

## YOUNG INVESTIGATOR PERSPECTIVES

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# Emerging Functions Of Gonadotropin-Releasing Hormone II in Mammalian Physiology and Behaviour

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Key words: GnRH II, reproduction, sexual behaviour, food intake, luteinizing hormone, cancer, type-2 receptor.

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### Abstract

Gonadotropin-releasing hormone (GnRH) is the central neuroendocrine regulator of the hypothalamic-pituitary-gonadal axis. Multiple structural variants of GnRH are present in vertebrates. The first isoform isolated in the mammalian brain (GnRH I) was shown to regulate the release of pituitary gonadotropins. Recently, a second form has been discovered in mammals (GnRH II), both in the brain and periphery. Although it is unlikely to be a primary regulator of gonadotropin release, the highly conserved GnRH II variant appears to have a wide array of physiological functions. In the periphery, GnRH I and II have similar roles in regulating cell proliferation and mediating hormonal secretion from the ovary and placenta in an autocrine/paracrine manner. In the brain, GnRH I and II apparently modulate mammalian reproductive behaviours in different but complementary ways: GnRH I stimulates luteinizing hormone/follicle-stimulating hormone secretion (and thus gonadal steroids) and promotes sexual behaviour in *ad libitum* fed animals. By contrast, GnRH II acts as a permissive regulator of female reproductive behaviour based on energy status, as well as a modifier of short-term food intake. GnRH II has also been implicated in the regulation of calcium and potassium channels in nervous systems of amphibians, functions which may also be present in mammals. Increasing evidence suggests that the effects of GnRH II in both the periphery and brain may be mediated by GnRH receptor subtypes distinct from the type-1 GnRH receptor. It is likely that this evolutionarily conserved peptide has been co-opted over evolutionary time to possess multiple regulatory functions in a broad range of biological aspects, including, but not limited to, reproduction. Here, the proposed actions of both neural and peripheral GnRH II in affecting physiology and behaviour are summarized, and an outline of critical directions for future research is proposed.

Gonadotropin-releasing hormone (GnRH) is an evolutionarily conserved decapeptide that plays a critical role in the regulation of reproduction in a wide range of vertebrate classes. Although it was originally discovered more than 30 years ago, the biology of GnRH is still an active field of research, both because it is an important peptide for the regulation of the hypothalamic-pituitary-gonadal axis, and because recent advances in molecular biology have enabled it to be studied at multiple functional levels. Moreover, numerous structural isoforms of the peptide have been identified, the functions of which are largely unknown. Indeed, many animals have multiple forms of GnRH in the brain and periphery, as well as multiple types of GnRH

receptors, indicating that the biology of GnRH is not as simple as was once thought.

This review focuses on the emerging functions of the most widely conserved GnRH variant, GnRH II, an isoform that has been identified in numerous species from almost every extant vertebrate class. Although GnRH II was originally discovered in birds and has a wide phylogenetic distribution, this review is limited to its effects in mammals. Furthermore, because several authors have recently reviewed the molecular biology of GnRH II and its receptor (1–3), the review will focus specifically on the functional role of this peptide in regulating physiology and behaviour. The proposed actions of both neural and peripheral GnRH II in affecting physiology and

behaviour will be summarized and discussed; in most cases, the definitive functions of this evolutionarily conserved peptide are not conclusively known, although the data presently available are promising and offer testable hypotheses for future research.

## GnRH II structure, neuroanatomy and receptor binding

### *Structure and phylogenetic distribution*

GnRH was first identified in mammals, specifically in pig and sheep brains (4, 5), and subsequently isolated and sequenced from all major vertebrate classes. In all cases, the primary structure of this GnRH peptide was one composed of 10 amino acids (4–6). Originally called luteinizing-hormone (LH)-releasing hormone based on its ability to promote the release of LH from the pituitary (7), this hypothalamic peptide was subsequently renamed GnRH when it was shown to also stimulate the release of follicle-stimulating hormone (FSH) (5, 8, 9). This GnRH form is now designated GnRH I, to distinguish it from other more recently discovered structural variants. In addition to stimulating pituitary gonadotropin release, GnRH I also plays a role in mammalian reproductive behaviour. Administration of GnRH I to ovariectomized female rats significantly increases sexual receptivity (10, 11) and GnRH I can facilitate mating behaviour in sexually naive male hamsters after their vomeronasal organ has been removed (12).

In addition to GnRH I, there are at least 22 other structural forms of GnRH present in animals (1, 2, 13); in most cases, these variants were originally named for the species in which they were first discovered. Studies employing radioimmunoassay (RIA), high pressure liquid chromatography (HPLC), and immunocytochemistry (ICC) have shown that most vertebrates have multiple forms of GnRH present in the brain, usually in distinct but overlapping regions (14–18). In every vertebrate species examined except jawless fish, one form of GnRH is a variant originally isolated in chicken brains (denoted chicken GnRH-II or GnRH II, to distinguish it from the GnRH I variant) (1, 19). In 1989, GnRH II was reported in mammalian brain of several marsupial species (20, 21). Four years later, Dellovade *et al.* (22) observed the presence of a form of GnRH in the brains of musk shrews

that appeared to be GnRH II; this was the first placental mammal reported to possess GnRH II. Subsequently, GnRH II has been identified in a wide-range of mammalian species including tree shrews (23), moles (23), capybaras (24), mice (25, 26), sheep (1), cows (1) and several primates, including humans (15, 17, 27, 28). The omnipresence of GnRH II across vertebrate and mammalian species suggests that this form of GnRH is evolutionarily conserved and likely to have an important biological function (1, 29).

The amino acid sequence of GnRH II is 70% similar to that of GnRH I, differing in three amino acids (at positions 5, 7, and 8) (Table 1). The DNA coding sequences for the GnRH I and GnRH II preprohormones have been determined. In humans, the genes for GnRH I and II are located on chromosomes 8 and 20, respectively (30). Both genes have the same modular structure, comprising three introns and four exons, which encode a precursor polypeptide consisting of a signal peptide, the GnRH decapeptide, and the GnRH-associated peptide (GAP), whose function is not known (6, 30).

### *Neuroanatomy and receptor binding of the GnRH II system*

In mammalian species, most GnRH I cell bodies are localized to the forebrain, principally in the preoptic area, medial septum, arcuate nucleus and the terminal nerve (associated with the olfactory system) (6, 31); these GnRH I cells originate in the olfactory placode and migrate to various forebrain regions during embryonic development (32). By contrast, the majority of GnRH II cell bodies are located in the midbrain, hindbrain, and extra-hypothalamic regions (17, 22, 23) (Fig. 1). GnRH II cells appear to derive from the germinal zone of the third ventricle and thus have a different embryonic origin than GnRH I cells (32). In musk shrews, the highest concentrations of GnRH II peptide are found in neurones located in the midbrain, with moderate amounts located in the medial habenula, midbrain central grey, hypothalamus and medial septum (33). Several studies have found little to no overlap in neurones containing the two variants of the peptide (23, 33), including those cells located in the hypothalamus (26, 27). In the musk shrew, in which the neural presence of GnRH II peptide has been well characterized, no one neurone contains both GnRH forms

TABLE 1. Gonadotropin-Releasing Hormone (GnRH) Isoforms Present in Mammalian Brain.

GnRH isoform	Amino acid sequence	Gene identified?	Forms identified	Mammalian orders	Reference
GnRH I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly	Yes (h, r, m, hm, rh, ts)	Peptide, mRNA	Ubiquitous (all orders)	(4–6, 13, 20, 21, 24, 28, 31)
GnRH II	pGlu-His-Trp-Ser- <b>His</b> -Gly- <b>Trp</b> -Tyr-Pro-Gly	Yes (h, rh, ts, msk)	Peptide, mRNA	Marsupialia, Insectivora, Rodentia, Scandentia, Primates, Artiodactyla	(17, 22–28, 88)
l-GnRH III	pGlu-His-Trp-Ser- <b>His</b> - <b>Asp</b> - <b>Trp</b> -Lys-Pro-Gly	No	Peptide	Rodentia, Primates	(8, 50, 89)
s-GnRH	pGlu-His-Trp-Ser-Tyr-Gly- <b>Trp</b> - <b>Leu</b> -Pro-Gly	No	Peptide	Rodentia, Primates	(100, 101)

The known GnRH variants present in mammals are listed (l = lamprey; s = salmon). An additional variant is found exclusively in guinea-pigs (gp-GnRH) and is not shown. The amino acid sequence of each peptide is 60–80% similar to that of GnRH I; the amino acids in **bold** vary from those at the same position in GnRH I. The genes for GnRH I and GnRH II have been identified in several mammalian species (h = human; r = rat; m = mouse; hm = hamster; rh = rhesus monkey; ts = tree shrew; msk = musk shrew), and both mRNA and protein for these two isoforms have been found in the brains of species from many mammalian orders. By contrast, neither the gene nor the message of l-GnRH III or s-GnRH has been discovered in any mammalian species, although peptide staining in the forebrain has been reported for both rodents and primates; the presence of these two GnRH forms in mammals remains putative.

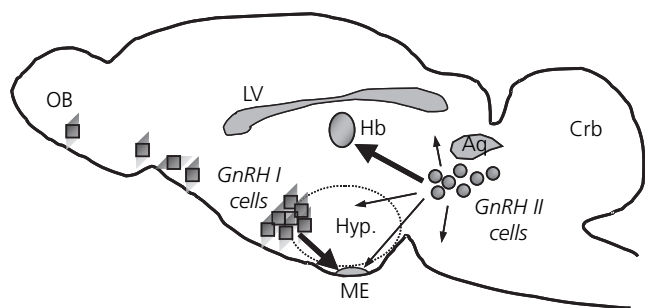


FIG. 1. Schematic diagram of a mammalian brain (sagittal view) depicting the locations of the primary clusters of gonadotropin-releasing hormone (GnRH) I (squares) and GnRH II (circles) neurones. Most GnRH I cells are located in the preoptic area and project primarily to the median eminence/pituitary (indicated by the thick arrow). Additional GnRH I fibres are present in other regions of the brain (not shown). By contrast, most GnRH II cells are located in the midbrain. A majority of the putative GnRH II projections lead to medial habenula (thick arrow), although additional fibres project to other areas throughout the brain (thinner arrows), including the median eminence. Aq, Cerebral aqueduct; Crb, cerebellum; Hb, medial habenula; Hyp, hypothalamus; LV, lateral ventricle; ME, median eminence; OB, olfactory bulb.

and GnRH II cells comprise 60% of the total brain GnRH (22).

In addition to the different locations of GnRH I and II neurones in the brain, the two GnRH cell-types also project to primarily separate neural target tissues (Fig. 1). As much as 75% of GnRH I fibres aggregate in the median eminence; fewer GnRH I axons have terminal fields in the organum vasculosum of the lamina terminalis, the main and accessory olfactory bulbs, the amygdala, midbrain central grey and the dorsal raphe nucleus (31). Although the fibres from neurones containing GnRH II are equally diffuse, only a small minority are found in hypothalamic and pituitary regions that play a role in LH/FSH activation (33). In the musk shrew, the majority of GnRH II-containing fibres terminate in sites outside the median eminence; abundant fibres are noted in the medial and lateral habenulae, medial septum, amygdala, midbrain central grey and regions of the hypothalamus (33). The main target area for GnRH II in musk shrews appears to be the medial habenula where presynaptic vesicles containing GnRH II have been identified at the ultra-structural level (33). GnRH II has also been found in several mammalian species (notably, primates) in high concentrations outside of the central nervous system; peripheral GnRH II mRNA and/or peptide is present in a wide range of tissues including kidney, bone, prostate, placenta, breast, endometrium and ovary (1). The possible functions of this peripheral GnRH II are discussed in further detail later in this review.

In addition to their different sites of synthesis and release, the two GnRH peptides bind selectively to two different GnRH receptors (1, 34). GnRH I binds a seven-transmembrane, G protein-coupled receptor (type-1 GnRH receptor) that is expressed in pituitary gonadotrope cells (2, 34). Recently, a second membrane receptor exhibiting high binding affinity for GnRH II (type-2 GnRH receptor) was isolated and sequenced in fish, amphibians and mammals, including humans (1, 2, 34–36). The mammalian type-2 GnRH receptor shares 40% homology in gene sequence with the type-1 GnRH receptor but, unlike the type-1 receptor, the former possess a

cytoplasmic carboxyl-terminal tail which may promote its rapid internalization and down-regulation (2, 34, 35).

Both GnRH receptor types have a wide distribution in the brain and periphery, and both are present in hypothalamic areas associated with regulation of reproductive behaviour (29, 34–36). Specifically, significant amounts of neural type-2 GnRH receptors have been reported in the preoptic area, arcuate nucleus, ventromedial hypothalamus, infundibular stalk and pituitary, medial habenula, midbrain central grey, cingulate cortex, occipital pole and cerebellum (1, 29, 35). Type-1 receptors are located mostly in the pituitary, olfactory bulb, lateral septum, arcuate nucleus, midbrain central grey, hippocampus (dorsal and ventral subiculum) and amygdala (medial, lateral, and cortical nuclei) (31). Although the two GnRH peptides can bind either receptor, GnRH I binds type-1 receptors with higher affinity than GnRH II, whereas GnRH II has a higher affinity for type-2 receptors (35, 36). Therefore, in addition to having specificity in their sites of synthesis and projections, the two GnRH variants may also confer different biological functions based on their affinities for these two receptor subtypes; these functions are discussed in more detail in the following sections.

## Physiological functions

### *Gonadotropin release*

When GnRH II was first discovered in the brains of avian, and later, mammalian species, it was hypothesized that this peptide would play a similar biological role as GnRH I (i.e. stimulation of gonadotropin release). Initial studies in chickens found that GnRH II was indeed a potent releaser of LH, both *in vitro* from dispersed pituitary cells and *in vivo* (37, 38). Subsequently, it has been determined that GnRH II also can promote LH secretion in a variety of mammalian species; however, it does so with a much lower potency than GnRH I (approximately 2% as effective) (39, 40). Similarly, additional studies found that GnRH II stimulates ovulation but with a lower potency than GnRH I (approximately 10% as effective) (33). Because exogenous GnRH II is only effective at stimulating LH release and ovulation at high doses, this peptide may be acting through pituitary type-1 GnRH receptors (which it binds with low affinity). This conjecture has recently been supported by studies in both sheep and primates. Although type-2 GnRH receptors are reportedly present in mammalian gonadotropes, GnRH II stimulation of pituitary gonadotropins is completely blocked with administration of type-1 GnRH receptor antagonists, both *in vitro* and *in vivo* (41–43). Thus, GnRH II apparently promotes LH release by binding to type-1 GnRH receptors in the pituitary. In support of this, GnRH II-induced ovulation in female musk shrews is similarly blocked by pre-treatment with the type-1 receptor antagonist, Antide (Kauffman and Rissman, unpublished results). Furthermore, spontaneous mutant hypo-gonadal (HPG) mice lacking a functional GnRH I peptide do not ovulate or secrete LH; GnRH II-immunoreactive staining has been reported in the brains of HPG mice (25), suggesting that the endogenous levels of GnRH II in these animals are not sufficient to promote LH secretion or ovulation.

In mammals, GnRH II has been hypothesized to preferentially activate FSH (an 'FSH releasing peptide') (35, 44) but there is currently little supporting evidence for this function. Multiple physiological studies have invoked the existence of an FSH-releasing factor (FSH-RF) to account for differential secretion in LH and FSH and the fact that some GnRH antagonists can suppress pulsatile release of LH without altering FSH pulses (45, 46). Initial studies addressed the possibility that the FSH-RF might be an analogue of GnRH (47); the presence of type-2 GnRH receptors in pituitary gonadotropes, and the fact that GnRH II does not potently stimulate LH, supports the possibility that GnRH II could be a selective FSH-RF. Experiments using dispersed chicken pituitary cells suggest that GnRH II has a minor preferential FSH-releasing activity compared to GnRH I (38). In sheep, an exogenous bolus administration (10 µg) of GnRH II produced a higher ratio of FSH to LH secretion than that achieved with GnRH I, although the latter peptide was significantly more effective than GnRH II in stimulating either of the gonadotropins individually (35). Yu *et al.* (48) showed that GnRH II had a slight preferential ability to induce FSH release in ovariectomized rats. However, more recent evidence contradicts these earlier findings; studies performed *in vitro* using rat hemi-pituitaries found that GnRH II had no significant FSH or LH release except for at very high doses, and no selective releasing activity for FSH (48, 49). Similarly, other recent studies performed *in vivo* in sheep and Rhesus monkeys, as well as an investigation using cultured primate pituitaries *in vitro*, have reported no selective FSH-releasing activity for GnRH II (41–43).

The present evidence implicates another isoform of GnRH, lamprey GnRH III (lGnRH III), as the most likely FSH-RF candidate. Although its gene has yet to be found in mammalian species, immunohistochemical staining for lGnRH III has been reported in the hypothalamus and median eminence of rats and primates, and its administration stimulates FSH release with minimal LH-releasing activity, both *in vitro* and *in vivo* (49–51). Because bound biotinylated lGnRH III in the pituitary was not displaced by GnRH I, there is speculation that this form of GnRH has its own receptor (8, 50). Thus, the FSH-releasing effects of GnRH II (which shares 80% homology with lGnRH III) previously observed in rats and sheep may be mediated by this putative lGnRH III receptor. Taken together, the physiological data currently suggest that GnRH II is unlikely to be the primary FSH-RF under normal conditions, although further experiments analysing the role of pituitary type-2 GnRH receptors (as well as other putative receptors) are needed before this issue can be fully resolved.

Finally, the neuroanatomical evidence also fails to support a gonadotropin-releasing role for GnRH II. In mammals, the majority of GnRH II cell bodies reside in the midbrain, with few cells present in hypothalamic and extra-hypothalamic regions (17, 22, 23). In addition, only a minority of the putative GnRH II fibres in the mammalian brain are present in regions that regulate gonadotropin secretion; in the musk shrew, the majority of GnRH II-containing terminals are present in the medial habenula (33). Collectively, these findings suggest that the primary role of neural GnRH II is not to stimulate gonadotropin hormone release but rather to act as neurotransmitter (6).

### *Role in peripheral reproductive physiology*

The expression of GnRH II mRNA and peptide has been observed in the periphery of mammals in several types of reproductive tissues, including the placenta, ovary, uterine endometrium, prostate and breast tissue (1, 52–56). Similar peripheral expression of type-2 GnRH receptors have been reported in the mammalian ovary, oviduct, uterus, placenta, testis and prostate gland (1, 3, 36).

In primates, including humans, peripheral GnRH II has been proposed to play a critical role in placental physiology, perhaps relating to the regulation of hormone release and embryonic development during pregnancy (53, 54). Early studies demonstrated the presence of a GnRH-like peptide in the placenta and postulated an autocrine/paracrine placental GnRH axis (57, 58). In support of this, exogenously administered GnRH analogs stimulated both human chorionic gonadotropin (hCG) and progesterone release from placental tissue *in vitro* (57–59). However, recent evidence casts doubt on the likelihood that GnRH I plays a significant role in this placental paracrine axis; very high doses of GnRH I are needed to stimulate placental hormone release and the endogenous placental GnRH peptide exhibits weak immunoreactivity with several GnRH I antisera, indicating a non-identity with GnRH I (57, 60). By contrast, data from humans suggests that GnRH II may be secreted in a pulsatile fashion from the placenta and may bind a specific high-affinity receptor (53); whether or not this receptor is the type-2 GnRH receptor remains to be determined.

Although the specific function of placental GnRH II remains unclear, it has been shown, similar to GnRH I, to stimulate hCG release *in vitro* from the placenta (53, 61). This hCG release may be mediated, in part, by an indirect effect of GnRH-stimulated release of placental leptin, which can in turn stimulate hCG secretion (62). Interestingly, GnRH II has a greater ability to stimulate placental leptin release *in vitro* than does GnRH I (62). Additional studies in humans have determined that peripheral GnRH II present in first-trimester placenta and uterine endometrial tissue (52, 63) can modify the transcription and translation of several biochemical factors known to be important in regulating trophoblast invasion of the endometrium at the maternal–fetal interface (64–66). Specifically, GnRH II in human placental extracts increased mRNA and protein expression of matrix metalloproteinases and urokinase-type plasminogen activator and decreased mRNA expression of plasminogen activator inhibitor. These proteins are critical to trophoblast invasion and embryonic implantation, indicating that peripheral GnRH II may also play an important regulatory role in the preparation for and maintenance of pregnancy. Although similar regulatory effects on placental mRNA and protein expression were also reported for GnRH I, Cetrorelix, a type-1 GnRH receptor antagonist, blocked these effects of GnRH I but not those induced by GnRH II (64–66); this suggests that the actions of these two peptides at the maternal–fetal interface are mediated by different receptors or different signalling pathways.

The mammalian ovary also produces a GnRH-like peptide (67, 68) but the affinity of the ovarian receptors for GnRH I or its analogues is much lower than that of the type-1 GnRH

receptor in the pituitary (54). Recent studies performed *in vitro* in baboons and humans have demonstrated the presence and release of GnRH II from ovaries (55, 69), and a GnRH II analogue binds with high affinity to a specific ovarian GnRH receptor with a potency more than 100-fold greater than that of GnRH I (54). Furthermore, administration of GnRH II suppressed progesterone secretion *in vitro* from both baboon and human granulosa cells (54, 69); GnRH I can also inhibit progesterone secretion (70), but higher than physiological doses are required. Both GnRH I and II treatment resulted in down-regulation of FSH receptor and LH receptor levels in human ovaries, suggesting that peripheral GnRH II may exert its antigonadotropic effects by down-regulation of ovarian gonadotropin receptors. Interestingly, treatment of human granulosa cells with FSH or hCG up-regulated GnRH II mRNA levels while it decreased expression of GnRH I mRNA, suggesting opposite regulation of these two peptides in the ovary (69).

The conformational structure of GnRH II makes this peptide more stable than GnRH I and therefore less susceptible to peptidase degradation (54). As a result, GnRH II has an increased bioavailability in the periphery, which could prove critical to its functions in reproductive physiology. Although the findings discussed in this section suggest interesting clinical implications regarding the use of GnRH II in human fertility, hormonal regulation and pregnancy, additional studies are needed to further elucidate the function(s) and mechanisms of action of peripheral GnRH II in mammalian reproductive tissues, and its interaction, if any, with peripheral GnRH I.

#### *Role in cancer and cell proliferation*

While the expression and function of GnRH II in mammalian tumours is currently becoming a topic of intense investigation, the study of the role of GnRH I in cancer has been ongoing for almost two decades. GnRH I and the type-1 GnRH receptor mRNA have been identified in human ovarian carcinoma, breast cancer tissue, endometrial carcinoma and prostate tumours (30, 70–73). In tumours of the reproductive tract, the type-1 GnRH receptor has also been identified at the protein level (30). Multiple studies have documented an antiproliferative action of GnRH I and GnRH I agonists on various types of reproductive cancer cells, both *in vivo* and *in vitro* (30, 70, 73). Additionally, treatment with several GnRH I antagonists results in stimulation of tumour cell proliferation as well as growth of specific cancer cells (30, 73). GnRH I might inhibit tumour growth and proliferation by interfering with intracellular mechanisms mediating the activity of stimulatory growth factors, such as insulin growth factor-I and epidermal growth factor (30, 73). A recent review also cites preliminary results implicating of role for GnRH I in the negative regulation of cancer metastasis (30), a function which has far-reaching clinical implications but which at present requires more research.

Similar to GnRH I, GnRH II has been implicated in the regulation of mammalian reproductive cancers. GnRH II is produced by tumour cells (30, 73, 74) and, like GnRH I, it reduces the proliferation of ovarian, breast, and endometrial cancer cells (30, 55, 56, 70, 73, 74). In human endometrial and

ovarian cancer cell lines, the antiproliferative effects of GnRH II were significantly greater than those of similar doses of GnRH I (74). Furthermore, in specific ovarian cancer cell lines (SK-OV-3) that are type-2 GnRH receptor mRNA-positive but type-1 receptor mRNA negative, GnRH II had a significant antiproliferative effect whereas GnRH I did not (74). This finding suggests that effects of GnRH II in cancer cell lines are not due to cross-reaction with the type-1 GnRH receptor. Similarly, after type-1 GnRH receptor knockdown in two different human ovarian tumour cell lines (EFO-21 and OVCAR-3), the antiproliferative effects of GnRH I were abolished while the effects of GnRH II were still present (73).

Collectively, these data indicate that GnRH II has strong antiproliferative actions on mammalian cancer cells and may act through a GnRH II-specific receptor, rather than the type-1 GnRH receptor, to achieve these effects. Whether this GnRH II-specific receptor is the previously characterized type-2 GnRH receptor is unclear; the presence of a GnRH receptor with low affinity for GnRH I in several human cancer cell lines has been confirmed with reverse transcriptase-polymerase chain reaction using primers derived from mammalian type-2 receptor mRNA as well as Southern blot analysis (73, 74). Furthermore, several studies have found that the type-1 GnRH receptor antagonist Cetrorelix inhibits tumour cell proliferation (70, 75–77), a paradoxical effect that may be explained by the fact that some GnRH I antagonists have been shown to confer agonist activity at the type-2 GnRH receptor (1, 35). Curiously, the type-2 GnRH receptor gene is apparently disrupted in humans by a premature stop codon (1). It is unknown whether this disrupted gene is able to encode a partial yet functional GnRH receptor. An alternate possibility is that there is another distinct as-yet-unidentified membrane receptor in humans that binds GnRH II with relatively high affinity. Future research on GnRH receptor structures and binding in human tumour cells will help illuminate these issues. Although the action of GnRH II in regulating human cancer offers promising clinical applications, it will also be interesting to determine whether such effects of GnRH II are naturally occurring in animals and, if so, whether this peptide has antiproliferative actions in brain as well.

#### *Neuromodulatory actions*

The first function ascribed to GnRH II in vertebrates was as a neuromodulator in amphibian nervous systems; in the sympathetic ganglia of bullfrogs, GnRH II was 100-fold more potent than salmon GnRH, and at least 1000-fold more potent than chicken GnRH I and mammalian GnRH I, in mediating the late, slow postsynaptic potential of select neurones (78, 79). It has been shown that GnRH II initiates this long-term increase in postsynaptic excitability by inhibiting a voltage-dependent potassium ( $K^+$ ) channel conductance, the so-called 'M' current (78, 79). The result is the facilitation of fast excitatory transmission by ganglia neurotransmitters, a mechanism which could generalize to other neural systems in other vertebrate species. Supporting such a role is the presence of GnRH II in sympathetic ganglia and its ability to bind GnRH receptors there; a homologue of the primate type-2 GnRH receptor was recently cloned in

*Xenopus* sympathetic ganglia, providing strong evidence that GnRH II activates this receptor to inhibit K<sup>+</sup> channels (1).

In addition to modulating K<sup>+</sup> channels, GnRH II may also play a role in regulating calcium (Ca<sup>2+</sup>) channels in neuronal systems. In adult bullfrog sympathetic ganglia, application of GnRH II significantly up-regulates Ca<sup>2+</sup> channel density, both *in vitro* and *in vivo* (80). Furthermore, *in vivo* removal of all sources of ganglionic GnRH II caused a 28% reduction in Ca<sup>2+</sup> current density (80). A similar role for GnRH II in modulating Ca<sup>2+</sup> release in neurones in the brains of fish was also reported (81). Because of the strong correlation between Ca<sup>2+</sup> influx and neurotransmitter release, increased Ca<sup>2+</sup> conductance may augment activity at synapses, providing a critical regulatory role in nervous system functioning (80). Collectively, these results indicate that GnRH II exerts direct control of calcium and potassium channel expression in neurones, critical functions that may also generalize to other species, including mammals.

**Behavioural functions**

*Facilitation of sexual behaviour*

Based on its neuroanatomical distribution and its ability to modulate synaptic ion channels in the ganglia of several vertebrates, it has been hypothesized that neural GnRH II might play a role as a behavioural neurotransmitter, perhaps in the regulation of reproductive behaviour (6, 29, 35, 82). For example, GnRH II injections reportedly increased courtship behaviour of sexually unresponsive salamanders within 15–30 min (83) and also increased the initiation of courtship song in ring doves (6) but, to date, the details for these two ‘observations’ have not been published.

The first published study of the possible role of GnRH II in sexual behaviour was in 1997 in birds, in which intracerebroventricular (i.c.v.) infusion of GnRH II facilitated a modest increase in courtship displays in female song sparrows (82). The role of GnRH II in mammalian behaviour was not addressed until several years later by Rissman and colleagues using the primitive mammalian species, the musk shrew (*Suncus murinus*) (29, 84, 85). These researchers proposed an intriguing regulatory function for mammalian GnRH II, which is to coordinate energy availability and reproduction. Similar to most female mammals, female musk shrews experiencing low food availability exhibit significant decreases in mating behaviours (86). Central i.c.v. infusion of GnRH II, but not GnRH I, significantly reversed the

inhibitory effects of food restriction on female musk shrew sexual behaviour (Fig. 2) (29). By contrast, GnRH II infusions did not stimulate increased mating behaviour in *ad libitum* fed female shrews whereas GnRH I did (84). It is unknown whether higher doses of GnRH II in *ad libitum* animals would have also increased mating because there was a trend for increased sexual behaviour at the highest dose tested (1 µg). However, the fact that lower doses of GnRH I (100 ng) increase sexual behaviour in *ad libitum* fed shrews

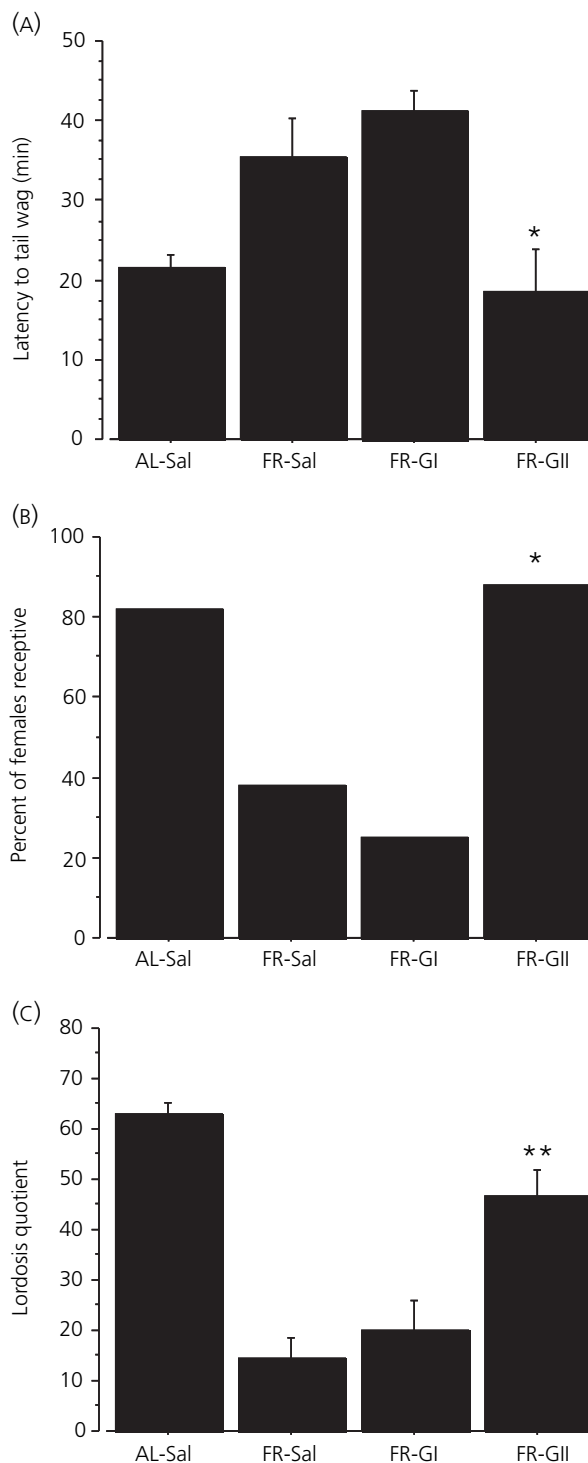


Fig. 2. The effects of gonadotropin-releasing hormone (GnRH) II (and GnRH I) on female sexual behaviour in underfed musk shrews and mice. (A) Latency for female musk shrews to tail wag (a receptive mating behaviour) in *ad libitum* (AL) or food restricted (FR) animals given an i.c.v. infusion of saline (Sal), GnRH I (GI) or GnRH II (GII). \*Significantly different from other food restricted groups but not different from *ad libitum* group. (B) Percent of female musk shrews displaying receptive mating behaviour (rump present and tail wag). Similar group designations as in (A). \*Significantly different from other food restricted groups but not different from *ad libitum* group. (C) Lordosis quotient of *ad libitum* fed or food deprived female mice given infusions of saline, GnRH I, or GnRH II. \*\*Significantly different from other food restricted groups and *ad libitum*-saline group. Data are taken from previous studies (29, 87).

(84) precludes the possibility that the lack of stimulatory effect of GnRH II in such animals was due to their sexual behaviour already being maximal (i.e. a ceiling effect).

To determine whether the role of GnRH II in musk shrew sexual behaviour extends to other mammals, we recently tested the effects of GnRH II in mice (*Mus musculus*), which differ from musk shrews in several critical ways (87). Female mice, unlike shrews, are spontaneous ovulators and exhibit regular cycles of hormonal and behavioural oestrus. Furthermore, mouse sexual behaviour is dependent on circulating gonadal steroids (i.e. oestrogen and progesterone), which is not the case for sexually responsive female musk shrews. We found that a single GnRH II infusion (1 µg) administered i.c.v. significantly increased mating behaviour in sexually experienced, food-deprived female mice (C57BL/6J strain) (87). By comparison, GnRH I had no stimulatory or restorative effect on lordosis behaviour in underfed females. The lordosis quotient (i.e. sexual receptivity) of underfed female mice given GnRH II was more than 30% higher than that of saline- or GnRH I-infused animals, but was slightly lower than that of *ad libitum* fed females (indicating submaximal restoration of behaviour) (Fig. 2). Conversely, in sexually experienced, *ad libitum* fed females, GnRH II did not further elevate lordosis behaviour above the level of sexual behaviour observed in saline-infused control mice (87). As was the case with musk shrews, this lack of GnRH II-mediated increase in lordosis in *ad libitum* fed mice was not simply due to sexual behaviour already being maximal because GnRH I infusion further increased lordosis in similarly fed females.

These findings in mice, in conjunction with those in musk shrews, indicate that GnRH II can rapidly and significantly promote sexual behaviour in females that would otherwise show minimal receptivity. However, because GnRH II does not have additional stimulatory effects on mating in *ad libitum* fed females, its actions are apparently permissive to reproduction, rather than merely stimulatory. Thus, we proposed that under conditions when food is readily available, endogenous GnRH II is likely released at a basal rate which permits mating. In contrast, when food is unavailable and energy status becomes compromised, GnRH II release decreases, temporarily reducing the expression of female receptive behaviour, thereby limiting reproduction. In support of this hypothesis, food restricted female shrews have significantly more GnRH II-immunoreactive cell bodies in the anterior midbrain compared to *ad libitum* fed females, as well as greater densities of GnRH II-containing fibres in the forebrain (possibly indicating increased peptide storage and decreased release) (29). Thus, GnRH II may be a neurochemical signal of the energetic state of a female, acting as a permissive factor to allow mating only if sufficient energy is available.

GnRH II peptide has been identified in shrew and rodent brains, and in immortalized mouse hypothalamic cell lines (GT1-7) using RIA, HPLC and immunohistochemical analyses (24–26, 88, 89); however, the rodent data remain controversial because the gene for the GnRH II peptide has not yet been identified in the mouse genome (90) and several earlier studies were unable to find the peptide in brain (22, 91). Discrepancies in the rodent literature regarding the

immunohistological presence of GnRH II peptide may, in part, be due to most studies being performed in *ad libitum* fed animals. In musk shrews, GnRH II release and storage appear to be contingent on food availability, with increased immunoreactivity revealed in GnRH-II containing neurones of underfed animals (29). Based on these findings, re-examination of the localization (and function) of GnRH II in rodents should be undertaken with animals that are on food restricted or food-deprived regimens. If future data corroborate previous findings that the GnRH II gene, message, and protein are truly absent in rodents, then the findings of GnRH II effects in mice (87) are likely pharmacological rather than physiological. In this case, additional mammalian models such as musk shrews and nonhuman primates may prove most useful for further GnRH II studies.

Numerous other mammals, including primates and humans, possess GnRH II mRNA and peptide in the brain (1, 23, 25, 27, 90), and the type-2 GnRH receptor has been located in areas of the brain associated with reproductive behaviours (2, 35); it is intriguing to speculate that GnRH II might have similar regulatory actions on reproductive behaviour in these species as well. Promising support for this speculation comes from a recent review which cites preliminary findings that GnRH II infusions can facilitate the sexual behaviour of female marmosets (2). Clearly, additional research is necessary to fully characterize the extent to which GnRH II regulates reproductive behaviour in various mammalian species, both in *ad libitum* and underfed conditions. Additional studies examining the function, if any, of GnRH II in male reproductive behaviour might also prove fruitful.

#### *Modulation of feeding and satiation*

Because GnRH II immunoreactivity varies with energy status in musk shrews, we recently tested the hypothesis that, in addition to its permissive role in regulating reproduction, GnRH II might also act as a modulator of feeding and energy intake (85). Specifically, we postulated that if food is unavailable and a female is in negative energy balance, lower levels of GnRH II release might be a signal to increase feeding and energy intake. Conversely, if there is abundant food available, the resulting higher levels of GnRH II secretion might be a signal to eat less (or at a baseline level). Thus, GnRH II may act as a feeding modulator, with higher levels of GnRH II release signalling the animal to decrease feeding behaviour. We tested this possibility in musk shrews; this species is an excellent model to study the effects of GnRH II on feeding because the presence of this peptide in musk shrew brain is well documented. Furthermore, shrews have a high metabolic rate and little body fat, even under *ad libitum* access to food, and therefore lack sufficient energy stores to use as a metabolic buffer in times of low food availability. Consequently, these animals eat a lot and their physiology is very sensitive to small reductions in food intake (29, 86, 92).

We administered GnRH I or II to adult female musk shrews and measured food intake 90 min, 3 h, 6 h and 24 h later. A single GnRH II infusion significantly decreased short-term food consumption in both *ad libitum* fed musk shrews and food-restricted females that were returned to *ad libitum* feeding (85). The decreased food intake was

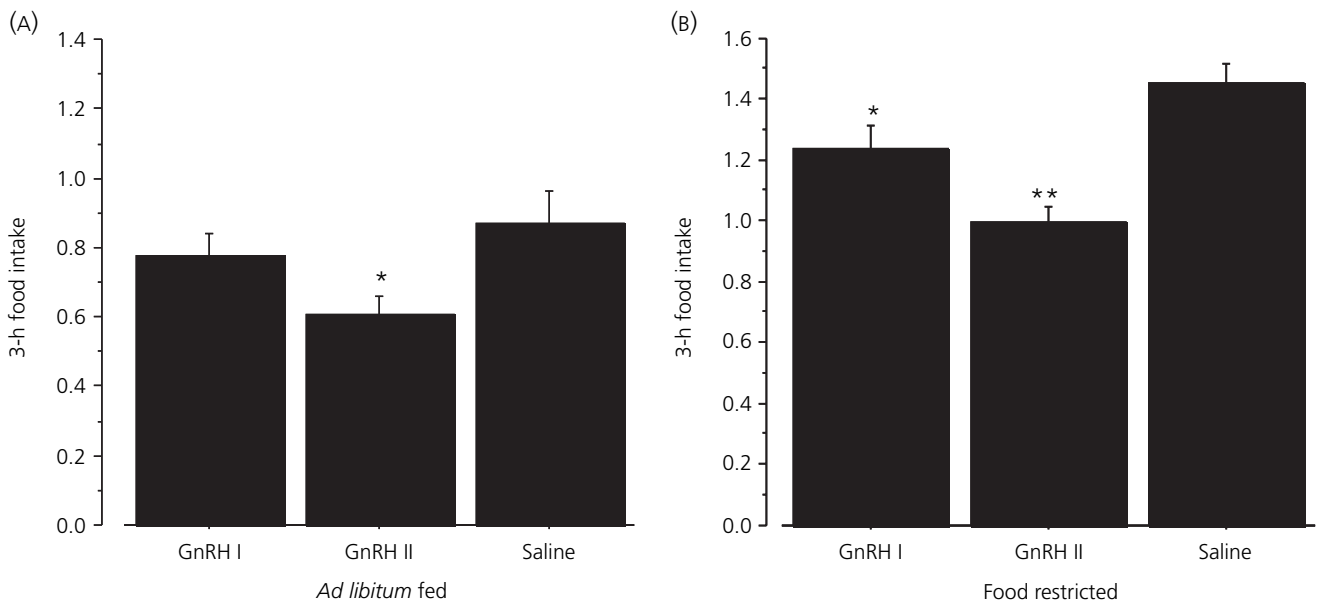


FIG. 3. The effects of gonadotropin-releasing hormone (GnRH) II (and GnRH I) on short-term food intake in female musk shrews. (A) 3-h food intake of *ad libitum* fed musk shrews after an i.c.v. infusion of GnRH I, GnRH II, or saline. \*Significantly different from saline-infused group. (B) 3-h food intake of previously food-restricted female shrews that were returned to *ad libitum* feeding immediately after an infusion of GnRH I, GnRH II, or saline. Food restriction was implemented by giving each animal only 60% of its *ad libitum* 24-h food intake on two consecutive days. \*Significantly different from saline-infused group. \*\*Significantly different from GnRH I and saline-infused groups. All infusions were administered i.c.v. into the lateral ventricle 30–60 min before lights off. Data taken from a previous study (85).

greatest during the first several hours after GnRH II infusion (a 28–33% reduction) (Fig. 3) and, in all cases, waned 3 h after administration. GnRH I or saline administration did not cause significant decreases in short-term feeding (although GnRH I had a mild effect in underfed animals). Twenty-four hour food intake was moderately reduced by a single GnRH II infusion in *ad libitum* fed shrews and less strongly affected by GnRH II given to previously underfed animals that were returned to *ad libitum* feeding after infusion. The 10% decrease in overall 24-h food intake in *ad libitum* fed females was entirely due to the inhibitory effects of GnRH II during the first several hours following infusion; after the first 3 h, GnRH II-treated animals did not eat less than saline-infused controls for the remainder of the day (85). These findings indicate that GnRH II can rapidly and significantly regulate short-term food intake in female mammals, and that its effects on feeding are short in duration, lasting several hours at most. Such a regulatory function compliments the permissive role of GnRH II in regulating female sexual behaviour according to food availability.

#### *Molecular mechanisms and neural sites mediating the behavioural effects of GnRH II*

Two GnRH receptors, type-1 and type-2, have been identified in mammals (1, 34, 35). Although GnRH II can bind both receptors, it has a 24-fold higher affinity for the type-2 compared with the type-1 GnRH receptor (35). GnRH II may be regulating mammalian behaviour through several possible receptor mechanisms. The first is that it binds to type-2 GnRH receptors which then directly affect behaviour, independent of GnRH I or type-1 GnRH receptors. However,

because GnRH II is also able to bind to type-1 GnRH receptors, albeit with lower affinity than for type-2 receptors, a second possible mechanism is that GnRH II binds type-1 receptors, which then directly regulate behaviour. Furthermore, the report that type-2 GnRH receptors are located in specific hypothalamic nuclei known to also contain GnRH I cells (32) suggests that GnRH II may also exert its behavioural effects by binding type-2 receptors of cells containing GnRH I hormone; in this case, type-2 receptor activation might then cause the release of GnRH I, which could subsequently bind its own type-1 receptor to affect behaviour.

It is unlikely that the permissive effects of GnRH II on reproductive behaviour are mediated through the type-1 GnRH receptor. GnRH I, which binds this receptor with high affinity, had no positive effect on sexual behaviour in underfed female mice or underfed female musk shrews (29, 85, 87). Similarly, GnRH I had little effect on food intake in musk shrews (85). Second, pretreatment of females with Antide, a potent type-1 GnRH receptor antagonist, did not prevent the restorative effects of GnRH II infusion on sexual behaviour in underfed mice (87) and underfed musk shrews (Kauffman and Rissman, unpublished data). These findings indicate that the actions of GnRH II on behaviour are likely mediated via the type-2 GnRH receptor or other as yet unidentified GnRH receptors. By contrast, stimulation of gonadotropin release by GnRH II in mammals is likely mediated via the type-1 GnRH receptor because blocking this receptor prevents any GnRH II-induced LH release in sheep and primates (41, 42). Intriguingly, in contrast to the type-1 receptor, the type-2 GnRH receptor possesses a cytoplasmic C-terminal tail that allows for fast desensitization and

down-regulation (2, 34, 35); such rapid down-regulation of type-2 receptors may be an additional mechanism by which the GnRH II system quickly responds to rapid fluctuations in environmental energy availability.

The gene and transcripts of the type-2 GnRH receptor have been identified in the brains of multiple mammalian species, including musk shrews, pigs, marmosets and rhesus monkeys (2, 29, 34–36). Although ICC analysis has confirmed the presence of the type-2 receptor protein in mouse brain (35), the gene encoding the type-2 receptor in mouse has not yet been elucidated, and there is some evidence that this gene has been disrupted by a premature stop codon in several mammalian species, including sheep, rats and humans (90). However, several studies have reported the expression of type-2 GnRH receptor mRNA in human cells, and have described effects of GnRH II in human tissue even in the presence of type-1 GnRH receptor blockers (64, 65, 73, 93). These findings indicate that GnRH II in humans is not acting through type-1 receptors, even though the type-2 receptor gene in humans appears to be incomplete (90). This raises the possibility that the disrupted type-2 receptor gene in humans

and other species is able to encode a truncated but functional receptor. Alternatively, the type-2 receptor may be encoded for by a different gene which remains to be identified. Finally, it is possible that the effects of GnRH II may be mediated by a receptor that has yet to be discovered or by an orphan receptor that is already known. Recent findings in fish and amphibians of a third class of GnRH receptors raises the possibility that a type-3 GnRH receptor or other GnRH binding receptor exists in mammals as well (94, 95); interestingly, GnRH II had a higher affinity for all three receptor subtypes than did GnRH I (94). Future genomic and molecular studies, and the development of type-2 GnRH receptor antagonists, will help to resolve these issues and determine which receptor(s) GnRH II binds to in order to achieve its effects in rodents, humans and other mammals.

It is currently unknown which neural sites mediate the actions of GnRH II on feeding or female mating behaviour in mammals. In musk shrews, the fibres of GnRH II-containing neurones are widespread and innervate numerous brain regions. Furthermore, type-2 GnRH receptors are located throughout the brain (1, 29, 35); i.c.v. infusion of GnRH II

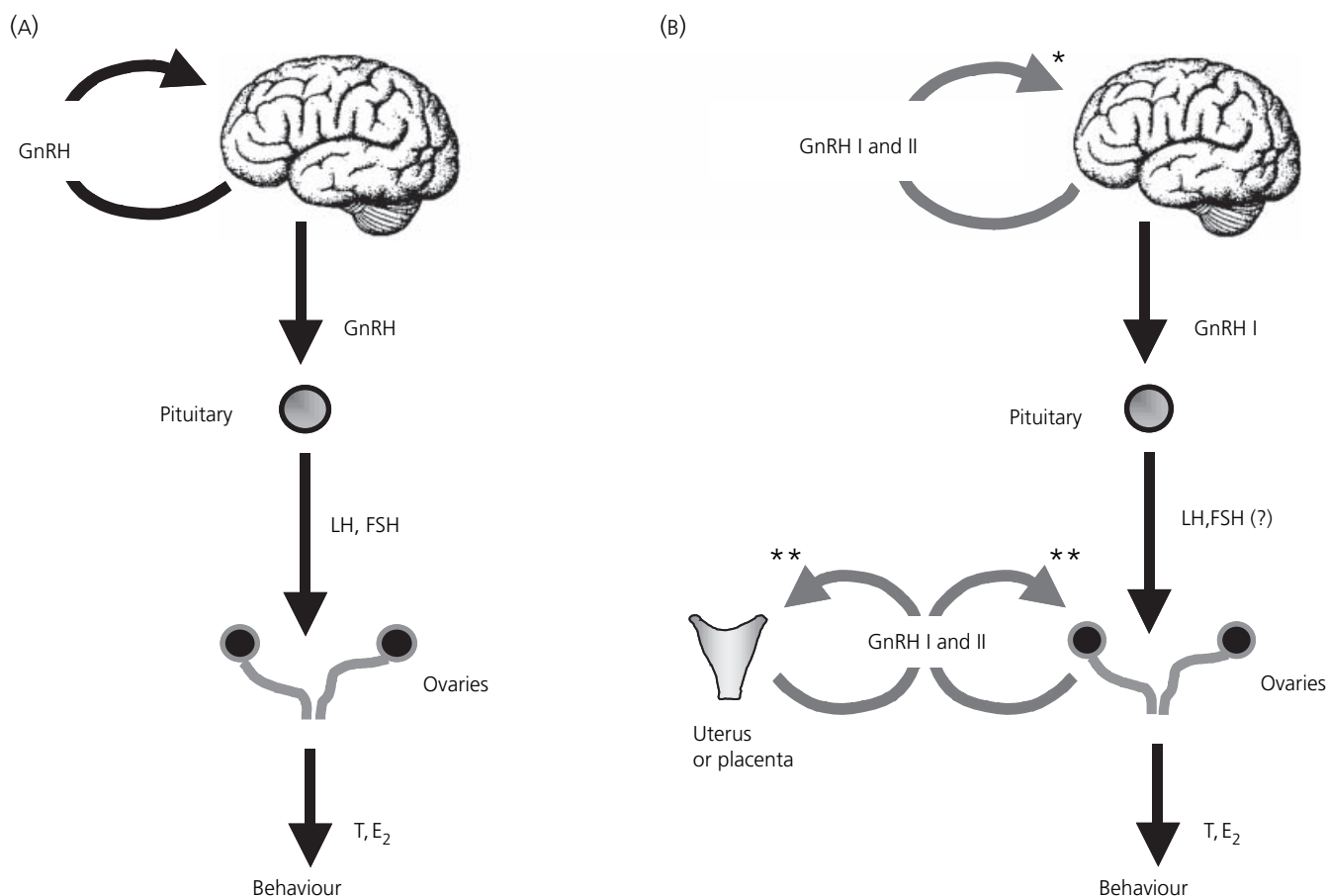


FIG. 4. Models of (A) old and (B) new views of gonadotropin-releasing hormone (GnRH) functions. It is now apparent that GnRH I is the primary stimulator of luteinizing hormone (LH) release from the pituitary. Although GnRH I also stimulates follicle-stimulating hormone (FSH) release, the presence of a selective FSH-releasing factor is still a topic of current research; current data implicate lamprey GnRH III as this putative factor. \*Both GnRH I and II have been implicated as regulatory neurotransmitters in the brain and may affect sexual and/or feeding behaviours; such effects may, in part, be mediated by the ability of GnRH II to regulate  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  channels at neural synapses. \*\*Both peptides are also produced by peripheral tissues, including ovary, placenta and endometrium. The functions of these peripheral GnRH peptides are uncertain, but may include regulation of progesterone and human chorionic gonadotropin release, anti-cell proliferation and regulation of implantation during pregnancy.

could potentially activate many of these regions. However, the best candidate sites are those containing type-2 GnRH receptors that have previously been implicated in the regulation of mammalian reproduction and/or food intake. In musk shrews and primates, type-2 GnRH receptors are present in reproductive areas such as the ventromedial hypothalamus, medial preoptic area, paraventricular nucleus and the medial habenula (1, 29). Similar type-2 receptor localization exists in areas implicated in feeding, including the paraventricular nucleus, ventromedial hypothalamus, arcuate nucleus, amygdala and nucleus of the solitary tract (1, 29, 35). One study has reported the presence of type-2 GnRH receptors in mouse pituitary (35); it remains to be determined where else in the mouse brain functional type-2 receptors are present, if at all.

### Conclusions and future directions

Gonadotropin-releasing hormone was first isolated in the mammalian hypothalamus and shown to regulate the reproductive system by stimulating the release of pituitary gonadotropins. However, GnRH I is but one of multiple structural variants which have evolved from a more ancient form (6). The most primitive and universal form of GnRH, GnRH II, may have a wide array of physiological functions (Fig. 4). GnRH II and GnRH I have similar roles in regulating cell proliferation and mediating peripheral reproductive physiology, both as autocrine/paracrine regulators of hormonal secretion and as transcriptional regulators of proteins involved in embryonic implantation. By contrast, GnRH I and II apparently modulate mammalian reproductive behaviours in different but complementary ways: GnRH I stimulates LH/FSH secretion (and subsequently gonadal steroid secretion) and promotes lordosis behaviour in *ad libitum* fed animals, whereas GnRH II acts as a permissive gate, allowing reproductive behaviour only when sufficient energy is available to support successful pregnancy and lactation. The primary behavioural function of GnRH II therefore appears to be as a neurochemical mediator between exogenous environmental conditions (availability of energy resources) and endogenous neuroregulatory processes (activation of mating).

Almost all of the proposed functions of GnRH II relate either directly or indirectly to reproduction. This may be a result of researchers' bias to associate the peptide with reproductive processes because its structure is so similar to GnRH I; however, anatomical and physiological evidence suggests that GnRH II may also play several roles in nonreproductive processes. For example, GnRH II and/or its receptor are present in high concentrations in kidney, muscle, and bone where its function remains unstudied. Additionally, GnRH II has been postulated to play roles in feeding modulation and ion channel regulation, as well as the regulation of cancer and T-cells. The term 'GnRH II' may itself be misnomer for this peptide, as it has minimal hormonal activity in the blood, appears to act primarily as a neurotransmitter or paracrine factor, and is not primarily responsible for gonadotropin-release. Future studies should not be limited to ascertaining only the reproductive roles of GnRH II. Although such functions are undeniably critical, it

is likely that this highly conserved peptide has been co-opted over evolutionary time to possess multiple regulatory functions in a broad range of biological aspects in addition to reproduction.

Although much has been learned in the past decade, there are still many areas of research to pursue regarding the function of GnRH II in mammals. Much of the current data indicate that the primary effects of GnRH II in mammals are not mediated by the classical type-1 GnRH receptor. However, it is still unclear which receptor(s) bind GnRH II to transduce its regulatory effects. Additional gaps in our knowledge include uncovering where GnRH II acts in the brain and which neural and hormonal factors control its synthesis and release. Acute refeeding reinstates sexual receptivity in underfed female musk shrews and female rodents in just 90 min and 3–6 h, respectively (87, 92, 96), indicating that the refeeding cues that restore reproduction are detected and processed relatively quickly. GnRH II infusions mimic the rapid restorative effects of brief refeeding on mating in underfed females. We therefore propose that the GnRH II system is one of the key factors involved in the mediation of food availability on sexual behaviour, although the precise mechanism(s) and signals that underlie this process are currently unknown. It is almost assured that these underlying mechanisms involve a complex interaction of a number of regulatory hormones and neurotransmitters. Several candidate factors include leptin, corticotropin-releasing factor, galanin, galanin-like-peptide and neuropeptide Y, and each of these has been implicated in the regulation of both food intake and/or sexual behaviour (8, 62, 97–99). Future studies addressing the interactions, if any, of these hormonal and neurotransmitter systems with the mammalian GnRH II system will better elucidate the functions and mechanisms of GnRH II in mammals.

### Acknowledgements

I would like to thank Irving Zucker and Emilie Rissman for their excellent guidance and mentorship, and for their exemplary strong inference approach to science. My research is supported by NIMH grants T32-HD07382, F32 MH070084 and R01 MH068729. I also acknowledge the University of Virginia Ligand Core Laboratory which is supported by NICHD/NIH through cooperative agreement U54 HD28934.

Accepted 29 June 2004

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