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Gonadotropin-Inhibitory Hormone (GnIH) and Its Mammalian Ortholog RFamide-Related Peptide-3 (RFRP-3): Discovery and Functional Implications for Reproduction and Stress

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Abstract

At the turn of the millennium, a neuropeptide with pronounced inhibitory actions on avian pituitary gonadotropin secretion was identified and named gonadotropin-inhibitory hormone (GnIH). Across bird species, GnIH acts at the level of the pituitary and the gonadotropin-releasing hormone (GnRH) neuronal system to inhibit reproduction. Since this initial discovery, orthologs of GnIH have been identified and characterized across a broad range of species. In many vertebrates, the actions of GnIH and its orthologs serve functional roles analogous to those seen in birds. In other cases, GnIH and its orthologs exhibit more diverse actions dependent on sex, species, season and reproductive condition. The present overview highlights the discovery and functional implications of GnIH across species, focusing on research domains in which the significance of this neuropeptide has been most explored.

Introduction

Since the discovery of gonadotropin-releasing hormone (GnRH) as a central regulator of the reproductive axis in the early 1970's (1-4), substantial progress has been made by the neuroendocrine community in advancing our understanding of the neurochemical circuits underlying reproductive function. It is generally accepted that the GnRH system acts as a point of convergence for the integration of intrinsic and extrinsic factors that guide reproductive effort based on appropriate developmental and metabolic states, time of day or year, current environmental conditions, hormonal status, and other variables relevant to reproductive success. In most cases, these signals are not deciphered directly by the GnRH

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system, but through signaling via upstream systems directly responsive to reproductively-relevant cues.

Prior to the recent advent of retrograde, viral tract tracing techniques permitting the identification of cell-phenotype-specific afferents, it was challenging to identify upstream regulators of the widely distributed GnRH network. As new reproductively-relevant candidate systems were identified, each was examined, in turn, for its influence on the GnRH neuronal system. Despite being less efficient than is now possible, this approach, combined with broad genomic and proteomic screening, led to the identification of a number of novel neuropeptides critical for reproductive function. In fact, quite unexpectedly, the 21st century saw the discovery of pronounced positive and negative regulators of the hypothalamo-pituitary-gonadal (HPG) axis (e.g., (5-7)), kisspeptin and gonadotropin-inhibitory hormone (GnIH), respectively, that caused neuroendocrinologists to question long-held beliefs about the neural control of reproduction. The present overview focuses on the discovery and functional significance of one of these neuropeptides, GnIH, and considers future directions aimed at further clarifying the neural targets, mechanisms of action, and commonalities/disparities among species for this neurochemical.

Discovery of GnIH in Birds

In the late 1970's, a cardioexcitatory peptide with a Phe-Met-Arg-Phe-NH_2 (FMRFamide) C-terminal motif was identified in the ganglia of the sunray venus clam (*Macrocallista nimbosa*). Over the next two decades, RFamide (Arg-Phe-NH_2) peptides serving central roles in neurochemical and hormonal communication were discovered across species. Given the significant role of this class of peptides across taxa, at the turn of the millennium Tsutsui and colleagues undertook a search for novel RFamide peptides in the central nervous system and identified a new hypothalamic RFamide peptide whose axons terminated at the level of the median eminence, pointing to a potential role in hypophyseal regulation. By applying this neuropeptide to cultured quail pituitary, Tsutsui and his group found that this peptide rapidly and dose-dependently inhibits gonadotropin release, thereby leading them to designate this peptide GnIH (5). These findings pointed to a novel mechanism of gonadotropin control independent of inhibitory actions on the GnRH system.

Following this initial discovery, the cDNA encoding the precursor polypeptide for GnIH was identified in quail (8) and other avian species (9-11), with the GnIH precursor encoding one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) that possess a common C-terminal LPXRFamide (X = L or Q) motif (reviewed in (9, 11, 12)). Subsequently, GnIH was isolated as a mature peptide in starlings (13) and zebra finches (14). In birds, GnIH is localized to the PVN with projections to the median eminence and to GnRH neurons (15). In birds, it is now well established that GnIH regulates reproduction by decreasing gonadotropin synthesis and release from anterior pituitary gonadotropes and/or by acting *via* the GnIH receptor (GnIH-R; GPR147, also called neuropeptide FF receptor 1 (NPFF1)) on GnRH neurons (reviewed in (9, 11, 12)).

The significance of GnIH in the control of reproduction in birds stimulated further research aimed at whether or not this neuropeptide was common across vertebrate species in terms of

its expression and function (16, 17). Indeed, peptides possessing the C-terminal LPXRFamide (X = L or Q) motif have been identified in a number of vertebrates, including mammals (for reviews, see (12, 16, 18-20)). In mammals, this neuropeptide precursor cDNA encodes three peptides [also known as RFamide-related peptides (RFRPs)], RFRP-1, -2, and -3, in bovine and human (for reviews, see (9-11). RFRP-1 and -3 are LPXRFamide peptides, but RFRP-2 is not. RFRP-3 is now considered to be the mammalian ortholog of avian GnIH, although effects of RFRP-1 on central and peripheral reproductive functioning have been reported, requiring further investigation to clarify the specific role of neuropeptide (e.g., (21-25)

GnIH as a regulator of mammalian reproductive function

Across mammals, including humans, RFRP-3, generally inhibits gonadotropin synthesis and/or release (e.g., (26-36)). However, RFRP-3 stimulates gonadotropin secretion in some contexts, dependent on sex, season, and/or reproductive status, (33, 37-39). These disparate effects of RFRP-3 are likely due to the fact that GPR147 can be coupled to either $G\alpha_i$, $G\alpha_s$, or $G\alpha_q$ proteins, and this coupling may differ by sex and/or reproductive status in some species (10, 40). Across mammals, RFRP-3 acts directly on GnRH cells; RFRP-3 cell terminal fibers are found in close apposition to GnRH cells, around 1/3rd of GnRH cells express GPR147 and application of RFRP-3 inhibits GnRH cellular activity (33, 41-45). RFRP-3 may also act through suppression of the positive stimulator of the reproductive axis, kisspeptin, as a subset of kisspeptin cells in the anteroventral periventricular and the arcuate nuclei express the RFRP-3 receptor (GPR147) (43, 46). Additionally, as discussed in greater detail below, RFRP-3 may also act directly on pituitary gonadotropes in some species.

Commonalities and Diversity of GnIH/RFRP-3

Although many commonalities exist, the locations and mode(s) of action of GnIH/RFRP-3 are not universal across vertebrate classes, findings not unexpected given the variability in reproductive physiology and strategies across species. The present overview explores these commonalities and discrepancies to common teleost models in GnIH research.

GnIH:GnRH interaction in the brain

As indicated previously, the first evidence of direct interaction of GnIH and GnRH in the brain came from a neuroanatomical study on birds (15). Putative contacts by GnIH/RFRP-3 cells onto GnRH cells in the hypothalamus have since been demonstrated in all vertebrates studied, including fish, frogs, hamsters, mice, rats, sheep, monkeys, naked mole-rats, and humans (30, 36, 44, 45, 47-49). Although there is putative contact between GnIH/RFRP-3 and GnRH cells in all vertebrates studied, a functional interaction remains to be demonstrated in some instances. However, in birds and mammals, GnRH neurons express GPR147 (13, 33). Thus, GnIH/RFRP-3 has the potential to influence HPG axis activity via action on GnRH neurons.

Examination of GnIH:GnRH actions in the fish brain have been particularly informative. In zebrafish (*Danio rerio*), an elegant study on the GnIH ortholog (LPXRFamide, or LPXRFa) demonstrated that in the preoptic area, LPXRFa fibers interact with gonadotropin-releasing

hormone 3 (GnRH3) cell bodies, and LPXRFa-3 reduced GnRH3 expression in brain slices (50). Further, zebrafish LPXRFa-2 and LPXRFa-3 antagonizes kisspeptin-2 (Kiss2) activation of kisspeptin receptor-1a (Kiss1ra) and kiss1ra-expressing neurons in the preoptic area are innervated by LPXRFa-immunoreactive (ir) fibers (see (51) for an overview of teleost kisspeptin). Thus, LPXRFa may act as a reproductive inhibitory neuropeptide in zebrafish via action uniquely on GnRH3 neurons in the brain (and not on the other GnRHs) while also potentially affecting the Kiss2/Kiss1ra pathway. As described further below, GnIH also acts on gonadotropes in the pituitary in zebrafish. In another teleost species, Nile tilapia (*Oreochromis niloticus*), although LPXRFa-R-immunoreactive (ir) fibers are distributed widely in the hypothalamus, neither LPXRFa-ir fibers nor LPXRFa-receptor are closely associated or co-expressed with GnRH1 or GnRH3 (52). Likewise, these peptides are not associated with kisspeptin (Kiss2) neurons, but they do appear to act on pituitary gonadotropes (see below). In contrast, LPXRFa dose-dependently suppresses tongue sole Kiss2-elicited cAMP responsive element-dependent luciferase activity in COS-7 cells (53). In general, these studies suggest that the role of GnIH as an inhibitory neuropeptide is maintained in fish species, although further studies of LPXRFa in other species are necessary to fully understand the role of this neuropeptide across fish.

GnIH/RFRP-3 action in the anterior pituitary

Equivocal findings have been obtained regarding whether RFRP-3 has direct actions on pituitary gonadotropes. For example, in hamsters, sheep, macaque, and humans, RFRP-3 projections have been observed in the outer layer of the median eminence (26, 27, 44, 45), a result not observed in rats (54). In other cases, fiber labeling in the median eminence is sparse or absent (17, 54-57). Consistent with the absence of RFRP-3 fibers in the outer layer of the median eminence in rats, for example, systemic injections of Fluorogold, a technique used to label hypophysiotropic cells via retrograde transport to cells contacting the portal blood supply, does not label RFRP cells in this species (54). In support of possible actions of RFRP-3 at the level of the hypophysis, RFRP-3 receptor is expressed in hamster (27) and human (45) pituitary and RFRP-3 inhibits gonadotropin synthesis and/or release in sheep (32), cattle (29), and rat (58) cultured pituitaries. Additionally, in ewes, RFRP-3 is detected in hypophyseal portal blood, with pulse amplitude and frequency being higher during the non-breeding season, suggesting direct actions on the pituitary in the seasonal control of reproduction ((59); and see below). However, one recent study found that intravenous infusion of RFRP-3 does not suppress plasma luteinizing hormone (LH) pulsatility in ewes or the estrogen-induced LH surge, drawing into question whether or not RFRP-3 acts on pituitary gonadotropes to suppress LH secretion in ewes, despite being detectable in portal blood (60). The GnIH homolog in teleost fish, LPXRF, acts on the anterior pituitary gland across species. In pituitary explants of zebrafish, LPXRFa-3 downregulates LHβ subunit and gonadotropin common α subunit expression (50). In tilapia, actions of GnIH on the anterior pituitary are likely more widespread, with LPXRFa-receptor expressed in LH, adrenocorticotropin-releasing hormone (ACTH), and α-melanocyte-stimulating hormone (α-MSH) cells (52), and LPXRFa in this species appears to be a positive regulator of reproduction (61).

GnIH/RFRP-3 control of the ovulatory cycle and female reproduction

In spontaneously ovulating mammals, estrogen secretion from maturing ovarian follicles maintains LH at low concentrations through estrogen negative feedback during the follicular phase of the ovulatory cycle. During the ovulatory phase, estrogen negative feedback fails and estrogen acts through positive feedback to stimulate the LH surge that initiates ovulation. Our group hypothesized that RFRP-3 cells might represent a locus at which estrogen acts to drive estrogen negative feedback and that the transient inhibition of this negative feedback is necessary for the preovulatory LH surge (27, 30). For these studies, we used Syrian hamsters due to the precision in the timing of their LH surge. We first found that RFRP-3 cells express estrogen receptor-α (ERα) and appear to mediate estradiol negative feedback (30), with similar expression of ERα later shown in mice (62, 63) and rats (64). Estradiol negative feedback was examined by removing the ovaries of hamsters to release the reproductive axis from sex steroid negative feedback and RFRP-3 peptide was administered in the early part of the light cycle, a time when negative feedback is maximal (30). These injections led to a rapid, dose-dependent suppression of LH, pointing to a likely role in estradiol negative feedback regulation. Additionally, our studies found that the removal of estradiol negative feedback at the time of the LH surge is mediated, at least in part, through circadian-controlled inhibition of the RFRP-3 system (27), with analogous findings recently reported in mice (65). These studies showing coordinated suppression of RFRP-3 cellular activity with proestrus were conducted in an estrogen-clamped LH surge model. However, RFRP-3 cellular activity is suppressed during proestrus in naturally cycling hamsters as well (66).

We next explored whether or not that the same cell phenotype(s) by which the master circadian clock in the suprachiasmatic nucleus (SCN) stimulates the LH surge (i.e., vasopressin (AVP) and vasoactive-intestinal polypeptide (VIP)) simultaneously coordinate the removal of RFRP-3 inhibition. We found that both AVP- and VIP-ergic SCN fibers project to RFRP-3 cells in the DMH (67). Additionally, central VIP injections suppress RFRP-3 cellular activity at the time of the LH surge, but not prior to the LH surge (67). RFRP-3 cells express the core clock protein, Period 1, in a rhythmic fashion, suggesting that autonomous RFRP-3 cell timekeeping underlies this time-dependent sensitivity to VIP signaling (67). RFRP-3 suppresses VIP-induced GnRH secretion from GT1-7 cells, further pointing to the necessity of inhibiting RFRP-3 at the LH surge (68). Together, these findings suggest that the SCN serves a dual role, both coordinating gonadotropic axis stimulation with negative feedback disinhibition to initiate the LH surge and ovulation and point to a novel, circadian-controlled neural circuit that participates in the transition from estradiol negative to positive feedback.

It is striking that the RFRP-3 system projects broadly to limbic structures driving motivated behavior, in addition to directly projecting to the GnRH system (30). RFRP-3 infusions on the day of proestrus grossly reduce sexual motivation in Syrian hamsters (69), suggesting that suppression of RFRP-3 system activity on the day of proestrus is not only necessary for the LH surge, but for sexual motivation as well. RFRP-3 reduces sexual motivation even when animals are primed with estradiol-17 β/progesterone (69), pointing to actions on motivational circuitry rather than through inhibition of the HPG axis. RFRP-3 administration

also alters cellular activity in key neural loci implicated in female reproductive behavior, including the medial preoptic area, medial amygdala and bed nucleus of the stria terminalis, independent of changes in circulating gonadal steroids (69). These results are consistent with additional reports of GnIH/RFRP-3 induced inhibition of sexual behavior in avian and rodent species (28, 47, 70, 71). Together, these data point to a neuromodulatory role for RFRP-3 in female sexual motivation, independent of downstream alterations in sex steroid production.

The suppression of RFRP-3 at the time of the surge appears to be common across mammalian species. In mice, RFRP-3 inhibits LH secretion when administered at the time of the preovulatory LH surge in intact mice, or during an estradiol-induced LH surge in ovariectomized mice (38). In rats, *Rfrp* mRNA is lower during proestrus than during diestrus (72) and treatment with RFRP-3 during proestrus suppresses GnRH cellular activity (73). In ewes, RFRP-3 expression is reduced during the preovulatory period and an 8 h infusion of RFRP-3 was shown to block estrogen-induced LH surges (74). In contrast, one recent study showed that 24 h infusion of RFRP-3 had no effect on the estradiol-induced LH surge in in ewes (60). Whether or not this disparity between studies is due to dose and time of administration of RFRP-3 requires further investigation. Unlike findings in hamsters, however, RFRP-3 infusion did not suppress female sexual behavior in this species. These findings indicate the need for suppression of RFRP-3 activity at the time of the LH surge in spontaneously-ovulating species in which RFRP-3 is inhibitory.

GnIH in the seasonal control of reproduction

Seasonal control of reproduction in birds

Given the pronounced effects of GnIH/RFRP-3 on the reproductive axis across species, researchers interested in the neural mechanisms driving seasonal changes in reproduction began to explore the possibility that the GnIH/RFRP-3 system represents a key locus at which transduced day length information is decoded to appropriately drive the GnRH system. Initial studies of GnIH in avian seasonal cycles of reproduction examined the pattern of GnIH across the seasons to determine whether any changes observed were consistent with a role for GnIH in the seasonal involution and regrowth of the reproductive axis. Early studies in house sparrows and song sparrows found that GnIH-ir neurons are larger in birds at the end of the breeding season than at other times of year, consistent with an inhibitory role for this neuropeptide in seasonal breeding (15). Similarly, GnRH mRNA levels and GnRH-ir cell numbers are higher during the non-breeding period and in short days relative to the breeding season and long days in Eurasian tree sparrows (75). In contrast, GnIH-ir cell number and size and *Gnih* mRNA do not differ between the breeding and non-breeding seasons in wild Australian zebra finch populations (76). This disparity is likely due to the fact that sparrows have distinct breeding seasons whereas zebra finches adopt an opportunistic breeding strategy based on water availability.

Photoperiod is transduced into a melatonin signal inversely proportional to day length (i.e., secreted at night) and inhibits the reproductive axis in birds (e.g., (77, 78). Removal of the major sites of melatonin production, the pineal gland and eyes (79), decreases the expression of *Gnih* precursor mRNA and the GnIH peptide content in the hypothalamus in quail (80). In

contrast, melatonin administration to quail leads increases *Gnih* mRNA expression and peptide. Melatonin likely acts directly on GnIH cells as Mel1c, a melatonin receptor subtype, is expressed on GnIH-ir neurons in this species (80). Additional support for direct actions of melatonin comes from the observation that melatonin administration dose-dependently increases GnIH release from quail hypothalamic explants *in vitro*, with GnIH release exhibiting a nightly peak when melatonin is produced (81). Moreover, LH release is inversely correlated with the pattern of GnIH, providing further evidence for a stimulatory role of melatonin on the GnIH network. Finally, GnIH release is increased in explants from short-day (SD) animals relative to explants from long-day (LD) quail. Together, these findings point to the GnIH system as a significant mediator of seasonal reproduction in birds, with melatonin acting directly on GnIH cells to inhibit the reproductive axis during the non-breeding season.

In addition to acting on cells upstream of the GnRH system, including GnIH, to regulate avian seasonal cycles of reproduction, melatonin may also act directly on the gonads to regulate local GnIH activity. In European starling, for example, the testes express mRNA for GnIH, GnIH receptor and the melatonin receptors, Mel1b and Mel1c (82). Consistent with a role in regulating seasonal breeding, GnIH and GnIH receptor expression levels in the testes are higher during the non-breeding season. Additionally, *in vitro*, melatonin administration dose-dependently increases *Gnih* mRNA and decreases testosterone release from gonadotropin-stimulated testes (82). Together, these results suggest that sex steroid secretion is regulated seasonally in starling testes by direct actions of melatonin on gonadal GnIH production ((82); reviewed in (83, 84)).

Seasonal control of reproduction in mammals

As in birds, given the conserved nature of RFRP-3 across species, this peptide emerged as an attractive candidate system guiding seasonal reproduction in mammals. Because increased negative feedback by gonadal steroids is required for seasonal reproductive inhibition (85-87), prior to the discovery of RFRP-3, studies of photoperiodic rodents searched for target sites at which melatonin signaling might alter sex steroid receptors to modify negative feedback. In Syrian hamsters, the dorsomedial hypothalamus (DMH), the site where RFRP-3 cells are localized in rodents, was found to express both sex steroid and melatonin receptors and be essential for short-day (SD) or melatonin-induced reproductive regression (88-91). These findings pointed to the possibility that melatonin signaling is decoded in the DMH, possibly through RFRP-3 neurons, to alter negative feedback across the seasons. However, despite being necessary for reproductive regression in Syrian hamsters, the DMH is not essential for enhanced gonadal steroid negative feedback following SD exposure in this species (92). Although these findings do not rule out a role for the DMH as a locus participating in seasonal changes in Syrian hamster reproductive function, they do suggest that melatonin acts upstream of the DMH to drive changes in RFRP-3, a possibility supported by findings described further below (93, 94).

As was the strategy in birds, initial studies of seasonally-breeding rodents explored the effects of photoperiod on RFRP-3 expression in Syrian and Siberian hamsters to determine whether or not patterns observed are consistent with a role for this neuropeptide in seasonal

breeding. Contrary to expectation, extended exposure to inhibitory day lengths leads to *reductions* in RFRP-3 peptide and mRNA (95, 96) as well as decreased fiber density and projections to GnRH cells in Syrian and Siberian hamsters (33, 95). Analogous seasonal patterns of RFRP-3 expression are seen in European (97) and Turkish (98) hamsters, as well as the semi-desert rodent, jerboa (99). Recently, a study in Syrian hamsters revealed that expression RFRP-3 receptor, GPR147, also varies seasonally (23). In females, *gpr147* expression is decreased under SD in multiple hypothalamic loci, mirroring SD suppression of the RFRP-3 peptide. However, hypothalamic *gpr147* expression in males was comparatively stable across photoperiods.

In contrast to LD-breeding rodents, exposure to SD stimulates the HPG axis in sheep. RFRP-3 expression, RFRP-3 projections to GnRH neurons, and release of RFRP-3 into the hypophyseal blood portal system are all reduced in SD animals in this species (36, 59, 100). As in hamsters, photoperiodic changes in RFRP-3 are not a result of altered sex steroids in sheep (36). This pattern of RFRP-3, in sheep, is consistent with a role in reproductive disinhibition during SD, whereas the pattern of expression in rodents is at odds with such a clear-cut role for this neuropeptide. As discussed previously, however, RFRP-3 can bind to both stimulatory and inhibitory G-proteins. Although G-protein binding to RFRP-3 receptors has not been examined in a seasonal context, suggestive evidence points to differential G-protein coupling in hamsters based on sex and season. For example, RFRP-3 inhibits release of LH in female Syrian hamsters, but stimulates LH in males (30, 37, 101). The stimulatory effect of RFRP-3 on LH secretion in males was recently also observed in mice (38). In male Siberian hamsters, RFRP-3 is inhibitory in long-day (LD) animals but stimulatory in SD hamsters (33). Consistent with these stimulatory effects, chronic treatment of SD male Syrian hamsters with RFRP-3 reactivates the HPG axis and accelerates gonadal growth (37, 101). Thus, there are species-, sex-, and photoperiod-specific aspects to RFRP-3 and control of the HPG axis. These contextual changes in the effects of RFRP-3 may be essential to its role in mediating photoperiodic control of reproduction and potentially explain why both sheep and hamsters experience SD-induced suppression of RFRP-3 despite the opposing effects of SD on their breeding condition (93). A complete understanding of the role of RFRP-3 in photoperiodism requires continuing investigation into the effects of species, sex, and photoperiod on RFRP-3 in the control of the HPG axis.

As more mammalian species were investigated, it became apparent that SD does not universally inhibit the RFRP-3 system. For example, expression of RFRP-3 does not differ between the breeding and non-breeding seasons in mares or either sex of red deer (102, 103). Likewise, the brushtail possum, a SD-breeding marsupial, displays *increased* expression of RFRP-ir under SD photoperiod (55). Furthermore, although Siberian hamsters reduce *Rfrp* under SD photoperiods, *Rfrp* expression is increased by intermediate day lengths, relative to LD (104). Thus, although RFRP-3 is regulated by photoperiod in the majority of species studied, continued investigation is necessary to elucidate the role of this peptide in seasonal reproduction. Studies in which RFRP-3 cells are genetically manipulated to examine the impact of RFRP-3 communication to cell-specific targets are needed to help clarify the role of this peptide seasonal breeding, as pharmacological manipulations may have off-target effects that make interpretation incomplete.

In sheep and rodents, SD suppression of RFRP-3 is driven by melatonin and not by downstream changes in circulating sex steroids; treatment with melatonin to mimic SD release reduces RFRP-3 expression (33, 96). Conversely, removing the source of endogenous melatonin via pinealectomy before transfer into SD prevents changes in RFRP-3 expression (33, 96). The effects of melatonin on RFRP-3 are consistent with the pattern of reproductive changes under natural conditions, with RFRP-3 expression unchanged by 3 weeks of SD exposure (95) and daily melatonin treatment leading to maximal RFRP-3 suppression after 60 days (96). Although RFRP-3 cells express estrogen receptor-α (30), neither hormone replacement nor gonadectomy influences RFRP-3 expression in SD or LD animals, indicating that changes in *Rfrp*/RFRP-3 are not the result of changing sex steroid feedback (33, 36, 95, 96, 101). Finally, in photorefractory hamsters (i.e., animals undergoing reproductive recrudescence that are insensitive to melatonin), *Rfrp* expression is suppressed, reflecting photoperiod rather than reproductive state (96). Thus, despite the observation that treatment with RFRP-3 can hasten hamster reproductive recrudescence (37, 101), this finding argues against a stimulatory role for RFRP-3 in spontaneous regrowth of the reproductive axis in spring, or at least in the initial neuroendocrine cascades initiating recrudescence.

Because the pars tuberalis (PT) of the anterior pituitary has the highest density of melatonin receptors across species (105-109) and has been implicated in seasonal breeding (110-113), this site has received significant attention as a locus at which melatonin acts to drive seasonal cycles of reproduction. In hamsters, melatonin acts on the PT to inhibit thyroid-stimulating hormone (TSH) synthesis which, in turn, acts in the mediobasal hypothalamus to alter the expression of type 2 deiodinase (Dio2), an enzyme that catalyzes the conversion of thyroxine (T_4) prohormone into the more biologically active triiodothyronine (T_3) hormone, and type 3 deiodinase (Dio3), which converts T_3 into the bio-inactive 3,5-diio-L-thyronine (T_2) (111). Exposure to SD photoperiod decreases the ratio of Dio2:Dio3 and decreases local concentrations of T_3, whereas transfer to LD increases Dio2:Dio3 (114-119). As spring approaches and melatonin duration is decreased, increased T_3 acts on downstream circuitry, presumably including RFRP-3 cells, to modify GnRH secretion and guide the transition from the winter to summer breeding phenotype (120). The precise means by which thyroid hormones signal the RFRP-3 system remains to be determined. Chronic central administration of TSH or T_3 to SD hamsters for several weeks restores the LD pattern of RFRP-3, indicating that RFRP-3 cells are either direct or indirect targets of this thyroid hormone pathway (94, 121).

Although not specifically investigated in the context of seasonal breeding in mammals, it is likely that, as in birds, local gonadal RFRP-3 contributes to seasonal changes in gonadal functioning. For example, RFRP-3 and GPR147 are expressed in the gonads of Syrian hamsters and mice (122-124). RFRP-3 is expressed in spermatocytes and in round to early elongated spermatids in hamster testis, whereas GPR147 protein is seen in myoid cells, pachytene spermatocytes, maturation division spermatocytes, and in round and late elongated spermatids (122). In mice, testicular RFRP-3 synthesis is increased during reproductive senescence and may contribute to the decline in testicular GnRH at this time (123). Finally, in mice, RFRP-3 and GPR147 are present in ovarian granulosa cells of

healthy and antral follicles during proestrus and estrus and in luteal cells during diestrus (124), suggesting participation in follicular development and atresia.

Interactions between the stress axis and GnIH/RFRP-3 Systems

Unfavorable environmental conditions and psychosocial stress suppress reproductive function by acting at all levels of the HPG axis (125, 126). Given that transient adverse environmental conditions can temporarily inhibit breeding during spring and summer in birds, Calisi et al. examined whether or not GnIH participates in stress-induced reproductive suppression in house sparrows (127). Indeed, GnIH-ir neuron numbers are increased in response to capture-handling stress in spring and cellular activation is increased by this stressor in both spring and fall. These findings pointed to GnIH as a mechanism contributing to the transient suppression of reproductive function during unpredictable, ‘stressful’ conditions (127). Further evidence for a role of GnIH in the avian stress response comes from studies showing that, in 14-day old chicks, exposure to high temperatures leads to increased, diencephalic *Gnih* precursor mRNA at 24 and 48 h (128). In mice, *Rfrp* gene expression is reduced by cold temerpatures, suggesting that temperature might alter *Rfrp* mRNA independent of effects on the stress axis (129). In zebra finches, restraint stress reduces GnIH-ir cell numbers concomitant with lower *Fshβ* mRNA, together suggesting that stress enhances GnIH release in this species (130). Glucocorticoids likely mediate this temporary reproductive inhibition through direct actions on GnIH neurons; glucocorticoid receptors (GR) mRNA is expressed in GnIH neurons in quail and treatment with corticosterone (CORT) increases *Gnih* mRNA expression this species (131). Finally, stress might also influence reproductive function through direct actions on the gonads; in European starlings, CORT up-regulates GnIH expression in the testis and metabolic stress up-regulate GnIH expression in the ovaries (132). Likewise, restraint stress increases *Gnih* mRNA expression in the testes of zebra finches (130).

As in birds, stress has pronounced inhibitory actions on the HPG axis of mammals. Analogous to findings in sparrows and quail, acute immobilization stress increases *Rfrp*/RFRP-3 mRNA and protein expression rapidly following stress in rats, returning to baseline 24 hours later (133). Stress-induced glucocorticoid secretion appears to act directly on GR-expressing RFRP-3 cells, as observed in birds (133). Similar findings are seen following mild foot shock stress in rats, with this stressor increasing FOS protein expression in RFRP-3-ir cells (134). Conversely, administration of RFRP-3 to control animals increased anxiety-like behavior, as measured by an open field test, in this same study (134). The impact of stress in rodents is likely conserved as restraint stress also increases RFRP-3 cell activation and expression in female mice (135). In addition to CORT increasing RFRP-3 cell activity, RFRP-3 appears to, in turn, further potentiate the stress response through increasing CORT release in mice, potentially via increases in corticotropin-releasing hormone (136). Consistent with this finding, RFRP-3 infusions induce anxiety like behavior that can be blocked by co-infusion with the recently discovered RFRP-3 receptor antagonist, GJ14 (136). Immune activation stress can also stimulate the RFRP-3 system; administration of lipopolysaccharide (LPS) to female rats elevates *Rfrp* and *Gpr147* mRNA expression (137). Finally, in one study, stress did not affect RFRP-3/*Rfrp* peptide or mRNA expression in sheep (138). However, a subsequent study by the same group showed that pseudostress

through daily intramuscular injections of adrenocorticotropin increased RFRP-3 cells numbers, gene expression/cell, and close contacts of RFRP-3 fibers onto GnRH cells (139). Together, these findings point to an evolutionarily conserved mechanism of control across species (140).

Recent findings using an RFRP-3-expressing neuronal cell line, rHypoE-23 cells derived from rat hypothalamus further confirm that glucocorticoids act directly on RFRP-3 cells to mediate the effects of stress (131, 141). rHypoE-23 cells express GR mRNA and increase *Rfrp* and *Gpr147* mRNA expression in response to CORT treatment. DNA deletion analysis revealed a CORT-responsive region upstream of the *Rfrp* precursor coding region that includes two GC response elements (GREs) at -1665 and -1530 bp. Mutation of the -1530 bp GRE abolished CORT responsiveness. These results provide a molecular basis by which glucocorticoids alter transcriptional activation of RFRP-3 cells during times of stress (131).

It is well established that chronic stress negatively affects reproductive functioning in females (142). However, whether female reproduction continues to be negatively affected following cessation of the stress is virtually unexplored. Geraghty et al. (143) recently found that even under conditions where stress is terminated 4 days *prior to* mating in female rats, there is persistent and marked reproductive dysfunction, with fewer successful copulation events, fewer pregnancies in those that successfully mated, and increased embryo resorption (143). Remarkably, genetic silencing via shRNA of *Rfrp* during stress completely rescues stress-induced infertility in female rats, resulting in mating and pregnancy success rates indistinguishable from non-stress controls (143). Importantly, genetic silencing of *Rfrp* occurred only during the stress procedure, establishing that stress-induced increases in RFRP-3 prior to pregnancy mediate the stress-induced dysfunction. Thus, these findings reveal that chronic stress has long-term effects on pregnancy success, even post-stressor, that are mediated by RFRP-3.

VI. Conclusions and Considerations

The present overview points to GnIH/RFRP-3 as an important modulator of the reproductive axis across species (Figure 1). In birds, the data consistently establish GnIH as an inhibitor of the reproductive axis through actions on the GnRH system and anterior pituitary gonadotropin regulation. In other species, the actions of GnIH/RFRP-3 are more complex, varying in function based on species, sex, season, and reproductive condition. These differences reveal an exciting opportunity to explore the marked diversity across mammalian species in the role(s) of RFRP-3. Likewise, within mammals, the disparity across studies in the actions of RFRP-3 at the level of the pituitary, including those within the same species, require further investigation to clarify the putative hypophysiotropic role of RFRP-3 in some cases. Researchers working together and sharing resources to standardize investigation within a species would help to clarify some of these discrepancies.

Investigating the influence of RFRP-3 in behavior also represents an important area for further investigation. In addition to the suppressive effects on sexual behavior described above, GnIH/RFRP has also been found to modulate displays of aggression in white crown sparrows and Japanese quail (71, 144, 145), and is associated with aggression in mice (146).

Additionally, RFRP/GnIH stimulates feeding or food hoarding in a variety of avian and mammalian species (28, 74, 147-150). This has led some to the proposition that RFRP may act to balance reproductive and metabolic needs (74, 151, 152). However, additional exploration of the effects of GnIH/RFRP-3 at specific neural loci remains necessary, as micro-infusion experiments suggest that effects of RFRP-3 on feeding may depend on the targeted region (153).

Whereas rapid progress has been made uncovering the functional significance of GnIH/RFRP-3 since its discovery almost two decades ago, numerous opportunity exists to further explore the roles played by this novel peptide across species. While most studies to date have examined the impact of GnIH/RFRP-3 on neural functioning, physiology and behavior, or patterns of GnIH/RFRP-3 expression associated with changes in physiology and behavior, there are fewer findings directly confirming the role of this peptide in normal functioning. This challenge represents further opportunity for researchers working across levels of analysis to combine their efforts to uncover the specific roles played by GnIH/RFRP-3 in typical physiological and behavioral conditions. Finally, the recent finding that RFRP-3 inhibits LH secretion in women (34) points to potential translational significance in the treatment of infertility in humans and the importance of continued investigation of this novel peptide.

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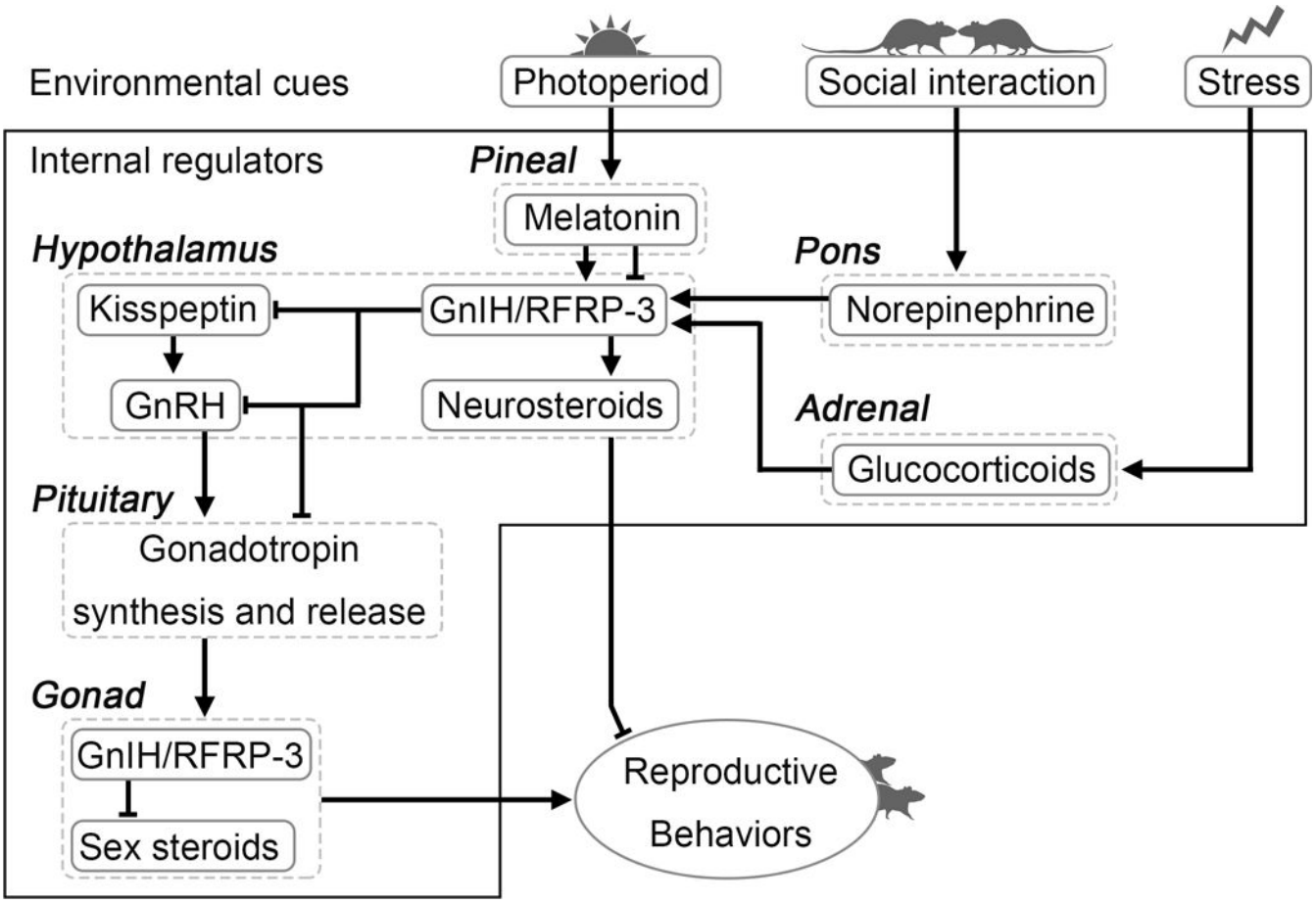

Figure 1.

Model of GnIH/RFRP-3 actions as described herein in birds and mammals. The GnIH/RFRP-3 system acts as a central locus at which internal and external factors relevant for reproduction converge. In turn, GnIH/RFRP-3 acts on the reproductive axis to regulate physiology and behavior via actions at the GnRH and kisspeptin systems, the anterior pituitary, and through alterations in neurosteroid synthesis.

Table 1

Primary structures of avian and mammalian GnIHs [LPXRFamide (X = L or Q) peptides].

	Animal	Name	Sequence	Reference
Birds	Quail	GnIH	SIKPSAY**LPLRFa**	Tsutsui et al. (2000)
		GnIH-RP-1*	SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVAN**LPLRFa**	Satake et al. (2001)
		GnIH-RP-2	SSIQSLLN**LPQRFa**	Satake et al. (2001)
	Chicken	GnIH	SIRPSAY**LPLRFa**	McConn et al., 2014
		GnIH-RP-1*	SLNFEEMKDWGSKNFLKVNTPTVNKVPNSVAN**LPLRFa**	Ikemoto et al. (2005)
		GnIH-RP-2*	SSIQSLLN**LPQRFa**	Ikemoto et al. (2005)
	Sparrow	GnIH*	SIKPFSN**LPLRFa**	Osugi et al. (2004)
		GnIH-RP-1*	SLNFEEMEDWGSKDIIKMNPFTASKMPNSVAN**LPLRFa**	Osugi et al. (2004)
		GnIH-RP-2*	SPLVKGSSQSLLN**LPQRFa**	Osugi et al. (2004)
	Starling	GnIH	SIKPFAN**LPLRFa**	Ubuka et al. (2008)
		GnIH-RP-1*	SLNFDEMEDWGSKDIIKMNPFTVSKMPNSVAN**LPLRFa**	Ubuka et al. (2008)
		GnIH-RP-2*	GSSQSLLN**LPQRFa**	Ubuka et al. (2008)
	Zebra finch	GnIH	SIKPFSN**LPLRFa**	Tobari et al. (2010)
		GnIH-RP-1*	SLNFEEMEDWRSKDIIKMNPFAASKMPNSVAN**LPLRFa**	Tobari et al. (2010)
		GnIH-RP-2*	SPLVKGSSQSLLN**LPQRFa**	Tobari et al. (2010)
Mammals	Human	RFRP-1	MPHSFAN**LPLRFa**	Ubuka et al. (2009)
		RFRP-3	VPN**LPQRFa**	Ubuka et al. (2009)
	Macaque	RFRP-1*	MPHSVTN**LPLRFa**	Ubuka et al. (2009)
		RFRP-3	SGRNMEVSLVRQVLN**LPQRFa**	Ubuka et al. (2009)
	Bovine	RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAAN**LPLRFa**	Fukusumi et al. (2001)
		RFRP-3	AMAHLPLRLGKNREDSLSRWVPN**LPQRFa**	Yoshida et al. (2003)
	Ovine	RFRP-1*	SLTFEEVKDWGPKIKMNTPAVNKMPPSAAN**LPLRFa**	Clarke et al. (2008)
		RFRP-3*	VPN**LPQRFa**	Clarke et al. (2008)
	Rat	RFRP-1*	SVTFQELKDWGAKKDIKMSPAPANKVPHSAAN**LPLRFa**	Ukena et al. (2002)
		RFRP-3	ANMEAGTMSHFPS**LPQRFa**	Ukena et al. (2002)
	Hamster	RFRP-1	SPAPANKVPHSAAN**LPLRFa**	Ubuka et al. (2012)
	(Siberian)	RFRP-3	TLSRVPS**LPQRFa**	Ubuka et al. (2012)
	Hamster	RFRP-1*	SPAPANKVPHSAAN**LPLRFa**	Kriegsfeld et al., (2006)
	(Syrian)	RFRP-3*	TLSRVPS**LPQRFa**	Kriegsfeld et al., (2006)

*Putative peptides. The C-terminal LPXRFamide (X = L or Q) motifs are shown in bold.