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Molecular Co-evolution of Gonadotropin-releasing Hormones and Their Receptors

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Abstract: Gonadotropin-releasing hormone (GnRH), synthesized in the hypothalamus, plays a pivotal role in the regulation of vertebrate reproduction. Since molecular isoforms of GnRH and their receptors (GnRHR) have been isolated in a broad range of vertebrate species, GnRH and GnRHR provide an excellent model for understanding the molecular co-evolution of a peptide ligand-receptor pair. Vertebrate species possess multiple forms of GnRH, which have been created through evolutionary mechanisms such as gene/chromosome duplication, gene deletion and modification. Similar to GnRHs, GnRH receptors (GnRHR) have also been diversified evolutionarily. Comparative ligand-receptor interaction studies for non-mammalian and mammalian GnRHRs combined with mutational mapping studies of GnRHRs have aided the identification of domains or motifs responsible for ligand binding and receptor activation. Here we discuss the molecular basis of GnRH-GnRHR co-evolution, particularly the structure-function relationship regarding ligand selectivity and signal transduction of mammalian and non-mammalian GnRHRs.

Key words: GnRH, receptor, G protein-coupled receptors, ligand selectivity, signal transduction, co-evolution

Gonadotropin-releasing hormone (GnRH) is a decapeptide that plays a key role in the control of reproduction (Mason et al., 1986). GnRH is synthesized in the hypothalamus and regulates the synthesis and secretion of luteinizing hormone and follicle-stimulating hormone. GnRH has also been found in peripheral tissues related to reproduction and the immune response, indicating that GnRH can play diverse roles in different tissues, either as an autocrine or a paracrine factor (Azad et al., 1991; Oikawa et al., 1990). Further, GnRH is known to have a direct action on prostate, ovarian, and breast tumors (Chen et al., 2002a; Grundker et al., 2004; Maiti et al., 2005).

The potential clinical applications of GnRH are being actively investigated using mammalian system. Identification of GnRH isoforms in both non-mammalian vertebrate and invertebrate species has enhanced the understanding of the function of GnRH peptides. Currently, 14 GnRH isoforms in vertebrate species and 10 isoforms in invertebrate species have been identified (Kah et al., 2007). This demonstrates that the functions of GnRH peptides have been evolutionarily conserved over 600 million years in addition to divergent species/class-specific functions.

GnRH exerts its action through the GnRH receptor (GnRHR). The GnRHR is a member of the rhodopsin-like G-protein coupled receptor (GPCR) family, structurally characterized by an extracellular amino terminus and an intracellular carboxyl terminus, linked by seven transmembrane helices connected by three extracellular and intracellular loops (Tsutsumi et al., 1992). As the number of GnRH peptides has increased with evolution, the number of GnRHR has also increased. Examining the co-evolution of the GnRH-GnRHR pair provides an excellent model for understanding the process of evolution of a neuropeptide ligand-receptor pair (Kah et al., 2007). This article will compare the molecular structures and functions of non-mammalian and mammalian GnRHs and their receptors. Understanding the co-evolution of this pair will deepen the understanding of the human GnRH-GnRHR system.

Evolution of the GnRH peptide

The cDNA corresponding to the GnRH precursor polypeptide have been isolated in a broad range of vertebrate species, enabling the construction of phylogenetical trees (Fernald and White, 1999). Most trees support the hypothesis that these cDNA sequences represent the division of the GnRH

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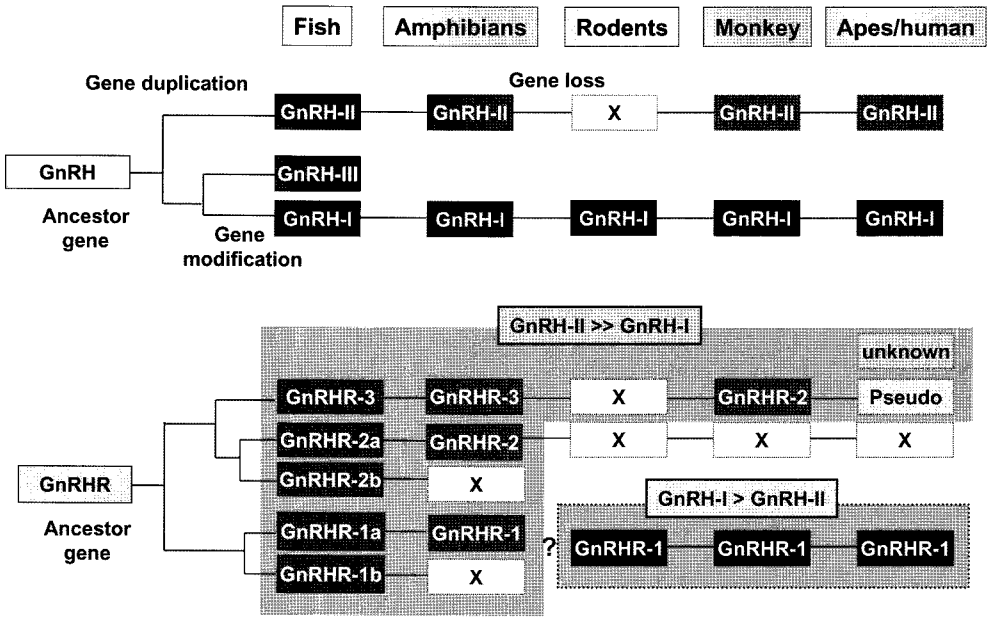

Fig. 1. Co-evolution of GnRH and GnRH receptor (GnRHR) genes. Either GnRHs or GnRHR may originate from a common ancestral gene and diversify through evolutionary mechanisms, including gene/chromosome duplication, modification and deletion. Such events generate families of related yet distinct peptides and receptors. Fish possess three forms of GnRH: GnRH-I, GnRH-II, and GnRH-III. Other vertebrates lack GnRH-III. Similarly, fish possess five forms of GnRHR, while amphibians possess three forms of GnRHR. In mammals, rodents have only one form of GnRHR, while monkeys have two forms of GnRHR (GnRHR-1 and GnRHR-2). In humans, the genes for the GnRHR-2 are thought to be nonfunctional pseudogenes due to the introduction of a premature stop codon. However, the presence of an uncharacterized human GnRH-II receptor cannot be ruled out. Nonmammalian GnRHR and mammalian GnRHR-2 have higher affinities to GnRH-II than GnRH-I, while mammalian GnRHR-1 has a stronger affinity to GnRH-I than to GnRH-II.

peptide into three distinct branches, GnRH-I, GnRH-II, and GnRH-III (Fig. 1).

GnRH-I was first identified in the mammalian hypothalamus (Matsuo et al., 1971). This initial discovery facilitated the isolation of GnRH peptides in other vertebrate and invertebrate species. The sequences of GnRH-I vary at positions 5 and 8 across vertebrate species (Kah et al., 2007; Yoo et al., 2000), while GnRH-II ([His^5, Trp^7, Tyr^8]GnRH) is fully conserved in almost all vertebrates (Fernald and White, 1999; White et al., 1998). GnRH-I is found predominantly in the hypothalamus. In contrast, GnRH-II has a widespread distribution in the brain, most prominent in the hindbrain and spinal cord (White et al., 1998). Although the exact function of GnRH-II is unclear, it was proposed that GnRH-II contributes to neuromodulation and sexual behavior (Troskie et al., 1997). Comparison of the cDNA sequences of GnRH-I and GnRH-II suggests that these two peptides have derived from a common ancestor (Fernald and White, 1999). GnRH-III, often called salmon GnRH ([Tyr^5, Trp^7, Leu^8]GnRH), is found only in fish species (Powell et al., 1994). Thus, with the exception of fish, most vertebrate species contain GnRH-I and GnRH-II. Like GnRH-I neurons, GnRH-III neurons in fish originate from the olfactory placode and migrate to the hypothalamus (Kah et al., 2007). These observations suggest that GnRH-I and GnRH-III may be generated through gene/chromosome duplication of a common ancestor (Fig. 1).

Evolution of the GnRH receptors

The presence of two or more forms of GnRH in a single species implies the presence of multiple forms of the GnRHR. Recently, we and other groups identified two or three different types of GnRHR in non-mammalian species, fish (Illing et al., 1999) and frogs (Wang et al., 2001). Furthermore, a second form of GnRHR has been cloned in the monkey (Millar et al., 2001). Currently, it is known that fish have five different GnRHRs (Moncaut et al., 2005), while amphibians have two or three (Troskie et al., 2000; Wang et al., 2001). Two forms of GnRHR exist in higher mammals: the type-I receptor (GnRHR-1) and the type-II receptor (GnRHR-2) (Fig. 1). In some mammals (e.g. human, chimpanzee, sheep, cow, rat and mouse), the GnRHR-2 gene has been inactivated or deleted (Gault et al., 2004).

The structure and function of mammalian GnRHR-2 closely resemble those of non-mammalian GnRHRs. However, similarities do not hold between mammalian GnRHR and non-mammalian GnRHR. Non-mammalian GnRHR and mammalian GnRHR-2 contain a C-terminal cytoplasmic tail that is important for receptor down-regulation and internalization (Heding et al., 1998). In contrast, mammalian GnRHR-1 does not have a C-terminal cytoplasmic tail

(Tsutsumi et al., 1992). Non-mammalian GnRHR and mammalian GnRHR-2 have conserved $Asp^{2.50}$/$Asp^{7.49}$ residues, while mammalian GnRHR-1 contains $Asn^{2.50}$/$Asp^{7.49}$ residues at these positions (Flanagan et al., 1999). The mammalian GnRHR-1 has higher affinity for GnRH-I than GnRH-II (Flanagan et al., 1994), while non-mammalian GnRHR and mammalian GnRHR-2 show a higher affinity to GnRH-II. Most fish GnRHRs exhibit highest sensitivity to GnRH-II, moderate sensitivity to GnRH-III, and poor sensitivity to the fish form of GnRH-I (Bogerd et al., 2002; Illing et al., 1999). Whether there are unidentified fish GnRHRs with high affinity for the fish form of GnRH-I remains uncertain. Like fish GnRHRs, none of the amphibian GnRHRs displays high affinity for GnRH-I, although bullfrog (bf) GnRHR-1 binds GnRH-I with a relatively high affinity (Seong et al., 2003). Thus, although GnRH-I is present in most vertebrate species, only mammals possess a high affinity receptor for GnRH-I. While mammalian GnRHR-1 is highly expressed in the pituitary, non-mammalian GnRHR and mammalian GnRHR-2, except for bfGnRHR-1, are expressed in the brain. Thus, these receptors may participate in neuromodulation regulated by either GnRH-I or GnRH-II.

The structural and functional origins of mammalian GnRHR-1 are currently uncertain. We propose that bfGnRHR-1 may be a functional analogue of mammalian GnRHR-1. Like mammalian GnRHR-1, bfGnRHR-1 is mainly expressed in the pituitary, where it receives hypothalamic GnRH inputs. bfGnRHR-1 has a relatively high affinity for GnRH-I compared to other non-mammalian receptors (Seong et al., 2003; Wang et al., 2001). Further, just two amino acid mutations ($Gln^{7.32}$ to Glu and $Ser^{7.31}$ to Pro) in bfGnRHR-1 greatly increases its sensitivity to GnRH-I and decreases its sensitivity to GnRH-II, behaving like mammalian GnRHR-1 (Wang et al., 2004). Thus, it appears that bfGnRHR-1 is an evolutionary intermediate between non-mammalian and mammalian GnRHR-1.

Signal transduction of mammalian and non-mammalian GnRHRs

Several studies regarding GnRH-mediated signal transduction have proposed that mammalian GnRHR-1 exclusively activates phospholipase C (PLC) via $G_{q/11}$ coupling (Grosse et al., 2000). PLC, in turn, hydrolyzes phosphatidylinositol 4, 5-bisphosphate to generate inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). However, other studies have shown that activation of GnRHRs produces cAMP via G_s/adenylate cyclase (AC) activation (Arora et al., 1998). Thus, the signaling cascade of the mammalian GnRHR-1 is still under debate. Recently, we demonstrated that mammalian GnRHR-1 prefers a PLC/protein kinase C (PKC)-linked signaling pathway, while bfGnRHRs, the representative non-mammalian GnRHRs, couple to both the PLC/PKC- and AC/PKA-linked signaling pathways with similar strengths or with a higher preference for the AC/PKA-linked pathway (Oh et al., 2003). Since the most striking structural difference between mammalian GnRHR-1 and non-mammalian GnRHR is the presence or absence of the C-tail, we presumed that this difference may influence which signal transduction pathway is preferred. The deletion of the C-tail from bfGnRHR-1 remarkably decreased its ability to induce the AC/PKA-linked signaling pathway. Further dissection of the C-tail revealed that a His-Phe-Arg-Lys (HFRK) motif in the membrane-proximal sequence of bfGnRHR-1 is a minimal requirement for activation of the AC/PKA-linked signaling pathway. The addition of this motif to mammalian GnRHR-1 or deletion of it from bfGnRHR-1 significantly affected the ability of the receptors to induce the AC/PKA-linked signaling pathway (Oh et al., 2005). Taken together, these results indicate that the HFRK motif in the membrane-proximal region confers the differential signal transduction pathways of action between mammalian GnRHR-1 and non-mammalian GnRHR.

Motifs responsible for ligand selectivity of GnRHRs

Non-mammalian GnRHR and mammalian GnRHR-2 each have a greater affinity to GnRH-II than to GnRH-I. The opposite is true for mammalian GnRHR-1. In addition, cetrorelix, a highly potent mammalian GnRHR-1 antagonist, exhibits poor potency at non-mammalian GnRHR and mammalian GnRHR-2. Further, trptorelix-1 and trptorelix-2, newly developed GnRH-II antagonists, display high affinity at non-mammalian GnRHR and mammalian GnRHR-2 (Maiti et al., 2003; Wang et al., 2003). These observations propelled us to identify the motifs or amino acid residues of the receptors responsible for such differential ligand selectivity.

A number of residues involved in ligand binding have been identified using mutagenesis in combination with computational modeling. The GnRH binding residues (i.e. Asp^{98}, Asn^{102}, Lys^{121}, Asn^{212}, Trp^{280}, Trp^{289}, and Tyr^{290}) of the human GnRHR-1 (Davidson et al., 1996; Flanagan et al., 2000; Hoffmann et al., 2000; Hövelmann et al., 2002; Millar et al., 2004) are similarly conserved in non-mammalian GnRHR and mammalian GnRHR-2 (Millar et al., 2001; Wang et al., 2001). Thus, these residues may be responsible for interaction with the amino acid sequence conserved between GnRH-I and GnRH-II. A Glu/$Asp^{7.32}$ residue in the extracellular loop 3 (ECL3) of the mammalian GnRHR-1 is known to interact with Arg^8 of GnRH-I, which may confer preferential ligand selectivity for GnRH-I (Flanagan et al., 1994). However, some non-mammalian GnRHRs possess Glu/Asp residue at the same position, yet

respond better to GnRH-II than GnRH-I (Wang et al., 2001). Amino acids neighboring Glu/Asp$^{7.32}$ are differentially arranged such that mammalian GnRHR-1 and non-mammalian GnRHR have an S-E/D-P motif and P-X-S/Y motif, respectively. We hypothesized the position of Ser$^{7.31}$ or Pro$^{7.33}$ of mammalian GnRHR-1 to be potential determinants of ligand selectivity. Either placing Pro prior to Glu$^{7.32}$ or placing Ser after Glu$^{7.32}$ significantly decreased the sensitivity and/or efficacy of GnRH-I but slightly increased the sensitivity and efficacy of GnRH-II in several mutant receptors. Further, mammalian GnRHR-1 mutants with a PEV, PES, or SES motif exhibited a marked decrease in sensitivity for GnRH-I. In these mutants, GnRH-II had a higher potency than GnRH-I (Wang et al., 2004). A bfGnRHR-1 mutant with the SEP motif showed a higher affinity to GnRH-I than GnRH-II. These results indicate that the position of Pro and Ser near Glu$^{7.32}$ in ECL3 is critical for the differential ligand selectivity between mammalian GnRHR-1 and non-mammalian GnRHR.

However, ligand selectivity was unaffected when the Pro-Glu-Tyr (PEY) motif in bfGnRHR-2 was replaced by SEP. This suggests the involvement of other residues in conferring ligand selectivity in this receptor. It is noteworthy that affinity for GnRH-I was greatly enhanced in a chimeric green monkey (gm) GnRHR-2 containing ECL3 and the ECL3-proximal TMD7 region of rat GnRHR-1 (Li et al., 2005). Point mutation analyses indicate that four amino acids, Leu/Phe$^{7.38}$, Leu/Phe$^{7.43}$, Ala/Pro$^{7.46}$, and Pro/Cys$^{7.47}$ in TMD7 are critical for ligand selectivity and receptor conformation. A combinatory mutation in gmGnRHR-2 (Pro$^{7.31}$-Pro$^{7.32}$-Ser$^{7.33}$ in EL3 to Ser-Glu-Pro and Leu$^{7.38}$, Leu$^{7.43}$, Ala$^{7.46}$, and Pro$^{7.47}$ in TMD7 to those of rat GnRHR-1) enhanced GnRH-I affinity by ~500-fold (Li et al., 2005), suggesting that multiple residues in ECL3 and TMD7 are involved in discriminating GnRH-II from GnRH-I.

Does the human receptor for GnRH-II exist?

In humans, the genes for GnRHR-2 are localized in chromosomes 1 and 14. These genes are expressed in a variety of tissues including the brain (Faurholm et al., 2001). However, these genes are thought to be nonfunctional pseudogenes resulting from the introduction of a premature stop codon (Millar et al., 2004; Morgan et al., 2003). Thus, humans have two GnRH forms, GnRH-I and GnRH-II, but only one receptor, the mammalian type-I receptor (Fig. 1). This raises various questions. Is GnRH-II a nonfunctional evolutionary reminiscence? Can the two GnRHs in humans act on a common receptor, the mammalian GnRHR-1? Does a functional receptor for GnRH-II exist? The function of GnRH-II in mammals is not clear, since the GnRH-II gene is not present in rodent species that serve as excellent models reflecting the function of a certain gene in humans. In humans, the function of GnRH-II has been demonstrated in immune tissues and reproductive tissue-derived cancer cells. GnRH-II is more potent than GnRH-I in inhibiting proliferation of human endometrial and ovarian cancer cells (Grundker et al., 2004). GnRH-II produced by human T cells triggers laminin receptor gene expression and cell migration (Chen et al., 200b). Due to the absence of a functional GnRHR-2 in humans, the action of GnRH-II is proposed to be mediated by GnRHR-I (Millar et al., 2004). In the monkey, GnRH-II is expressed in the hypothalamus, which may allow GnRH-II to reach the pituitary gonadotropes (Urbanski et al., 1999). Indeed, GnRH-II potently stimulates gonadotropin release when injected *in vivo* (Densmore and Urbanski, 2003). This effect is likely the result of an action on mammalian GnRHR-1, as a GnRH-I antagonist attenuated both GnRH-I- and GnRH-II-induced LH release (Okada et al., 2003). However, no evidence for such action has been provided in a human model. Some pharmacological studies suggest the presence of functional GnRH-II receptor that is uncharacterized and distinct from the conventional GnRHRs. For instance, GnRH-II-induced laminin receptor gene expression is not blocked by the GnRH-I antagonist cetrorelix (Chen et al., 200b). The antiproliferative effect of GnRH-II on ovarian cancer cells was not abolished by knock-down of the GnRHR-1 in these cells (Grundker et al., 2004). Recently, we demonstrated that human prostate cancer cells may possess a GnRH-II binding protein. Radio-labeled GnRH-II substantially bound to a variety of prostate cancer cell lines; this binding was displaced by cold GnRH-II but not by GnRH-I. Using photoaffinity labeling with ^{125}I-[azidobenzoyl-D-Lys^{6}]GnRH-II, we observed that an 80-kDa protein specifically bound to GnRH-II (Maiti et al., 2005). Considering that the molecular weight of mammalian GnRHR-1 is 44~55-kDa, this GnRH-II binding protein could not have been the conventional GnRHR-1. Taken together, human GnRH-II could have roles either distinct from or similar to those of GnRH-1. However, whether these GnRH-II actions are meditated by the conventional GnRHR-1 or a novel, uncharacterized receptor remains to be further investigated.

Concluding remarks

During evolution, the amino acid sequences of GnRH and its receptor have diverged from a common ancestral source. Sequence divergences of neuropeptides and their receptor families may explain how distinct ligand selectivity has been conserved while maintaining core ligand-receptor interaction sites. Molecular and comparative studies of GnRHs and their cognate receptors may enhance understanding roles and clinical applications of GnRHs and their receptors in humans.

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