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Regulation of the GnRH neuron during stress

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Abstract:

The effect of stress on reproduction and gonadal function has captivated investigators for nearly 100 years. Following the identification of GnRH 50 years ago, a niche research field emerged fixated on how stress impairs this central node controlling downstream pituitary and gonadal function. It is now clear that both episodic GnRH secretion in males and females, and surge GnRH secretion in females, are inhibited during a variety of stress types. There has been considerable advancement in our understanding of numerous stress-related signaling molecules and their ability to impair reproductive neuroendocrine activity during stress. Recently, much attention has turned to the effects of stress on two populations of kisspeptin neurons—the stimulatory afferents to GnRH neurons that regulate pulsatile and surge-type gonadotropin secretion. Indeed, future work is still required to fully construct the neuroanatomical framework underlying stress effects, directly or indirectly, on GnRH neuron function. The objective of this review is to evaluate and synthesize evidence that stress-related signaling molecules act directly on GnRH neurons. Here, we review the evidence for and against the action of a handful of signaling molecules as inhibitors of GnRH neuron function, including corticotropin-releasing hormone, urocortins, norepinephrine, cortisol/corticosterone, calcitonin gene-related peptide, and arginine-phenylalanine-amide-related peptide-3.

Main Text:

1. Introduction:

As the GnRH neuron is central to the orchestration of pulsatile luteinizing hormone (LH), surge LH as well as the coordination of reproductive cycles in the female, each of these processes is vulnerable to impairment by stress. We begin by briefly discussing important studies which distinguish effects on pulses versus surge secretion, across stress models and species, and expand to highlight studies of reproductive cyclicity (e.g. menstrual cycles in women or primates and estrous cycles in other mammals). Although addressed separately, we acknowledge that distinguishing the relative importance of impaired pulsatile and surge GnRH secretion is challenging due to the interdependence of these modes of GnRH secretion in manifestation of ovarian cycles. Indeed, pulsatile secretion of LH supports gametogenesis and steroidogenesis in both sexes, and in females, a rise in estradiol (E2) is necessary for triggering the preovulatory LH surge.[1] Therefore, stress suppression of LH pulses can have profound effects on the LH surge, ovulation and the reproductive cycle and health in general (E2 and testosterone support musculoskeletal,[2] metabolic,[3,4] and mental[5] health). One important caveat is that much of the evidence supporting our understanding of stress actions on the GnRH neuron is based on the assessment of LH secretion, which does not always directly reflect GnRH secretion, such as in situations of diminished pituitary responsiveness (i.e. during stress or during the surge).[6] Despite this limitation, assessment of LH secretion remains a robust and economical method of assessing GnRH secretion indirectly. As such, much of the data discussed below utilize LH concentrations to infer GnRH secretion patterns. Additionally, different stress types impair reproduction via different pathways; thus, identifying the neural systems and central signaling molecules whereby distinct stress types interfere with the secretion of GnRH remains an exciting and expanding field of research. The objective of this review is to examine the effect of stress on GnRH neurons; in particular, the evidence that stress-related signaling molecules act directly on GnRH neurons will be evaluated.

1.1 Pulses: A variety of stress models have been used to investigate the effects of stress on LH pulsatile secretion including psychosocial, metabolic and immune/inflammatory. For psychosocial stress, physical restraint suppressed LH pulses in monkeys,[7] sheep,[8] rats,[9] and mice.[10,11] Various models of metabolic stress including insulin-induced hypoglycema,[12-16] glucoprivation,[17,18] lipoprivation[19] and feed restriction[20-22] each suppressed LH pulses. Immune/inflammatory stress modeled with either endotoxin (lipopolysaccharide)[23-25] or administration of cytokines[26,27] also suppressed LH pulses in many species. Together these

data, from a variety of stress models and species, demonstrate potent inhibitory effects of stress on pulsatile LH secretion, in both males and females.

1.2. Surge: Stress has also been shown to interfere with the generation of the preovulatory GnRH/LH surge in two major manners. First, stress can prevent or delay the rise in E2 necessary for triggering the LH surge. A delay in the rise of E2 and subsequent LH surge, likely reflecting an inhibition of LH pulses, has been demonstrated in sheep during psychosocial (transport stress),[28] immune/inflammatory[29] and metabolic stress.[30] Interestingly, in sheep exposed to metabolic stress in the early follicular phase, although the rise in E2 and LH surge was delayed, timing of estrous behavior was not altered.[30] This raises the possibility that if ovulation did occur, it may not have been correctly timed with mating to facilitate fertilization. Second, stress can interfere with the ability of E2 to induce surge-type GnRH and LH secretion. Thus, E2-induced surge models are necessary to isolate and distinguish the effects on the surge generation circuitry from the masking effects on pulsatile LH or ovarian E2 production. Indeed, immune/inflammatory stress also blocked the E2-induced GnRH/LH surge in sheep[31] and rats.[32,33] In mice, metabolic stress (chronic feed restriction) blocked an E2-induced LH surge.[20] Collectively, these data demonstrate multiple central mechanisms whereby stress interferes with generation of the LH surge.

1.3. Reproductive cycles: In theory, suppression of either pulsatile or surge-type GnRH/LH secretion could inhibit reproductive cycles. Functional hypothalamic amenorrhea is an anovulatory disorder in women, resulting from insufficient GnRH and LH secretion, which is often associated with a variety of life experiences constituting metabolic and/or psychosocial stressors.[34] Similarly in monkeys, a combined psychosocial stress, feed restriction and exercise paradigm, inhibited menstrual cycles.[35] In mice, both chronic psychosocial stress (daily restraint stress)[36] and mild feed restriction[20] suppressed estrous cyclicity, as evidenced by persistent diestrus-like vaginal lavage consisting primarily of leukocytes and an absence of cornified cells indicating low E2 levels. However, not all stress paradigms result in suppression of the estrous cycle. For example, altered estrous cyclicity was not identified in either a two-week unpredictable chronic mild stress protocol[37] or a daily restraint (homotypic) stress model[38] in mice. Determination of estrous cyclicity in rodents is routinely performed by analysis of cells collected from vaginal lavage. Importantly, morphology of these cells is primarily dictated by E2 levels and does not necessarily indicate that ovulation occurred.[39] Indeed, normal estrous cyclicity has been observed in mice, as determined by vaginal lavage, that did not produce natural or E2-induced LH surges and had ovaries with few or no corpora lutea, suggesting

impairment not revealed by vaginal cells *per se*.[40] In sheep, although some psychosocial stressors (such as 4 hours of transportation stress) delayed and suppressed the LH surge,[28] other repeated acute stressors including isolation, restraint and predator sounds did not disrupt follicular phase events.[41] Whether application of more intensive stressors would disrupt estrous cycles remains an outstanding question. Overall, these data demonstrate that normal reproductive cycles can be sensitive to the inhibitory effects of stress, though some stress types (immune or metabolic) may be more effective in suppressing cycles than psychosocial stress. These important phenomenological data have provided rationale for investigation of the specific neural substrates and processes responsible for suppression of GnRH secretion and thereby reproductive suppression during stress. In the following sections, the function of a handful of important mediatory molecules with implications for direct *vs.* indirect actions on GnRH will be reviewed (see Figure 1).

2. Effect of stress mediators on GnRH cells and secretion:

2.1. Corticotropin-Releasing Hormone (CRH) and related peptides: Since its discovery in 1980, the neuropeptide CRH, which regulates the hypothalamic-pituitary-adrenal (HPA) axis, has been postulated to be the integrator of the reproduction and stress axes. Investigation of CRH as a possible mediator of stress-induced suppression of gonadotropin secretion continues to evolve with understanding of species differences and functional arrangement of neurons that produce CRH receptors and ligands. In ovariectomized (OVX) monkeys, intravenous injection or infusion of CRH suppressed pulsatile LH secretion.[42-44] The effects of CRH in sheep, however, are varied. Indeed, CRH delivered intracerebroventricularly (ICV) to ewes in the early follicular phase of the estrous cycle suppressed LH pulse frequency.[45] However, in OVX ewes (with or without gonadal steroid replacement) ICV CRH has been reported to have either no effect[46] or stimulate LH pulse frequency.[46,47] Similarly, in orchidectomized (ORCHX) or testosterone-replaced ORCHX rams, ICV CRH increased mean LH concentrations.[48] In rats, ICV but not IV CRH reduced LH in OVX rats and OVX rats treated with estradiol benzoate (an LH surge model).[49] Additionally, in anesthetized rats ICV CRH blocked the GnRH surge on the evening of proestrus.[50] In OVX mice, chemogenetic activation of CRH neurons in the paraventricular nucleus (PVN) suppressed LH pulses,[51] indicating that some molecule(s) released from these neurons is sufficient to inhibit gonadotropin secretion. These varied results likely demonstrate some species differences and highlight the necessity of carefully considering gonadal steroid hormone status of experimental animals; additional experimental details such as dose and route of administration may also influence these varied observations. Importantly, though inhibitory

effects of CRH on gonadotropin secretion have been reported, numerous conditions exist in which CRH did not suppress gonadotropin secretion which raises the possibility that other signaling molecules are critical for suppression of reproduction during stress.

2.1.a. Urocortins: In mammals, there are three urocortin peptides (UCN1, UCN2 and UCN3) that are structurally similar, but distinct from CRH. All three urocortin peptides are produced in the brain and have been investigated for their roles in stress responses. The major site of UCN1 expression is within and adjacent to the Edinger-Westphal nucleus of the midbrain.[52,53] UCN2 is produced in the PVN, arcuate (ARC), and supraoptic nuclei of the hypothalamus, as well as the brainstem and spinal cord.[54] UCN3 is produced in the medial amygdala and hypothalamus (preoptic area and perifornical region).[55] UCN2 injected ICV into E2-replaced OVX rats suppressed LH pulse frequency.[56]

2.1.b. CRH and urocortin signaling mechanisms: CRH signals via its receptor corticotropin-releasing hormone receptor 1 (CRHR1). Corticotropin-releasing hormone receptor 2 (CRHR2) shares approximately 70% sequence homology with CRHR1, though it is encoded by a distinct gene. UCN2 and UCN3 have much higher affinity to CRHR2, whereas UCN1 has approximately equal affinity for both receptors. Consideration of both CRHRs and their ligands is important because some commonly used CRHR antagonists (e.g. alpha-helical CRH) are not specific to CRHR1, and thus physiological actions of CRHR2 ligands have been attributed to CRH. Indeed, non-specific CRHR antagonists prevented (or partially reversed) the inhibitory effects of acute metabolic stress on LH in monkeys[57] and rats.[58] With the advent of specific receptor antagonists the roles of the receptor subtypes have been investigated. For example, CRHR1 blockade prevented the suppression of LH pulses following psychosocial stress, but not metabolic or immune/inflammatory stress in rats.[59] In monkeys, a combined psychosocial and metabolic stress paradigm that suppressed pulse frequency was reversed with a specific CRHR1 antagonist.[60] Conversely, specific CRHR2 antagonists partially reversed the inhibitory effect of acute metabolic and immune/inflammatory stress on LH pulses in rats.[59] Thus, CRH and urocortins are necessary for the suppression of LH during various stress paradigms, though their relative importance varies.

2.1.c. Evidence for direct action at GnRH neurons: CRHR1 and CRHR2 have heterogenous expression patterns in the brain, including in the vicinity of GnRH neurons. In mice, approximately 30% of GnRH neurons contained immunoreactivity for CRHRs (the antisera could not distinguish type of CRHR).[61] Consistent with this finding, ~25% of GnRH neurons in the mouse were found to contain mRNA for *Crhr1* via microarray and confirmed with single-cell

RT-PCR, yet, no evidence of *Crhr2* was found in GnRH cells.[61] Additionally, CRH terminals are observed in close contact with GnRH neurons in humans[62] and rats,[63] and in mice, CRH terminals have been observed in close contact with GnRH fibers in the ARC.[51] Inhibitory actions of CRH have been documented *in vitro* as CRH treatment decreases GnRH transcription in GN11 cells (a model of immature GnRH neurons)[64] and decreases GnRH mRNA in GT1-7 cells (model of mature GnRH neurons). Thus, there exists an anatomical framework for actions of CRH directly on GnRH neurons as well as functional evidence for actions of CRH on GnRH cell lines in culture.

2.1.d. Evidence against direct action at GnRH neurons: Despite reports of CRH terminals in close contact with GnRH neurons in multiple species, retrograde tracing agents delivered to the vicinity of GnRH neurons in the POA did not label CRH neurons in the PVN in rats.[65] In sheep, cells located in the PVN that project to the POA were not activated by a psychosocial stress paradigm, despite robust activation of other (non-POA projecting) cells in the PVN.[66] Moreover, in contrast to reports of *Crhr1* mRNA in GnRH cells, no colocalization was found between GnRH immunoreactivity and CRHR1 using a transgenic mouse with GFP under the CRHR1 promoter[67,68] or between mRNA for *Gnrh* and *Crhr1* via dual-label *in situ* hybridization.[68] Moreover, genetic deletion of CRHR1 from GnRH neurons did not prevent suppression of LH following restraint stress or LPS administration.[68] Together these anatomical and functional data do not support a major role for CRH acting directly on GnRH neurons.

The effect of CRH on the electrical properties of GnRH neurons are varied but largely support the hypothesis that CRH does not act directly on GnRH neurons. First, in OVX[69] and ORCHX[68] mice no effect of CRH on GnRH firing rate was observed. In another study, CRH was found to stimulate firing in a sub-set of GnRH neurons in OVX mice.[51] Acute brain slices from mice in the diestrous phase of the estrous cycle[51] or OVX[69] mice treated with a dose of E2 sufficient to induce an LH surge (OVX+E2) showed an increase in firing rate in 20-40% of GnRH neurons. These stimulatory effects of CRH on GnRH neuron firing are likely indirect because CRH treatment did not alter potassium currents nor excitability of GnRH cells but did increase the frequency of GABA post synaptic currents,[67] indicating an increase in GABA release from other nearby cells. (Note, often inhibitory elsewhere in the central nervous system, GABA is generally stimulatory upon GnRH cells due to their relatively high intracellular chloride concentrations[70]). Finally, in OVX+E2 mice treated with a higher dose of CRH, an inhibition of GnRH neurons was observed.[69] The inhibitory effect of high doses of CRH was attributed to an action on CRHR2, because application of UCN3 (highly specific to CRHR2) also suppressed GnRH cell firing in

OVX+E2 mice.[69] It is likely that these CRHR2 mediated inhibitory effects are not directly on GnRH cells, because GnRH cells do not contain *Crhr2* mRNA.[61] Another caveat pertains to the effects of CRH on GnRH soma described above. Considering evidence that pulsatile secretion of LH (and presumably GnRH) can be induced by activation of GnRH fibers in the median eminence, CRH actions upon the GnRH soma may be applicable only to modulation of surge-type LH secretion.[71] Analysis of calcium flux in GnRH fibers in the lateral ARC and median eminence revealed no effect of CRH treatment nor was CRH able to alter the increase in calcium flux (i.e. change in fluorescence) induced by exogenous kisspeptin treatment.[51] Based on this collective work, it is likely that CRH and the urocortin peptides act on neurons afferent to GnRH cells to suppress gonadotropin secretion (Figure 1A).

The site(s) of action for CRH in the suppression of gonadotropin secretion remains an outstanding question. One possibility is the KNDy cell population in the ARC which forms the GnRH pulse generator and co-expresses kisspeptin (encoded by Kiss1), neurokinin B (encoded by Tac2), and dynorphin,[72,73] since these cells express one of the CRHRs in rats (the antisera could not distinguish CRHR1 from CRHR2[74]). CRH inhibited MUA volleys in the MBH of rhesus monkeys[42] (an assessment of GnRH pulse generator activity, likely emanating from KNDy cells) and reduced *Kiss1* mRNA abundance in rats.[75] However, CRH did not alter firing rate in ARC Tac2 cells from female mice[67] (highly colocalized with kisspeptin in the ARC, thus KNDy cells), nor did optogenetic activation of CRH terminals in the ARC alter ARC kisspeptin cell firing in female mice,[51] which collectively support CRH actions on neurons afferent to the KNDy cells. An alternative site of CRH action on GnRH/LH pulsatility is in the locus coeruleus (LC) as discussed below. In contrast, deletion of CRHR1 or CRHR2 from all neurons and glia did not prevent the suppression of LH secretion following restraint stress or LPS administration, which would support a hypothesis that neither CRH nor the urocortin peptides have a major role in suppression of LH during stress.[68] However, these unexpected findings, which are at variance with vast pharmacological data, may be explained by incomplete knockdown of the receptors, potentially spurious effects related to nestin-cre line itself,[76] or developmental compensation, and thus should be interpreted cautiously.

2.2. Catecholamines (Norepinephrine and Epinephrine): The catecholamines, norepinephrine (NE) and epinephrine (EPI), have long been recognized as important mediators of stress responses, both peripherally (released from adrenal medulla) and centrally. In the brain, EPI and NE are primarily produced in the brainstem, largely in three stress-responsive nuclei: ventral lateral medulla (VLM; A1 population), nucleus of the solitary tract (NTS; A2 population),

and the LC (A6 population).[77] The A1 and A2 populations receive rich interoceptive inputs (e.g. area postrema, vagus nerve), central inputs (e.g. amygdala, hypothalamus), and contain steroid hormone receptors. Anatomically, neurons in the A1 and A2 populations project widely throughout the brain, including the hypothalamus; thus, they are well positioned to survey the brain and body and transmit stress signals to the hypothalamus to regulate neuroendocrine function. Indeed, both the A1 and A2 neuron populations are implicated in the activation of PVN CRH during immune/inflammatory stress.[78,79] Neurons in the LC receive input largely from the brain, including from CRH terminals arising from the amygdala, bed nucleus of the stria terminalis, and to a lesser degree the PVN.[80] CRH injection in the LC induces ACTH and corticosterone secretion,[81] stress-like behaviors[82] and suppresses pulsatile LH secretion,[83] thus demonstrating the capacity to mediate several stress-related responses.

2.2.a. Evidence for direct action on GnRH cells: Biosynthesis of catecholamines, NE and EPI, occurs via successive action of enzymes, which serve as markers for the neurons that produce catecholamines. The enzymatic pathway includes, phenylalanine hydroxylase (phenylalanine → L-tyrosine), tyrosine hydroxylase (L-tyrosine → L-dopa), aromatic amino acid decarboxylase (L-dopa → dopamine), dopamine β-hydroxylase (DBH; dopamine → NE), phenylethanolamine N-methyltransferase (NE → EPI). NE and EPI signal via the adrenoreceptor family of G-protein coupled receptors, of which several members are expressed throughout the brain. Low to moderate expression of the α1, α2, and β1 adrenoreceptors have been detected in some pools of mouse GnRH neurons.[84,85] DBH immunoreactive terminals have been observed in close contact with GnRH soma[86] and dendrites.[87] Utilizing a pseudorabies tracing virus to label afferents to GnRH cells, tyrosine hydroxylase-immunoreactive neurons were identified in the NTS and LC at time points corresponding to primary afferents, and in the VLM at a later time point (possibly a secondary afferent).[88] It should be noted that timing of pseudorabies spread has been shown not to be a reliable method of distinguishing primary vs. higher order afferents.[89] Never-the-less, these data provide an anatomical framework by which catecholamine neurons in the brainstem act on GnRH cells. Consistent with an action on GnRH neurons, NE and adrenoreceptor agonists (α1 and β receptors) suppressed GnRH cell firing in acute brain slices collected from male and female mice.[90] Moreover, this suppressive effect occurred in the presence of glutamate, GABA, and voltage-gated sodium channel blockade indicating a direct effect on GnRH neurons.[90] Administration of NE into the third ventricle suppressed LH pulses in rats.[91] Thus, electrophysiological and pharmacological data raise the possibility of a direct inhibitory action of NE and (possibly EPI) on GnRH cells.

2.2.b. Evidence against direct action on GnRH cells: Administration of NE or agonists for its receptors into specific brain regions has yielded support against action directly on GnRH neurons during stress. Injection of NE or adrenoreceptor agonists into the POA in E2 replaced OVX rats[92] and sheep[93] stimulated LH secretion, whereas no effect was observed in the absence of E2.[93] These stimulatory effects may be related to the facilitation of LH surge secretion. In contrast, NE and adrenoreceptor agonists injected into the PVN potently suppressed LH pulses in rats.[94] Interestingly, the inhibitory effect of adrenoreceptor activation can be blocked by non-specific CRH receptor antagonists,[94] which raises the possibility that brainstem catecholamine neurons project to the PVN to suppress LH secretion in an indirect manner. Immunotoxic ablation of DBH terminals in the PVN resulted in depletion of catecholamine neurons in the brainstem (primarily the NTS region), and importantly blocked the suppressive effect on chronic glucoprivation on estrous cyclicity in rats.[95] The same DBH ablation technique revealed that decimation of brainstem catecholamine neurons also blocked activation of PVN neurons and attenuated the rise in corticosterone following psychosocial[96] and immune/inflammatory[79] stress. These data support the hypothesis that brainstem catecholamine neurons project to the PVN to activate the HPA axis and suppress the hypothalamic-pituitary-gonadal axis during stress, thereby suppressing GnRH neurons indirectly (Figure 1B).

2.3. Calcitonin Gene-Related Peptide (CGRP): CGRP is produced in several brain regions including the stress-responsive parabrachial nucleus (PBN) of the brainstem. CGRP neurons in the PBN are activated during a variety of stress types; moreover, these CGRP neurons are innervated and regulated by NE neurons in the A2. CGRP administration induced HPA axis activation[97] and stress-related behaviors.[98] Importantly, ICV infusion of CGRP suppressed pulsatile LH secretion in OVX+E2 rats,[99] and a CGRP receptor antagonist blocked the inhibitory effect of metabolic stress (hypoglycemia) on LH secretion in rats. CGRP likely has many roles in mediating stress responses, including regulation of gonadotropins during, at least, some types of stress.

2.3.a. Evidence for direct action on GnRH cells: Although the effects of CGRP on gonadotropin secretion are striking, much is still to be learned about the mechanisms for these effects. CGRP terminals are abundant in the POA. Even though the origin of these fibers is not known, PBN neurons are known to project to the POA. Pharmacological data support a role for this neuropeptide to act in the POA as CGRP microinfused into the POA (vicinity of GnRH neurons), but not other regions, suppressed LH pulses in rats.[100] Though it is not known if GnRH neurons

in vivo contain the receptor for CGRP, the GT1-7 cell line does,[101] and CGRP treatment reduced the abundance of mRNA for *Gnrh*.[101]

2.3.b. Evidence against direct action on GnRH cells: Although CGRP neurons project to and act in the vicinity of GnRH neurons, pharmacological evidence suggests an indirect action of CGRP *in vivo.* The suppressive effect of ICV CGRP on LH pulses can be blocked by a CRHR1 antagonist,[102] indicating that CGRP acts via activation of CRH neurons. CGRP administration increased *Crh* mRNA in both the PVN and amygdala, supporting a role for either or both populations.[102] It is not clear how a CRHR1 dependent action of CGRP might suppress LH during metabolic stress, since a CRHR1 receptor antagonist did not block the suppressive effect of metabolic stress.[59] Thus, much is still to be learned about the role of CGRP in mediating the effects of stress and its interactions with GnRH neurons (Figure 1C).

2.4. Cortisol/Corticosterone: The adrenal steroid cortisol (or corticosterone in rodents) is a potential mediator of the inhibitory effect of stress on gonadotropin secretion. Hydrocortisone acetate suppressed LH pulses in both OVX pigs[103] and ORCHX monkeys[104] demonstrating sufficiency of cortisol to inhibit gonadotropin secretion in a E2-independent manner in some species. However, in female sheep[105,106] and mice,[107] the ability of cortisol or corticosterone, respectively, to suppress LH pulse frequency is dependent on E2. In OVX sheep, a cortisol treatment that achieved a stress-like level of cortisol, suppressed LH pulse amplitude,[108] without altering GnRH pulse amplitude, LH pulse frequency or GnRH pulse frequency[109] indicating an effect in the pituitary, not hypothalamus. In contrast, in ovary-intact ewes during the early or mid-follicular phase of the estrous cycle (before the LH surge)[110] or OVX ewes treated with E2 and progesterone to mimic an estrous cycle,[106] cortisol suppressed GnRH and LH pulse frequency, demonstrating a role for E2 to sensitize the hypothalamus to the effect of cortisol.

The molecular mechanisms by which E2 permits the inhibitory effect of glucocorticoids on GnRH pulse frequency remains a significant outstanding question. Intriguingly, glucocorticoids also interfere with the actions of E2 during the LH surge. Corticosterone blocked the E2-induced LH surge in mice,[111] and cortisol delayed and blunted the amplitude of the E2-induced LH surge in sheep.[112] Since GnRH neurons do not contain glucocorticoid receptors, it is likely that any effects of cortisol or corticosterone are mediated via afferents to GnRH neurons. In sheep[113] and mice,[107] glucocorticoid receptor is present in the majority of KNDy cells (as well as the AVPV/PeV population in mice) and corticosterone inhibits activation of either kisspeptin population in female mice[107,111] supporting the potential for glucocorticoids to act directly upon kisspeptin cells (Figure 1D). Some species differences are also at play, as corticosterone

treatment does not alter pulsatile LH secretion in OVX rats with or without gonadal steroid replacement.[75] Interestingly, although corticosterone suppressed *Kiss1* mRNA in rats,[75] kisspeptin neurons do not appear to contain glucocorticoid receptors in this species;[74] the relevance of this decrease in transcript levels is unclear since pulsatile LH secretion was not altered. In mice, although corticosterone suppressed KNDy cell activation, the abundance of mRNA for *Kiss1 and Tac2* were not altered.[107] Interestingly, corticosterone modestly suppressed the abundance of *pDyn* (mRNA for dynorphin),[107] whereas an increase in *pDyn* would be expected concurrent with decreased LH pulse frequency. In mice, although the majority of ARC kisspeptin cells contain mRNA for dynorphin, there are some non-kisspeptin neurons that contain pDyn, [114] and the method employed in the above work could not resolve which population of neurons was altered by corticosterone. Collectively, these findings support the idea that glucocorticoid-induced inhibition of the pulse generator and the resulting decrease in LH pulse frequency may not be mediated by changes in transcription of KNDy related genes.[107] Clearly, future investigation is required to understand how glucocorticoids suppress gonadotropin secretion in many, but not all species, through cells and signaling pathways afferent to GnRH neurons.

2.5. Arginine-Phenylalanine-Amide-Related Peptide-3 (RFRP-3): RFRP-3 is the mammalian ortholog of the avian neuropeptide, gonadotropin-inhibitory hormone. Unlike the actions of gonadotropin-inhibitory hormone in birds, RFRP-3 appears to act predominantly within the brain to regulate gonadotropin secretion in a variety of physiological contexts, including stress. The RFRP-3 receptor, GPR147 (NPFFR1), is a G-protein coupled receptor that is expressed in GnRH neurons.[115] RFRP-3 is a member of the RFamide peptide family which also includes kisspeptin, neuropeptide FF, prolactin-releasing peptide, 26RFa and others.[116] These peptides have structural similarity and as a result have some affinity for each other's receptors.[116] A previously used antagonist for GPR147 (RF9) also acts as a partial agonist of the kisspeptin receptor;[117] thus, pharmacological approaches to investigating these signaling pathways can be difficult to interpret. Despite these challenges, several lines of evidence support the hypothesis that RFRP-3 is an important regulator of LH secretion during stress. First, the inhibitory effect of fasting on LH secretion was partially reversed in NPFFR1 knock-out mice.[118] Second, knockdown of *Rfrp3* prevented infertility caused by repeated restraint stress in female rats.[119] Third, ablation of RFRP3 neurons prevented restraint stress-induced suppression of LH pulses in female mice.[120] Evidence for direct action of RFRP-3 on GnRH neurons include findings that GnRH cells contain the receptor for RFRP-3 (NPFFR1)[115,121] and that RFRP-3 terminals are found in close contact with GnRH cell bodies.[115,122,123] Additionally, RFRP-3 was shown to inhibit

GnRH cell firing, an effect maintained following GABA and glutamate receptor blockade, suggesting a direct effect on GnRH cells.[124,125] RFRP-3 may also act on ARC as well as AVPV/PeV kisspeptin cells, since these cells contain GPR147 and also receive close contacts from RFRP-3 neurons.[115] Thus, RFRP-3 likely influences GnRH secretion via direct and indirect actions on GnRH cells (Figure 1E).

3. Future Directions and Perspectives:

In this review, we highlighted much of the work performed to address the question: *how is GnRH secretion suppressed during stress*? Theoretically, the response to stress involves three principal actions: detection of the stressor (the stimuli), transmission of signal(s), and action on some element(s) of the reproductive axis. Here, we focused on the specific matter of whether or not a handful of signaling molecules act directly on GnRH neurons to suppress gonadotropin secretion during stress. We suggest that this is the perfect time to evaluate the evidence supporting direct actions of stress mediators on GnRH neurons as recent advancements have demonstrated the importance of two populations of kisspeptin-containing cells in the hypothalamus that organize pulsatile and surge-type GnRH secretion. With the discovery of these cells, alternative sites of action for stress-related signaling molecules have been revealed yet remain to be fully-tested. The implication is that earlier papers should be read with the understanding that the kisspeptin systems (and subsequent importance of the ARC and rostral hypothalamic [AVPV/PeV in rodents, preoptic area in ruminants and primates] cell populations[126]) were not known or fully appreciated at the time of publication. Thus, there is still much work to be done to determine the exact pathways, including identifying upstream neural sites, cell types and mediators, by which GnRH cells are inhibited during stress.

Here, we evaluated the evidence for either direct or indirect action of several signaling molecules that have been investigated as mediators of impaired GnRH cell function, including CRH, the urocortin peptides, norepinephrine, CGRP, cortisol/corticosterone and RFRP-3. Though all of these molecules ultimately reduce GnRH cell function, the preponderance of evidence discussed above indicates that most of these signaling molecules do not act directly on GnRH neurons. The exception is RFRP-3, in which data are currently limited. Anatomical and electrophysiological data support the possibility that RFRP-3 acts directly on GnRH neurons,[115,121-125] though it is possible that RFRP-3 also acts on kisspeptin neurons to alter GnRH cells indirectly as well.[115] Rigorous testing of the hypothesis that RFRP-3 acts directly in GnRH neurons will require generation of animals that lack the RFRP-3 receptor (NPFFR1) in GnRH neurons, which has not been done. Although anatomical and electrophysiological

evidence similarly support the hypothesis that catecholamines act directly on GnRH neurons,[84-88] functional *in vivo* data contradict this possibly. First, microinjection of NE into the POA (site of GnRH) neurons does not suppress LH pulses,[94] and second, the inhibitory effect of NE is reversed by CRHR antagonists,[94] indicating NE acts via CRH or urocortin peptides. The current evidence suggests that the others likely directly or indirectly suppress KNDy cell activity, which ceases to stimulate pulsatile GnRH secretion (summarized in Figure 1).

As the suppression of LH pulses has the potential to blunt or delay the preovulatory rise of E2, any of these mediators, acting directly or indirectly to inhibit pulsatile GnRH secretion, are one potential mechanism whereby stress can also suppress surge GnRH/LH secretion. A second mechanism is interference with the GnRH/LH surge mechanism in the presence of sufficient E2. Indeed, discriminating between direct actions on GnRH cells versus afferent pathways, such as the rostral population of kisspeptin neurons, during stress-induced suppression of the surge remains an open question, with the exception of glucocorticoid-induced suppression of the positive-feedback response to E2 (Figure 1D). It is clear that greater resolution of the upstream circuits controlling GnRH neuron function during either pulsatile or surge modes of secretion will enable clarification of direct versus indirect actions of stress-activated signaling factors on both GnRH neurons as well as the kisspeptin populations afferent to this indispensable cell population.

3.1. Influence of estradiol: One area of future investigation of particular interest to us is the role of E2 in sensitizing the reproductive axis to the inhibitory effects of stress, which has been demonstrated in many mammalian species. In mice, we (K.M.B. laboratory) have shown that some stimuli (glucocorticoid treatment and immune/inflammatory stress) are E2-dependent; however, other stimuli are not (psychosocial and metabolic stress). Both theoretically and technically this is an important observation. From a technical standpoint, detection of LH pulses in ovary-intact mice is challenging because of low LH pulse frequency and low baseline concentrations. Moreover, although LH pulses and synchronized calcium events in KNDy neurons occur throughout the estrous cycle (except on day of estrus), inter-pulse interval can vary between 20 and 80 min among pulses,[127,128] which further complicates identifying a *bona fide* decrease in LH pulses. Therefore, experimentation on OVX animals is attractive since it permits detection of frequent pulses in the control condition; however, this approach opens critique to a 'lack of physiological relevance' and importantly the possibility of missing E2-dependent effects.

An alternative approach is to OVX and replace physiological-like levels of E2 in silastic capsules, as has been performed in other species. We (K.M.B. laboratory) recently published an E2-replacement paradigm that generated diestrous-like levels of E2, using uterine weight as a proxy for circulating E2 concetrations. Moreover, this dose of E2 reduced LH pulse frequency compared to OVX mice and also reversed other physiological effects of OVX including weight gain and loss of circadian corticosterone rhythms, further demonstrating the physiological relevance of this dose.[107] Importantly, this dose of E2 permitted the inhibitory effects of immune/inflammatory stress[26] and chronic corticosterone treatment[107] on LH pulse frequency, demonstrating that this dose of E2 is sufficient to sensitize the neuroendocrine system to stress. Despite the physiological evidence supporting the utility of this dose of E2, it is clear that LH concentrations and pulse frequency in this OVX+E2 model[107] are substantially greater than those observed in intact females during diestrus.[128] One explanation for this discrepancy is that some ovarian factor other that E2 contributes to the suppression of gonadotropin secretion. Although potentially interesting and illustrative of the many outstanding mysteries of the estrous cycle, the importance of reliable methods to clamp E2 concentrations during experimentation cannot be overstated. In addition to enabling reliable detection of LH pulses, OVX+E2 models offer numerous practical advantages, including overcoming the technical challenge of generating a cohort of animals that can be used for experimentation on the same day, since there are no reliable methods of synchronizing estrus cycles in mice. Furthermore, since E2 regulates GnRH/LH secretion and GnRH/LH secretion in turn regulates E2 concentrations, clamping E2 at a fixed level is necessary for removing the confounding effect of altered E2 whilst studying other regulators of GnRH/LH secretion. Indeed, ovary-intact animals will reveal the full sequalae of stress effects on reproduction with greater physiological relevance; however, OVX+E2 models are necessary for reducing this incredibly complex biologic system into isolated components for detailed analysis of the underlying neural circuits.

The physiological mechanism for E2 to potentiate the inhibitory effects of stress on gonadotropin secretion remains a significant outstanding question. In rats, the observation that E2 delivered into the NTS or PVN (but not the ARC, POA, LC, or VLM) allowed 48 hours of fasting to suppress pulsatile LH secretion, an effect observed in OVX+E2 but not OVX rats,[129] offers some clues to sites of action. In mice, E2 treatment did not alter the number of cells that expressed cFos in the brainstem or PVN in response to IL1B.[26] Whether this indicates that E2 does not potentiate activity of neurons in these areas or that cFos immunoreactivity is not sufficiently sensitive to detect changes in activity of these cell populations remain outstanding questions. Moreover, although robust suppressive effects of metabolic and psychosocial stress

in OVX mice have been documented, it is not known if E2 can heighten the responses to these stress types. Whether E2 would cause suppression of LH pulses in response to more moderate metabolic or psychosocial challenges, or if E2 would prolong the duration of impaired pulsatile LH secretion is unknown. Molecularly, E2 could enable changes in sensitivity to stress in a variety of ways including altered synaptic connectivity of neural circuits, changes in ligand or receptor expression, remodeling epigenetic modifications, altered ion channel abundance or conductivity underlying the excitability of cells or their sensitivity to stimuli. Thus, key in understanding the neurobiology of stress will be deciphering the many actions of E2 (and testosterone) in the brain.

3.2. Influence of species differences: In reflecting on the past 50 years of literature in this review, some topics are noteworthy for the future. First, as the field of stress effects on reproduction continues to flourish it is clear that differences among species will persist. Hopefully these differences will provide unique and insightful comparisons that enable us to better understand the neurobiology of stress responses. One interesting example of an anatomical-functional correlation is the observation that glucocorticoids suppress LH secretion in OVX and E2 replaced mice[107] and sheep,[106,110] species in which glucocorticoid receptor has been detected within KNDy cells. In contrast, in rats, which do not express glucocorticoid receptor in KNDy cells, LH secretion is not altered by glucocorticoid treatment.[75] As E2 is required for glucocorticoid-induced inhibition of LH in sheep and mice, future work remains in which glucocorticoid levels are clamped in order to tease out the role of other mediators or neuronal populations, potentially influenced by E2. Neuroendocrine research has included many diverse species; this review contained data from humans, monkeys, pigs, sheep, goats, hamsters, rats and mice. Indeed, each bring valuable advantages and physiological contexts, though there has been a trend for increased use of mice in the study of stress on reproduction. Low animal cost, availability of transgenic and molecular approaches, and recent advances in serial blood sampling for analysis of pulsatile LH secretion contribute to the appeal of the mouse model. Application of this species warrants keen understanding of mouse physiology, since this species displays clear differences from other species, including rats.

3.3. Influence of technical advancement: A second topic is the power of advancing technologies, particularly multi-label immunohistochemistry and *in situ* hybridization as well as RNA-sequencing, which will provide greater ability to identify and localize important signaling molecules and receptors. These approaches will allow high-throughput screening and targeted analysis of numerous signaling candidates that will accelerate our understanding of stress

neural circuits. Hopefully these techniques will permit analysis of heterogenous cell populations, and shine new light on diverse, and at times conflicting roles of neural populations. For example, NE produced in the brainstem is critical for suppression of LH secretion during stress, as discussed above, but is also necessary for the LH surge (i.e. enhanced GnRH outflow).[130-132] There is also great heterogeneity in the CRH neurons in the PVN.[133] Whether distinct populations regulate circadian rhythms and stress effects or whether different stress types activate different subpopulations of these PVN CRH cells remain to be fully understood. It is likely that sub-populations of neurons will be identified, and refined approaches will allow us to target cell populations with enhanced precision.

3.4. Final thoughts: A final topic of increased importance will be integrating the effects of the numerous stress-related signaling molecules, of which only a portion are presented here. In the last several decades, several peptides and transmitters have been identified and tested (reviewed above). Though some interactions between signaling molecules have been uncovered, there is still much work to be done in integrating these studies to discover the complete neural pathway between sensing a challenge of or threat to homeostasis all the way to suppression of GnRH neurons. Thus, in the 50 years since the discovery of GnRH, tremendous advancements have been made uncovering how GnRH secretion is regulated, and we are optimistic that future work will continue to expand this theoretically interesting and clinically important field.

4. References

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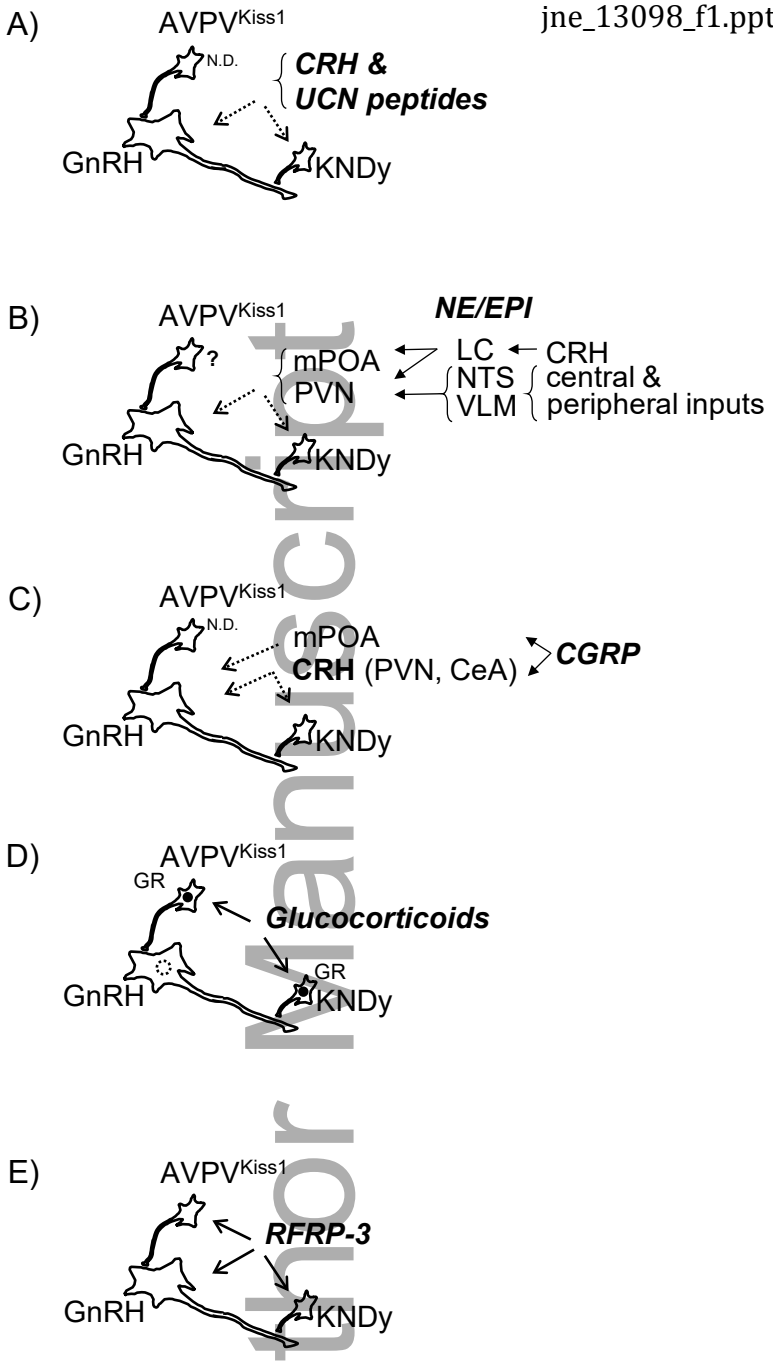

Figure 1: Schematic representation of speculated interactions of key inhibitory stress mediators upon GnRH neurons and/or upstream AVPV^{Kiss1} and KNDy cells. A) CRH & UCN peptides, B) NE/EPI, C) CGRP, D) Glucocorticoids (in sheep and mice, but not rats, see text for details); GR expression is indicated in AVPV^{Kiss1} and KNDy cells (closed circle) and absent in GnRH cells (dashed circle), E) RFRP-3. Solid arrows indicate evidence supporting direct regulation. Dashed arrows indicate evidence supporting indirect regulation. Filled Arrow heads indicated positive regulation. Open arrow heads indicate negative regulation. ND indicates no data available. Question marks indicate evidence supporting regulation of unclear directionality. Note, cartoon schematics largely based on data from rodents.