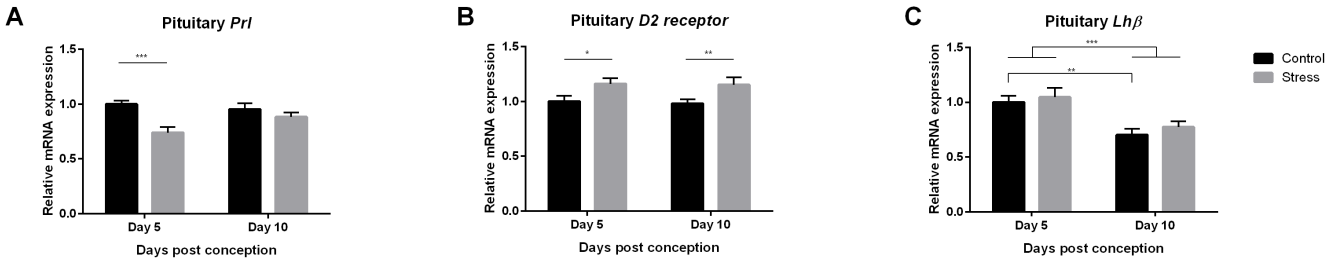

Figure 5. The impact of stress on pituitary mRNA expression. A: pituitary *Prl* mRNA expression is attenuated by stress in early-, but not mid-pregnancy. B: pituitary *D2 receptor* mRNA expression is increased in both early- and mid-pregnancy following stress exposure. C: pituitary *LHβ* mRNA expression is unaffected by stress, but exhibits a reduction as pregnancy progressed. Data are presented as mean ± standard error of the mean. * $p < 0.05$; ** $p < 0.03$; *** $p < 0.01$.

Steroid Hormones

No significant difference was found in baseline corticosterone concentrations over time ($P > 0.05$; Figure 6A). Chronic stress elevated baseline corticosterone at all pregnancy stages (main effect for Treatment (F (1, 33) = 20.32, $P < 0.0001$); planned contrasts: early: $t = 3.264$, $P < 0.014$; mid: $t = 3.262$, $P < 0.002$).

Chronic stress resulted in lower circulating progesterone in early- and mid-pregnancy (main effect for Treatment (F (1, 34) = 18.27, P < 0.0001; Figure 6B); planned contrasts: early: $t = 3.793$, $P < 0.0007$; mid: $t = 2.195$, $P < 0.02$). Main effect for Days Post Conception (F (1, 34) = 8.39, P < 0.006) is driven by the higher impact of stress on progesterone concentration in early-pregnancy compared to mid-pregnancy ($t = 3.08$, $P < 0.003$).

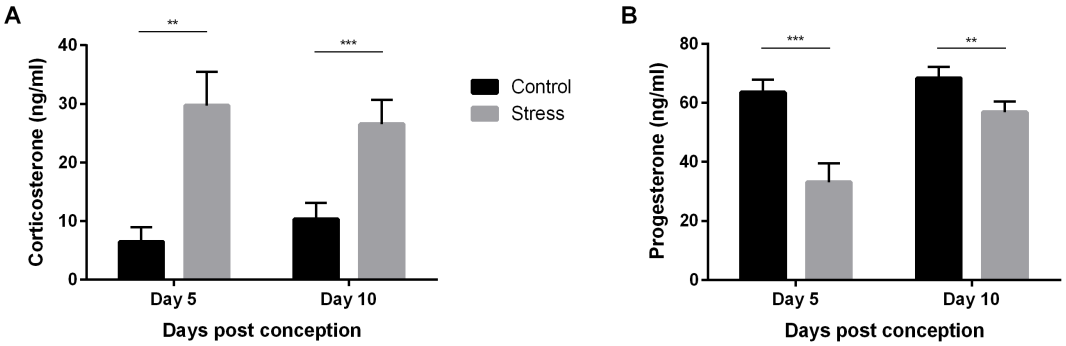

Figure 6. Steroid hormones are modulated by chronic stress in early- and mid-pregnancy. A: baseline corticosterone concentrations are elevated in chronically stressed mice in both early- and mid-pregnancy, compared to non-stressed mice. B: circulating progesterone concentrations are suppressed in chronically stressed mice in both early- and mid-pregnancy, compared to non-stressed mice. Data are presented as mean ± standard error of the mean. ** $p < 0.03$; *** $p < 0.01$.

Pituitary Hormones

For both stressed and control animals, prolactin was assessed at the morning and late afternoon during the expected times of the morning and afternoon prolactin surges. As expected, circulating prolactin decreased as pregnancy progressed (main effect for Days Post Conception in both morning ($F(1, 34) = 18.27$, $P < 0.0001$) and afternoon ($F(1, 34) = 7.35$, $P < 0.01$); Figure 7A). Prolactin concentrations did not differ between chronically stressed and control groups across the different stages of pregnancy or at different time points (morning and evening; $P > 0.05$ for all).

Circulating LH were unaffected by stress nor by stage of pregnancy ($P > 0.05$; Figure 7B).

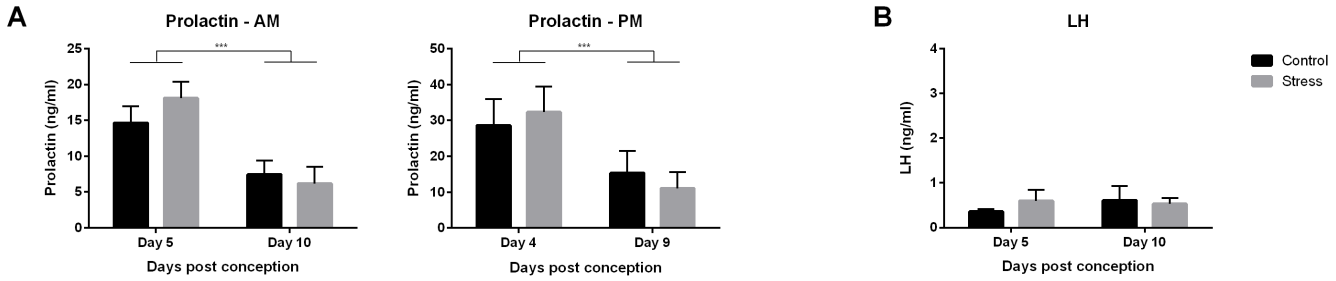

Figure 7. Circulating pituitary hormones are unaffected by chronic stress in early- or mid-pregnancy. A: circulating prolactin decreases as pregnancy progressed, but concentrations do not differ between chronically stressed and non-stressed mice at either pregnancy stage, in the morning (A, left) nor in the afternoon (A, right). B: circulating LH are unaffected by stress nor by stage of pregnancy. Data are presented as mean ± standard error of the mean. *** $p < 0.01$.

4.4 Discussion

The present findings indicate that, contrary to expectation, the RFRP-3 system is not affected by stress during pregnancy in the same manner as has been observed in non-pregnant females. Specifically, the number, intensity, and size of RFRP-3-ir cells were unaffected by stress in either early- or mid-pregnancy. These findings are surprising given several findings in non-pregnant mice and rats indicating altered RFRP-3 cell activation and increased gene expression in response to chronic and acute stress [299-301]. The disparity relative to non-pregnant rodents could be indicative of neuroprotection resulting from changes in neuroendocrine status occurring in pregnancy. Mammals, including women, experience substantial, central morphological and functional changes during pregnancy, including (but not limited to) a transient reduction in brain size [390, 391], reduced volume and suppressed hippocampal cell proliferation [392, 393], increases activation of the oxytocin and prolactin systems [394-397], and attenuated HPA response to a variety of stressors [398-400]. The attenuated stress response is a result of the progressive rise in placental CRH, which leads to elevation in baseline glucocorticoid secretion from the adrenal gland (i.e., hypercortisolism), that in turn suppress maternal hypothalamic CRH production [175]. It is possible that an additional outcome of this reduced stress response is the downstream effect on the RFRP-3 system.

Whereas the number and intensity of RFRP-3-ir cells were unaffected by stress, these measures were reduced as pregnancy progressed. Pregnancy-stage-related dynamic changes are not surprising, and have been previously reported in other contexts [397]. For example, ovarian mRNA expression of steroidogenic acute regulatory protein (*StAR*) and P450 cholesterol side-chain cleavage enzyme (*SCC*), involved in progesterone synthesis, is attenuated by chronic stress in early pregnancy, but markedly downregulated and unaffected by chronic stress by mid-pregnancy [383]. Likewise, pregnant rodents exhibit superior performance in spatial memory and reference memory tasks in early- and mid-pregnancy that then declines in late-pregnancy [401-404]. Taken together, these finding emphasize the numerous adaptations that occur throughout pregnancy and might explain why in mice the RFRP-3 system is protected from perturbation at this time.

The present findings also suggest a potential novel pathway between RFRP-3 and TIDA neurons. In non-stressed pregnant females, a small percentage of TIDA neurons received close appositions from RFRP-3 axons, providing a potential mechanism for the inhibition of prolactin secretion by stress. Furthermore, in early-pregnancy, stress increased the percentage of these contacts, suggesting that increased RFRP-3 cell input to TIDA neurons, as a result of stress, is possibly responsible for dysregulated *Prl* mRNA expression at this time point. Notably, in our pilot work, a similar pattern of results was found, with the percentage of RFRP-3-TIDA contacts increasing even more substantially from ~12 to ~23% in early pregnancy. However, these finding were from a small sample size (n=3-4/group) and the higher percentage of RFRP-3-TIDA contacts was not seen in a larger sample. Nonetheless, these findings identify a potential pathway of control for the impact of stress on neuroendocrine factors critical to pregnancy success.

In agreement with the neuroprotection observed for the RFRP-3 system, circulating prolactin was unaffected by stress in the morning and in the afternoon. These times were chosen because twice daily prolactin surges occur at those time points and we predicted that during chronic stress

will suppress prolactin release at these times [381]. The lack of effect of stress on prolactin concentrations is surprising given that stress attenuated pituitary *Prl* and *D2 receptor* mRNA expression. One possible explanation for this discrepancy is the pulsatile manner of prolactin release. With a pulsatile secretion pattern, a single time point may not be representative of the dynamic changes in hormone secretion [405]. Alternatively, stress can alter mRNA levels that are not reflected in translated proteins, consequently limiting impact on hormone release. Higher temporal resolution mRNA and hormone measures (e.g., serial sample collection) will help select among these possibilities and permit a more substantial characterization of potential stress-induced hormone dysregulation.

Finally, stressed mice exhibited elevated baseline concentrations of corticosterone that were associated with reduced progesterone concentrations. These findings are consistent with other rodent studies showing a negative correlation/association between baseline corticosterone and progesterone production during pregnancy [364, 381, 383]. One possible link between corticosterone and progesterone is via GnRH neurons and pituitary lactotrophs. Although lactotrophs are directly regulated by glucocorticoids, we found no impact of chronic stress on circulating LH or prolactin in pregnant mice. It is also possible that stress does not impact progesterone secretion through alterations in prolactin. For example, previous studies of chronic stress in pregnant mice suggest that glucocorticoids do not inhibit progesterone release during pregnancy via a top-down mechanism of control as seen in non-pregnant females [383]. Direct effects of glucocorticoids on ovarian physiology are also unlikely because of local glucocorticoid regulation within the ovaries [297, 298]. To better understand the underlying mechanism through which glucocorticoids inhibit progesterone release and subsequently impair pregnancy success, additional progesterone- and glucocorticoid-regulating pathways should be considered, including placental lactogens and 11β-hydroxysteroid dehydrogenase type 1 and 2 (11β-HSD1 and 11β-HSD2).

In the present investigation, non-pregnant mice were not included. As a result, comparisons between the impact of stress on pregnant and non-pregnant mice cannot be directly made. Thus, it is possible that the methods employed to examine the impact of stress on the RFRP-3 system were not sensitive enough capture subtle changes. Likewise, whether or not TIDA neurons express the RFRP-3 receptor, GPR-147, was not examined and neurochemicals co-released from this cell phenotypes may act on TIDA neurons to influence their activity. Comparing RFRP-3 profiles and examining RFRP-3-TIDA contacts as well as GRP147 in both pregnant and non-pregnant mice and under stress and no-stress conditions will help further elucidate the role of RFRP-3 in reproductive regulation.

Despite numerous studies attempting to understand the mechanism underlying reproductive failure, and specifically the pathways through which stress negatively impacts pregnancy outcomes, the interactions between stress and reproductive dysregulation are still not fully understood. The impact of stress during pregnancy appears to occur via mechanisms distinct from those seen in non-pregnant animals, thus underscoring the need for further studies examining these alternative pathways at different stages of pregnancy.

4.5 Acknowledgements

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5 Long-Term Effects of Chronic Stress on Embryo and Placenta Development in a Mouse Model

5.1 Introduction

A myriad number of human and animal studies have demonstrated the negative impact of stress on pregnancy outcomes and fetal development. In humans, maternal stress is associated with increased risk for miscarriages, preterm birth, and low birth weight, particularly if stress is occurring in the first trimester of pregnancy [281-284]. Likewise, psychological stress in the form of depression and anxiety is associated with increased risk for preeclampsia, lower birth weight, and reduced head circumference [282, 283, 285, 286]. In animal models, stress exposure or administration of glucocorticoids result in higher rates of embryo resorption (the rodent equivalent of a miscarriage), reduced litter size, and intrauterine growth restriction (IUGR) [287-289]. The impact of stress on offspring development persists beyond pregnancy and birth; maternal stress is associated with increased risk for adverse health outcomes later in life, affecting offspring's metabolism, cardiovascular system, and immunity [290-292], as well as increasing the risk for neurodevelopmental disorders [293-296].

Prenatal effects of stress are thought to be mediated by increased maternal HPA activity and the consequential rise of circulating glucocorticoids [297, 298]. Stress and elevated glucocorticoid concentrations lead to the suppression of progesterone, a hormone critical for pregnancy maintenance [351-354, 383] which, in turn, can lead to adverse pregnancy outcomes (e.g., implantation failure and delayed fetal development) [209, 348-350]. Despite numerous studies exploring the mechanisms underlying the negative impact of stress on pregnancy outcomes, the factors mediating these effects are less well understood. In our previous efforts to fill in these gaps we investigated how chronic stress and consequently increased glucocorticoids are acting on neural and endocrine networks to suppress progesterone production at different stages of pregnancy [383 and Chapter 3]. We found that chronic stress inhibited ovarian progesterone synthesis in early- but not mid-pregnancy, despite baseline corticosterone concentrations being elevated throughout. Interestingly, the suppression of progesterone in these pregnant mice did not occur via a top-down mechanism of the HPG axis [383]. Similarly, we found no evidence supporting a stress-induced activation of the inhibiting neuropeptide RFamide-related peptide-3 (RFRP-3; the mammalian ortholog of avian gonadotropin-inhibitory hormone (GnIH)), as it is the case in non-pregnant animals [7, 299-301]. This finding further suggests that alternative mechanism/s other than the top-down regulation. Several questions arise from those findings: (a) how do the early-pregnancy changes affect the embryo, (b) are those effects maintained across pregnancy and do they persist past recovery, and (c) if so, how do these changes impact fetal development? To address these questions we subjected pregnant mice to chronic psychological stress for the first half of gestation and allowed them to recover until late gestation. During late gestation, the long-term impact of stress on the developing embryos and their placentae was examined.

The placenta is considered to be one of the mediators by which stress effect fetal development, responding indirectly as well as directly via the glucocorticoid receptor (GR) [406]. The placenta is a unique, transient organ sitting at the interface of the maternal and fetal vascular systems, and mediating maternal-fetal exchange of nutrients and waste to support *in utero* development. In both

humans and rodents, its impact on the developing conceptus is immense; a functional placenta is critical for embryogenesis, transmission of the maternal milieu to the developing fetus, and long-term fetal programming [407, 408]. Consequently, placental impairments can result in poor pregnancy outcomes, including fetal growth restriction and miscarriage [409]. Indeed, many obstetrical syndromes, including pre-eclampsia, IUGR, stillbirth and preterm birth, frequently involve a defective placenta [410]. Furthermore, in many cases of placental deficiencies that manifest in late gestation (e.g., deficiencies in nutrient transport capacity), the underlying causes reside early in development, when the anatomical foundations of placentation are initially established [411].

The mouse and human placenta are similar in some aspects of form and function, but are structurally different. In humans and rodents, the placenta is hemochorial, as the maternal vascular space is in direct contact with differentiated trophoblasts. Widely conserved between mice and humans is the progesterone and estrogen dependency in the process of decidualization, the process in which maternal endometrial stromal cells to undergo a specialized reaction to blastocyst implantation [412, 413]. Likewise, analogous cell types are found among human and rodent trophoblasts, including proliferative trophoblast cells, invasive trophoblast cells and cells differentiating into syncytium. However, the placental morphogenesis and endocrine functions differ between human and rodent placentae. Unlike the human placenta, the mouse placenta achieves its definitive structure only halfway through gestation. In mice, the placenta is formed as extra-embryonic blood vessels invaginate into the chorionic trophoblast layer and stimulate vascularized trophoblast cells to differentiate to form different structures of the placenta. Two of these structures, the labyrinth zone (LZ) and junctional zone (JZ), are responsible for the exchange of nutrients between the mother and embryo and hormone production, respectively. Placentation deficiencies involving the LZ or the JZ may result in detrimental outcomes on fetal development and survival. Specifically, LZ impairments may results in an imbalance between the metabolic demands of the fetus and the ability of the placenta to meet those needs. If the LZ insufficiently mediates nutrient and oxygen transfer to the embryo, fetal growth may be hindered as a consequence [414]. Likewise, JZ anomalies are associated with IUGR and the size of the JZ (rather than the placenta as a whole) affects fetal growth [415, 416]. Despite numerous findings pointing to the involvement of placental deficiencies in adverse pregnancy outcomes, the underlying mechanism leading to these outcomes are not well understood.

In the placenta, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) protects the embryo from maternal glucocorticoids by catalyzing the conversion of cortisol and corticosterone to their inactive form. In contrast, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) reverses this conversion. Altered activity of 11β-HSD2 is thought to contribute to the impact of stress on the embryo as stress decreases the expression of 11β-HSD2 [417] and birth weight correlates with placental 11β-HSD2 activity [406, 418, 419]. Likewise, some IUGR fetuses exhibit reduced placenta 11β-HSD activity and mRNA expression with reduced cortisone:cortisol ratios in the umbilical artery [420]. Taken together, these findings suggest the involvement/contribution of altered placental 11β-HSD in delayed development.

Similar to 11β-HSD, fetal growth restriction is also associated with altered expression of pleckstrin homology-like domain, family a, member 2 (*Phlda2*) and Trophoblast-specific protein alpha (*Tpbpa*) and reduced placental lactogens. The imprinted gene *Phlda2* regulates the size of

the JZ. Mice lacking the *Phlda2* gene exhibit a disproportionately large JZ, whereas overexpression is associated with apoptosis of trophoblast cells and reduction of the JZ [421, 422] and fetal growth restriction [423, 424]. In addition to regulating placental and fetal growth, *Phlda2* also controls the production of placental lactogens, secreted throughout gestation to support development [425]. Finally, the gene *Tpbpa*, expressed in the spongiotrophoblast layer of the mature placenta, is involved in maternal blood delivery to the placenta [426] and responsible for trophoblast differentiation [427, 428]. The present study explores the long term impact of chronic stress on fetal and placental development following recovery from stress while considering the mechanisms required for proper placental functioning. We specifically examine LZ and JZ absolute size and proportion, and the expression of genes involved in placental and hormone regulation (i.e., *11β-HSD1* and *11β-HSD2*, *GR*, *Phlda2, PL2, and Tpbpa)*.

5.2 Materials and Methods

Animals

C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA) and housed in ventilated cages on a 14:10 light/dark cycle (lights on at 07:00, lights off at 21:00) with food and water available *ad libitum*. Experimental animals were pair-housed with an age-matched male throughout the experiment. All animals were allowed to acclimate for at least 1 week prior to commencing the experiment. Females used in these experiments were 10-12 weeks old. All protocols were approved by the UC Berkeley Office of Laboratory Animal Care and were consistent with NIH guidelines for the care and use of laboratory animals.

Experimental procedures

Virgin, female mice were paired with sexually-experienced males and examined for vaginal copulatory plugs every morning. The morning on which a plug was found was considered gestational day 1. Females were then randomly assigned to chronic stress (n=10) or control (unstressed; n=9) groups such that assignment between groups was balanced across the length of the experiment. All females were weighed each morning prior to treatment. Animals assigned to the chronic stress group were moved each morning, beginning on day 1, to a separate room where they were restrained in a modified 50 mL centrifuge tube (Corning, Corning, NY, USA). Animals were also exposed to predator odor during restraint: each day, 15 uL of predator odor (undiluted fox urine, Minnesota Trapline, Inc; Pannock, MN) was freshly soaked into a new cotton ball and placed in the cage with each mouse during restraint. Daily stress exposure lasted four hours from 09:00 to 13:00 and was repeated daily for 9 days. Unstressed females remained in their home cages.

On day 15 of pregnancy mice were euthanized via intraperitoneal injection of sodium pentobarbital (200 mg/kg) followed by rapid decapitation. Trunk blood was collected into 1.5 mL Eppendorf tubes (Fisher Scientific) and the number of fetuses for each side of the uterus was counted and fetal developmental abnormalities or resorption sites were recorded by an observer unaware of the individual's treatment. The fetuses were individually weighed and a subset of them were assessed for developmental stage based on Theiler Staging [429](Table 1) by 2 independent observers who were unaware of the dams' treatment. Placentae were collected and flash frozen in

isopentane on dry-ice. Tissues were stored at -80°C until extraction and analysis. Blood was centrifuged at 1300 g for 15 min at 4°C and plasma removed. Plasma was centrifuged a second time for 1 min. and then aliquoted and stored at -80°C. Blood samples were collected in less than 3 min from lifting the cage.

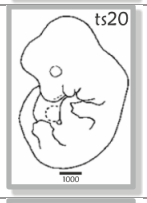
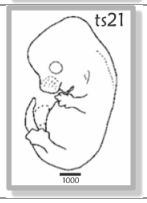
Theiler Stage	Days post conception	Figure	Features
19	11.5		Lens vesicle completely separated from the surface epithelium, Anterior, but no posterior, footplate. Auditory hillocks first visible
			Absent: retinal pigmentation and sign of fingers
20	12		Earliest sign of fingers, (splayed-out), posterior footplate apparent, retina pigmentation apparent, tongue well-defined, brain vesicles clear
			Absent: 5 rows of whiskers, indented
21	13		Anterior footplate indented, elbow and wrist identifiable, 5 rows of whiskers, umbilical hernia now clearly apparent
			Absent: hair follicles, fingers separate distally
22	14		Fingers separate distally, only indentations between digits of the posterior footplate, long bones of limbs present, hair follicles in pectoral, pelvic and trunk regions
			Absent: open eyelids, hair follicles in cephalic region
23	15		Fingers & Toes separate, hair follicles also in cephalic region but not at periphery of vibrissae, eyelids open
			Absent: nail primordia, fingers 2-5 parallel
24	16		Reposition of umbilical hernia, eyelids closing, fingers 2-5 are parallel, nail primordia visible on toes
			Absent: wrinkled skin, fingers & toes joined together
25	17		Skin is wrinkled, eyelids are closed, umbilical hernia is gone
			Absent: ear extending over auditory meatus, long whiskers

Table 1. On gestational day 15, the fetuses were individually weighed and a subset of them were assessed for developmental stage based on Theiler Staging by 2 independent observers who were unaware of the dams' treatment. This table is adapted from The House Mouse: Atlas of Embryonic Development (Theiler, 1989). For the complete table refer to [429].

Hormone analyses

Progesterone was quantified using the Cayman Chemical Progesterone ELISA (Item No. 582601, Ann Arbor, MI). Intra-and inter-assay variations for progesterone were 3.6% and 6.8%, respectively. Baseline corticosterone was quantified using the Enzo corticosterone ELISA kit (ADI-900-097; Enzo Life Sciences, Inc., Farmingdale, NY) according to the manufacturer's protocol for small sample volumes. Intra-and inter-assay variations were 6.19% and 5.08%, respectively. Prolactin was assayed using the mouse prolactin ELISA kit from Abcam (ab100736, Cambridge, MA). Intra-and inter-assay variations were 3.6% and 4.2%, respectively. Some samples did not have sufficient plasma to quantify all hormones, thus sample sizes may vary for different hormone measures.

Gene expression analysis

Total RNA was extracted from LZ and JZ samples using ISOLATE II RNA Mini-kit (BIO-52073, Bioline USA Inc., Taunton, MA). RNA concentration and purity were assessed by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific). The RNA quality of a random subset of samples (n = 16 and 8 for LZ and JZ, respectively) were analyzed on an Agilent Technologies Bioanalyzer and yielded an average RNA integrity number (RIN) of 9.4 or higher. Reverse transcription was performed using Takara Bio PrimeScript RT Reagent Kit with gDNA Eraser (cat. no RR047A, Mountain View, CA) and then frozen at -20°C until RT-qPCR was performed.

Analysis of relative gene expression via qRT-PCR was performed using SSOAdvanced SYBR Green supermix (BIO-RAD, 1725272, Hercules, CA, USA). Samples were run on a BIO-RAD CFX384 machine with 10µl reaction volumes with a 2-step amplification for 40 cycles followed by a melt curve. Primers were designed from published sequences for *mus musculus* using NCBI Primer BLAST software (Table 2). Primer sets were validated for specificity using positive, negative, no reverse transcriptase, and no template controls, and confirmed with a single-peak melt curve and correct product length. Efficiency of each primer set was determined by standard curve; primers were 96.5-104.8% 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 resulting data were analyzed in Microsoft Excel following the delta delta Cq method [324].

The geometric mean of 2 housekeeping genes' expression was used for reference. Because the expression of housekeeping genes was found to vary with treatment between tissues, samples from different tissue types were analyzed with different reference genes. *Rplp* and *TBP* were used as reference genes for the. Housekeeping genes were confirmed to not be significantly different between all groups, and in all genes replicate group Cq Standard deviation was smaller than 0.2. All data are expressed as a fold-change over early-pregnancy, control individuals. Some samples did not have sufficient cDNA to quantify the expression of all genes, thus sample sizes vary for different genes measured.

Primer	Forward	Reverse
11β-HSD1	TCTACATAACCAAGGTCAACGTGT	TCCCAGAGATTTCCTTCATAGCTG
11β-HSD2	GAACCTCTGGGAGAAACGCA	CGTGCACGTGCTCAATGTAG
GR	CCAAAGCCGTTTCACTGTCC	CGGCTGGTCGACCTATTGAG
Phlda2	TGATCGACTTCCAGAACCGT	CTGGTTCCCGGCTCAACTG
PL2	GGACAGCTTGGACCTATGGC	CCGATGTTGTCTGGTGGACT
Tpbpa	GCTATAGTCCCTGAAGCGCA	TCTATGTTGGAGCCTTCCGTC
Rplp	ATCTACTCCGCCCTCATCCT	GCAGATGAGGCTTCCAATGT
TBP	GGGAGAATCATGGACCAG	CATGATGCTTGATCACATGTCTCG

Table 2. Primers used for qRT-PCR.

Placental histology

H&E staining

Each placenta was sectioned at 20 μm for hematoxylin and eosin staining and histological assessment and at 60 μm for dissection of the junctional and labyrinth zones for further RNA extraction and gene expression analysis. Additional 40 μm sections were used for determining sex of the fetus for each placenta.

Slides containing placenta sections were placed in 4% PFA for 30 min. Slides were then moved to PBS for 5 min followed by Hematoxylin (80 sec) and water (1 min). The slides were then incubated with the following solutions: Acid 70% EtOH (30 sec), DW (10 quick dips), Scott's water (1 min), DW (3 min), Eosin Y (40 sec), DW (1 min), 70% EtOH (1 min), 70% EtOH (1 min), 80% EtOH (1 min), 95% EtOH (1 min), 100% EtOH (2 min), histoclear (10 min). Finally, slides were coverslipped with Permount (SP15-500, Fisher Scientific) and allowed to dry overnight.

Microscopy and quantification of placentae

Slides of placenta sections were scanned using brightfield light microscopy on a Zeiss AxioScan.Z1 microscope at the CRL Molecular Imaging Center, supported by the Biological Faculty Research Fund at the University of California, Berkeley. Images were taken at 5x and individual tiles were automatically stitched together in the Zeiss ZEN 2 Slidescan application (blue edition) to create a single picture of the whole organ cross-section. Cross-section photos were then converted from proprietary .czi file types to .tiff file types in the Zeiss ZEN 3.1 application (blue

edition). Four representative photos from each placenta were selected by an investigator blind to their experimental condition.

A custom MATLAB script was created to process the selected images and quantify the total cross-section area, LZ area, JZ area, percentage of each zone containing tissue, and length of the LZ-JZ border.. For the full script, please refer to the GitHub repository: https://github.com/Kriegsfeld-Lab/Placenta-Morphology-Analysis-2021

Briefly, the MATLAB script employed a color analysis to divide the placental zones - labyrinth, junctional, and decidua- according to their characteristic colors following H&E staining (see above). Four sections from each placenta were randomly selected for the morphological quantification and analysis. First, each original photograph was divided into 8 different clusters based on a CMYK color map, controlling for the intensity and contrast of the stain on each tissue section. The user, unaware of the treatment assignment, was prompted to choose which of the 8 CMYK clusters is the brightest in all placental zones and the script then focused on that cluster for the remainder of the analysis. Next, the chosen CMYK cluster was divided into 8 different segmentations based on distance between color peaks on an HSV color map. As the different placental zones absorb the stain differently, each zone is best represented by a different color. The 8 largest color peaks on the HSV map were divided such that each zone was most visible in the color segmentation that was closest to the stain color of that zone. This information was used to assign a color segmentation to each zone. The user was then prompted to roughly outline each zone, LZ, JZ, and DC, on the color segmentation chosen for each zone, respectively. This rough outline directed the script to the location to be analyzed and the perimeter of each placental zone to be defined. A separate image was generated for each zone (Figure 1) and the perimeter outline was used to calculate the area of the region, as well as subtract the area that does not contain tissue (to account for potential apoptosis). The length of the junction of the LZ and JZ was calculated by detecting where their perimeters overlap. Finally, the images of the zone sub-region perimeters, tissue, the LZ:JZ junction line were compiled.

In some instances where the chorionic plate tissue or remnants of the yolk sac were adhered to the placenta, they were excluded from the analysis using an accessory script that used the images and calculations created from the main script to recalculate the final data based on user input. This code is also in the GitHub repository (see link above).

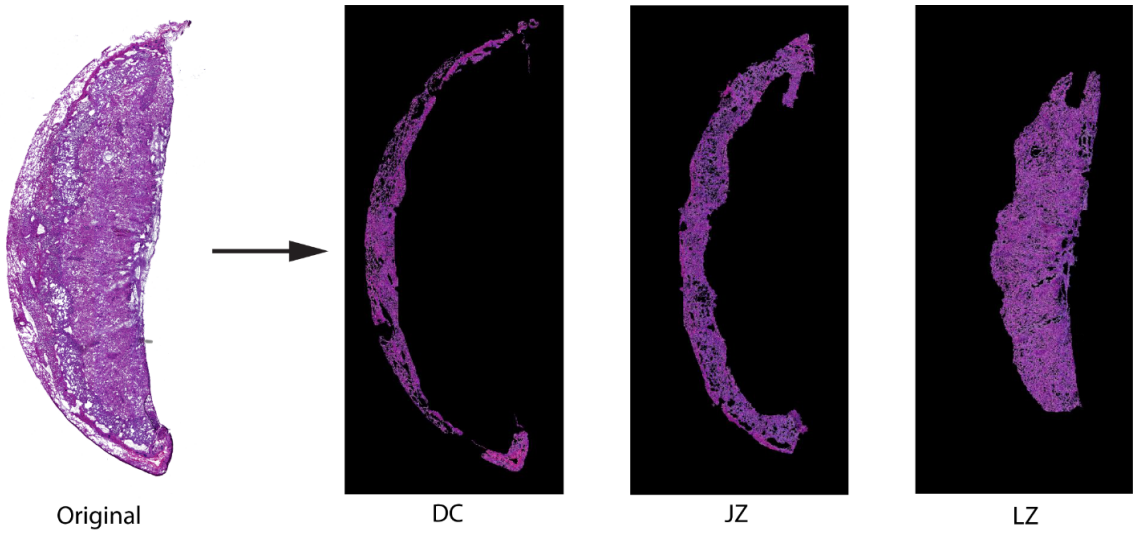


Figure 1. Representative images of each placental zone, generated by a custom MATLAB script. Each image was used to calculate the area of the region, as well as subtract the area that does not contain tissue. The length of the junction of the LZ and JZ was calculated by detecting where their perimeters overlap. Finally, the images of the zone sub-region perimeters, tissue, the LZ:JZ junction line were compiled. DC: maternal decidua. JZ: junctional zone. LZ: labyrinth zone.

Determination of sex

Placenta sections (40 µm) were incubated overnight at 56°C in 200 µL of Proteinase K and lysis buffer diluted at 1:100 (200mM NaCl, 100mM Tris (pH 8.5), 5mM EDTA (pH 8.0), and 0.2 percent SDS). The digested samples were centrifuged at 14,000 g at room temperature for 10 minutes, and the supernatant was poured off into a tube containing 200 µL of isopropanol and subsequently inverted to precipitate the DNA pellet. After a second round of centrifugation (14,000 g, room temperature, 10 minutes), the isopropanol was aspirated and the DNA pellet for each sample was diluted with 50 µL of TE Buffer (pH 7.6). The polymerase chain reaction (PCR) designed as a probe for sex genotyping was adapted from Simon James Tunster (2017) to yield a 269 bp product from the X chromosome and a 353 bp product from the Y chromosome (Forward: CACCTTAAGAACAAGCCAATACA; Reverse: GGCTTGTCCTGAAAACATTTGG) [430]. This probe was used to detect the two-copy Y-linked Rbm31y and the single-copy X-linked Rbm31x in the DNA samples. PCR was performed using MyTaq 2X Red Mix from Bioline (cat. no BIO-25044, Memphis, TN), and 10 µM of Rbm31x/y forward and reverse primers. Thermocycler conditions were 94°C for 2 minutes, followed by 30 cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 30 seconds with a final elongation period of 72°C for 5 minutes. PCR reaction were mixed with 6X Blue Gel Loading Dye and then loaded onto a 1.5 percent agarose gel.

Statistical analysis

Placenta gene expression and histology data were analyzed in R 3.6.3 using mixed models in the lme4() packages. Post-hoc comparisons were made using the lmerTest() package. In all models, dam ID was included as a random effect. Maternal weight and hormone analysis were

performed in Prism (San Diego, CA, USA). Main effects and interactions were examined using a two-way analysis of variance (ANOVA), with repeated measures where applicable. Unpaired, one-tailed t-test with Welch's correction, were used for planned comparisons with a hypothesized direction of effect based on previous literature. Significant main effects were examined via post hoc contrasts by Tukey's multiple comparisons test. All data are reported as mean ± standard error of the mean (SEM) with $P < 0.05$ considered statistically significant.

5.3 Results

Maternal weight

Chronically stressed mice exhibited a different pattern of weight gain throughout gestation, compared to non-stressed mice, with a magnitude of difference increasing as pregnancy progressed (Figure 2; significant interactions of Treatment by Gestational Day; $F(13, 221) = 5.637$, $P < 0.0001$)). Overall, stressed females exhibited smaller weight gains than non-stressed females (main effect of Treatment; $F(1, 17) = 7.37$, $P < 0.01$)), and both groups experienced increased in body weight as pregnancy progressed (main effect of Gestational Day; $F(13, 221) = 297.0$, $P < 0.0001$)).

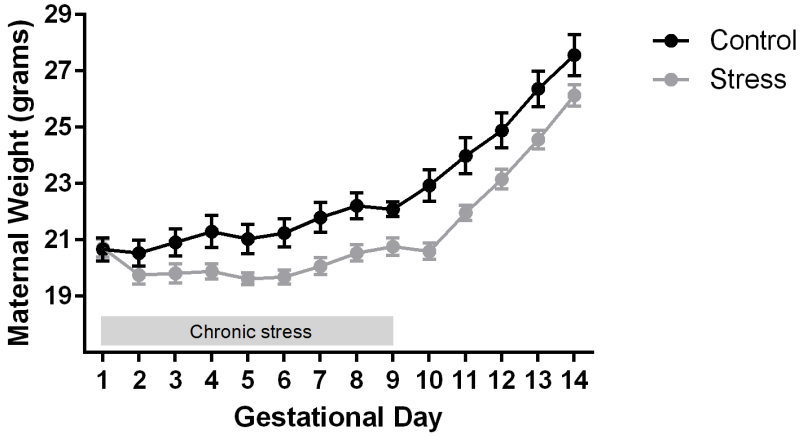


Figure 2. The impact of chronic stress on maternal weight. Pregnant mice that were exposed to chronic stress for the first 9 days of gestation exhibited a different pattern of weight gain throughout gestation, compared to non-stressed mice, with a magnitude of difference increasing as pregnancy progressed.

Hormones

No significant difference was found in baseline corticosterone concentrations over time ($P > 0.05$; Figure 3A). A significant interaction of Treatment by Days Post Conception was found ($F(2, 50) = 3.369$, $P < 0.04$), indicating that the magnitude of the difference between stress and control animals was reduced over stage of pregnancy (i.e., baseline corticosterone concentrations decreased in the stressed group but not in the controls). Chronic stress elevated baseline corticosterone at all pregnancy stages (main effect for Treatment ($F(1, 50) = 24.92$, $P < 0.0001$); planned contrasts: early: $t = 3.264$, $P < 0.014$; mid: $t = 3.262$, $P < 0.002$; late: $t = 2.586$, $P < 0.009$).

A significant interaction of Treatment by Days Post Conception was found for progesterone concentrations (F (2, 51) = 6.12, $P < 0.004$; Figure 3B). Specifically, chronic stress resulted in lower circulating progesterone in early- and mid-pregnancy but not in late-pregnancy (main effect for Treatment (F (1, 51) = 13.68, P < 0.0005); planned contrasts: early: $t = 3.793$, $P < 0.0007$; mid: $t = 2.195$, $P < 0.02$; late: $t = 0.14$, $P > 0.05$).

As expected, circulating prolactin decreased as pregnancy progressed (main effect for Days Post Conception. F (2, 50) = 28.7, $P < 0.0001$, Figure 4). Prolactin concentrations did not differ between chronically stressed and control groups across the different stages of pregnancy ($P > 0.05$ for all).

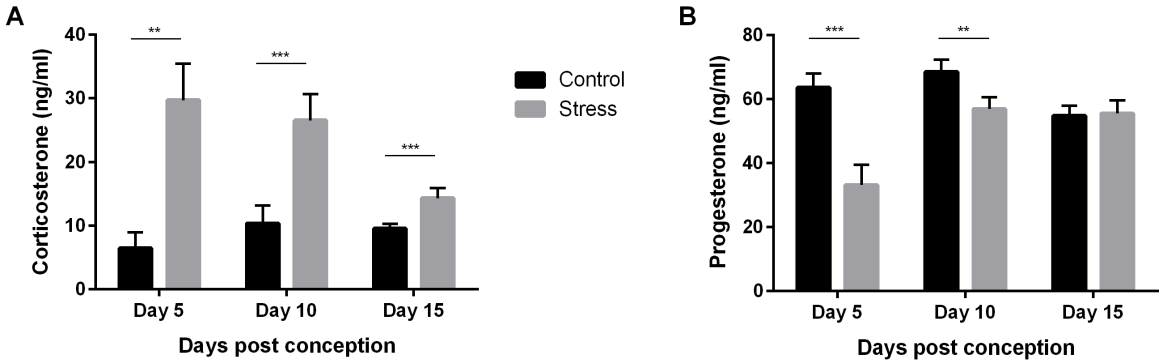


Figure 3. Steroid hormones are modulated by chronic stress. A: baseline corticosterone concentrations are elevated in chronically stressed mice in early- and mid-pregnancy, as well as in late-pregnancy, 6 days following the final stress exposure. B: circulating progesterone concentrations are suppressed in chronically stressed mice in both early- and mid-pregnancy, compared to non-stressed mice. Data are presented as mean ± standard error of the mean. ** $p < 0.03$; *** $p < 0.01$.

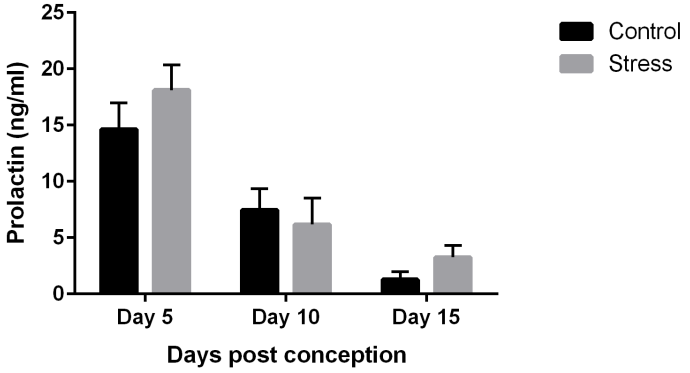


Figure 4. Circulating prolactin decreases as pregnancy progresses, but concentrations do not differ between chronically stressed and non-stressed mice at either pregnancy stage. Data are presented as mean ± standard error of the mean.

Embryos

Stress did not significantly affect the total number of embryos or number of resorption sites per dam ($P > 0.05$; Table 3). Developmental stage (TS) was assessed in embryos from a subset of dams. Whereas developmental stage was highly consistent among embryos from non-stressed mice (no embryos at TS<22), embryos from chronically stressed mice exhibited a high variation in which 35% of embryos were at TS<22 and 50% of dams had embryos at TS<22. The number of males and females embryos per dam exhibited a trend, with a female:male ratio of 4.2:3.5 and 4.8:2.8 for non-stressed and stressed dams, respectively, but did not reach statistical significance ($P < 0.07$). Finally, for statistical purposes, we used a single value of the average fetal weight by sex per dam, and found that stress significantly attenuated average fetal weight (main effect for Treatment ($F(1, 35) = 6.6$, $P < 0.01$, Figure 5); planned contrasts: females: $t = 1.88$, $P < 0.03$; males: $t = 1.87$, $P < 0.03$).

	Control (n=9)	SEM	Stress (n=10)	SEM
Mean number of embryos	7.78	0.42	7.9	0.34
Mean number of resorption sites	0.89	0.19	0.7	0.28
Mean embryo weight (grams)	0.201	0.01	0.154	0.01
Median embryo weight	0.199	0.01	0.150	0.01
Mean developmental stage (Theiler Staging; n=4, 6)	22.63	0.09	20.833	0.42
Percent of embryos < TS22 (n=4, 6)	0		35	
Percent of dams with embryos <TS22 (n=4, 6)	0		50	
Female : male ratio	4.2 : 3.5		4.8 : 2.8	

Table 3. Descriptive parameters of embryo development in chronically stressed and non-stressed (control) dams. Pregnant mice were exposed to stress for the first 9 days of gestation, 4 hours per day, and allowed 6 more days to recover before sacrifice at gestational day 15. Non-stressed pregnant mice remained in their home cage.

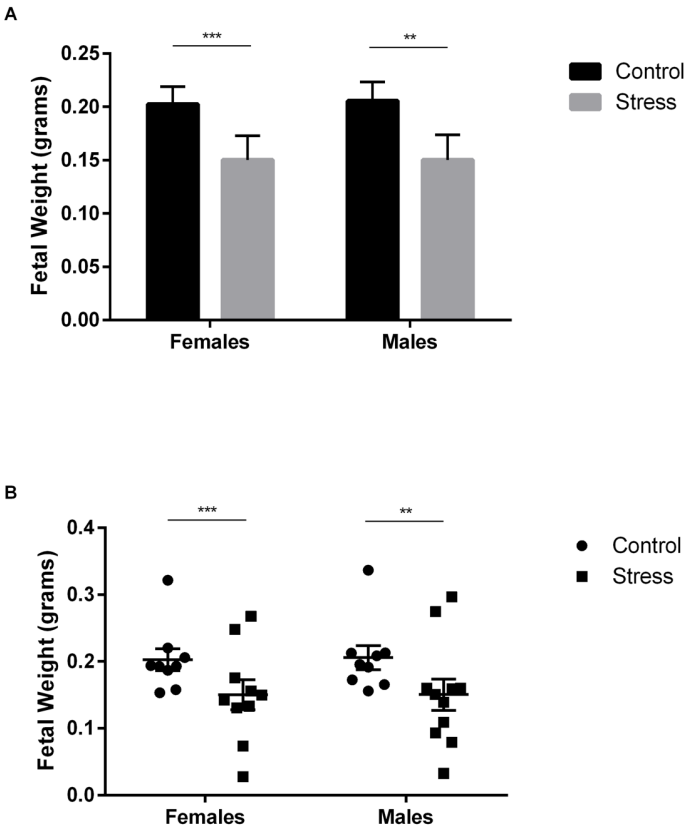


Figure 5. Prenatal chronic stress decreases body weight in female and male embryos. A: bar graph. B: scatter plot with each data point representing the average fetal weight by sex per dam. Data are presented as mean ± standard error of the mean. ** $p < 0.03$; *** $p < 0.01$.

Placental Histology

To examine the long term impact of chronic stress on placental morphology, we quantified the total area (mm^2) of each zone, the percent of the area that did not contain tissue ("void"; as an indicator of potential apoptosis), and the length of the curve separating the LZ and the JZ (Figure 6).

In female embryos from chronically stressed dams, the total area of both the LZ and the JZ was reduced (LZ: 1.86 vs. 1.62 mm^2 in control vs. stress, respectively, $t = 1.77$, $P < 0.04$; JZ: 1.07 vs. 0.86 mm^2 in control vs. stress, respectively, $t = 2.85$, $P < 0.005$. Figure 7A). No significant difference was found in the percent void of either zone.

In male embryos, the total area and the percent void in both the LZ and the JZ did not significantly differ between embryos from chronically-stressed and non-stressed dams (Figure 7B).

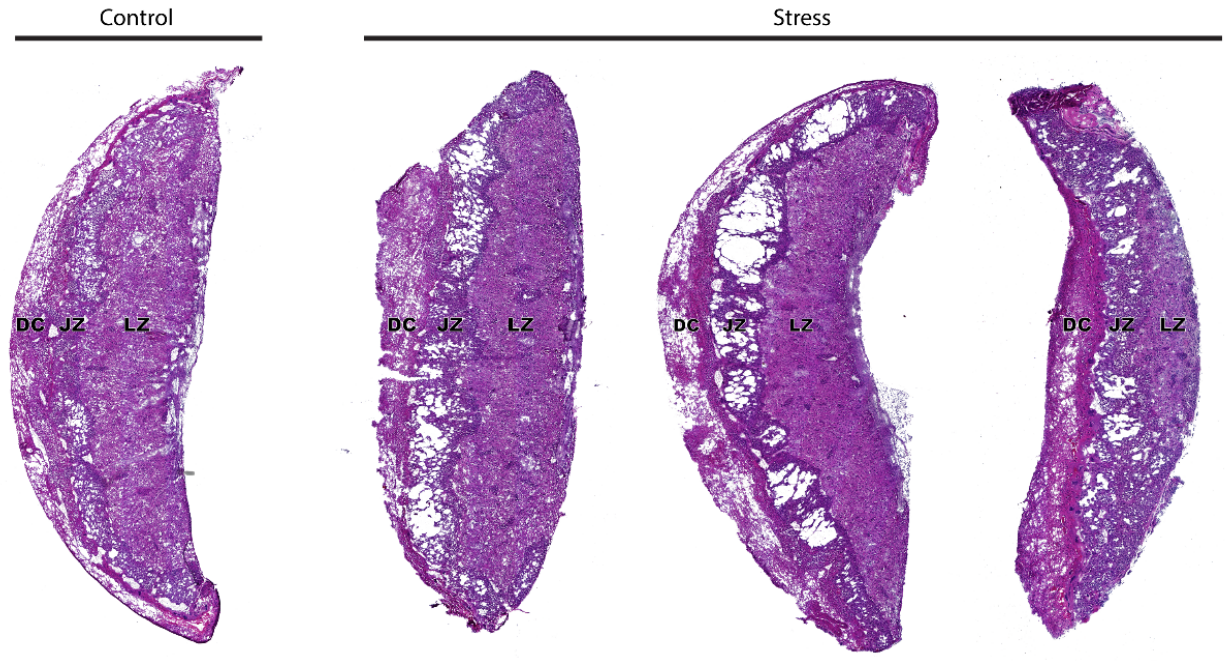


Figure 6. A representation of placentae from stressed and non-stressed embryos. To examine the long term impact of chronic stress on placental morphology, we quantified the total area (mm^2) of each zone, the percent of the area that did not contain tissue ("void"; as an indicator of potential apoptosis), and the length of the curve separating the LZ and the JZ. DC: maternal decidua. LZ: labyrinth zone. JZ: junctional zone.

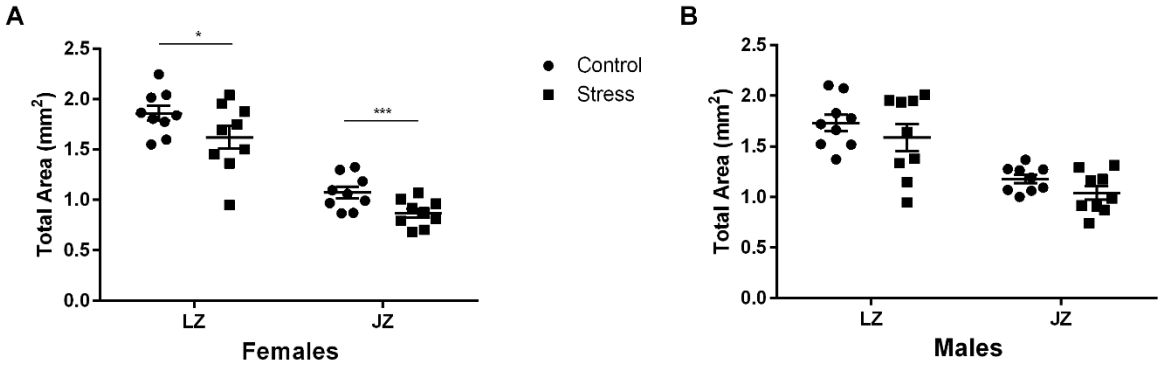


Figure 7. Chronic stress attenuated the size of both the LZ and JZ in female (A), but not male (B), embryos. Each data point representing the average fetal weight by sex per dam. Data are presented as mean ± standard error of the mean. * $p < 0.05$; *** $p < 0.01$.

Placental Gene Expression

We examined the long term effects of chronic stress on the mRNA expression of *11β-HSD1* and *11β-HSD2*, *GR*, *Phlda2, PL2, and Tpbpa* in the LZ and JZ.

LZ

In female embryos, chronic stress significantly modulated the mRNA expression of *11β-HSD1, GR*, *PL2* in the LZ (*11β-HSD1*: $t = 2.11$, $P < 0.02$; *GR:* $t = 1.99$, $P < 0.03$*; PL2:* $t = 2.02$, $P < 0.02$).

In male embryos, chronic stress significantly affected the mRNA expression of *GR* in the LZ ($t = 1.88$, $P < 0.04$).

JZ

In the JZ of placentae from female embryos, chronic stress significantly modulated the mRNA expression of *11β-HSD1* ($t = 2.06$, $P < 0.02$).

In the JZ of placentae from male embryos, chronic stress significantly modulated the mRNA expression of *Phlda2* ($t = 2.05$, $P < 0.02$).

Correlations

Correlations were evaluated within the stressed group only. Mid-pregnancy maternal weight (day 10) was positively correlated with mid-pregnancy progesterone concentrations (r = 0.653, $P < 0.02$; Figure 8). Surprisingly, progesterone at this time point did not correlate with any other measure. However, in female embryos maternal weight at day 10 was negatively correlated with day 15 11bHSD2 in both the LZ (r = -0.69, $P < 0.01$) and the JZ (r = -0.64, $P < 0.02$) and with total size of the JZ (r = 0.79, $P < 0.005$), correlations that were not evident in male embryos.

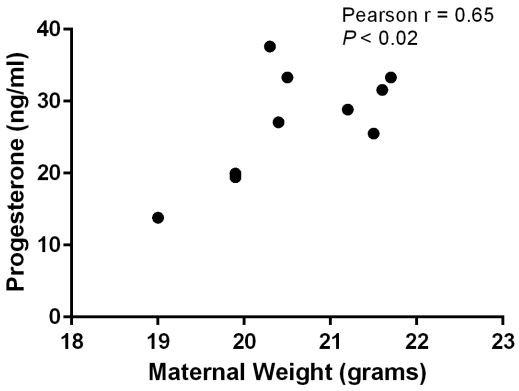


Figure 8. In chronically stressed mice, mid-pregnancy (day 10) progesterone concentrations were positively correlated with maternal weight at mid-pregnancy (day 10). Pearson's r = 0.65, $P < 0.02$.

5.4 Discussion

The present study sought to investigate whether stress-related effects occurring in early pregnancy persist past recovery and how stress affects fetal and placental development. The present findings suggest that the impact of stress persists after the exposure to the stressor has ceased in chronically stressed dams. Interestingly, the elevation in corticosterone in late pregnancy was not associated with a reduction in circulating progesterone, as observed in early- and mid-pregnancy. Nonetheless, the early exposure to chronic stress led to persistent developmental delays, reduced embryo weight, and abnormal placental histology and function.

Chronic stress can lead to a host of detrimental effects on implantation, litter size, embryo resorption, fetal growth, and survival rates [289, 299, 431-433]. In the present study, the impact of the chronic stress is reflected in maternal weight, with chronically stressed dams exhibiting lower weight gain, surpassing their pre-pregnancy weight after an initial decline following the cessation of stress. This difference could potentially result from embryo resorption and/or fewer implantations, though, in contrast to some reports, we did not find evidence to support this notion. Studies in which litter size was not impacted by stress-exposure report a significant increase in postnatal mortality of pups born to chronically and intermittently stressed mothers [434]. The altered weight gain, along with the inconsistency in the relation of corticosterone and progesterone, raises the notion that the progesterone suppression is not a direct outcome of the elevation in glucocorticoids, but may be more closely related to, or mediated by, attenuated weight gain. Supporting this notion is the positive correlation between maternal weight and progesterone concentrations at mid-pregnancy, though causation remains to be determined. This finding is in line with previous studies reporting a reduction in food intake in prenatally chronically stressed mice [435, 436]. As stressed mothers reduce their energetic intake and increase energy expenditure while resources are being depleted, the energetic allocation to maternal investment is decreased. Most likely, there are many endocrine and metabolic factors being altered by stress and contributing to progesterone suppression and downstream effects.

The negative impact of chronic stress is also evident in fetal weight and developmental stage. Although difficult to tease the two measures apart, body weight of chronically stressed fetuses were markedly reduced compared to those of non-stressed fetuses, and a subset of embryos also exhibited an earlier developmental stage than their gestational age. These findings are in line with other reports of stress related reduction in fetal weight and developmental delays [434, 435, 437]. It appears that the impact of stress in the first half of gestation is detrimental, even if progesterone levels or maternal weight gain are seemingly unaffected when evaluated at a late-pregnancy time point. Major developmental processes take place in the first half of gestation, and alterations in food intake, weight maintenance, metabolism, and energy consumption can support or impair these processes.

Across mammals, stressed mothers are less likely to birth males than females [438-440]. For example, a recent study assessing the psychological and physiological profiles of pregnant women found that healthy women gave birth to 56% boys whereas psychologically and physically stressed women gave birth to 40 and 31% boys, respectively [439]. The present findings exhibit are in agreement with this notion, with a female:male ratio of 4.2:3.5 for non-stressed dams and 4.8:2.8 for stressed dams, although this trend did not reach statistical significance. Notably, our

chronically stressed dams exhibited a high variance in sex ratio, with some dams carrying only 1 male (with 6-7 females) and others maintaining a roughly 1:1 ratio. None of the chronically stressed dams, however, gestated more males than females, in contrast to several non-stressed dams. It is likely that the type, duration, and timing of stress differentially influences each sex [437].

Despite not reaching statistical significance for sex ratio, males and females exhibited distinct placental alterations in response to stress. Whereas placental morphology did not differ between stressed and non-stressed male embryos, the LZ and the JZ (and, consequently, the placenta as a whole) were smaller in stressed, compared to non-stressed, female embryos. This is in seeming contrast to the notion that males are more vulnerable to stress (in terms of sex ratio at birth). However, the placenta is not a passive organ, it can morphologically and functionally adapt to optimize nutrient and oxygen supply. It is possible that the placentae of female embryos were more successful in supporting their survival because of their morphological (and functional) adaptation to environmental and physiological demands (e.g., limiting their size). Despite the common view of a smaller placenta as a negative pregnancy outcome, it may be the very outcome that enables survival, even if associated with lower birth weight, though this possibility requires further examination. Regardless, it is likely that the ability of the placenta to adapt to changing demands plays a role in embryo resilience and vulnerability.

The placenta of female embryos did not only differ morphologically, but also functionally. Stressed females exhibited lower mRNA expression of *PL2* in the LZ, which is in agreement with their reduced placenta size, as *PL2* expression is highly associated with placental development [441]. Likewise, stressed females exhibited lower mRNA expression of *11β-HSD1* and *GR* the LZ and lower *11β-HSD1* mRNA expression in the JZ. Surprisingly, stress did not affect the expression of *11β-HSD2*, which is considered the glucocorticoid "barrier". Although *11β-HSD1* holds bidirectional conversion abilities (between the active and inactive forms of glucocorticoids), it is thought to predominantly increase the activity of glucocorticoids in target tissues [442, 443], though this has not been specifically assessed in the placenta. If the increase in local glucocorticoid activity also applies to the placenta, then reduced *11β-HSD1* mRNA expression, along with the reduction in *GR* expression, suggests that the placentae of chronically stressed females limited their local glucocorticoid exposure. Interestingly, stressed males, but not females, exhibited an increase in JZ *Phlda2* mRNA expression. Phlda2 modulates the placenta's demands for maternal resources, and increased expression is associated with fetal growth restriction [423, 425, 444]. Taken together, these findings reveal a distinct impact of stress on males and females and the differential mechanisms underlying these outcomes.

Notably, regardless of the statistical significance of the effects of stress on a given measure, the present study found a remarkable variability in the impact of stress on dams as well as embryos. Whereas some dams who were chronically stressed exhibited deficiencies in a variety of measures (e.g., fetal developmental stage), other chronically stressed dams appeared to be more resilient to stress. Individual differences in stress response are surprising given that the mice in this study are inbred, hence 100% genetically identical. This suggest that the variation in response is, at least partially, due to epigenetic modifications. Indeed, in humans, methylation in the promoter of the GR NR3C1 were found in offspring of mothers exposed to prenatal stress [445-447]. Likewise, maternal circadian disruption, which activates the HPA, was found to be associated with

differential methylation in the placenta [448]. In stressed rats, increased DNA methylation within the *11β-HSD2* promoter corresponded with neural changes [449].

Importantly, in the present study maternal body weight and hormonal status have been measured periodically, whereas fetal and placental observations are limited to late gestation. Pregnancy is characterized by dynamic changes in neural, endocrine, immune, and metabolic networks (as well as others). Thus, the impact of chronic stress observed in measures from late gestation does not represent these dynamics but merely the outcome of converging factors acting long before specimen collection. For example, in the present study, chronically stressed embryos exhibited reduced weight at late pregnancy but progesterone concentrations at that time point did not differ between stressed and non-stressed dams. Earlier measures of progesterone indicated that this hormone was reduced in stressed dams despite the later recovery. Similarly, alterations in placental morphology and function occur before any changes in fetal growth are detected [450, 451], which can potentially explain some of the discrepancies between findings from the present study and previous reports (e.g., the lack of stress-induced alteration in *11β-HSD2* mRNA expression, which might be time and context dependent).

Overall, the present findings suggest that the impact of stress is evident well past its cessation, and that some aspect of reproductive function seemingly recover. However, these early, stressful experiences result in persistent developmental delays, reduced embryo weight, and abnormal placental histology and function. These findings underscore the need for higher temporal resolution to characterize the kinetics of placental morphology and function and their relation to fetal development in normal and atypical pregnancies.

5.5 Acknowledgements

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6 Conclusions

Each phase of the female reproductive cycle, from ovulation, pregnancy and fetal development, to parturition, is regulated by complex interactions of neural and endocrine networks operating synergistically and adjusting in response to physiological needs. Disrupting this multifaceted orchestration can lead to marked, negative consequences for female reproductive health. The overarching goal of this dissertation research was to shed light on the neural and endocrine mechanisms regulating the female reproductive system, focusing on ovulation and pregnancy, and how these mechanisms are affected by disruption to homeostasis: circadian misalignment and stress.

Chapter 2 revealed time-dependent responsiveness of the reproductive axis to RFRP-3 inhibition, suggesting a variation in the sensitivity of arcuate nucleus kisspeptin neurons to this neuropeptide. This study supports the notion that the reproductive axis is most sensitive to RFRP-3 inhibition in the afternoon (around the time of the LH surge) and suggests that the mechanisms driving LH secretion differ depending on the time of day and neurochemical environment, at least in Syrian hamsters. This study highlights the importance of timed suppression of the RFRP-3 system at the appropriate time of day to allow for the LH surge and ovulation to take place. Furthermore, it raises the possibility that, in cases of circadian disruption (e.g., irregular sleep patterns, nighttime exposure to light-emitting devices, shift work), mistimed RFRP-3 release may be responsible for compromised fertility seen across species, including humans.

In many cases, compromised fertility and adverse pregnancy outcomes are associated with psychosocial or physiological stress. Chapters 3-5 investigated the mechanisms underlying the impact of chronic stress in early- and mid-gestation, at the level of multiple corresponding systems, namely, the endocrine system, ovaries, brain and pituitary gland, placenta, and embryo, and the lasting outcomes past the cessation of stress. These studies revealed that many of the effects of stress in pregnancy occur via mechanisms distinct from the top-down pathways within the reproductive axis involved in stress-related effects in non-pregnant mammals. Specifically, progesterone suppression does not occur via alterations in LH or prolactin secretion, and the RFRP-3 system is not affected by stress during pregnancy in the same manner as has been observed in non-pregnant females, indicative of potential neuroprotection resulting from changes in neuroendocrine status occurring in pregnancy. Significantly, these studies suggest a potential novel pathway between RFRP-3 and TIDA neurons in the regulation of prolactin and pregnancy maintenance. Despite the seeming recovery of some aspects of reproductive function in late gestation, the early exposure to chronic stress leads to persistent developmental delays, reduced embryo weight, and abnormal placental histology and function. Major developmental processes take place in the first half of gestation, and alterations in food intake, weight maintenance, metabolism, and energy consumption can support or impair these processes. Interestingly, males and females are distinctly affected by stress with differential mechanisms underlying these outcomes. It is likely that the type, duration, and timing of stress differentially influences each sex. Taken together, these studies emphasize the numerous adaptations that occur throughout pregnancy and demonstrate that chronic stress impacts neural and endocrine networks differently in different stages of pregnancy as well as in pregnant and non-pregnant mammals.

Nonetheless, understanding the mechanisms involved in female reproductive success in general, and in pregnancy maintenance specifically, is hindered by a lack of some basic but essential information. Much of our current knowledge stems from studies measuring various variables at a given time point (of day, of cycle, or of pregnancy). This practice provides a glimpse into the physiological state at a very specific time point. Physiological processes, however, are dynamic, and exhibit rhythmic fluctuations, and in the context of pregnancy they also change with its progression. Taking a 'snapshot' of a dynamic process may be unrepresentative of the process as a whole and can lead to erroneous inferences. In my view, many of the discrepancies in the scientific literature stem from insufficient temporal resolution that leads to interpretations that may only hold true in a static environment. If the studies described in this dissertation had examined a single time point in late gestation, erroneous conclusions regarding the role of progesterone would have been made. To better understand how disruptions to homeostasis impact pregnancy, we first need to characterize the typical physiological changes occurring in healthy pregnancy throughout its progression. Thus, there is a need for a better understanding of the kinetics of normal and atypical pregnancies and the mechanisms contributing to these outcomes. The importance of considering a higher temporal resolution goes beyond the study of pregnancy and holds true to any physiological aspect, both *in vivo* and *in vitro*. For example, a developing conceptus in cultured conditions of *in vitro* fertilization (IVF) is typically exposed to a static environment, whereas the *in vivo* milieu within the maternal oviduct and uterus is changing rhythmically. Understanding how it is changing and mimicking this rhythmicity *in vitro* may increase IVF success rates.

The modern world makes stress and circadian disruption virtually inescapable, underscoring the importance of developing safe and effective strategies to maximize reproductive health (and health in general) in the face of pervasive disruptions to homeostasis. Likewise, educating couples about the importance of circadian and mental health represents an important consideration for health professionals providing counseling during family planning.

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