Linking Stress and Infertility: A Novel Role for Ghrelin

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ABSTRACT Infertility affects a remarkable one in four couples in developing countries. Psychological stress is a ubiquitous facet of life, and although stress affects us all at some point, prolonged or unmanageable stress may become harmful for some individuals, negatively impacting on their health, including fertility. For instance, women who struggle to conceive are twice as likely to suffer from emotional distress than fertile women. Assisted reproductive technology treatments place an additional physical, emotional, and financial burden of stress, particularly on women, who are often exposed to invasive techniques associated with treatment. Stress-reduction interventions can reduce negative affect and in some cases to improve *in vitro* fertilization outcomes. Although it has been well-established that stress negatively affects fertility in animal models, human research remains inconsistent due to individual differences and methodological flaws. Attempts to isolate single causal links between stress and infertility have not yet been successful due to their multifaceted etiologies. In this review, we will discuss the current literature in the field of stress-induced reproductive dysfunction based on animal and human models, and introduce a recently unexplored link between stress and infertility, the gut-derived hormone, ghrelin. We also present evidence from recent seminal studies demonstrating that ghrelin has a principal role in the stress response and reward processing, as well as in regulating reproductive function, and that these roles are tightly interlinked. Collectively, these data support the hypothesis that stress may negatively impact upon fertility at least in part by stimulating a dysregulation in ghrelin signaling. ***(Endocrine Reviews 38: 432 – 467, 2017)***

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Infertility, defined as the inability to achieve a viable pregnancy after 12 months of unprotected intercourse (6 months if the woman is over age 35), affects a remarkable one in four couples worldwide (1, 2). Female and male factors both contribute to infertility, with 30% to 50% attributable to male factors, such as low sperm count, poor sperm quality, hypogonadism, and other abnormalities. Physiological causes of female infertility include ovulation disorders and tubal damage (~50%), as well as 10% to 30% of all cases remaining unexplained (3–5). Recent data, particularly from animal models (including nonhuman primates) suggest psychological stress may be a major contributor to both male and female infertility (6–12). Thus, stress can compromise every aspect of fertility, including libido, sperm quality, ovulatory capacity, and implantation (13–15) (see Fig. 1). Indeed, stress can be so detrimental to fertility that in at least one species, it is used as a deliberate strategy to suppress fecundity in competitors (16).

Although the link between stress and reproductive dysfunction has been well-established and extensively investigated in nonhuman animals, where additional variables can be tightly controlled, the question of whether stress directly impedes fertility in humans remains difficult to conclusively examine (17–20). Psychological stress experienced by female or male partners of infertile couples has been associated with lower conception rates in at least some cases (21–24). However, there have been a number of methodological challenges and conflicting findings facing research in the area. These include the lack of

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ESSENTIAL POINTS

- Infertility affects a remarkable one in four couples worldwide
- Psychological stress is a major contributor to male and female infertility, at least for some individuals
- For those individuals who are affected by life stress to a greater extent than others, understanding the underlying mechanisms and the reasons for their increased susceptibility is essential
- We propose that ghrelin is a compelling link between stress and infertility that may partially explain the individual differences in the way stress affects fertility

prospective longitudinal studies on the general population (18), the use of nonstandardized measures of stress, and the fact that the majority of human studies have been conducted on couples attending fertility clinics (18, 25), with, likely, underlying physical and/or hormonal causes of infertility. There is also the important consideration that stress is a normal facet of life, thereby making nonstressed control groups very difficult to source. Stress is usually dealt with by an appropriate and regulated neuroendocrine response, but although psychological stress affects us all at some point, individuals differ in the impact this has on their physiology, including fertility. These individual differences may explain why some studies have demonstrated substantial reproductive implications of psychological distress (13, 15, 23, 24, 26–31), whereas others have found small effect sizes or no association (32–38). It is likely that individuals experiencing extreme or long-term stress or those more vulnerable to its effects may be particularly vulnerable to fertility consequences.

Infertility itself can be highly stressful. Many of those who struggle with infertility seek help from assisted reproductive technology treatments. These treatments, including *in vitro* fertilization, can involve invasive techniques that place an additional burden of stress on the couple in addition to the stress associated with an inability to conceive (39). The effects of stress may therefore be particularly detrimental for couples undergoing assisted reproductive technology, leading in some couples to more treatment cycles to conceive (28, 39–41), resulting in discontinuation of treatment before achieving pregnancy (42–44), and potentially contributing to the low overall success rates (45). Similarly, pre-existing psychological conditions, such as anxiety, depression, and high levels of distress, can have a negative effect on assisted reproductive technology outcomes (24, 28, 46–50) [but see (51–54)]. For instance, Smeenk *et al.* (48) show anxiety and depression were significantly negatively correlated with pregnancy outcome in a multicenter prospective study in 2001. The same group was not able to replicate this finding in a separate study in 2009 (36). However, patients who are prone to anxiety, depression, or high stress levels may particularly benefit from psychosocial support during the treatments.

Some human evidence suggests intervention to reduce stress may improve the chances of successful pregnancy. A meta-analysis of 21 studies reviewing the efficacy of psychological interventions for infertile patients, from which 13 studies reported pregnancy outcomes, shows a significant positive impact of

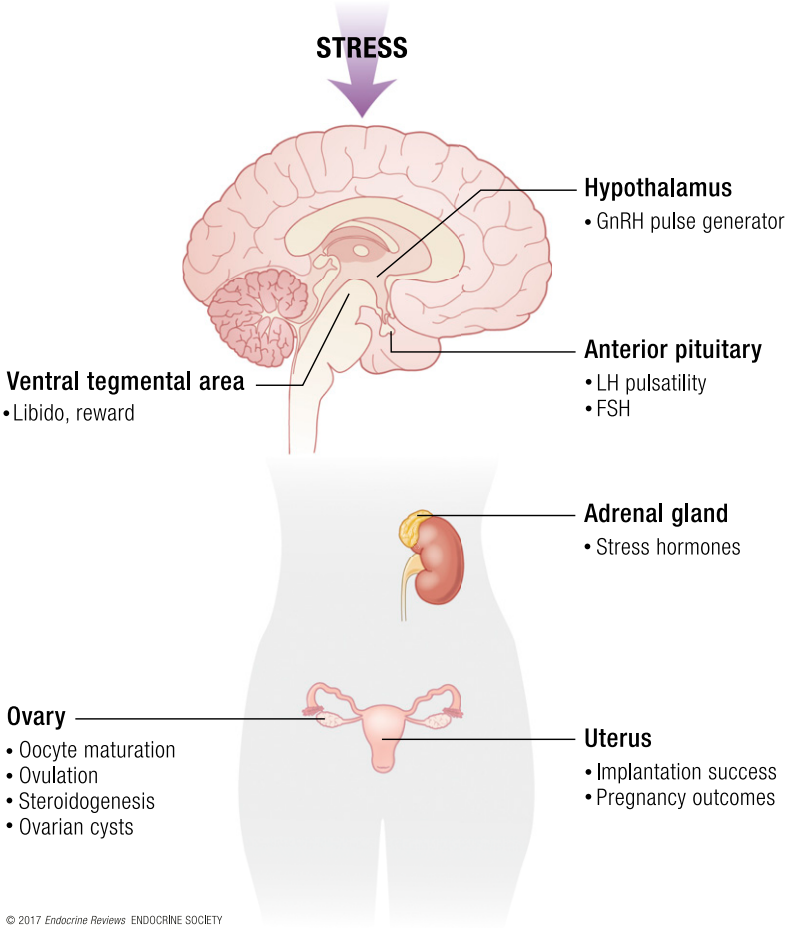


Figure 1. Stress influences reproductive function at all levels. Stress can interfere with reproductive function at all levels of the reproductive axis. It can suppress libido, reward, and mating behavior at the level of the brain, particularly the ventral tegmental area. It interferes with the hypothalamic GnRH pulse generator and LH and FSH release from the anterior pituitary. It suppresses oocyte maturation, ovulation at the level of the gonads, as well as increasing the likelihood of ovarian cysts and affecting both ovarian and testicular steroidogenesis. Stress is also detrimental to pregnancy outcomes postconception, reducing the likelihood of successful blastocyst implantation. Adapted from Servier Medical Art under Creative Commons CC-BY license.

interventions, including counseling, cognitive-behavioral therapy, mind or body-oriented relaxation, education, psychodynamic or psychoanalytic therapy on pregnancy rates, despite these interventions having no measurable effect on mental health. These improved pregnancy success rates were only evident for couples not receiving assisted reproductive technology treatments (55), highlighting that many factors additional to stress contribute to infertility. Another meta-analysis of 22 studies has also indicated a positive effect of psychotherapy on conception. In this analysis, 45% of subjects in the intervention group reported pregnancy by study completion, compared with 14% of controls, with similar rates of pregnancy achieved in patients receiving assisted reproductive technology treatments and those who were not in specific medical care (56).

As a consequence of these studies, attention is given to stress reduction management in individuals and couples undergoing fertility treatments. The Australian Assisted Reproductive Treatment Act (2008) stipulates a mandatory pretreatment counseling session that includes a discussion of areas of potential stress and strategies for managing these (57). Similar guidelines are implemented in other countries (58). We should note that other studies have shown that short-term attempts to reduce stress levels may have limited or no effect on pregnancy outcomes (35, 59, 60), highlighting the complexity and multifaceted origins of infertility in humans. It will be useful to see large-scale long-term stress intervention studies in infertile humans with accompanying measures of stress perception and circulating stress hormones.

Given the complexity of the interaction between the stress and reproductive axes, links between stress, stress-reduction approaches, and successful conception in humans are inconsistent. Likewise, attempts to isolate single causal links between stress and infertility have not yet been successful. However, stress management has important potential to improve reproductive success rates if we can identify the correct strategies for those people most likely to benefit. It is therefore essential that we begin to identify why fertility may be markedly affected by stress in some individuals, and less so in others. In this review, we discuss the current literature in the field of stress-induced reproductive dysfunction and the multifaceted nature of this interaction, based on nonhuman animal models and human studies. Here we will particularly focus on an important gut hormone, ghrelin, as a compelling link between stress and infertility.

Stress and the Hypothalamic-Pituitary-Adrenal Axis

"Stress" itself has been a controversial concept since its first description in physiology by Cannon and Selye early last century (61–65). However, a stressor (the stimulus) can broadly be considered an intense, nonroutine challenge to homeostasis resulting in a nonspecific response that includes general activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathomedullary systems. Each stressor can also activate a more specific signature response. Here we will refer to "stress" as the stimulus and "the stress response" as the body's reaction to the stimulus. Although there are myriad types of stress, they can be loosely categorized into "physical" and "psychological" stress based on the immediacy of the impact on the body and the endocrine and neuronal responses they elicit. Physical stressors (interoceptive, homeostatic, systemic) are those involving an immediate disturbance of tissue integrity and a specific activation of the central amygdala and rostral A1 and A2 brainstem noradrenergic cells in addition to general HPA axis and sympathomedullary activation. Psychological stress (neurogenic, psychogenic, emotional) involves a threat to tissue disturbance rather than a direct injury and a specific medial amygdala and caudal A1 and A2 brainstem noradrenergic pattern of neuronal activation (66–69).

Acutely, within seconds to minutes, both physical and psychological stress generally activates the sympathomedullary system leading to the release of adrenaline and noradrenaline, which increase heart rate, blood pressure, respiration, and blood glucose levels to facilitate attention and action directed at combatting the stress. Within minutes the HPA axis is activated, with corticotropin-releasing hormone (CRH)- and arginine vasopressin-expressing cells in the medial parvocellular region of the paraventricular nucleus of the hypothalamus stimulating the release of CRH from axonal terminal boutons in the median eminence, which, in turn, stimulates corticotrophs in the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into systemic circulation. By approximately 20 to 30 minutes after the onset of the stress (70, 71), ACTH is acting at the melanocortin-2 receptors on the adrenal cortex to stimulate the synthesis and release of glucocorticoids into the circulation. Glucocorticoids remain elevated for ~60 to 120 minutes after the onset of an acute stressor (70, 72) and have roles in immunosuppression, glucose uptake and mobilization, fat storage, and memory consolidation, among others. Glucocorticoids also negatively feed back onto glucocorticoid and mineralocorticoid receptors, chiefly in the hippocampus and hypothalamus, to inhibit further activation of the paraventricular nucleus of the hypothalamus (PVN) so suppressing ongoing

activation of the HPA axis once the stressor has been resolved (73–76) (see Fig. 2).

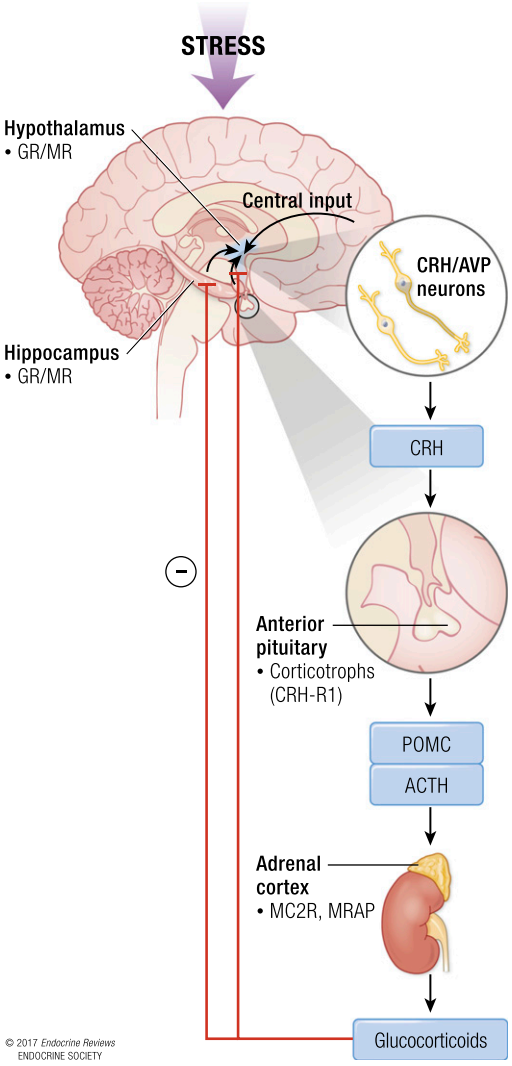
The response to acute stress is typically a highly adaptive phenomenon, enabling the individual to appropriately combat the stressor and recover (73–76). However, if a stressor becomes chronic, due to prolonged infection or exposure to substantial life challenges, such as infertility, negative complications can ensue. Chronically elevated glucocorticoids can lead to a variety of complications, including excess weight gain, memory impairments, and mood disorders, and as we will discuss, this also has consequences for fertility.

As outlined in the introduction, although much controversy exists in human research examining the effects of stress on fertility, causal relationships have been demonstrated in animal models. Thus, chronic unpredictable stress in mice has been shown to impair oocyte developmental potential through severe apoptosis and oxidative stress (7–9). In our own work, we have seen that exposure to psychological stress has an inhibitory effect on copulatory behavior and suppresses the hormonal surge during mating in female rats (6). We have also shown that animals that are programmed to be hyperresponsive to stress demonstrate impaired sexual development and detrimental changes to their ovarian or testicular gametogenesis (6, 77), indicating stress and the HPA axis interact significantly with the reproductive axes.

Stress and the Hypothalamic-Pituitary-Gonadal Axis

Reproduction is an essential function for the perpetuation of the species and, as such, is controlled by a sophisticated regulatory network of neuroendocrine signals that are originated and integrated by the hypothalamic-pituitary-gonadal (HPG) axis. The HPG hormonal cascade begins in the medial preoptic area (mPOA) of the hypothalamus, with the release of gonadotropin-releasing hormone (GnRH) from GnRH neurons. This collection of neurons, known as the GnRH pulse generator, sends axons to the median eminence from where GnRH is released in a synchronized pulsatile manner and is the central control mechanism for the reproductive cycle (78–80). This pulsatile GnRH release is driven by several specific mechanisms, including calcium and cyclic adenosine monophosphate signaling (81), electrical activity of GnRH neurons (82), autocrine regulation of the GnRH receptor (83), coupling of the GnRH receptor to G-related proteins (84), expression of G protein-coupled receptor 54 and its endogenous ligand, kisspeptin (85), as well as positive and negative regulation by gonadal steroids (86). The GnRH peptide is secreted from the nerve endings into the hypophyseal portal system, to stimulate the synthesis and the release

Figure 2. The HPA axis. Upon encountering a stressor, medial parvocellular CRH and arginine vasopressin (AVP) neurons of the hypothalamus receive input from several brain regions, including the amygdala, brainstem, and prefrontal cortex, and stimulate CRH release into the median eminence. CRH acts on the anterior pituitary through CRH receptor type 1 (CRH-R1), leading to the release of ACTH into circulation, a peptide hormone derived from pro-opiomelanocortin (POMC). ACTH stimulates glucocorticoid release to regulate glucose and fat utilization and storage, memory, immune function, and other stress-coping strategies, as well as negatively feeding back on glucocorticoid and mineralocorticoid receptors in the hypothalamus and hippocampus to suppress further HPA axis activation. Adapted from Servier Medical Art under Creative Commons CC-BY license.



of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary (87). FSH and LH are released in pulses into the blood stream to stimulate the gonadal production of gametes and the release of sex steroids, including estrogen, progesterone, and testosterone (88). The levels of each of the HPG axis hormones are regulated by complex positive and negative feedback loops, and

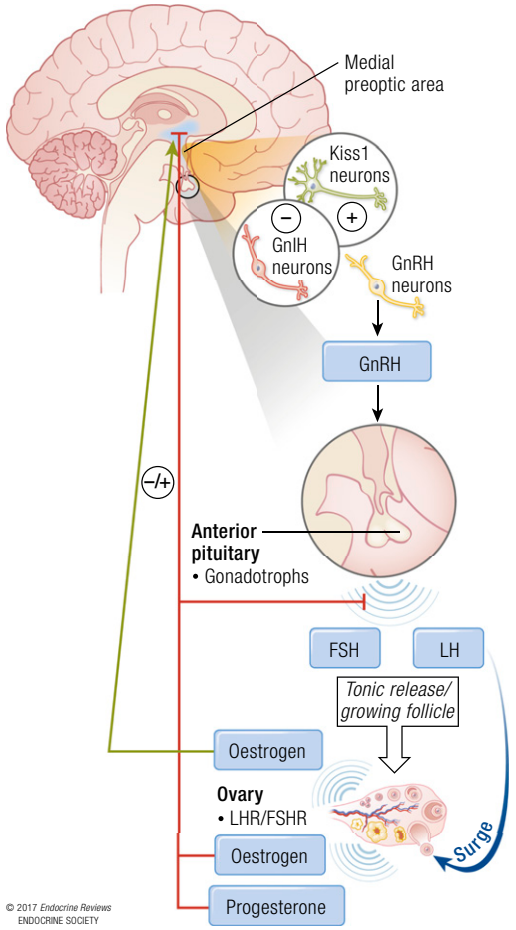


Figure 3. The HPG axis. Hypothalamic mPOA kisspeptin (Kiss1) neurons stimulate and gonadotropin inhibitory hormone (GnIH) neurons inhibit GnRH pulsatile release into the median eminence. GnRH release stimulates the release of gonadotropins LH and FSH from the anterior pituitary. This in turn leads to the release of ovarian steroids mainly estrogen and progesterone, that can carry out both negative- and positive-feedback actions depending on the stage of the ovarian cycle. In males, gonadal release of testosterone produces inhibitory actions on GnRH/gonadotropin secretion (negative feedback). Pituitary gonadotropins also stimulate steroidogenesis. Adapted from Servier Medical Art under Creative Commons CC-BY license.

are also influenced by other neuroendocrine signals (see Fig. 3). As such, the HPA and the HPG axes are well known to coregulate one another both centrally and peripherally, with the extent of stress (acute vs chronic), species, sex, and individual differences in resilience affecting the ability of stress to influence reproduction (89–91) (see Fig. 4). One of the major structures affected by stress is the hypothalamic GnRH pulse generator, with stress diminishing its ability to stimulate pulsatile release of gonadotropins. This loss of pulsatility may lead to hypothalamic amenorrhea (92), and can be accompanied by reproductive dysregulations associated with other targets along the HPG axis.

The effects of stress on hypothalamic GnRH neuronal signaling

The role of CRH in stress-induced suppression of GnRH signaling

The inhibitory effects of stress are initiated by CRH-mediated suppression of the GnRH pulse generator, diminishing the subsequent pituitary release of gonadotropins in rats (93), sheep (91, 94), nonhuman primates (95), and humans (96). These inhibitory effects of CRH can be, at least partially, reversed by CRH antagonists as has been shown in rats (97–99) and nonhuman primates in response to a mild psychological and metabolic (100), as well as an inflammatory stress (101), although inconsistencies are seen in the primate research, with other studies demonstrating that CRH receptor antagonism paradoxically increases cortisol release, inducing further LH release suppression (12).

The CRH family of neuropeptides integrates the neuroendocrine stress responses in the brain through two distinct receptor subtypes, CRH receptor type 1 (CRH-R1) and type 2 (CRH-R2), with higher binding affinity of CRH to CRH-R1 (102). CRH-R1 is highly expressed in the brain and pituitary and to a lesser extent in peripheral tissues (102, 103). Although the actions of CRH to drive HPA axis activation during stress are primarily mediated by CRH-R1 (104), stress-induced suppression of the GnRH pulse generator is mediated by both CRH-R1 and CRH-R2 (98, 99, 105).

The vulnerability of the GnRH pulse generator to stress has been suggested to be influenced by CRH-GnRH connectivity in the mPOA, a GnRH-rich area. The existence of synaptic connectivity between CRH and GnRH neurons (106) and expression of CRH-R1 receptors in mouse GnRH neurons in this area (107) indicate the possibility of direct actions of CRH on the mPOA GnRH system. In contrast, although the PVN plays a central role in autonomic and neuroendocrine regulation of stress responsiveness (108), PVN CRH neurons do not appear to directly coordinate stress-induced reproductive dysfunction. Lesions of the PVN are unable to block stress-induced suppression of gonadotropin release (109), and no direct connectivity between PVN CRH and GnRH neurons has been detected (110). Furthermore, infusion of calcitonin gene-related peptide, which is known for its role in stress-induced suppression of the HPG axis (111), suppresses LH pulse frequency when infused directly into the mPOA, but not into the PVN (112). As we will discuss in the Ghrelin Signaling in the Stress Response, Reward, and Mood Disorders section, ghrelin can regulate the expression of CRH in stress-sensitive areas including hypothalamus (113) and ghrelin, administrated both centrally and peripherally, mediates its anxiogenic effects via the PVN CRH neurons (114, 115). However, because the inhibitory effects of ghrelin on central reproductive function have been shown in

other regions, such as the mPOA (116, 117), it is possible that ghrelin also acts via the activation of CRH neurons in these areas to suppress LH pulsatility.

Additional regulatory mechanisms may influence stress-induced GnRH pulse frequency. Other major stress-regulatory areas, such as the amygdala, particularly the medial and central nuclei, have been implicated in stress-induced suppression of the GnRH pulse generator (118–120), with ghrelin, at least indirectly, also influencing these regions during stress (113, 121).

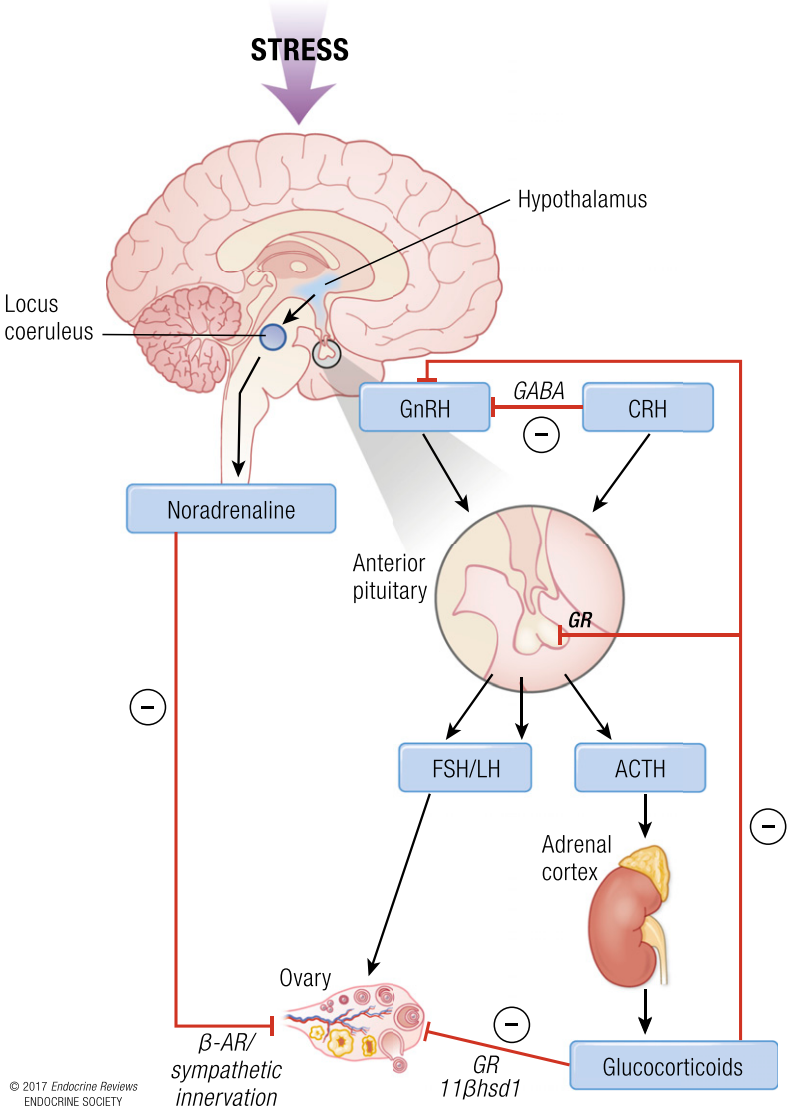
The role of γ-aminobutyric acid in stress-induced suppression of GnRH signaling

In addition to CRH, the inhibitory effect of stress on the GnRH pulse generator activity is likely to be mediated via γ-aminobutyric acid (GABA)-ergic signaling. Most studies have reported inhibitory effects of GABA on GnRH/LH pulsatility (122–125), with the inhibitory actions of GABA on GnRH neurons emerging at the time of vaginal opening in the mouse, demonstrating a switch from their postnatal depolarizing profile (126). These inhibitory effects of GABA-ergic signaling have been proposed to be mediated by $GABA_A$ receptor activation (122, 124, 125). In female nonhuman primates, blockade of $GABA_A$ receptor increases GnRH release and accelerates the onset of puberty (127). In regards to the effects of stress, GABA-ergic signaling is modulated by central administration of CRH (128), by different stressors (120, 129), and by ghrelin (114). $GABA_A$ and $GABA_B$ receptors in the mPOA are differentially involved in mediating the effects of stress on LH pulsatility, and antagonism of both receptors has been shown to block the CRH-induced inhibition of LH release in rats (130, 131). At the level of the median eminence GABA can act on the GnRH nerve terminals, leading to disruption of estrous cyclicity in rats (123).

Kisspeptin and stress-induced suppression of GnRH signaling

Another key regulator of GnRH signaling that can be influenced by stress and ghrelin is kisspeptin. Kisspeptin is a neuropeptide that regulates fertility by conveying information on systemic levels of sex steroids to GnRH neurons, and thus regulates both tonic and pulsatile GnRH release, playing a critical role in the onset of puberty (85, 132–135). The most abundant populations of kisspeptin-expressing neurons are found in the arcuate nucleus of the hypothalamus and preoptic area, particularly the anteroventral periventricular nucleus (134, 136). Kisspeptin neurons are also located in the PVN, and project to limbic structures (137, 138), which are involved in stress-induced suppression of the GnRH pulse generator (120, 128). Different stress paradigms that have been shown to suppress GnRH release also result in down-regulation of hypothalamic expression of the kisspeptin gene (Kiss1) and its receptor (Kiss1r) in the mPOA and the arcuate nucleus (139, 140), suggesting that kisspeptin may play a critical role in the stress-induced suppression of GnRH pulsatility and LH release. Similar to stress, ghrelin also has an inhibitory effect on kisspeptin-mediated GnRH pulsatility and LH release (116, 141), as we will discuss in the Ghrelin's Local and Systemic Role in Hypothalamic-Pituitary Reproductive Control section.

Figure 4. Intersection of HPA and HPG axes. CRH can directly inhibit the GnRH pulse generator leading to suppression of pituitary gonadotropins (LH and FSH), in turn resulting in diminished oocyte maturation and steroidogenesis. Glucocorticoids can act directly at the level of the pituitary to suppress gonadotropin release, as well as to exert suppressive effects within the gonads. CRH release from the paraventricular nucleus of the hypothalamus activates locus coeruleus (LC) neurons increasing noradrenaline production. This stress-induced increase in sympathetic activity can then increase sympathetic innervation of the ovary, contributing to the development of ovarian cysts. Adapted from Servier Medical Art under Creative Commons CC-BY license.



The role of gonadotropin-inhibitory hormone in stress-induced suppression of GnRH signaling

A negative regulator of the HPG axis that has also been implicated in stress-induced reduction of GnRH activity is the gonadotropin-inhibitory hormone, also known as RFamide-related peptide in rodents and humans (142). In rats, exposure to stress leads to hypothalamic upregulation of RFamide-related peptide expression, and this increase is associated with a significant reduction in circulating LH (143). Another recent study has demonstrated that chronic stress-induced reproductive dysfunction in rats is completely resolved by targeted knockdown of hypothalamic RFamide-related peptide-3 (144).

The effects of ghrelin on the activity of RFamide-related peptide-containing neurons have not yet been fully characterized (145). RFamide-related peptide neurons do not express ghrelin receptors, suggesting any effects of ghrelin are indirect or via a yet unidentified receptor (146). RFamide-related peptide neurons, however, coexpress CRH-R1 and glucocorticoid receptors (143), and CRH increases RFamide-related peptide receptor Gpr147 gene expression and suppresses GnRH mRNA levels in GnRH cells *in vitro* (147), whereas adrenalectomy abolishes the effects of stress on RFamide-related peptide/GnRH signaling (143). Gpr147 is also expressed by kisspeptin neurons (148), and RFamide-related peptide inhibits kisspeptin-induced activation of GnRH neurons (149). These findings suggest that gonadotropin-inhibitory hormone acts directly and indirectly on the GnRH network to mediate the inhibitory effects of stress on reproduction. It is also evident that due to the extreme complexity of the neural networks involved in the control of reproduction, the multiple signaling mechanisms reviewed previously work in conjunction to mediate the effects of stress on fertility.

Stress and pituitary and gonadal function

Glucocorticoids and the regulation of pituitary gonadotropins

Although stress-induced decreases in gonadotropin release are primarily controlled centrally and are influenced by changes to the activity of the hypothalamic GnRH pulse generator, direct subtle changes may also occur locally. Early studies, in several species including humans, demonstrated that glucocorticoids act directly on the pituitary to inhibit its responsiveness to GnRH and reduce the release of gonadotropins *in vivo* and *in vitro* (150–154). More recent evidence suggests inhibition of the pituitary's sensitivity to GnRH may also be regulated directly at the pituitary by ghrelin (113, 155, 156), as we will discuss further in the Ghrelin's Local and Systemic Role in Hypothalamic-Pituitary Reproductive Control section. The effects of glucocorticoids at the pituitary are possibly divergent for LH and FSH, because *in vitro* release of FSH is increased from rat pituitary cells when treated with glucocorticoids, but LH production is suppressed (157). The potential for direct effects of glucocorticoids on the pituitary are further supported by the evidence that glucocorticoid receptors are expressed by rat (158) and mouse (159) gonadotrophs, and glucocorticoids can act directly upon the anterior pituitary gonadotrophs to suppress GnRH-induced LH subunit β gene expression (159). Synthesis of the β-subunit is the rate-limiting step in LH production (160). Consistent with a direct action of glucocorticoids at the level of the pituitary, studies in ovariectomized ewes have demonstrated that cortisol treatment induces suppression of LH, and this suppression occurs in the absence of a reduction in GnRH pulsatility (161), whereas GR antagonism reverses cortisol- and psychosocial stress-induced suppression in pituitary responsiveness to GnRH (162, 163). Overall, these findings suggest that glucocorticoid-induced suppression of pulsatile LH release may be driven by a reduction of pituitary responsiveness to GnRH. However, the full extent of glucocorticoid (and ghrelin's) role in compromising the GnRH-driven FSH and LH surges may be poorly estimated due to the technical difficulties in sampling blood sufficiently frequently to obtain accurate surge profiles, particularly in small animal models. Sheep, nonhuman primate, and human studies will prove to be particularly informative in this regard. Another important pituitary hormone known to be influenced by stress and ghrelin is prolactin, and we discuss its role in mediating the effects of stress and ghrelin on reproductive function in Ghrelin as a Regulator of the HPG Axis section.

The effects of glucocorticoids on gonadal function

Exposure to stress is usually accompanied by decreased gonadal steroid production (89, 164). This decreased steroid production may be a result of a stress-induced attenuation of the pituitary reproductive hormone release affecting the gonadal output (165–167), as well as by a direct effect of glucocorticoids and sympathetic innervation on the gonadal activity (168, 169), as discussed here and in the the Effects of Catecholamines on Gonadal Function section.

Several studies in humans have demonstrated that increased levels of glucocorticoids due to an exposure to stress can reduce the release of gonadal steroids, with and without a concomitant reduction in plasma LH levels (164, 170–172). Bereavement stress (173), as well as experiences of war (174, 175) have been associated with reduced quality of sperm. However, these effects are likely to be related to the perceived degree of stress. As such, there are mixed findings regarding the impact of chronic mild stress, such as job strain, on semen quality. Although some studies have found no association between work-related stress and semen quality or sex steroids (173, 176, 177), others

found that higher job strain does lead to significant impairment of sperm quality (178, 179) and decreased levels of testosterone (180). These discrepancies suggest that although occupational stress may be detrimental to male fertility, different measures of job strain such as effort-reward imbalance need to be considered to determine whether work stress has necessarily a negative impact on wellbeing (181). Particularly because job loss and unemployment are also associated with poor sperm quality (177) and lower testosterone (182), these data suggest these effects are likely to be related to increased anxiety and depression, independently linked to poor fertility (22, 171).

The impact of stress on testosterone levels in adult males may be influenced by exposure to early life stress, and thus program increased vulnerability to the effects of adult stress (183). In the testis, glucocorticoids act directly on Leydig cells that express glucocorticoid receptors (184). The access of glucocorticoids to their receptors is controlled by the type 1 isoform of 11β-hydroxysteroid dehydrogenase. In male gonads, this enzyme is exclusively and abundantly expressed in Leydig cells where it catalyses the oxidative inactivation of glucocorticoids. However, in response to severe stress, 11β-hydroxysteroid dehydrogenase1 is saturated and excessive glucocorticoids may cause rapid repression of testosterone production [reviewed in (185)]. Ghrelin has also been shown to modulate testicular function both directly at the testicular level and through its systemic administration, suppressing Sertoli and Leydig cell proliferation (186, 187).

A direct effect of glucocorticoids on the ovary has also been identified. Glucocorticoid receptors are expressed in different cell types within the ovary (188), and their expression is maintained during follicular maturation, ovulation, and pregnancy (189). Similar to glucocorticoid action in the testis, 11β-hydroxysteroid dehydrogenase regulates glucocorticoid action within the ovary. In cultured human granulosa-lutein cells, diminished 11β-hydroxysteroid dehydrogenase activity has been shown to mediate cortisol-induced inhibition of ovarian steroidogenesis (190). Ovarian steroidogenesis is also suppressed locally by ghrelin (191), where it inhibits the expression of other steroid pathway enzymes, such as 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and cytochrome P450 aromatase (192).

Glucocorticoids have also been implicated in oocyte maturation along the ovulatory cycle and species specific effects have been noted. Glucocorticoids suppress meiotic maturation in gilt oocytes (193). In contrast, in mouse oocytes, only supraphysiological levels of glucocorticoids inhibit follicle differentiation and oocyte maturation (194). In ewes, inconsistent effects of cortisol and dexamethasone on oocyte maturation have been demonstrated, with no effect of these glucocorticoids on the capacity of the oocytes to undergo fertilization (195). In humans, however, higher levels of cortisol have been detected in the follicular fluid of oocytes that were not fertilized than in the follicular fluid of successfully fertilized oocytes (196), and higher ovarian 11β-hydroxysteroid dehydrogenase 1 activity has been correlated with better fertilization rate (197).

The effects of catecholamines on gonadal function

In addition to the local ovarian effects of glucocorticoids, catecholamines, and in particular noradrenaline, have an important role in follicular maturation and steroidogenesis (198–200). β-adrenergic receptors are expressed by the theca-interna ovarian cells, and activation of these receptors leads to increased androgen production (201). A stress-induced increase in sympathetic activity, such as that induced by cold and restraint stress, can increase sympathetic innervation of the ovary, contributing to the development of ovarian cysts in rodents (202, 203), and these effects can be abolished by lesions to the noradrenergic nucleus locus coeruleus (204). Polycystic ovarian morphology and excessive androgen levels are common features of polycystic ovarian syndrome (PCOS). As we will discuss in the Role of Ghrelin Signaling in PCOS section, dysregulation in ghrelin signaling has also been implicated in the pathophysiology of PCOS.

Ghrelin and Its Receptors: Structure, Distribution, and Function

Ghrelin is a gut-derived hormone that was originally associated with feeding behavior and energy homeostasis (205, 206). It has lately been found to have important roles in such diverse biological functions as motivation, memory, vascular function, and neuroprotection after brain injury (207–211). Ghrelin is also a key regulator of the endocrine response to stress (212) and of reproduction (155, 191, 213–222).

Ghrelin is a 28-amino acid peptide (223). It is principally produced in the stomach as proghrelin where it undergoes posttranslational octanoylation by the enzyme ghrelin-*O*-acyltransferase (GOAT) to form acylated ghrelin (AG) (224, 225). Ghrelin thus exists in the circulation in at least two major bioactive forms: AG and des-acylated ghrelin (DAG), the unacylated form. There is some evidence that ghrelin is expressed in tissues other than the gut, including pancreas and kidney, pituitary, ovaries, and testes (226–229). However, data from studies in mice with the ghrelin reporter tagged to green fluorescent protein (230, 231) suggest that ghrelin in the brain is likely derived from circulating ghrelin having passed through the blood-brain barrier (232) rather than being specifically synthesized in ghrelin-containing neurons (212).

AG was first identified in 1999 as the endogenous ligand for the growth hormone secretagogue receptor (GHSR1a), through which it stimulates growth hormone release (223). GHSR1a is expressed in numerous tissues

Table 1. Distribution of GHSR in Rat and Mouse Hypothalamus and Pituitary

Organ	Abbreviation	Rat	Mouse
Hypothalamus			
Anterior hypothalamic area	AHA	−	+
Anteroventral periventricular nucleus	AVPe	+	++
Arcuate nucleus	ARC	++++	++++
Dorsomedial nucleus	DMH	+	++
Medial preoptic nucleus	MPOA	+	−
Paraventricular nucleus	PVH	+	++
Periventricular hypothalamic nucleus	Pe	−	+
Retrochiasmatic area	RCA	+	+
Suprachiasmatic nucleus	SCh	++	+++
Ventromedial nucleus	VMH	++	+
Pituitary gland			
Anterior pituitary			
Corticotrophs			+++
Gonadotrophs			+
Lactotrophs			++
Somatotrophs			++++
Posterior pituitary			−

Hypothalamic data are adapted from (234). Pituitary data are adapted from (235). Hypothalamic and pituitary GHSR expression has been identified in rodents (234–236), sheep (237), and human (238), as well as nonmammalian species including birds (239) and fish (240, 241). Symbols in this table are not comparable between organs. Abbreviations: −, no detectable expression; +, minimal detectable expression; ++, some detectable expression; +++, high expression; ++++, very high expression.

throughout the body, including the ovaries and testes (228, 229, 233) (see Table 1). It is also expressed in brain regions important in regulating the HPA and HPG axes, and in regions directly involved in these axes, including hypothalamus and pituitary (113, 231, 234). GHSR1a is a G protein-coupled receptor and activation by the endogenous ligand stimulates a phospholipase C/protein kinase C/inositol trisphosphate pathway that triggers inositol trisphosphate-dependent calcium release from intracellular stores. This intracellular calcium combines with calcium entering the cell via voltage-gated L-type calcium channels to stimulate the downstream response (242–244). In addition to its ligand-dependent effects, GHSR1a has high constitutive activity, and can signal at approximately 50% of its maximal capacity in the absence of ghrelin, at least *in vitro* (245). GHSR1a is also able to dimerize with other receptors, such as the dopamine receptor subtype 2 to modulate dopamine signaling (246) and the melanocortin-3 receptor to modulate melanocortin signaling (247, 248).

Although AG's interaction with GHSR1a has been well-characterized, the receptor for the more abundant form of circulating ghrelin, DAG (224), is currently unknown. DAG does not act at the GHSR. DAG is known to inhibit the effects of AG (249), but it also has important independent physiological effects, including in stress (250, 251) and potentially in fertility.

Ghrelin Signaling in the Stress Response, Reward, and Mood Disorders

Ghrelin in stress and mood, evidence from clinical studies

In a clinical setting, ghrelin is closely affected by stress, mood, and stress-related disorders, such as anxiety and depression. Women with high levels of interpersonal stress have higher serum ghrelin levels than their less-stressed counterparts (252), and ghrelin is acutely elevated after stress imposed in an experimental setting (253–255), or even an anticipation of stress (256). Interestingly, AG is elevated by stress in direct correspondence to the magnitude of stress, so that people with greater glucocorticoid responses to stress have also higher ghrelin levels (253). Several findings suggest ghrelin is involved in the stress response in this context. For instance, exogenous ghrelin stimulates the release of both ACTH and cortisol in humans (257–259). A continuous infusion of AG over 5 hours in female rhesus macaques also stimulates cortisol release (260, 261), whereas a bolus injection in the

same species has no such effect (262). Studies by Wilson and colleagues (263) further demonstrate that although subordinate female monkeys chronically exposed to social stress do not demonstrate differences in circulating AG, they do have increased sensitivity to the orexigenic effects of ghrelin, even in the fed state. This increased sensitivity stimulates increased consumption of a low caloric diet, when a choice between high and low caloric diets is available. Interestingly, dominant females have reduced consumption of this diet in response to ghrelin. Furthermore, CRH receptor antagonism over 4 days in subordinate and dominant monkeys is efficient at reducing hypercortisolism; however, it stimulates increased caloric intake by subordinates and has anorectic effects in dominant animals (262); again illustrating individual differences in response to stress.

Ghrelin-related dysregulation of the HPA axis can lead to mood disorders, particularly if ghrelin is chronically affected. Individuals with polymorphisms in the gene for preproghrelin can be at increased risk of panic (264) and major depressive disorders (265), and treatment-resistant depressive individuals have elevated ghrelin compared with controls, and those who do respond to treatment (266). With respect to depression, conflicting findings have been seen, which reflect the complexity of ghrelin's role. Several studies have shown ghrelin is elevated in depression (267–269), whereas others have shown no changes (270–272). These discrepancies may be accounted for by differences in body mass index or satiety status, because elevated body mass index suppresses, and fasting elevates, ghrelin (273, 274). Thus, Barim *et al.* (275) found depressive patients have a tendency to have less circulating ghrelin than controls, but body mass index was also lower in this group, potentially independently accounting for the findings. Degree or manifestation of the illness may also play a role because at least one study has found differences in circulating ghrelin depending upon whether the patients were treatment-resistant or -responsive (266).

There is now some support for the idea that elevated ghrelin in depression reflects a role for the peptide in combatting or reducing the effects of this disorder. Thus, treatment interventions that mitigate depression can suppress ghrelin. For instance, electroconvulsive therapy (267, 268), citalopram (275), mirtazapine (276), and other antidepressants (268) reduce circulating ghrelin; albeit that maprotiline elevates it (277). Furthermore, Kluge *et al.* (278) have shown in a small clinical sample that acute ghrelin administration has a tendency to improve depressive symptoms in men, but not women, with major depressive disorder. It also significantly improves sleep quality in both men and women with depression-related sleep disturbance (278). Transient suppression of catecholamines, which reliably and reversibly induces depressive symptoms, significantly reduces circulating ghrelin in healthy people (279), and a single exposure to exogenous ghrelin led to elevated mood in 30% of healthy people (three of nine) (259).

Ghrelin in stress and mood, mechanistic detail from animal models

Animal models have served to clarify some of the conflicting findings of the human studies. In animal models, as in humans, stress increases ghrelin. With acute stress, including tail pinch and water avoidance, circulating ghrelin and stomach ghrelin mRNA are increased (280–282). With ongoing stress, including chronic daily restraint, chronic unpredictable stress, and chronic social defeat, the same pattern is seen (283–286), but the increased ghrelin can persist. In mice, increased ghrelin as a result of 10 days daily social defeat was still evident at least 1 month after the last defeat session (286).

Stress-induced ghrelin targets the HPA axis at several levels of the axis, and in healthy individuals under normal conditions probably facilitates an immediate hormonal response that enables effective coping with the cause of the stress (212). GHSR is negligibly expressed in mouse PVN CRH neurons, and ghrelin thus indirectly activates these neurons to stimulate ACTH release from the pituitary (113, 234, 287). In ghrelin knockout ($ghr^{-/-}$) mice, the PVN c-Fos response to acute restraint is exacerbated compared with wild-type controls, but glucocorticoid release is reduced in the same animals. In the absence of ghrelin, the adrenals are capable of responding normally, because exogenous ACTH causes similar glucocorticoid release in the $ghr^{-/-}$ as in the wild-type. The PVN is also capable of responding normally without ghrelin, because mimicking glucocorticoid release with dexamethasone causes similar activation of this region in both groups (113). GHSR is widely expressed in the anterior pituitary (235). It is also coexpressed with ACTH in corticotropic cells (235), and exogenous ghrelin stimulates ACTH release *in vivo* (259) and *in vitro* (288). Thus, ghrelin likely facilitates HPA axis activation by directly stimulating ACTH release from corticotrophs in the anterior pituitary.

"Ghrelin is important for appropriate reproductive function, including development of the reproductive axis."

Although the PVN CRH cells are not directly activated by ghrelin, the peptide does increase CRH mRNA (280, 289) and does indirectly activate these neurons (287, 290). Among several sites potentially important in relaying stress signals to the PVN following ghrelin stimulation are the amygdala, centrally projecting Edinger Westphal nucleus, locus coeruleus, and the ventral tegmental area (212). Dysregulation of the amygdala is a hallmark of anxiety, depression, and dysfunctional HPA axis responses to stress making it a potentially important site for ghrelin's activity under stress conditions (291). It is also a key upstream regulator of the GnRH pulse generator (120, 128). Ghrelin signaling via the amygdala is likely indirect or

inhibitory. Although the medial amygdala contains abundant GHSR, projects directly to the PVN, and is highly activated following psychological stress, very few of the GHSR-expressing cells are those that express stress-induced c-Fos. Edinger Westphal nucleus urocortin GHSR-containing cells are activated by stress in the presence of ghrelin, and project to the medial amygdala suggesting a possible route by which ghrelin indirectly activates the PVN (113). Ghrelin, and fasting, both strongly activate amygdala activity, as well as increasing CRHR1 mRNA in the region (292–294). Intra-amygdala ghrelin injections can also influence the symptoms of depression, although in one study this only occurred in conjunction with calorie restriction (295).

The locus coeruleus also projects directly to the PVN, and these projections are activated by stress to stimulate PVN CRH neurons (296). Intracerebroventricular ghrelin binds to these locus coeruleus catecholaminergic neurons potentially influencing the HPA axis via this pathway (297). The increased sympathetic activity may then increase sympathetic innervation of the ovary, leading to the development of ovarian cysts (202, 203), and contributing to the PCOS phenotype (298, 299). Intracerebroventricular ghrelin similarly increases neuronal activation in the nucleus of the solitary tract, and catecholamine cells from this region can directly activate the PVN and are strongly implicated in regulating an appropriate stress response (67, 297, 300, 301). The ventral tegmental area is another candidate region expressing GHSR, responding to stress, and stimulating the PVN. Activation of the ventral tegmental area may therefore also mediate the effects of ghrelin on HPA axis function. In addition, it has particular significance in ghrelin's role in the rewarding aspects of reproductive function (302, 303).

Stress-induced ghrelin imbalance regulates brain reward circuitry

As well as, and integrated with, an important role for ghrelin in stress, ghrelin is a key player in responses to motivating stimuli and controlling the feelings of reward. This role has been particularly elucidated with respect to the rewarding effects of food, but also pertains to drugs of abuse and other pleasurable stimuli, likely including sex (304). Thus, conditioned place preference for a food reward in the context of chronic psychosocial stress is not evident in mice lacking the GHSR1a, suggesting ghrelin signaling is essential for such reward behavior (305). Similarly, conditioned place preference for food reward under basal conditions can be blocked with ghrelin antagonists (306). Central ghrelin also increases the preference for highly palatable rewarding foods, including fats and saccharin (307, 308). These ghrelin-induced changes in food preference do not occur in the absence of GHSR (306). The idea that ghrelin itself drives a feeling of reward comes from studies showing satiated mice develop a conditioned place preference to ghrelin alone (302).

To achieve this reward regulation, ghrelin interacts with cortical and mesolimbic areas, including nucleus accumbens, amygdala, and ventral tegmental area (309). GHSR is expressed on midbrain dopamine neurons in the substantia nigra (310) and ventral tegmental area (208), regions important for motivational aspects of multiple behaviors, including sexual behavior (311). Activity of the dopamine system in these regions is elicited by expectation of, or exposure to, pleasurable stimuli, such as sexual experience (312). Ghrelin administration directly into the ventral tegmental area leads to elevated nucleus accumbens dopamine levels (313) and increased feeding behavior (208, 209). Ghrelin directly into the ventral tegmental area also influences motivation to persevere at a task to obtain a food reward. Thus, rats given ventral tegmental area ghrelin had an increase in operant lever pressing and nose pokes to obtain sucrose compared with controls (309, 314). Blocking ghrelin signaling with a ghrelin antagonist suppresses operant performance in calorie restricted rats (314). These effects seem to be particular to the ventral tegmental area because ghrelin administered directly into the nucleus accumbens has no similar effect (309).

In addition to a role for ghrelin in food-related reward, ghrelin signaling can also promote alcohol consumption, and sensitization to other drugs of abuse including cocaine, amphetamines, and nicotine (315–322). Indeed, Kaur and Ryabinin (323) have identified that ghrelin's role in driving alcohol consumption critically involves the Edinger Westphal nucleus, with alcohol stimulating the Edinger Westphal nucleus and ghrelin suppressing this effect, implicating this region as a potential interface between stress and reward processes that can be modulated by ghrelin.

Whether ghrelin plays a specific role in rewarding behavior in mating remains to be explicitly tested. However, many of the mechanisms for such behavior overlap with those for reward in food and drugs of abuse. The mesolimbic dopamine pathway is clearly strongly involved in all types of rewarding behavior, including sexual attraction, sexual pleasure, and interpersonal attachment (324). Nucleus accumbens and ventral tegmental area are both activated in association with mating, and sexual experience can lead to remodeling in these regions, with increases in the number of dendrites and spines in the nucleus accumbens in sexually experienced rats compared with sexually naïve (325–327).

To date, few studies have directly examined ghrelin's role in the rewarding aspects of mating behavior. There is one study that has examined the effects of acute ghrelin on mating, and this found intraperitoneal ghrelin can suppress male ultrasonic

calling to receptive females and increase the latency to attack a rival male, evidence of reduced aggression, but preference for female odor was retained (328). These findings are likely to reflect the interplay between ghrelin's role in reproductive function and in feeding, with acute ghrelin preferentially stimulating feeding-related behavior over mating. In direct assessment of ghrelin's rewarding role in mating, ghrelin acutely administered peripherally or centrally into the ventral tegmental area and other areas of the dopamine reward system increases sexual motivation and behavior in male mice. These studies have also shown that acute peripheral or central pharmacological suppression of GHSR or genetic deletion of the receptor reduces sexual motivation and behavior in these animals (303, 329). However, persistent elevation of ghrelin induced by chronic stress may have an opposing effect on sexual motivation and behavior, similar to sexual impairment under negative energy balance (330), typically associated with increased ghrelin. We should note here that GHSR-, GOAT-, and ghrelin-knockout mice breed normally under laboratory conditions (Spencer, Sominsky, Andrews, unpublished observations, 2017). However, these conditions are necessarily relatively stress-free and unchallenged. It is likely the absence of an effective ghrelin system would affect motivation to mate as well as other reproductive factors under conditions of chronic stress. In this regard, it will be essential to directly examine mating behaviors, and other fertility factors, in the context of stress in inducible knockouts that have not had the opportunity to developmentally compensate for ghrelin absence.

Ghrelin as a Regulator of the HPG Axis

Ghrelin's local and systemic role in hypothalamic-pituitary reproductive control

In addition to its role in regulating the stress response and reward, ghrelin is important for appropriate reproductive function, including development of the reproductive axis. It regulates reproductive physiology through its systemic release and local expression, acting at all levels of the HPG axis (331). The local and systemic effects of ghrelin on the adult reproductive system are complex. In regards to its hypothalamic-pituitary reproductive action, AG has been shown to have predominantly inhibitory effects *in vivo*. Acute intracerebroventricular administration of AG in ovariectomized adult rats suppresses LH pulse frequency, but not the pulse amplitude, suggesting the inhibitory effects of ghrelin on LH release are mediated by its effects at the level of the hypothalamus (155, 332). In support of this, GnRH release by hypothalamic explants from ovariectomized adult rats is inhibited by AG (155). Similar decreases in LH pulse frequency, without concomitant differences in LH pulse amplitude, have been detected in ovariectomized rhesus monkeys subjected to chronic peripheral infusion of AG. These suppressive effects of AG on LH pulsatility are concomitant with an increase in circulating cortisol levels (260), and both of these effects are prevented by CRH receptor antagonist treatment (261), demonstrating the role of ghrelin in the interaction between the HPA and HPG axes. The inhibitory effects of AG on LH pulse frequency also occur in ovariectomized estrogen-replaced fed rats, after acute peripheral administration of AG, and these effects are further exacerbated by overnight fasting (116). Acute AG inhibits LH release throughout the estrous cycle in intact adult female rats (155), intact males, and gonadectomized male and female rats prepuberty, with no changes in FSH release (333). These changes are not apparent in prepubertal intact females (156, 333), indicating that ghrelin's suppressive effects on GnRH/LH release are reproductive maturity-dependent in females, but potentially not in males. Indeed, another study in male rats has demonstrated that both acute and chronic administration of AG or DAG, as well as an acute coadministration of both peptides, inhibits LH release in prepubertal and adult males, with a similar reduction of FSH in adults (141). The combined inhibitory effects of AG and DAG on LH release that were demonstrated by this group are contrary to the potential antagonistic effects of DAG on the metabolic effects of AG (334). In young adult men, AG has also been shown to suppress LH release, both its pulse frequency and amplitude (217). In women, repeated administration of AG suppresses both LH and FSH (219). Elevated ghrelin levels in exercising women, and women with anorexia nervosa that suffer from chronic energy deficiency, predict menstrual disturbances and hypothalamic amenorrhea (335–337). Ghrelin-induced suppression of LH release is similar to that induced by stress (92–95). Because energy deficiency is a metabolic stress that leads to an increase in glucocorticoids (338), it is likely that ghrelin and glucocorticoids together communicate inhibitory information to the HPG axis [reviewed in (145)].

Ghrelin has also an inhibitory effect on kisspeptin-stimulated LH release (141). Acute administration of AG, fasting, and their combination suppresses Kiss1 mRNA expression in the mPOA (116), possibly contributing to the inhibitory effects of ghrelin on LH release. However, despite the apparent central inhibitory effects of ghrelin on LH secretion and the mPOA expression of kisspeptin gene, Smith *et al.* (146) found minimal to no coexpression of GHSR on GnRH, kisspeptin, and tyrosine hydroxylase neurons in the anteroventral periventricular nucleus, or RFamide-related peptide neurons in the dorsomedial hypothalamus, using the GHSR-enhanced green fluorescent protein reporter mouse model. This study demonstrated that over 90% of the GHSR-expressing

cells in the anteroventral periventricular nucleus and the periventricular nucleus express estrogen receptor-α (ERα), suggesting that the central inhibitory effects of ghrelin on LH pulsatility may be mediated by estrogen (146). Another study has identified GHSR and ERα coexpression in neurons in distinct hypothalamic nuclei in female mice, including anteroventral periventricular nucleus, the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus, and the arcuate nucleus. Notably, only in the arcuate nucleus was this coexpression, as well as GHSR mRNA expression, mediated by high estrogen levels. The induction of GHSR mRNA expression in the arcuate nucleus by estrogen treatment in ovariectomized mice is specific to neurons expressing ERα and Kiss1 mRNA. Furthermore, direct application of ghrelin induces estrogen-dependent depolarization of arcuate nucleus Kiss1 neurons (117). Estrogen controls energy balance and expenditure, with a reduction in estrogen synthesis and its circulating levels leading to an increase in body weight and adiposity, as is typically seen in women who enter menopause [reviewed in (339)]. Estrogen is also known to regulate stress sensitivity, with reduction in endogenous estrogen enhancing anxiety, and estrogen replacement producing anxiolytic effects at least in some animal models of ovariectomy and in postmenopausal women (340–342). A series of studies in subordinate female rhesus monkeys demonstrate that chronic exposure to psychosocial stress modulates physiological responses to estradiol, inducing hypersensitivity to the negative feedback effects of estrogen on LH secretion (12), reduced sensitivity to the anorectic effects of estrogen (343), and impaired anxiolytic effects of estradiol in the context of sexual behavior, even at higher doses (344). These animals also exhibit heightened sensitivity to the orexigenic effects of postprandial AG (262). These effects of chronic stress on the responses to estrogen have been suggested to be at least partially explained by an increase in estrogen-induced GABA-ergic tone in the prefrontal cortex of subordinate females. This social status difference between subordinate and dominant females is reversed by CRH receptor antagonism (345). Studies examining the interactions between estrogen and stress, however, have shown contradictory findings in both animals (346–348) and humans [reviewed in (349)]. The different findings reflect differences in behavioral tasks, differences in endogenous and exogenous levels of estradiol and length of exposure to stress. The individual differences in responses to estradiol are particularly important to consider in light of negligible support for beneficial effects of estradiol on mood in postmenopausal women in the Women's Health Initiative studies (350, 351).

Estrogen-mediated regulation of the stress responses is likely to be mediated by both ERα and ERβ (352), whereas the effects of estrogen on energy balance are primarily mediated by ERα (353). Kiss1-expressing neurons in the arcuate nucleus, a critical region for food intake regulation, coexpress ERα and play a pivotal role in the integration of energy balance signaling and reproduction (354). Although some kisspeptin neurons in this region coexpress ERβ, this receptor does not play an important role in estrogen feedback regulation of GnRH activity (354). Nevertheless, the exact role that estrogen plays in mediating the effects of ghrelin on Kiss1 neurons and hence GnRH pulse generator remains to be established. It is also important to note that there are species differences in the expression of GHSR in brain areas involved in the regulation of reproductive activity, such as mPOA, because GHSR is expressed in this region in mice, but probably not in rats (117, 234), suggesting indirect mechanisms may be involved in the inhibitory effects of AG on Kiss1 expression in this region in rats (116). These differences warrant further species-specific investigation into the central effects of ghrelin on reproductive signaling.

In addition to its central effects on GnRH/LH release, there is a potential for ghrelin to directly influence pituitary LH and FSH secretion, as it does for ACTH (113). In the mouse pituitary, GHSR is exclusively expressed in the anterior pituitary, with higher expression in males than in females, as has been demonstrated using the GHSR-enhanced green fluorescent protein mouse model (235). The highest expression of GHSR is evident in somatotrophs, corresponding with ghrelin's potent growth hormone releasing activity. Modest expression of GHSR-enhanced green fluorescent protein is found in gonadotrophs and lactotrophs, indicating that ghrelin can act directly at the pituitary level to influence LH, FSH, and prolactin secretion (235), as it does for ACTH (113). Interestingly, the direct effects of AG on pituitary gonadotropins are opposite to its centrally mediated inhibitory effects, with an *in vitro* application of AG on male and female rat anterior pituitaries dose-dependently potentiating basal LH and FSH production (155, 156, 333). These direct stimulatory effects are estrogen-dependent, with an attenuation of these effects at estrus or after ovariectomy in adult rats, as well as in neonatally estrogenized females prepuberty (155, 156). Cyclic fluctuations in pituitary GHSR have also been reported, with decreased GHSR mRNA expression at estrus and metestrus (155). The involvement of estrogen in the effects of ghrelin on gonadotropin release is not surprising, because the majority of GHSR-expressing cells in the anterior pituitary of both males and females coexpress ERα (235). When AG and GnRH are added simultaneously to the incubation medium, the effects of AG appear to be age-dependent. In adult female rats, AG inhibits GnRH-stimulated LH release *in vitro* at all stages of the estrous cycle,

whereas it potentiates this GnRH-stimulated LH production by anterior pituitaries of intact and ovariectomized prepubertal females (155, 156). In contrast to these *in vitro* findings, continuous infusion of AG in men has been shown to inhibit spontaneous LH pulsatility, as well as the LH response to naloxone, but not to a GnRH stimulus (355). Naloxone is an opioid antagonist that acts centrally to induce gonadotropin secretion (356). Therefore, these results suggest that despite the ability of AG to directly influence the pituitary function *in vitro*, its *in vivo* effects on the release of gonadotropins are predominantly centrally mediated.

At least part of ghrelin's role in fertility is likely mediated through prolactin. Prolactin is produced by the lactotrophs in the anterior pituitary (357). Besides its crucial role in the initiation and maintenance of lactation, prolactin has a wide variety of physiological roles, including in reproduction, and stress responsiveness (358, 359). Prolactin attenuates the effects of stress, and is thus likely to at least partially mediate the diminished stress responsivity seen during pregnancy and lactation, when prolactin levels are upregulated (360, 361). Prolactin has been recently implicated in resilience to the effects of chronic stress in rats, with those animals that were more resilient to stress demonstrating higher plasma levels of prolactin than their more vulnerable counterparts (362). Hyperprolactinemia, however, is known to suppress GnRH/LH pulsatility (363–365), and is a major cause for infertility in males and females (366). This is particularly relevant to our discussion of the effects of ghrelin on fertility, because exogenous AG has been shown to stimulate prolactin release in men and women (257, 258, 367), as well as in human pituitary cells *in vitro* (368), with no effects of DAG on prolactin levels when administered alone or in combination with AG (369). On the other hand, intracerebroventricular administration of AG in sheep failed to induce changes in circulating prolactin (370). The effects of AG on prolactin release may also be dependent on reproductive maturity and cyclicity, because in prepubertal males and females, as well as in hyperprolactinemic aged female rats, both systemic and central administration of AG inhibited prolactin release, with no changes in prolactin production being evident when pituitary samples were challenged with increasing doses of AG *in vitro* (371). Other studies on primary cultures of female rat pituitary cells have demonstrated stimulation of prolactin release by GHSR1a agonists [reviewed in (372)]. Similarly, cortistatin, a neuropeptide that binds GHSR1a, was found to increase prolactin release in mice, *in vivo* and *in vitro*, and in primary pituitary cell cultures of nonhuman primates, an effect that is blocked by GHSR1a antagonists (373). In mice, GHSR deletion results in an important reduction in the mRNA expression of prolactin and fewer prolactin-positive cells in the pituitary. These changes are associated with reduced expression of the pituitary-specific transcription factor that is essential for differentiation of pituitary cells into somatotrophs, lactotrophs, and thyrotrophs (374). These data suggest that the effects of AG on the release of prolactin are likely to be at the level of the pituitary gland, with potential species and reproductive age-related differences. Because high prolactin inhibits LH release (365), a stimulatory effect of AG on prolactin levels may contribute to its inhibitory effects on LH pulsatility.

The ghrelin system in the gonads

The ghrelin system in the testis

Species-specific mRNA and protein expression of ghrelin and GHSR have been demonstrated in mammalian and nonmammalian ovary and testis [reviewed in (331, 375, 376); see Table 2]. The GHSR gene is persistently expressed in rat testis from infancy to adulthood (377), including after selective elimination of Leydig cells (378). On the other hand, specific GHSR1 AMRNA and protein expression becomes detectable only postpuberty in mature nonreplicating Sertoli and Leydig cells (377). In addition, GHSR and GHSR1 AMRNA are detected in the seminiferous epithelium at all stages of the spermatogenic cycle, with variable expression across the cycle. Specifically, minimal detection of GHSR and GHSR1 AMRNA appears in the rat seminiferous epithelium at stages VII to VIII (377), which are also characterized by the lowest expression of the FSH receptor and hence lower sensitivity to FSH (382, 383). In support of this

Table 2. Distribution of GHSR in Gonads

Ovaries	
Ovarian follicles (all developmental stages)	+
Granulosa cells	+
Theca cells	+
Luteal cells	+
Oocytes	−/+
Testes	
Germ cells	+
Sertoli cells	+
Interstitial tissue (Leydig cells)	+

Gonadal data are from sheep; adapted from (237). Gonadal GHSR expression has been identified in rodents (226, 377, 378), sheep (237), pigs (379), and humans (228, 229, 380, 381).
Abbreviations: −/+, detectable expression in some cells; +, some detectable expression.

potential relationship between the expression of GHSR/GHSR1a and FSH responsivity, stimulation of the testes with FSH *in vivo* significantly upregulates total GHSR and specific GHSR1a gene expression in adult rats, with no effect of human chorionic gonadotropin treatment that acts as LH superagonist *in vivo* or *in vitro* on the expression of these ghrelin receptor isoforms (377). In addition, *in vivo* intratesticular challenge with AG suppresses proliferation of differentiating immature Leydig cells, and is associated with decreased expression of stem cell factor, a primary regulator of Leydig cell development. This inhibitory activity of AG is dependent on FSH signaling, because it is absent in hypophysectomized rats, but restored upon FSH replacement (186). Chronic systemic administration of AG has also been shown to induce morphometric alterations and a reduction in the number and functional capacity of different spermatocytic cells in adult rats (187). *In vitro* challenge of rat testicular tissue with AG has been shown to increase the expression of GHSR and GHSR1a (377), and to inhibit human chorionic gonadotropin and cyclic adenosine monophosphate-stimulated testosterone secretion (378). Zhu *et al.* (384) has demonstrated GHSR1a expression in the mouse testis in steroidogenic Leydig cells, Sertoli cells, as well as in germ cells, particularly in transcriptionally inactive elongating/elongated spermatids, indicating that ghrelin may be involved in spermatogenesis/spermiogenesis. Indeed, abnormal spermatogenesis in the testis of leptin deficient *ob/ob* mice, characterized by arrest at the elongating spermatid stage, is improved by GHSR antagonist treatment (384). In human testis, GHSR1a has also been located in germ cells, but mainly in meiotic pachytene spermatocytes (228), as well as in Leydig and Sertoli cells (228, 385).

Ghrelin itself is also expressed in the testis. In rat testis, the ghrelin gene has been detected at all stages of development, and ghrelin's immunolocalization has been identified in mature fetal and adult Leydig cells (378, 386). Ghrelin protein expression in rat testis is specific to Leydig cells, because it is undetectable after selective Leydig cell elimination (386). Barriero *et al.* (386) has also shown that testicular ghrelin mRNA and protein expression in rats is dependent on pituitary LH, with a reduction in ghrelin's expression after hypophysectomy, and its partial restoration after human chorionic gonadotropin replacement. Furthermore, administration of human chorionic gonadotropin to intact male rats transiently increases testicular ghrelin mRNA levels, with no effect of FSH administration (386), contrary to the FSH-dependent regulation of GHSR1a expression and activity (186, 377). These findings suggest that Sertoli cells, primary responders to FSH, may not play a role in the regulation of ghrelin expression in rat testis (386). Ghrelin immunostaining is also detected in steroidogenic Leydig cells in mice, and is significantly increased in the testis of *ob/ob* mice that are obese and infertile. Inhibition of ghrelin signaling in these animals, in turn, restores steroidogenic activity, reduces germ cell apoptosis, and improves sperm production (384). Interestingly, however, ghrelin has also been shown to attenuate testicular dysfunction induced by ionizing radiation, heat, cadmium, and chemotherapy in mice and rats (387–390), potentially due to ghrelin's antioxidant properties (213). In human testis, ghrelin is strongly present in steroidogenic Leydig and to a lower extent in Sertoli cells, but is not present in germ cells (228, 385, 391). Ghrelin expression in Leydig cells is negatively correlated with serum testosterone levels, suggesting testicular ghrelin is involved in steroidogenesis (385). Moderate protein expression of ghrelin is also evident in the human rete testis, efferent ductules, epididymis, vas deferens, seminal vesicles, as well as in spermatozoa (391). Contrary to rodent and human testis, in adult sheep ghrelin is also detected in the germ cells, in addition to interstitial (Leydig) and Sertoli cells, with increased immunoreactivity in the germ cells prior to the first meiotic division of the spermatogenic cycle (237).

The ghrelin system in the ovary

GHSR1 AMRNA and protein expression has been described in pig, sheep, and human ovary (229, 233, 237, 380). In human ovary, GHSR1a has a wide distribution, and is immunolocalized to oocytes, cuboidal granulosa cells, theca cells, hilus interstitial cells, as well as steroidogenic luteal cells in young, mature, old, and regressing corpus luteum (380). GHSR1a is also expressed in ovarian surface epithelium and in the ciliated cells within the human fallopian tube epithelium at all phases of the cycle (229). *In vitro* stimulation of cultured human granulosa-lutein cells with AG has been shown to inhibit steroidogenesis, and this effect is prevented by GHSR1a, but not GHSR1b antagonism, despite the more abundant expression of the latter receptor in the granulosa-lutein cells (191). In sheep ovary, GHSR1a is similarly immunolocalized to ovarian follicles at all developmental stages, with stronger signal in the granulosa than the theca cells, low levels of detection in the oocytes, and positive immunostaining in the luteal cells of the corpus luteum (237). GHSR1a presence in the prepubertal porcine follicles was found to mediate ghrelin's stimulatory effects on estradiol secretion, aromatase activity, and cell proliferation. GHSR1a antagonism, however, did not affect ghrelin-induced suppression of cellular apoptosis suggesting this specific *in vitro* effect of ghrelin may be independent of GHSR1a binding (233). In rats, chronic *in vivo* treatment with AG induced a substantial decrease in the number of corpora lutea and in the ovarian volume, but increased the number of ovarian follicles with a reduced mean diameter (214), potentially reflective of the effects of AG on cell proliferation and

apoptosis. Another study in rats has demonstrated that chronic treatment with AG, DAG, or their combination during the peripubertal period delays follicular maturation and results in decreased ovarian weight, suggesting the inhibitory effects of ghrelin on ovarian development are only partly dependent on GHSR1a pathways (392). These effects of chronic AG treatment are similar to the deleterious effects of chronic stress on ovarian apoptosis and oocyte developmental potential (7).

Ovarian ghrelin expression has been demonstrated in the cytoplasm of the hilus interstitial cells and granulosa lutein cells in the young and mature corpus luteum, but is not detected in regressing corpus luteum, nor is it detected in oocytes or somatic cells of the ovarian follicles in human ovary (380). The ghrelin gene is also expressed in rat ovary, and its expression levels depend on the stage of the estrous cycle, with lowest detection in proestrus and highest expression in diestrus, during the luteal phase of the cycle. This cyclic expression of the ghrelin gene has been shown to be dependent upon gonadotropin signaling, because administration of GnRH antagonist induces a decrease in ovarian ghrelin mRNA levels throughout the luteal phase of the estrous cycle (226). Ghrelin immunostaining is not detected in growing and preovulatory follicles, but is strongly detected in the cytoplasm of steroidogenic luteal cells in corpus luteum of the current cycle, as well as in regressing corpus luteum (226), a finding that is different to the stage-dependent localization of ghrelin in the corpus luteum of the human ovary (380). Overall the strong and cycle-dependent expression of ghrelin in the corpus luteum suggests ovarian ghrelin may also play a direct role in the regulation of steroidogenesis, in addition to ghrelin's systemic suppressive effects on the HPG axis function.

Ghrelin's role in the regulation of puberty

In line with the local and systemic, mostly inhibitory, effects of ghrelin along the HPG axis, and its role in metabolism and energy balance, ghrelin has been shown to play a role in pubertal development. Although the critical role of the metabolic hormone leptin on puberty onset has been well established, the role of ghrelin in the regulation of puberty has not yet been fully elucidated. Few studies have demonstrated in rats that in contrast to the increased sensitivity of female puberty to the permissive effects of leptin (393), male puberty appears to be more sensitive to the effects of ghrelin. Although chronic administration of low doses of ghrelin induced a delay in the onset of puberty in male, but not female rats (394), only a high dose of ghrelin was able to induce a similar delay in vaginal opening females (392). These inhibitory effects on the onset of puberty have been induced by chronic treatment with both AG and DAG, suggesting GHSR1a-dependent and -independent mechanisms may be involved (141, 392). Human data indicate that circulating total ghrelin levels are significantly increased during early postnatal life and then decline with age until the end of puberty and early adulthood (395, 396). This relationship between the decrease in ghrelin and advanced pubertal age is more pronounced in boys than in girls, and is also associated with a negative correlation between ghrelin and insulinlike growth factor 1 (396). Insulinlike growth factor 1 plays an important role in the activation of GnRH pulsatility at puberty (397). These findings therefore suggest that the decline in circulating ghrelin levels during the peripubertal period may facilitate growth and act as a permissive signal on puberty onset. Additional limited information is available from studies in boys with constitutional delay of growth and puberty (CDGP). CDGP is characterized by short stature, delay in bone maturation, and delayed puberty (398). CDGP is also often associated with substantial psychological stress (399–401). Circulating ghrelin in boys with CDGP is negatively correlated with anthropometric parameters, such as body mass index, height, and weight (402, 403), as well as testicular volume, gonadotropins, and testosterone (403). It is important to note, however, that these studies report total ghrelin levels and do not distinguish between AG and DAG, which may play independent roles in human pubertal development. Therefore, further investigation is required to elucidate the function of the ghrelin peptides and their mechanisms of action in male and female puberty.

Ghrelin Signaling in Pregnancy

Ghrelin's role in fertilization and implantation

Ghrelin and its receptor expression in reproductive tissues has also been demonstrated in human, rat, and sheep placentae, endometria, and fallopian tubes (229, 404), as well as in fetal tissues (405, 406), indicating its potential involvement in the course of pregnancy (see Fig. 5). In addition, ghrelin gene expression has been detected in pregnant rat ovary, with higher expression in early, than in late gestation (226).

Pregnancy begins with successful fertilization and implantation, and these are highly dependent on endometrial receptivity (407). Several factors, such as ovarian steroids, cytokines, and neuropeptides, as well as glucocorticoids, regulate this complex interaction (408–411). Glucocorticoids play an important role throughout pregnancy, and maternal glucocorticoids are critical for fetal development [reviewed in (412)], but exposure to excess glucocorticoids due to chronic maternal stress is associated with the development of metabolic disorders and an increased risk of emotional and cognitive disturbances in later life [reviewed in (413)]. Ghrelin is also likely to be involved in the maintenance of pregnancy, including the implantation

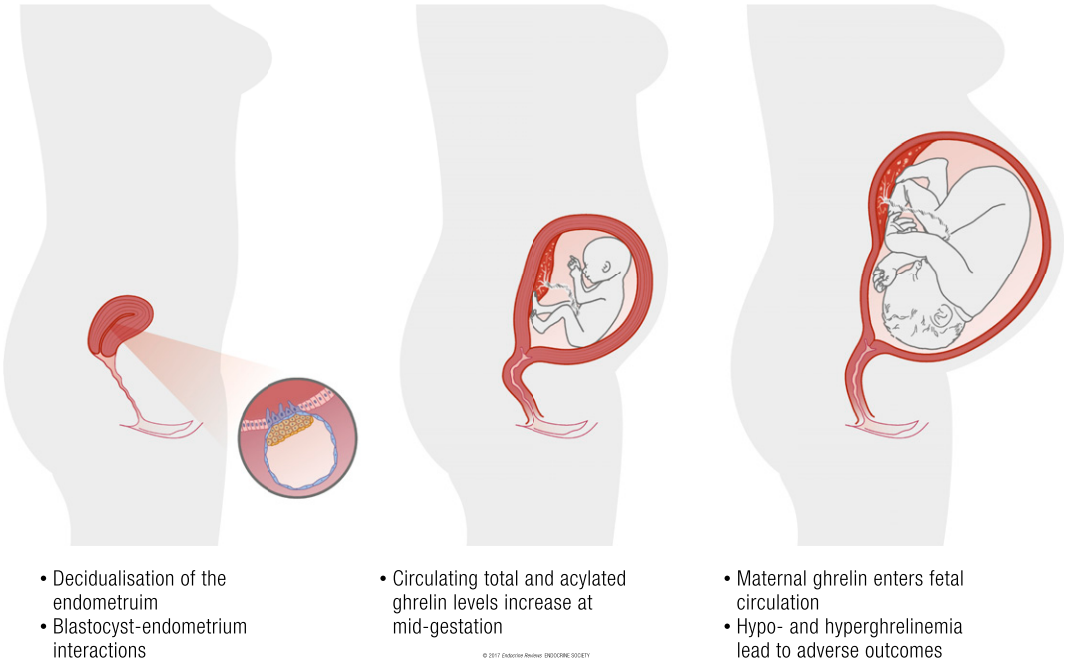


Figure 5. Ghrelin and pregnancy. Early in pregnancy, ghrelin regulates decidualization of the endometrium and blastocyst–endometrial interactions. Circulating total and acylated (AG) ghrelin levels increase at midgestation in human pregnancy, followed by declining levels during the peripartum period. Maternal ghrelin enters fetal circulation, with both hypo- and hyperghrelinemia leading to adverse effects on neonatal outcomes, including birth weight, neurodevelopment, and fertility. Adapted from Servier Medical Art under Creative Commons CC-BY license.

process (216, 414, 415). In women, ghrelin gene and protein expression are significantly increased in decidualized, compared with nonpregnant endometrium, whereas GHSR expression is evident in both cycling and pregnant endometrium (414), and is significantly increased in the midsecretory phase, the time of implantation (215). Interestingly, reduced midsecretory endometrium expression of ghrelin and GHSR1a, at the stage when implantation is likely to occur, is associated with reproductive dysfunction and infertility (215). Ghrelin mRNA and immunolocalization is also strongly detected in first trimester human placenta (414, 416), with negligible to no expression of GHSR transcripts in first trimester and at term placentae (381, 414, 417). In rat placenta, ghrelin mRNA detection is minimal during early gestation, peaking at gestational day 16, and followed by decreasing expression at the latest stage of gestation (416). Ghrelin immunolocalization has also been detected in ovine placentae throughout gestation, with maximal expression occurring toward mid gestation, as well as persistent GHSR1a immunoexpression, with no effect of gestational age on its placental levels (404). Ghrelin and GHSR1 AMRNA and protein levels are also detected in *in vitro* preimplantation sheep embryos (418). These patterns of expression indicate differences between species in the pregnancy-related time course of the ghrelin system expression, likely to reflect species differences in gestation length and energy demands (419).

Ghrelin mRNA expression has been found to be significantly increased in endometrial stromal cell coculture with first trimester human placenta, as compared with endometrial stromal cell primary culture alone. Moreover, addition of AG to the incubation media increases decidualization of endometrial stromal cells induced by cyclic adenosine monophosphate *in vitro* (414, 420), suggesting ghrelin may play role in the remodeling of the endometrium in preparation for pregnancy. Ghrelin immunostaining is also detected in human blastocysts, and ghrelin levels are present in blastocyst culture medium, suggesting ghrelin is secreted by the blastocysts and may be involved in the blastocyst–endometrium interaction (215). Low and medium, but not high doses of AG, have also been shown to stimulate proliferation and decrease apoptosis in human choriocarcinoma cell line JEG-3, common processes in placental formation (421). AG can also promote the rate of blastocyst formation *in vitro* (422, 423), but to exert inhibitory effects on the inner cell mass and trophectoderm cell numbers in blastocysts, negatively affecting potential embryo viability in sheep and mice (415, 422, 423). Corresponding to the latter inhibitory effects, AG has been shown to diminish the rate of meiotic maturation of the porcine oocytes *in vitro* (424). Importantly, the effects of AG on blastocyst formation and embryo quality are not directly dose-dependent, with some doses improving and others inhibiting blastocyst formation rate (425). These nonlinear effects of AG may reflect the differences between the exogenous doses to naturally circulating ghrelin under basal, overfed, fasting, or stressed conditions (256). Stress conditions, when glucocorticoid and AG levels are high (253, 426), have been reported to produce a negative effect on blastocyst formation and implantation (427).

Circulating ghrelin levels fluctuate during the course of pregnancy, with a substantial increase in AG

and total ghrelin levels at midgestation in human pregnancy compared with nonpregnant women, followed by declining levels in the third trimester (428, 429), as opposed to a peak in total (430), AG (236), and DAG (405) levels toward the end of gestation in rats, followed by a decline after parturition (430). Generally, circulating AG levels are lower in human pregnancy than postpartum (431), suggesting that AG may contribute to the adaptation to a positive energy balance in pregnancy. The decrease in AG cannot be attributed to its increased deacylation, because the activity of butyrylcholinesterase, the enzyme that deacylates circulating ghrelin (432), is also significantly reduced in pregnancy (431). The peripartum period is also associated with stress-hyporesponsiveness, in humans and nonhuman animals, vital for fetal development and for maternal mental health and wellbeing (433–439).

Although one study has shown a negative association between circulating and follicular fluid total ghrelin levels and embryo development (440), others have found no effect of ghrelin on human embryo quality and pregnancy success (441, 442). Circulating total ghrelin levels in early pregnancy have also failed to predict pregnancy viability in women undergoing *in vitro* fertilization procedures (443). However, *in vivo* studies in rodents have demonstrated the ability of exogenous ghrelin to interfere with pregnancy success. In mice, hyperghrelinemia induced by a high dose of AG, as well as GHSR1a antagonism during the periimplantation and early gestation periods, led to adverse effects on pregnancy outcomes, including diminished fertilization rate, and delayed embryo development (216). Similarly, in rats, chronic administration of AG during the first half of gestation results in reduced litter size at birth (394). Therefore, although variations in fluctuation of ghrelin in pregnancy may not be sufficient to predict pregnancy outcomes, disruption of this natural process may be detrimental to pregnancy viability and success. Similar to CRH or glucocorticoid excess, that may contribute to the risk of early miscarriage (13, 444), evidence presented previously suggests that stress-induced hyperghrelinemia is likely to lead to negative pregnancy outcomes. It is important to note that some studies have not found an association between stress and/or excess glucocorticoids and pregnancy failure (445, 446), thus the causal link between stress-induced changes in the availability of ghrelin and pregnancy outcomes need to be assessed in future studies. It is also important to specifically investigate the acylation status of circulating ghrelin in its relation to pregnancy, because some studies provide measures of total ghrelin, which include both AG and DAG (430, 440, 441, 443).

Ghrelin's role in fetal development

AG and DAG are present in the fetal circulation, with decreasing levels of both peptides at the end of gestation in rats, whereas amniotic levels of DAG remain high at this time (405). Nakahara *et al.* (405) has also demonstrated that maternal ghrelin (AG) rapidly crosses the placenta and enters fetal circulation, and that chronic treatment with AG, but not DAG at the end of pregnancy significantly and dose-dependently increases neonatal body weight at birth. On the other hand, exposure to maternal AG deficiency has been shown to reduce body weight at birth in rats (405), as well as to affect the fertility of the offspring, leading to abnormal endometrial function in these animals (447). Circulating total ghrelin levels in term infants negatively correlates with birth weight and body length. These correlations are absent in preterm infants (born between 23 and 36 weeks of gestation) (448, 449). Furthermore, total ghrelin levels are significantly increased in the circulation of intrauterine growth-restricted fetuses (450), suggesting that ghrelin may potentially begin to regulate neonatal growth and metabolism at a late gestational stage and may play a role in fetal adaptation to an adverse environment.

Ghrelin, GHSR1a, GOAT and prohormone convertase 1/3, that is responsible for the conversion of proghrelin to ghrelin (451), are all expressed in the human myometrium, indicative of its potential for autocrine and paracrine effects in this tissue (452). O'Brien *et al.* (452) has also shown down-regulation of ghrelin mRNA and protein expression, along with a substantial decrease in GHSR1, GOAT, and prohormone convertase 1/3 protein expression during labor. Because ghrelin has been shown to inhibit human myometrial contractility *in vitro* (453), this decrease in the myometrial expression of the ghrelin system at labor may potentially be necessary to allow successful parturition. This decrease in ghrelin expression coincides with stress-hyporesponsiveness and suppression of the HPA axis activity at parturition, driven by endogenous opioids, prolactin, and oxytocin that act together to suppress stress-responsivity in the peripartum period (433, 454, 455).

Maternal total ghrelin levels subside immediately after delivery and are lower in lactating rats (430), and in both breastfeeding and non-breastfeeding women postpartum (456), than in nonpregnant controls. Another study that assessed the acylation status of ghrelin in human mothers has shown that although total ghrelin concentrations begin to increase between days 4 to 180 of lactation, circulating AG levels continue to decrease (457). Fasting ghrelin levels, that are lower in postpartum than nonpregnant women, are negatively correlated with body mass index and fat mass in the mother (456). Interestingly, ghrelin levels in lactating rats do not appear to be dependent on prolactin and oxytocin, the hormonal regulators of lactation (430). Administration of dopamine agonist decreases

"We propose that elevated maternal AG is likely to influence the reproductive potential of the offspring."

circulating prolactin, and treatment with the dopamine antagonist increases prolactin levels, with no effect on the concentrations of ghrelin. Oxytocin antagonist has also been shown to inhibit the levels of oxytocin, without affecting circulating ghrelin levels (430). AG and total ghrelin are also present in human breast milk (457–460) and their levels correlate with circulating ghrelin concentrations in breastfed infants (457, 460). Ghrelin mRNA is present in the mammary gland, suggesting the source of ghrelin in breast milk may be both the mammary gland and the circulation (459, 461). Similarly, glucocorticoids are present in mother's milk, positively correlating with maternal circulating concentrations, and elevated levels of glucocorticoids in the milk have been found to influence offspring behavioral phenotype, including increased fear and negative emotionality in human infants and nonhuman primates (462–464).

Recent discoveries, including our own, have identified the role of AG and DAG in fetal and neonatal development. Both AG and DAG are involved in fetal neurogenesis within the hypothalamus and the spinal cord, with the proliferative effects of AG, but not DAG continuing after birth in rats (406). In the neonatal period, AG regulates hypothalamic development by limiting the leptin-induced growth of hypothalamic connectivity (465). Changes to circulating AG and DAG have been shown in neonatally overfed mice (466) and rats (467), respectively. These changes are associated with altered central responsiveness to exogenous ghrelin, and may in part explain the development of an obese phenotype in this model (466, 467). Although the role of ghrelin in human fetal and neonatal development has not yet been established, it is highly plausible that maternal ghrelin similarly influences critical developmental processes in infants. Alterations to ghrelin's naturally fluctuating circulating and breast milk levels during the peri- and postpartum periods may thus differentially program brain development. In this regard, a study in pregnant mice has demonstrated that chronic administration of AG during pregnancy increases an anxietylike phenotype, basal circulating CRH and fasting AG levels in adult offspring, along with a reduction in hypothalamic GHSR1a and neuropeptide Y gene expression (468). Interestingly, the authors have also demonstrated that exposure to chronic stress in pregnancy induces an increase in endogenous maternal AG and DAG, and this effect is then reflected in increased fetal AG levels (468). These data indicate that maternal ghrelin has a direct effect on the fetus, and these early alterations in the maternal–fetal ghrelin milieu induce long-lasting neuroendocrine and behavioral changes in the offspring. Although fetal development obviously has complex inputs from many factors additional to ghrelin and more research is required to establish the link between maternal AG, DAG, and neurogenesis in humans, the findings we presented previously are consistent with our hypothesis that stress-induced increases in maternal ghrelin levels may affect pregnancy success and program fetal and neonatal development. Because maternal AG can be transferred to the offspring during the peripartum period (405, 457, 460), and increased AG has mostly inhibitory effects on the reproductive axis (375, 392, 469, 470), we propose that elevated maternal AG is likely to influence the reproductive potential of the offspring.

The role of ghrelin in pregnancy-associated disorders

Clinical perspectives on ghrelin dysregulation in pregnancy: Who is at risk?

Overweight and obesity significantly increase the risk of pregnancy complications, including preeclampsia and gestational diabetes mellitus (GDM). Both AG and DAG are significantly decreased in obese individuals (266, 471) and high-fat feeding in mice has been shown to induce hypothalamic resistance to ghrelin (472, 473) that is reversed by calorie-restricted diet (474). As discussed in more detail in Preeclampsia and Gestational Diabetes sections, dysregulation of ghrelin signaling is associated with preeclampsia, GDM, intrauterine growth-restriction, and other complications, and is also independently implicated in adverse pregnancy outcomes. Although the pathophysiological relationship between metabolic disorders and pregnancy is well recognized, the influence of psychological stress and stress-related disorders on pregnancy outcomes is often overlooked (475). Chronic stress during pregnancy and higher levels of maternal CRH has been shown to predict preterm delivery (476). Depression and anxiety have been similarly demonstrated to be important risk factors for pregnancy loss, preterm delivery, and low birth weight (477–479). Chronic stress has also been suggested to increase the risk of preeclampsia (480). The role of perinatal stress in developmental programming of the brain, cognition, and behavior, as well as in developmental programming of metabolic dysfunction has also been established [reviewed in (481–483)]. As discussed earlier, chronic stress also contributes to the development of metabolic disorders (484), and is associated with elevated ghrelin levels (121, 280–282). Therefore, assessment of AG and DAG, cortisol, along with body mass index, blood pressure, glucose tolerance, and other stress and metabolic biomarkers, may help to predict the risk of pregnancy complications. Later we present the existing evidence for the role of ghrelin in preeclampsia and GDM, the common complications of pregnancy.

Preeclampsia

Gestational hypertension and preeclampsia affect 3% to 6% of pregnancies, with a higher incidence in first pregnancies (485, 486). Preeclampsia is associated with the risks of intrauterine growth-restriction, preterm delivery, and perinatal mortality (487–489). Although the underlying etiology remains uncertain and likely involves many contributing factors, obesity is considered to be a major risk factor for hypertension and preeclampsia (490). Leptin levels are increased in pregnancy, and are particularly increased in women with preeclampsia compared with women with a healthy pregnancy (491), and these levels are positively correlated with body mass index (492). In contrast, total ghrelin levels have been found to be significantly decreased in pregnant women with preeclampsia as compared with healthy pregnant women, and to negatively correlate with blood pressure (493–495). This inverse relationship between circulating ghrelin and blood pressure is also typically present in nonpregnant healthy and hypertensive individuals, as well as animals with hypertension [reviewed in (496, 497)]. Makino *et al.* (498), however, has shown that although total ghrelin levels negatively correlate with blood pressure, these levels are increased in preeclamptic as compared with healthy pregnant women. It is important to note the potential differences in the gestational stage at the time of ghrelin assessment between the studies, as well as in the methods of sample analysis and processing. Because rapid deacylation of ghrelin occurs in circulation (432), protection of ghrelin from deacylation upon blood sample collection is essential to evaluate physiological concentrations of AG and DAG (251, 499). Therefore, further investigation into the specific roles of AG and DAG in preeclampsia is required.

Psychological stress is also a substantial contributor to preeclampsia in women (500–504). Animal studies have shown chronic stress in pregnancy leads to hypertension accompanied by increased adrenal weight, as well as an increase in circulating catecholamines, CRH, and insulin levels in pregnant rats (505–507). Increased blood pressure and adrenal weight in the dam are associated with lower fetal weight and increased fetal adrenal weight and blood pressure (507), similar to changes that occur in human preeclampsia. The development of preeclampsia in conditions of chronic distress has been proposed to be mediated by increased levels of glucocorticoids that are independently associated with hypertension and endothelial dysfunction, common features of preeclampsia (480). With respect to hypertension and ghrelin levels, spontaneously hypertensive pregnant rats have significantly increased circulating total ghrelin levels compared with normotensive controls at the last day of gestation. However, placental expression of the ghrelin gene is significantly lower in hypertensive than in normotensive rats (508). In contrast, a study using Dahl salt-sensitive rats as a model of hypertension and intrauterine growth-restriction, has found that the levels of placental ghrelin are significantly higher in Dahl salt-fed pregnant rats than in Dahl pregnant rats fed a control chow diet (509). Although both these studies indicate a potential for an independent placental synthesis of ghrelin, the discrepancies in the direction of change between hypertensive to normotensive pregnancies may be related to specific strain differences, emphasizing the need for further research of ghrelin's role in human pregnancy, particularly in mediating the deleterious effects of psychological stress.

Gestational diabetes

Another common complication in pregnancy is GDM. GDM affects 2% to 10% of pregnancies and its prevalence has significantly increased over the past 20 years worldwide (510, 511) and is a substantial risk for the development of type 2 diabetes (512). Overweight and obesity prior to and during pregnancy predispose to GDM (513, 514). GDM exposes the fetus to maternal hyperglycemia, leading to fetal macrosomia and hyperinsulinemia (515, 516). Several studies in women with GDM have identified an association with psychological stress and mood disorders (517, 518); however, the causality of this direction is not clear, because the diagnosis of pregnancy complications is itself a stressful experience. Nevertheless, mood disorders are known to contribute to hyperinsulinemia and insulin resistance (519, 520), and exposure to perinatal stress has been shown to promote insulin resistance in the offspring and induce diabetes-related autoimmunity, independently of other risk factors (521, 522), suggesting psychological stress during pregnancy may contribute to the development of insulin resistance and GDM, with long-term developmental implications. Psychological stress has also been shown to increase the production of inflammatory markers in pregnant women (523), consistent with increased risk of pregnancy complications, including GDM (524, 525).

Several studies have assessed the role of ghrelin as one of several likely players in the development of GDM, with some inconsistencies regarding the change of total ghrelin vs specific changes in AG and DAG in women with GDM. AG has been found to be decreased (429), whereas others found no changes in AG, but an increase in DAG in women with GDM both during pregnancy and postpartum (431). Another study in a small sample of diabetic pregnant women, using glucose and insulin clamp techniques, has demonstrated that acute increases in glucose and insulin do not affect AG, but significantly decrease circulating DAG levels (526), suggesting DAG may be involved in the regulation of energy balance in

"Prolonged exposure to increased AG has overall negative impacts on psychophysiological state."

GDM. Although no differences have been found in circulating total ghrelin levels at baseline or after a glucose load between women with GDM and pregnant women with normal glucose tolerance (527, 528), ghrelin mRNA expression is significantly increased in the placental tissue of GDM women (528). Interestingly, a follow-up study for a period of 8 to 10 years of 98 women with GDM has revealed that 22.5% of the participants developed type 2 diabetes, and low total ghrelin levels at 12 weeks postpartum was a substantial risk factor for this complication (529).

Linking Ghrelin Imbalance and Stress in Reproductive Dysfunction: A Role for an Endogenous Hormone

The role of ghrelin signaling in PCOS

Our previous discussion has demonstrated that ghrelin and stress are both intimately and interactively involved in regulating all aspects of reproductive function, and their interaction typically leads to detrimental outcomes (see Fig. 6). One of the more researched reproductive disorders in the context of ghrelin signaling is PCOS. PCOS is typically characterized by polycystic ovarian morphology, and often accompanied by insulin resistance, hyperandrogenism, and chronic anovulation (530, 531). Women with PCOS demonstrate increased sympathetic nerve activity (298), increased sympathetic innervation of the ovaries (299), and an impairment in noradrenaline reuptake or deamination (532). Obesity significantly increases the risk of PCOS and contributes to other comorbidities of PCOS, including insulin resistance (533). As previously discussed, both stress and ghrelin activate the sympathetic nervous system (296, 297), contributing to the development of PCOS (202, 203, 298, 299). Altered cortisol metabolism has also been attributed to the pathophysiology of PCOS (534–536).

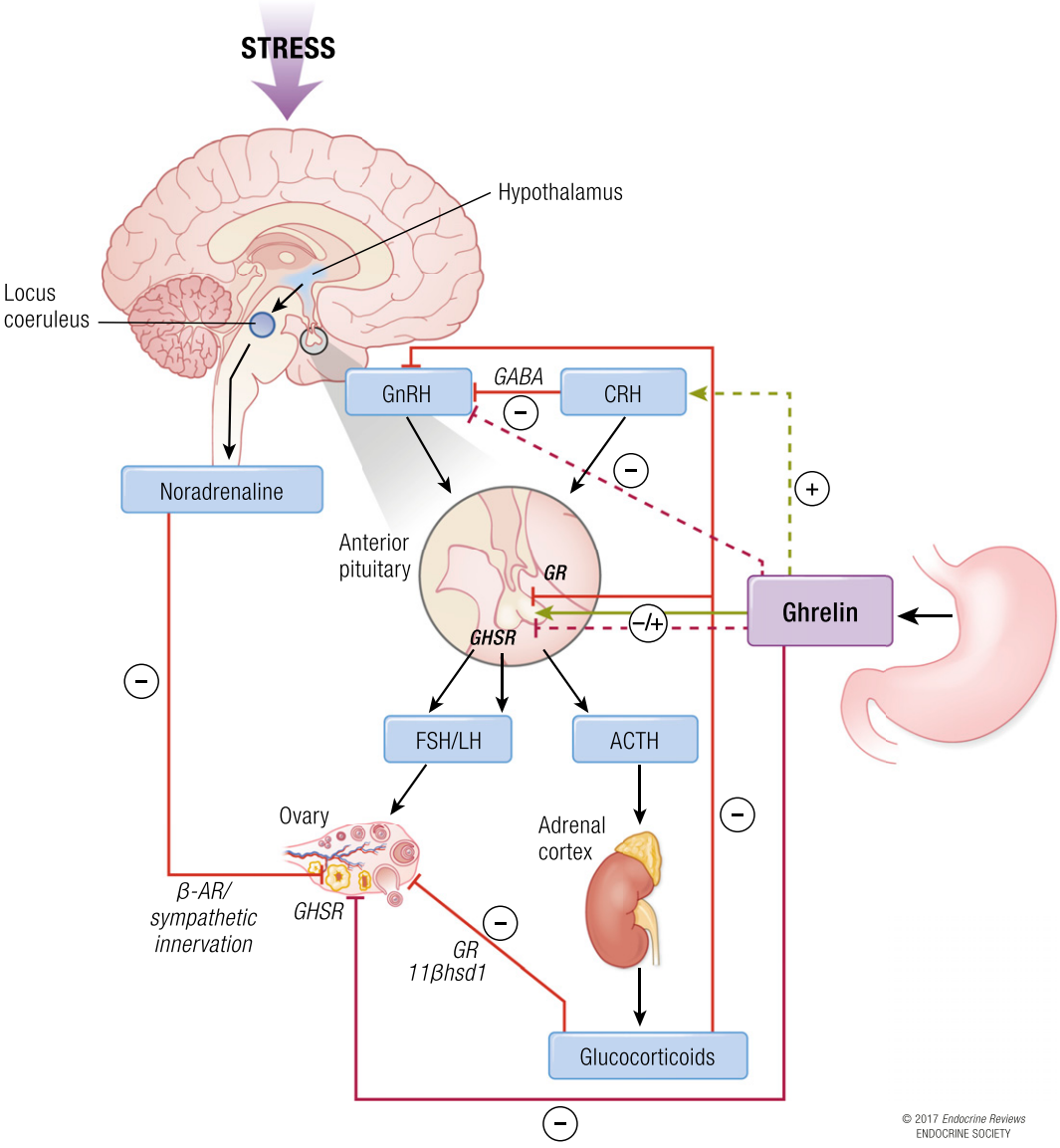
Total ghrelin levels that are typically reduced in obese individuals (273, 471, 537) are also reduced in women with PCOS, as has been demonstrated in several studies (538–541). There is a further reduction in ghrelin levels in obese PCOS as compared with lean PCOS patients (542, 543), or obese controls (544, 545). Some studies, however, have not found these differences (546, 547). A negative correlation has been demonstrated between total ghrelin levels and insulin in obese women with PCOS (544, 545, 548). However, Schöfl *et al.* (549) has found this relationship to be present only in patients with insulin-sensitive, but not in patients with insulin-resistant PCOS who displayed very low fasting ghrelin levels (549), similar to obese insulin-resistant individuals (550). Metformin treatment, a common therapy of choice in insulin-resistant women with PCOS, significantly increases circulating ghrelin levels (549). An inverse correlation between ghrelin and androgen levels has also been demonstrated (538, 541, 544, 545, 551), as well as evidence of a negative relationship between hirsutism and ghrelin levels (544). Treatment with antiandrogen drugs or oral contraceptives increases plasma ghrelin (552, 553). Although normalization of androgen levels also improves insulin sensitivity, changes in plasma ghrelin concentrations are mainly dependent on the declining androgen levels and not on changes in insulin (552), suggesting that androgens may play role in the regulation of ghrelin production in PCOS. Because increased ghrelin levels typically inhibit LH release (217, 219, 220), it is also possible that lower levels of ghrelin in patients with PCOS promote hypersecretion of LH, a characteristic feature of PCOS (554), leading to an increase in ovarian androgen production (470). Interestingly, one study has examined the psychological parameters and emotional state in patients with PCOS and their relation to total ghrelin levels (555). Unlike in other studies, this group has demonstrated that lean women with PCOS exhibit higher ghrelin levels as compared with their weight-matched controls (555, 556), whereas in obese patients with PCOS ghrelin is reduced (555). Moreover, Komarowska *et al.* (555) has shown that lean patients with PCOS exhibit poor resistance to stress and significantly increased ACTH levels, compared with obese patients with PCOS, and that overall anxiety state is positively correlated with total ghrelin levels in patients with PCOS. These latter data emphasize the complexity and the heterogeneous phenotype of this disorder, and the need for further evaluation of the role that ghrelin may play in regulating both the etiology of PCOS and the patients' emotional state.

Ghrelin's role in energy deficiency and reproduction

Sufficient and adequate energy reserves are required for reproduction. It is therefore not surprising that different states of metabolic imbalance, ranging from energy insufficiency to obesity, often lead to reproductive dysfunction. As a major orexigenic hormone that regulates energy homeostasis, ghrelin, has been suggested to mediate the influences of altered nutritional states on reproductive health (557). Although circulating ghrelin levels are decreased in obese patients and increased in patients with anorexia nervosa (558), both conditions contribute to ghrelin resistance (559).

As we discussed previously, obesity is an important contributing risk factor to the development of PCOS, as well as to the development of pregnancy complications. Anorexia nervosa is also associated with reproductive issues, such as amenorrhea (560). Although despite menstrual irregularities women with anorexia nervosa may become pregnant, the physiological and psychological demands of pregnancy and motherhood present a substantial challenge for women who

Figure 6. Ghrelin regulates the effects of stress on fertility. Exposure to stress involves the coordinated interaction between the sympathomedullary system, the HPA axis, the HPG axis, and the ghrelin system. Ghrelin regulates the stress response by acting indirectly on CRH neurons in the PVN and directly at the anterior pituitary gland to facilitate ACTH release. Ghrelin also binds to catecholaminergic neurons in locus coeruleus (LC) increasing noradrenaline production, and further influencing the HPA axis. The increased HPA axis and sympathetic activity exert negative effects along the HPG axis. Ghrelin has also an inhibitory and indirect effect on kisspeptin-stimulated LH release, and is able to act directly at the level of the pituitary influencing gonadotropin secretion. Within the ovary and the testis, ghrelin has predominantly an inhibitory effect on steroidogenesis. Adapted from Servier Medical Art under Creative Commons CC-BY license.



struggle with anorexia (561). Animal models of undernutrition have also shown a decrease in the expression of hypothalamic kisspeptin and delayed puberty onset (562), as well as a diminished ovarian reserve, when an exposure to suboptimal nutritional environment occurs early in life (563, 564).

Ghrelin levels are increased in women with anorexia nervosa and exercise-induced amenorrhea (335, 337, 565). In both anorexia and obesity, ghrelin levels are inversely correlated with body mass index (273, 566, 567). Therefore, increased ghrelin levels in the state of chronic energy deficiency may act as a compensatory mechanism in an attempt to increase food intake and normalize energy homeostasis, as well as to inhibit HPG axis signaling, both centrally and peripherally, until energy reserves are restored.

In rats, food deprivation has also been shown to be associated with increased noradrenergic release in the PVN and elevated plasma ACTH levels, whereas intracerebroventricular administration of AG further enhanced these effects (568). These findings further support the role of ghrelin in mediating neuroendocrine

responses to stress, and suggest that increased ghrelin levels in the state of energy deficiency enhance stress responsivity. Psychological stress, in turn, induces an increase in circulating ghrelin, specifically in AG, but not DAG, and this increase has been shown to have both anxiolytic and anxiogenic effects [reviewed in (250)]. These differences are likely to be related to the duration of stress (212, 569). AG release in situations of acute stress is likely to play an adaptive role, helping an individual to cope with stress (113). However, prolonged exposure to increased AG has overall negative impacts on psycho-physiological state (121, 266, 268, 280), and as we propose herein, this stress-induced increase in AG is detrimental to reproductive function.

Ghrelin's role in stress-induced reproductive dysfunction

As has been demonstrated with ghr$^{-/-}$ animals and with cases of infertility despite normal ghrelin activity, ghrelin is not strictly necessary (under nonstressed conditions) nor sufficient for reproduction. However, ghrelin modulates the stress response at almost all levels of the HPA and SAM axes, strongly influences reward, and is also an important player in successful functioning of the HPG axis, ovarian follicle maturation, and spermatogenesis. We therefore conclude ghrelin plays a substantial role linking stress with infertility.

The evidence we presented previously describes ghrelin's role in integrating stress responsivity, as well as its regulatory role in reproductive function. The role of ghrelin signaling in both of these neuroendocrine systems is undoubtedly complex and is intertwined with its role in energy homeostasis. Interestingly, although the hypothalamus is the main site of ghrelin's metabolic, stress-related, and reproductive actions, these effects appear to be regionally dissociated. Both the arcuate nucleus and PVN mediate the orexigenic effects of centrally administered AG (115, 297, 570), and these regions are also responsive to its anxiogenic effects (571). However, although intact arcuate nucleus signaling is essential for the metabolic actions of peripheral ghrelin (297, 572), ghrelin-induced activation of PVN CRH neurons is independent of the arcuate nucleus (114, 266, 268, 571). CRH neuronal signaling is also implicated in the central reproductive actions of ghrelin, because CRH antagonist has been shown to prevent the inhibitory effects of AG on LH pulsatility in nonhuman primates (261). However, because PVN CRH neurons are not directly involved in the suppression of LH pulsatility (109, 110, 112), these CRH-mediated effects of AG are likely be conveyed by CRH-GnRH connectivity in the mPOA (107), where AG exerts inhibitory (indirect) effects on Kiss1 neurons (116). In the periphery, administration of AG induces an increase in ACTH and glucocorticoid levels in animals and humans (258–261), and these inhibit reproductive function at all sites of the reproductive axis as we have discussed. It is therefore plausible that in response to stress, both AG and HPA axis hormones act synergistically to suppress reproduction. It is important to incorporate in this regard the role of ghrelin in reward, including in the rewarding aspects of mating. Acutely AG has been demonstrated to enhance sexual motivation and behavior in male mice (303, 329). However, in female mice, chronic calorie restriction, typically associated with increased AG, reduces sexual receptivity and this is reversed by administration of the GHSR antagonist (573), suggesting that persistent elevation of AG induced by chronic stress may also inhibit sexual motivation and behavior.

The ghrelin system is also critically involved in the regulation of pregnancy and fetal development, and although the evidence is limited, it appears that increased levels of AG, including those induced by chronic stress, can be transferred to the fetus, programming an anxietylike phenotype in the offspring (468). Ghrelin deficiency during pregnancy, however, also produces detrimental developmental outcomes (405), suggesting that balanced ghrelin levels are required to maintain healthy pregnancy and improve fetal outcomes. Altered levels of ghrelin have also been associated with complications of pregnancy and other reproductive disorders in humans [reviewed in (469, 470)], and although further research using experimental conditions that induce alterations in ghrelin levels is required, we propose that chronic stress may significantly contribute to these reproductive disorders, via an imbalance in the availability of ghrelin.

Current Limitations and Future Directions

It is important to note that although the evidence that we presented in this review strongly supports our hypothesis that stress negatively impacts fertility and pregnancy by stimulating dysregulation in ghrelin signaling, a direct role for ghrelin as a mediator between stress and fertility has not yet been shown. Therefore, much remains to be elucidated in regards to the mechanisms involved in the integration of stress, ghrelin, and reproductive function, and with respect to potential therapeutic implications of these discoveries.

Future experiments addressing this role will need to be mindful of the potentially independent, complementary, and antagonistic role of AG and DAG, as indicated by the research looking into the distinct roles of AG and DAG in metabolism (249, 574–577), neuroprotection, and cerebrovascular function (207, 211, 578, 579), as well as stress and anxiety (113, 251). Unfortunately, the vast majority of studies investigating the role of ghrelin in reproduction that we have presented in this review do not specify the acylation status of ghrelin.

Future studies will also need to assess the potential for commercially available pharmacological compounds targeting the availability of AG and DAG to remedy stress-induced infertility. Encouragingly, recently developed bioactive DAG analog, AZP-531 (Alizé Pharma, France), is a potent inhibitor of circulating AG (574, 580). This compound is currently undergoing clinical trials in patients with type 2 diabetes and in patients with Prader-Willi syndrome, who suffer from elevated AG and dysregulation of the ghrelin system. Thus far, AZP-531 has been shown to be safe, well tolerated, and its improved pharmacokinetic profile differentiates it from existing ghrelin antagonists (581). This compound may therefore provide a useful therapeutic in the context chronic stress-induced infertility. However, due to the involvement of ghrelin in multiple functions, extensive research is required to address the possibility of safely utilizing this compound in improving fertility. Nonetheless, the clear evidence of ghrelin's role in stress and fertility suggest targeting its action may provide a useful therapeutic to remedy infertility in some stress-susceptible couples.

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Abbreviations

ACTH, adrenocorticotropic hormone; AG, acylated ghrelin; CDGP, constitutional delay of growth and puberty; CRH, corticotropin-releasing hormone; DAG, des-acylated ghrelin; FSH, follicle-stimulating hormone; GABA, γ-aminobutyric acid; GDM, gestational diabetes mellitus; GHSR, growth hormone secretagogue receptor; GnRH, gonadotropin-releasing hormone; GOAT, ghrelin-*O*-acyltransferase; HPA, hypothalamic-pituitary-adrenal; HPG, hypothalamic-pituitary-gonadal; LH, luteinizing hormone; mPOA, medial preoptic area; PCOS, polycystic ovarian syndrome; PVN, paraventricular nucleus of the hypothalamus.