

# Evolution of gonadotropin-releasing hormone (GnRH) structure and its receptor

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## ABSTRACT

It is evident now that the gonadotropin-releasing hormone (GnRH) structure was already in existence very early in the evolution of animals and was co-opted in diverse ways to regulate reproduction. During 600 million years of animal evolution, the N and C termini of GnRH have been conserved as functional domains for binding and activating cognate receptors to accomplish its functions. About 400 millions years ago, a single substitution of the chiral amino acid in position 6 of GnRH in jawless fish by the achiral glycine facilitated a type II'  $\beta$ -turn conformation of GnRH to allow spatially close interaction of functional domains of GnRH with receptors, in contrast to the interaction of more extended GnRH structures with their cognate receptors in earlier-evolved species. GnRH II was preconfigured to this conformation through intramolecular interactions, which accounts for its high binding affinity and total conservation of primary structure over 400 million years of evolution. It is very surprising and fascinating that the coordinated evolutionary selection of amino acids participating in binding GnRH has resulted in such perfection, that no substitution with a natural amino acid in any position improves binding potency.

**KEY WORDS:** GnRH, GnRH receptor, GnRH gene, GnRH receptor gene, evolution, conformational constraint

## INTRODUCTION

GnRH is a key peptide of hypothalamic origin that initiates a hormonal cascade after binding to a specific receptor at the surface of pituitary cells. It

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is synthesized in the neurons in the preoptic area of the hypothalamus and enzymatically processed in hypothalamic neurons from a larger prohormone and packaged in storage granules that are transported down axons to the external zone of the median eminence (Kaiser et al., 1997; Millar et al., 2004; Cheng and Leung, 2005). The peptide is released in a pulsatile manner, in synchronized pulses every 30-120 min, from the nerve endings of about 1000 neurons into the hypophyseal portal circulation system through which the hormone is transported to the anterior pituitary gland. It stimulates the biosynthesis and secretion of LH and FSH from pituitary gonadotropes after binding to its cognate type 1 receptor. Each GnRH pulse stimulates the biosynthesis and secretion of LH and FSH from pituitary gonadotropes (Millar et al., 2004), but FSH pulses are less distinct. LH is stored in the anterior pituitary and its secretion depends on GnRH binding to its pituitary receptor. The frequency of pulses is highest at the ovulatory LH surge and lowest during the luteal phase of the ovarian cycle. The asynchronous patterns of LH and FSH release result from changes in GnRH pulse frequency, modulating effects of gonadal steroid and peptide hormones on FSH and LH responses to stimulation by GnRH, and differences in the half-life of these two hormones. The spectrum of different GnRH molecules and their cognate receptors in mammals and non-mammals, their relationships and differences, are now known and, therefore, it is possible to trace their evolution in the animal kingdom. The large body of knowledge on this subject makes it impossible to review all aspects of GnRH structure, action, and evolutionary changes. The most important aspects of this subject will, however, give us an idea on why this simple decapeptide is a very important, fascinating subject of research and a key to understanding many physiological processes.

#### GnRH AND ITS GENE

Mammalian GnRH (mGnRH; GnRH I; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH<sub>2</sub>) was first isolated from the ovine, bovine and porcine hypothalamus in several laboratories, among them also in our laboratory (Kochman, 1966, 1969; Kochman and Domański, 1969), but was finally characterized and its primary structure elucidated by two independent research groups in 1971 (Matsuo et al., 1971; Burgus et al., 1972). Professors Andrew V. Schally and Roger Guillemin were honored by being awarded the Nobel Prize in 1977 for this outstanding scientific achievement. At first, it was believed that GnRH is a unique structure in all animal species and in humans, with a primary and basic physiological role in regulating the release and biosynthesis of LH and FSH in the anterior pituitary. During subsequent years of intensive studies it became clear that many forms of this peptide exist in vertebrates and to date 30 structurally different forms of GnRH have been identified.

Fifteen structural variants of the GnRH molecule have been found in vertebrates, and 15, in invertebrates (Millar et al., 2004; Roch et al., 2011), nine different GnRHs were identified in prochordates, which are vertebrate progenitors (Adams et al., 2003; Millar et al., 2004). A further six GnRH sequences were determined in other invertebrates (Figure 1). Analysis of invertebrates that evolved before tunicates (echinoderms, mollusks and annelids) reveals that their GnRH-like peptides have two additional amino acid residues in positions 2 and 3, although critical amino acids are retained throughout the peptide (Roch et al., 2011). The first invertebrate protostomian GnRH sequence to be identified was from octopus (Iwakoshi et al., 2002). This and other sequences form a unique group of invertebrate GnRH peptides that are 11 or 12 amino acid residues in length (Figure 1). These sequences differ from vertebrate and tunicate GnRHs by insertion of two amino acids after position 1 and variation of the proline residue in position 9 of the sequence (Tsai and Zhang, 2008). Other GnRH gene sequences were identified from two mollusks: sea hare (*Aplysia californica*), owl limpet (*Lottia gigantea*), an annelid, the marine worm (*Capitella teleta*), an echinoderm, the sea urchin (*Strongylocentrotus purpuratus*), and another annelid, the leech (*Helobdella robusta*). The *Aplysia*, limpet, marine worm, and leech GnRHs lack the last glycine in position 10 (using vertebrate numbering), but maintain the amidation and dibasic cut site. Sea urchin, an invertebrate deuterostome, has a putative GnRH of 12 amino acids that represents a missing link between the 10-amino acid GnRHs of other deuterostomes (including tunicates and vertebrates) and the 11- or 12-amino acid GnRHs of the protostomes (including mollusks and annelids) (Roch et al., 2011; Figure 1).

In addition to their role in regulating reproduction, GnRHs can also function in a paracrine way in placenta and gonads, in an autocrine manner in GnRHs neurons and immune cells, and play neurotransmitter/neuromodulatory roles in the central and peripheral nervous systems, e.g., in sympathetic ganglions or the mid-brain (Millar et al., 2004). The mediation of GnRH action is never through communication by the peripheral circulatory system. One form of GnRH is able to perform all of these functions, but now it is well known that at least two, but probably even three, different GnRHs are present in most vertebrate species. Chicken GnRH II, isolated first from chicken brain, was found to be the most ubiquitous form of GnRH molecule. The chicken GnRH II structure is perfectly conserved and present in all species, from bony fish to man. This molecule is considered to be the earliest evolved GnRH form and has critically important physiological functions. This form of GnRH was designated later as GnRH II, while the hypothalamic GnRH was named GnRH I. In vertebrate species, a third, also well conserved, GnRH (salmon GnRH) was found, sequenced, and localized to the terminal nerve in the forebrain in teleost fish and then designated

		Amino acid sequence												
		1	2	3	4	5	6	7	8	9	10			
1	Mammal*	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH <sub>2</sub>		
2	Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH <sub>2</sub>		
3	Guinea Pig	pGlu	Tyr	Tyr	Ser	Tyr	Gly	Val	Arg	Pro	Gly	NH <sub>2</sub>		
4	Frog	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly	NH <sub>2</sub>		
5	Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH <sub>2</sub>		
6	Salmon***	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH <sub>2</sub>		
7	Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly	NH <sub>2</sub>		
8	Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH <sub>2</sub>		
9	Whitefish	pGlu	His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	Gly	NH <sub>2</sub>		
10	Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH <sub>2</sub>		
11	Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH <sub>2</sub>		
12	Lamprey II	pGlu	His	Trp	Ser	His	Gly	Trp	Phe	Pro	Gly	NH <sub>2</sub>		
13	Chicken II**	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH <sub>2</sub>		
14	Lamprey III	pGlu	His	Trp	Ser	His	Gly	Trp	Lys	Pro	Gly	NH <sub>2</sub>		
15	Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly	NH <sub>2</sub>		
16	Chelyosoma I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH <sub>2</sub>		
17	Chelyosoma II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly	NH <sub>2</sub>		
18	Ciona I	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly	NH <sub>2</sub>		
19	Ciona II	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly	NH <sub>2</sub>		
20	Ciona III	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly	NH <sub>2</sub>		
21	Ciona IV	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly	NH <sub>2</sub>		
22	Ciona V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly	NH <sub>2</sub>		
23	Ciona VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly	NH <sub>2</sub>		
24	Ciona VII	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly	NH <sub>2</sub>		
25	Octopus	pGlu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	His	Pro	Gly	NH <sub>2</sub>
26	Sea Urchin	pGlu	Val	His	His	Arg	Phe	Ser	Gly	Trp	Arg	Pro	Gly	NH <sub>2</sub>
27	Aplysia	pGlu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	Tyr	Ala	-	NH <sub>2</sub>
28	Limpet	pGlu	His	Tyr	His	Phe	Ser	Asn	Gly	Trp	Lys	Ser	-	NH <sub>2</sub>
29	Marine worm	pGlu	Ala	Tyr	His	Phe	Ser	His	Gly	Trp	Phe	Pro	-	NH <sub>2</sub>
30	Leech	pGlu	Ser	Ile	His	Phe	Ser	Asn	Ser	Trp	Gln	Pro	-	NH <sub>2</sub>

\* mammal - mammalian GnRH; mGnRH:GnRH I; \*\* chicken II - cGnRH II : GnRH II

\*\*\* salmon GnRH - sGnRH : GnRH III

Figure 1. Amino acids sequences of naturally occurring GnRH structural variants spanning approximately 500 million year of evolution. The encircled amino acid residues (on the left and on the right) show the conserved NH<sub>2</sub>- and COOH-terminal residues that play important functional roles. Nonconserved residues are either unimportant or convey ligand selectivity for a particular GnRH receptor. The GnRHs are named according to the species in which they were first discovered, and they may be represented in more than one species. For example, mammalian GnRH is widely present in amphibians and primitive bony fish. Chicken GnRH II (chicken II) is present in most vertebrate species, including man, salmon GnRH (GnRH III) is probably present in all teleost fish (Millar et al., 2004; Roch et al., 2011 - modified). Numbers: 1-15: vertebrates; 16-24: invertebrates - prochordates, tunicates (Chelyosoma and Ciona); 25-30: invertebrates: octopus, mollusks; annelids and echinoderm

as GnRH III. GnRH III has complete sequence conservation, but is present only in teleosts, indicating that its gene evolved after the divergence of teleosts from the vertebrate evolution path. It is interesting to note that the two genes coding two forms of GnRH (GnRH III) exist in the genome of the sockeye salmon. The general classification of the GnRHs into three family forms has its background in the structural analysis of genes encoding these peptides and further confirmed by a very extensive study of phylogenetic parameters (Millar et al., 2004).

The NH<sub>2</sub>-terminal amino acid sequence (pGlu-His-Trp-Ser) and carboxyl terminal amino acid sequence (Pro-Gly-NH<sub>2</sub>) have been totally conserved over 400 million years of chordate evolution, only with the exceptions of two cases of conservative Tyr substitutions (Figure 1). The type I GnRHs exhibit important variations in positions 5, 7 and 8, which are very essential for ligand selectivity (Millar et al., 2004).

The gene of the sea squirt (*Ciona*), a prochordate (chordate ancestor), shows an extraordinary feature having the three GnRH forms coded in tandem within its single gene (Millar et al., 2004). The most ancient of the GnRH forms that has been identified is the GnRH molecule found in the octopus. This GnRH shows the characteristic pGlu and Pro-Gly-NH<sub>2</sub> ends but also possess the two additional amino acid residues in the middle of the sequence. This GnRH is able to stimulate LH release from the pituitary cells of quail (Millar et al., 2004).

The GnRHs in the primitive jawless lamprey, in prochordates, and in octopus, do not contain the conserved glycine in position 6, which is present in jawed vertebrates (Figure 1). The presence of chiral amino acid residues in the position of achiral glycine in position 6, which prevents the  $\beta$ -turn conformation, results in the low binding affinity of these GnRHs to the mammalian pituitary receptor. This indicates that the receptors in these lower organisms do not require GnRH to be in folded conformation and that this important feature evolved first in the receptors of bony fish (Millar et al., 2004; Barran et al., 2005). These basic problems in evolution will be discussed in more detail later in this article.

The structure of the human GnRH I gene is presented in Figure 2. It contains four exons, three introns and is located as a single gene copy on chromosome 8p11.2-p21 (Yang-Feng et al., 1986; Radovick et al., 1990). The first exon of the gene is untranslated and consists of 61 bp in mRNA expressed in the hypothalamus. The second exon encodes the signal sequence, the GnRH decapeptide, the cleavage site (GKR) processing signal and the first 11 GnRH-associated peptide (GAP) residues. The third exon codes for the next 32 GAP residues. The fourth exon codes the remaining GAP residues and contains the translation termination codon and also the entire 3'-UTR (Adelman et al., 1986; Radovick et al., 1990).

The human GnRH II gene is located on chromosome 20p13 (White et al., 1998) (Figure 2). It contains four exons and three introns. The GnRH II prohormone

is organized identically to the GnRH I precursor. The human GnRH II gene (2.1 kb) is shorter than the GnRH I gene (5 kb) because introns 2 and 3 in GnRH I gene are larger (White et al., 1998). The GAP is 50% longer in the GnRH II precursor (84 vs 56 amino acid residues) than in the GnRH I precursor. A similar GAP was discovered in the placental mammal tree shrew (76 vs 50 amino acid residues), suggesting that a larger GAP is characteristic for mammalian GnRH II precursors (Cheng and Leung, 2005).

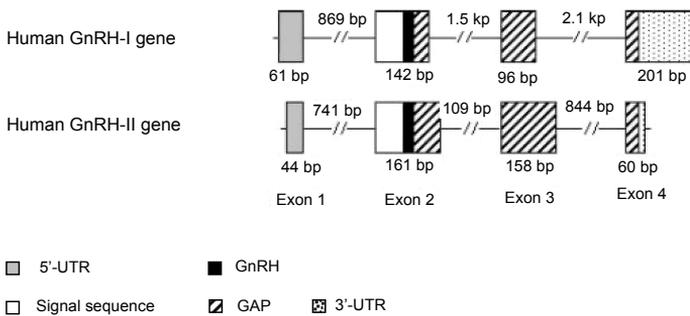


Figure 2. cDNA and genomic structures of human GnRH genes. In humans, two forms of GnRH, termed GnRH-I and GnRH-II, encoded by separate genes on chromosome 8p11.2-p21 and 20p13 are identified. Both genes are composed of four exons (*boxes*) interrupted by three introns (*thin lines*), and their encoded preprohormones are organized identically such that they all have a signal sequence, followed by a GnRH decapeptide, a conserved GKR cleavage site, and a GAP is found in the human body (Cheng and Leung, 2005 - modified)

The most prominent difference in the tissue distribution of GnRH I and GnRH II in humans, is that GnRH II is expressed at the highest level outside the brain (White et al., 1998). The GnRH II mRNA level in kidney is about 30-fold higher than in any brain region. The expression of its gene in the bone marrow and prostate is about 4-fold greater than in the brain (White et al., 1998). GnRH I gene expression was not observed at a high level outside the brain (White et al., 1998). The cell bodies of GnRH I neurons in humans are concentrated in the preoptic area and basal hypothalamus. They can also be found in the septal region and anterior olfactory area and in the cortical and medial amygdaloid nuclei (Stopa et al., 1991). They predominate in the median eminence and infundibular stalk, although many projections are to the neurohypophysis (Anthony et al., 1984; King and Anthony, 1984; McArdle et al., 2002; Cheng and Leung, 2005).

The GnRH II gene in the human brain is expressed mainly in the periaqueductal region of the midbrain (Chen et al., 1998), high in the caudate nucleus and, to a lesser extent, in the hippocampus and amygdala (White et al., 1998).

The human placenta expresses GnRH I mRNA *in vitro* and secretes the GnRH I decapeptide, which is identical with that synthesized in the hypothalamus (Siler-Khodr and Khodr, 1979; Khodr and Siler-Khodr, 1980). Expression of GnRH I mRNA has been demonstrated in all human uterine compartments (Irmer et al., 1994; Chegini et al., 1996; Kobayashi et al., 1997; Dong et al., 1998; Raga et al., 1998; Cheng and Leung, 2005). GnRH I immunoreactivity was found in all endometrial cell types, with the most intense staining during the luteal phase (Raga et al., 1998). Expression of GnRH I and GnRH II mRNAs is identical in all human ovarian tissues, including granulosa-luteal cells (Kang et al., 2000, 2001; Millar, 2001; Cheng and Leung, 2005).

The presence of GnRH II in humans (White et al., 1998), together with an apparent absence of a functional full-length GnRH type II receptor (Millar, 2003; Morgan et al., 2003) and high binding affinity of GnRH II for the GnRH type I receptor (Sun et al., 2000; Fromme et al., 2001; Pflieger et al., 2002), suggests that this receptor has adopted the role of the cognate receptor for GnRH II (Millar, 2003; Millar et al., 2004).

### *Structure of the GnRH receptor and its gene*

The GnRH receptor belongs to the rhodopsin-like G protein-coupled receptor (GPCR) superfamily, which contains a characteristic seven transmembrane (TM)-domain structure (Stojilkovic et al., 1994; Sealfon et al., 1997; Cui et al., 2000). Unlike other members of the GPCR family, however, the mammalian GnRH receptor lacks the entire carboxyl-terminal tail (Stojilkovic et al., 1994; Sealfon et al., 1997), which is known to participate in various functions of GPCR regulation through interaction with a complex network of receptor-associated proteins (Ferguson, 2001; Bockaert et al., 2003). The amino acid sequence of the GnRH receptor was established first for the mouse receptor cloned from the pituitary  $\alpha$ T3 gonadotrope cell line and was subsequently confirmed (Reinhart et al., 1992; Tsutsumi et al., 1992; Perrin et al., 1993). After the identification of the mouse GnRH receptor sequence, similar pituitary cDNAs were found in five other mammalian species, including humans, and in one non-mammalian vertebrate: humans (Kakar et al., 1992; Chi et al., 1993), rats (Eidne et al., 1992; Kaiser et al., 1992; Perrin et al., 1993), sheep (Brooks et al., 1993; Illing et al., 1993), cows (Kakar et al., 1993), pigs (Weesner and Matteri, 1994) and catfish (Tensen et al., 1997). The identified amino acid sequence for these GnRH receptors has more than 85% conserved structure overall in the six mammalian species and is nearly identical within the transmembrane domains (TM). The cow, sheep and human receptors have a structure 328 amino acids long, while the mouse and rat receptors have 327 amino acid residues due to the absence of one residue in the

second extracellular domain; the catfish receptor has 370 amino acid residues and this difference is because it has a 49 amino acids in the cytoplasmic C-terminal domain, which is not present in mammalian receptors (Tensen et al., 1997).

Homologs of many mammalian GnRH receptors were cloned from a marsupial (possum) (King et al., 2000; Cheung and Hearn, 2002), two forms from the goldfish (Illing et al., 1999), bullfrog, brown frog and clawed toad (Troskie et al., 2000; Wang et al., 2001; Seong et al., 2003), chicken (Sun et al., 2000), medaka (Okubo et al., 2001), striped bass (Alok et al., 2000), trout and salmon (Madigou et al., 2000; Jodo et al., 2003), cichlid (Robinson et al., 2001), Japanese eel (Okubo et al., 2000), amberjack, rubber eel, and sea squirt (Millar et al., 2004). The non-mammalian receptors with the greatest homology to the mammalian pituitary receptors have 42-47% amino acid identity with them, but 58-67% identity among each other. These receptors are all designated as type I GnRH receptors. It is not altogether clear from homology comparisons that classifying mammalian and non-mammalian type I together is very precise and correct, but similarities in micro-domains support this classification (Millar et al., 2004).

Because the evolutionary time separating amphibians and bony fish is similar to that separating amphibians and mammals, the poor sequence conservation of the mammalian type I GnRH receptor with the non-mammalian receptors implies a sudden acceleration in evolutionary change in mammals. This may have been driven by the loss of the carboxyl-terminal tail in the mammalian GnRH type I receptor, which is unique among G protein-coupled receptors (Millar et al., 2004).

In the goldfish (Illing et al., 1999), zebra fish (Troskie et al., 1998), catfish (Blomenrohr et al., 1997; Bogerd et al., 2002) and salmon there are two isoforms (type Ia and type Ib) that have 70% amino acid identity (Millar et al., 2004). In the goldfish, they differ in type Ia having a putative SH3 binding domain (poly proline sequence) in the carboxyl tail, which potentially conveys the possibility of coupling to mitogen-activated protein kinase (MAPK) (Millar et al., 2004).

The presence of three GnRH forms in most vertebrate species suggests that three cognate GnRH receptor sub-types also exist. The extracellular coil domain 3 (EC3) in the receptor is a major determinant of receptor selectivity for the different GnRH structural variants. Degenerate oligonucleotides from conserved boundary TM from genomic DNA from different vertebrates were used to amplify this domain (Troskie et al., 1998). The GnRH type II receptor sequences from mammalian species were subsequently used to identify a human type II receptor (Millar et al., 1999; Conklin et al., 2000; Faurholm et al., 2001; Millar, 2003) and to clone these sequences also from bullfrog and clawed toad (Wang et al., 2001; Millar et al., 2004). Marmoset, macaque and green monkey (Millar et al., 2001; Neill et al., 2001) type II receptors were also identified. The same approach also

permitted the cloning of the GnRH type III receptor from the bullfrog (Wang et al., 2001). The results of these procedures, along with the cloning of many other GnRH receptors, indicate and show the early evolution in the tree of receptor subtypes in vertebrates that occurred during the same evolutionary time as the tree of the GnRH ligands for receptors. Type III GnRH receptors may arise from duplication of an ancestral gene in lower vertebrates. The numerical naming of individual cloned receptors in the Genbank database frequently does not comply with the phylogenetic relations because the researchers have named them by pharmacological characteristics, order of discovery, or tissue expression (Okubo et al., 2001; Wang et al., 2001; Bogerd et al., 2002; Seong et al., 2003; Millar et al., 2004). In order to create a reliable systematics tree for receptors, a more systematic and consistent approach in this area of research is necessary (Millar et al., 2004).

The transcript of a human GnRH type II receptor lacks a frame-shift and internal stop codon in proper position. The transcripts of this gene are, therefore, incapable of being translated to a full-length G protein-coupled receptor (GPCR). This apparent silencing of the type II receptor in humans is very paradoxical in light of the extraordinary conservation of the cognate GnRH II peptide, which exists unchanged from bony fish to man (Millar et al., 2004).

The human GnRH receptor molecule consists of a 328 amino acid chain and has TM connected by extracellular coils (ECs) and intracellular coils (ICs). The binding site residues and binding pocket formation are very important structural elements. These include disulphide bond formation and glycosylation sites. The structure contains residues involved in receptor activation and residues that are highly conserved throughout the rhodopsin family of GPCRs. Residues involved in coupling to G proteins, protein kinase C (PKC), and protein kinase A (PKA) phosphorylation sites have also been identified in its structure (Millar et al., 2004).

Unlike most GPCRs, which are intronless, the human GnRH receptor gene is a single copy on chromosome 4q21.2 (Albarracin et al., 1994; Leung et al., 1995; Kakar, 1997) and is composed of three exons separated by two introns and spans more than 15 kb along the chromosome (Fan et al., 1995; Kakar, 1997; Cheng and Leung, 2005) (Figure 3). Exon-1 contains the 5'-UTR and the first 522 nucleotides of the open reading frame encoding the first three TM domains and a part of the fourth TM domain. Exon 2 is responsible for encoding the next 220 nucleotides of the reading frame, which contains the rest of the fourth TM domain, the fifth TM domain and also a part of the third intracellular loop. Exon 3 has the rest of the coding sequence and the 3'-UTR. The location of all of the exon-intron boundaries of the human GnRH receptor gene is conserved very well in rodent and ovine sequences, but the first intron of the human gene is much

smaller (Reinhart et al., 1992; Albarracin et al., 1994; Campion et al., 1996). Five transcription start sites were discovered for the GnRH receptor gene in the human brain and 18 in the human pituitary (Fan et al., 1995; Kakar, 1997). All of these start sites are clustered into two regions, which are 579-819 and 1348-1751 bp upstream of the ATG initiation codon. Five polyadenylation signals are within an 800-bp area in a cluster-like format located in the 3'-UTR of the human GnRH receptor gene (Fan et al., 1995). The 3'-UTR contains several ATTTA motifs, which are responsible for mRNA instability and are present in many other RNAs that are rapidly degraded (Ross, 1996; Gay and Babajko, 2000). The size of the GnRH receptor mRNA calculated from the length of the 5'- and 3'-UTRs is about 5.5 kb, which is in close agreement with the size of the major transcript (4.7-5 kb) expressed in the human pituitary (Cheng and Leung, 2005).

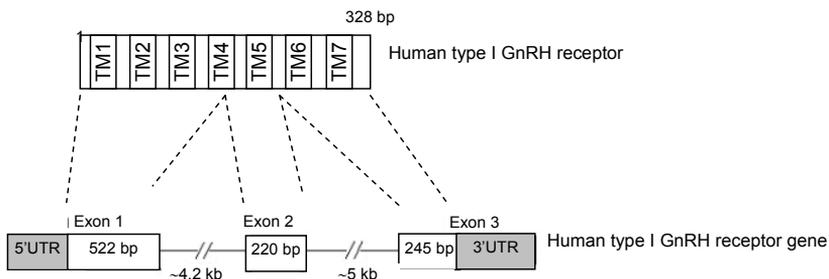


Figure 3. cDNA and genomic structures of human GnRH type I receptor gene. In humans only one conventional GnRH receptor subtype (termed type I GnRH receptor) is found. The gene coding for the type I GnRH receptor lies on chromosome 4q21.2 and consists of three exons separated by two introns. Exon 1 contains the 5'-UTR and encodes the first three TM domains and a portion of the fourth TM domain. Exon 2 is 220 bp in length and encodes the remainder of the fourth TM domain, the fifth TM domain, and part of the third intracellular loop. Exon 3 encodes the rest of the open reading frame and contains the 3'-UTR (Cheng and Leung, 2005 - modified)

The proximal 5'-flanking region of the human GnRH receptor gene exhibits a great homology with that of the rodent and ovine sequences (Albarracin et al., 1994; Fan et al., 1995; Campion et al., 1996; Reinhart et al., 1997). Nonetheless, the human gene possesses certain specific differences in structure from that in other species. The significant difference between the human and rodent genes is the location of their transcription start sites. The start sites for the rodent genes are within 100 nucleotides from the initiation codon (Albarracin et al., 1994; Reinhart et al., 1997), while the human gene has more than 703 bp (Fan et al., 1995). The second difference is that the human sequence contains multiple TATA and CAAT boxes residing in close proximity to each other near the transcription start sites (Fan et al., 1995; Kakar, 1997). The presence of consensus TATA boxes is unusual

among all the GPCRs sequences to date, as many of these genes contain GC-rich promoter regions (Kamura et al., 1997; Moro et al., 1999). It should be stressed that only the human GnRH type I receptor uniquely lacks a carboxyl-terminal tail. The human type II receptor holding gene homolog carries a frameshift and premature stop codon, suggesting that a full-length type II receptor does not exist in humans, as was already mentioned earlier in this article (Cheng and Leung, 2005). Studies on the transcriptional regulation of the human GnRH receptor gene indicate that tissue-specific gene expression is mediated by differential promoter usage in various cell types. Functionally, GnRH type I and GnRH type II are very important as autocrine and paracrine regulatory molecules in many extrapituitary cells and tissues (Cheng and Leung, 2005).

Molecular cloning of the marmoset, macaque and green monkey GnRH type II receptor cDNAs indicated that they consist of a typical seven-TM-domain GPCR (Millar et al., 2001; Neill et al., 2001). The primate type GnRH receptors, like all non-mammalian GnRH receptors cloned (Tensen et al., 1997; Troskie et al., 1998; Illing et al., 1999; Okubo et al., 2001; Wang et al., 2001), have a C-terminal tail that is responsible for the receptor's susceptibility to rapid desensitization and subsequent internalization (King et al., 1986; Heding et al., 1998; Hislop et al., 2001; McArdle et al., 2002; Neill, 2002; Pawson et al., 2003). The tail present in the receptor structure plays an important role in agonist binding and is essential in expression and regulation of the receptor (Lin et al., 1998; Blumenrohr et al., 1999). An interesting feature of the GnRH type II receptors is the presence of an Asp/Asp microdomain in the helices of the second and seventh TM and the substitution of VPPS sequences for the LSD/EP sequence in the third EC (Flanagan et al., 1994, 1999; Sealson et al., 1997; Millar et al., 2001).

The mRNA of the GnRH type II receptor is expressed in the marmoset brain (Millar et al., 2001). Pharmacological studies have shown that the marmoset GnRH type II receptor is highly selective for GnRH-II, and, to a smaller degree, for salmon GnRH and [d-Arg<sup>6</sup>]-GnRH-II (Millar et al., 2001; Neill et al., 2001). The human type I and marmoset type II GnRH receptors couple to G<sub>q/11</sub> and activate extracellular signal-related kinase 1/2 (ERK1/2), however, they stimulate the p38 MAPK pathway in different ways (Millar et al., 2001). The GnRH type II of marmoset may be activated by a GnRH I antagonist, which was originally known as a full antagonist of the human GnRH type I receptor (Millar et al., 2001). This result is possibly helpful in the understanding and correct explanation of the fact that certain GnRH-Is exhibit antagonist properties (Emons et al., 1993; Yano et al., 1994; Millar et al., 2001).

## EVOLUTION OF GnRH AND ITS COGNATE RECEPTOR

The GnRH structure was already present very early in the evolution of animals and participated in the regulation of reproduction in multiple ways (King and Millar, 1995). This molecule was present in earlier vertebrates, which appeared approximately 500 million years ago. The similarity among the different GnRH genes suggests that they are derived from a common ancestor. This parent gene underwent duplication to create at first an additional protein coding region, from which a second form of GnRH originated and subsequently evolved further. All vertebrate species possess at least two, but more often three, forms of GnRH that are expressed, which means that the duplication occurred before to the appearance of jawless fish. During evolution considerable divergence occurred in the GnRH genes (King and Millar, 1995).

In the primitive jawless fish (*Agnatha*), lamprey GnRH I was identified (Sherwood et al., 1986), and several years later, a second form: lamprey GnRH III (Sower et al., 1993) was found. In cartilaginous fish (*Chondrichthyes*), chicken GnRH II, dogfish GnRH, and a third GnRH form were identified (Powell et al., 1986a; Sherwood, 1987; Lovejoy et al., 1991, 1992). In the bony fish (*Osteichthyes*), chicken GnRH II is the most universal form of GnRH (Powell et al., 1986a; Yu et al., 1988; Sherwood and Lovejoy, 1989; King et al., 1990; Okuzawa et al., 1990; Sherwood et al., 1991; Amano et al., 1992; Ngamvongchon et al., 1992). Many bony fish species have either mGnRH, sGnRH, catfish GnRH, or another form of GnRH in addition to chicken type GnRH (Sherwood et al., 1984, 1991; King and Millar, 1985; Yu et al., 1988; King et al., 1990; Amano et al., 1992). In amphibians (*Amphibia*) three different GnRHs are also usually present: chicken GnRH II, mGnRH, and sGnRH (King and Millar, 1986; Conlon et al., 1993; Licht et al., 1994). In reptiles: chicken GnRH II and chicken GnRH I predominate, and a third form is also present in some species (Powell et al., 1986b; Lovejoy et al., 1991).

Chicken GnRH II and chicken GnRH I are generally present together in birds (King and Millar, 1982a,b; van Gils et al., 1993). In most eutherian (placental) mammals only mammalian GnRH has been detected (King et al., 1988; Kelsatl et al., 1990; Gautron et al., 1991), but chicken GnRH II has also been identified in early-evolved species (Dellovade et al., 1993; King and Millar, 1995). In most metatherian (pouched) species, mGnRH and chicken II are present together (King and Millar, 1995).

Two or three forms of GnRH are present in most species, with GnRH II being the most universal form (King and Millar, 1995). These facts mean that gene duplication took place in the earliest vertebrates and that chicken GnRH II remained a highly conserved molecule during the entire history of vertebrate

evolution and plays a very important functional role. The eight different GnRH molecules underwent evolutionary changes, and at least two forms are expressed together in most vertebrate species: chicken GnRH II and a second form, which varies depending on the species. The cell bodies with GnRH are located in different areas of the forebrain and midbrain, and processes project from them to almost all areas of the central nervous system (CNS), the vasculature, and the cerebrospinal fluid (King and Millar, 1995).

Over the entire period of evolution, GnRH performed pituitary reproductive functions but also many different extrapituitary functions. Within the CNS, GnRH acts as a neurotransmitter or as a neuromodulator. GnRH also exhibits direct effects on reproductive behaviour and affects the function of amphibian sympathetic ganglion neurons. Outside the CNS, GnRH plays a paracrine role in the gonads, where it affects steroid hormone production, and in the placenta, where it stimulates the secretion of chorionic gonadotropin (Hsueh and Schaeffer, 1985; King and Millar, 1995). The action of GnRH in different tissues is achieved through anatomic arrangements that allow discrete delivery of GnRH in physiologically effective concentrations to target cells. Hypothalamic GnRH is transported *via* portal vessels to the anterior pituitary, and its great dilution in the general circulation, at least in mammals, results in its concentrations being too low to allow effective binding to GnRH receptors in other tissues. Gonadal and placental GnRHs are secreted by specific cells to act on proximal cells in a paracrine way, and the same privacy of communication is present when GnRH plays a role of a neurotransmitter or an autocrine regulatory peptide (King and Millar, 1995).

GnRH in prochordates (Powell et al., 1996; Craig et al., 1997) is secreted from neurons and directly acts and regulates the gonads' function (Powell et al., 1996; Craig et al., 1997; Terakado, 2001) in these representatives of vertebrate progenitors. GnRH receptors have also been found to directly affect vertebrate gonadal function (Hsueh and Schaeffer, 1985), possibly reflecting the earliest role of GnRH as exemplified in prochordates (King and Millar, 1997; Millar et al., 1997). The GnRHs have an ancient physiological and evolutionary role as regulators of reproduction, at first through their direct neural delivery to the gonads and in later evolutionary times, as hypothalamic neuroendocrine regulators of the gonads through gonadal stimulation by gonadotropins. These GnRHs have conserved N-terminal residues (pGlu-His-Trp-Ser) and C-terminal residues (Pro-Gly-NH<sub>2</sub>), with the exception of two conservative substitutions (Figure 1). All of the GnRHs are further characterized by the presence of a glycine residue in position 6 in cartilaginous and bony fish, amphibians, reptiles, birds and mammals. The glycine residue, being achiral, permits the peptide to assume a type II'  $\beta$ -turn conformation, which is important for high affinity and binding activity in mammals (Karten and Rivier, 1986; Sealfon et al., 1997; Pflieger et al., 2002; Millar et al., 2004).

The GnRHs in the ancient jawless lamprey and prochordate species (with the exception of *Ciona* VI) are all characterized by the presence of chiral amino acids in position 6 (Figure 1). This limits the formation of a type II'  $\beta$ -turn conformation and these GnRHs are expected to have correspondingly low binding affinities and biological activities at GnRH receptors of higher vertebrates (King and Millar, 1997; Millar et al., 1997, 2004; Sealfon et al., 1997; Pflieger et al., 2002).

Earlier in evolution, GnRH interacted with the GnRH receptor in a more linear conformation. As the evolution of jawed fish progressed, the structural changes in GnRH molecule and in its cognate receptor required GnRH to be in the folded type II'  $\beta$ -turn conformation for binding to its receptor. A combination of ion mobility mass spectrometry (IM-MS) and molecular modeling (MM) methods applied in the study of the precise structure of the GnRH peptides gave very good results and documented that this structure has an excellent correlation of biological activity at vertebrate GnRH receptors with the ability of these peptides to form the more folded and compact conformation.

These results clearly point to the co-evolution of GnRH structure and the structure of its cognate receptor; the receptors of organisms that evolved early, bound GnRH when it had an extended, relaxed structure, whereas the GnRH receptors of higher organisms required GnRH in a folded type II'  $\beta$ -turn conformation, in which the N and C termini are very close during binding to receptors (Sherwood et al., 1993; Millar et al., 2004; Barran et al., 2005).

The importance of having the achiral glycine in position 6 in mammalian GnRH for effective biological activity was demonstrated in empirical studies a long time ago (Monahan et al., 1973; Karten and Rivier, 1986; Sealfon et al., 1997). More recent studies using N- and C-terminal-directed antibodies, fluorescence spectroscopy, MM and NMR confirmed the earlier findings that Gly<sup>6</sup> was essential to allow GnRH to assume the II'  $\beta$ -turn conformation (Karten and Rivier, 1986; Sealfon et al., 1997; Millar et al., 2004). All of these findings together supported numerous structure-activity studies indicating that mammalian GnRH interacts with its cognate receptor in the type II'  $\beta$ -turn conformation through the amino (pGlu-His-Trp-Ser) and carboxyl (Arg-Pro-Gly-NH<sub>2</sub>) terminal domains (King and Millar, 1997; Millar et al., 1997, 2004; Sealfon et al., 1997; Barran et al., 2005). The identification of GnRH structural variants in vertebrates and prochordates demonstrated the conservation of these domains over more than 400 million years of evolution (Barran et al., 2005).

The stringent requirement of jawed vertebrate GnRH receptors for a glycine residue in position 6 to allow presentation of the N- and C-terminal domains in a folded conformation to the receptor, made the presence of chiral amino acids in this position in jawless fish and prochordate GnRHs unexpected. This finding suggests that the GnRH receptors of these species are able to interact with GnRH

in an extended conformation. The similar low binding affinities of mammalian, Ciona I and Ciona III GnRHs at the Ciona A receptor supports this interpretation. The ability of the endogenous prochordate GnRHs and mammalian GnRHs to stimulate spawning in prochordate species (Powell et al., 1996; Craig et al., 1997; Adams et al., 2003) also indicates that prochordate GnRH receptors do not distinguish mammalian and prochordate GnRHs (Barran et al., 2005). GnRHs having an achiral amino acid in position 6 were active in mollusk species (Pazos and Mathieu, 1999; Barran et al., 2005). Interestingly, one of the prochordate GnRHs (Ciona VI) has Gly<sup>6</sup> and is active in stimulating spawning in *C. intestinalis* (Adams et al., 2003). This result supports the data demonstrating that the Ciona GnRH receptor binds GnRHs with chiral (Ciona I and III) and achiral (mGnRH) amino acids in position 6 equally well (Barran et al., 2005). On the basis of these experiments, it is evident that GnRH forms with chiral and achiral amino acids in position 6 can assume both a relaxed linear and extended conformation (Barran et al., 2005).

Ciona I GnRH exhibits a looser and less configured structure than mammalian GnRH and has poor binding affinity at vertebrate receptors. By comparing the collision cross sections from experimental and calculated structures, it is apparent that l-Ala at position 6 induces steric hindrance to the formation of the more compact folded structure, as was evidenced using molecular modeling and IM-MS methods. This is supported by the finding of larger Trp<sup>3</sup>-Leu<sup>7</sup> distances in Ciona GnRH I than the Gly<sup>6</sup> or d-Ala<sup>6</sup> formed *in vacuo*, as revealed using the IM/MS collision method. The d-Trp form adopts a much more compact geometry, with a type II'β-turn, whereas the l-Trp variant is more extended due to steric effects caused by the bulky side chain in the naturally occurring form (Barran et al., 2005). Molecular modeling (Guarnieri and Weinstein, 1996) revealed that the conformational preference for a type II' β-turn in the backbone of mammalian GnRH is significantly diminished by an Arg<sup>8</sup>→Lys<sup>8</sup> substitution. These results show the functional importance of Arg in the mammalian form. Glycine residues are often found at turning points in protein structures, as the absence of a side chain enables a right turn to be made, but such turns are only successful if additional non-covalent interactions are present, as promoted here by Arg. This combination of Gly<sup>6</sup> and Arg<sup>8</sup> in mGnRH produces a peptide configured with high affinity for the mammalian receptor. Substitution with d-Ala<sup>6</sup> or Gly<sup>6</sup> in Ciona I increases the affinity for the human receptor, since the peptide can now form a tighter turn. If, however, Ser<sup>8</sup> is present rather than Arg<sup>8</sup>, the very high affinity exhibited by mGnRH at the mammalian receptor is precluded, due in part to the additional stabilization of the compact structure by Arg and the interaction of Arg with an acidic residue in extracellular loop 3 of the GnRH receptor, which contributes to configuration of the ligand at mammalian receptors

(Flanagan et al., 1994; Fromme et al., 2001; Barran et al., 2005). When the chiral amino acid (Ala) in position six of Ciona I GnRH was substituted with the achiral glycine or with d-Ala, which enhances the type II'  $\beta$ -turn conformation, there was a marked increase in the binding affinity of the peptides at the vertebrate GnRH receptor (Barran et al., 2005).

These studies suggest that the early evolved GnRH receptors of protochordates bind GnRH in a more extended configuration and that after the subsequent evolution of the receptors in jawed vertebrates, they required a more compact configuration of GnRH for binding (Barran et al., 2005). The conservation of the N- and C-terminal domains of GnRHs in invertebrates and vertebrates (protochordates, jawless fish, amphibians, birds and mammals) indicates that these domains are functionally important for binding and activating GnRH receptors. Most of the binding sites for the N- and C-terminal GnRH residues in human and vertebrate receptors are present in the tunicate GnRH receptor (Barran et al., 2005).

Direct activation of the gonads in Ciona by GnRH appears to play an important role as a pheromone in a prochordate, *Saccoglossus*, and in the mollusk, chiton (Barran et al., 2005).

The different and multiple actions of GnRHs in the animal reproductive system are realized through its effects on the pituitary, gonads, placenta and nervous system, and represent an impressive fact of excellent conservation of its function within an important physiological network of a regulatory system and ensuring its effective work. It is possible that GnRH has an early origin in life history as a regulator of reproduction, since yeast  $\alpha$  mating factor has 80% amino acid homology with mammalian GnRH and stimulates gonadotropin release from the mammalian pituitary (Loumaye et al., 1982; King and Millar, 1995).

A fluorescence study of Trp<sup>3</sup> suggested that His<sup>2</sup> and Tyr<sup>5</sup> are in close proximity to Arg<sup>8</sup> in mammalian GnRH (Chou et al., 2003). The techniques of conformational memories and nuclear magnetic resonance (NMR) provided direct and precise structural evidence for mammalian GnRH being in a type II'  $\beta$ -turn conformation (Kiesel et al., 2002; Sakamoto et al., 2003).

Mammalian GnRH interacts with mammalian GnRH type I receptors in a type II'  $\beta$ -turn conformation, involving its residues 5-6. This conformation can be further constrained by substitution of a d-amino acid at position 6 or by a 6,7  $\gamma$ -lactam insertion involving residues 6 and 7, thereby increasing receptor binding affinity. Pflieger et al. (2002) proposed that this is not the case for non-mammalian GnRH receptors. This conformational constraint increases the binding affinity of mammalian, chicken and salmon GnRH, both for the chicken and catfish receptors and for the mouse receptor. The type II'  $\beta$ -turn conformation enhances ligand binding for non-mammalian GnRH type I receptors as well as for mammalian GnRH type I receptors (Pflieger et al., 2002; Millar et al., 2004; Barran et al., 2005).

There is a question whether the structure of the GnRHs and their receptors in invertebrates conserved their structure during evolution in a sufficient degree to support the homology with the structure of GnRHs and their receptors in vertebrates. An essential role of invertebrate GnRHs in the reproduction of these animals is often considered, having in mind that function can be a good criterion for the placement of these peptides and their receptors in the GnRH family.

Functionally, only one tunicate receptor was able to activate the  $G_{q/11}$  pathway and trigger inositol triphosphate ( $IP_3$ ) accumulation (Tello et al., 2005). In response to *Ciona*-specific GnRHs, three receptors stimulated cyclic AMP synthesis by stimulation of  $G_s$ . This is a minor GnRH-stimulated pathway in vertebrates (Cheng and Leung, 2005) and its use in *Ciona intestinalis* may reflect the disruption of the tunicate genome and signaling pathways during evolution (Roch et al., 2011). After the characterization of GnRH receptors from tunicates, a GnRH receptor was isolated from octopus (Kanda et al., 2006). This discovery established that the GnRH family had deep evolutionary roots stretching back before the protostome/deuterostome split, long before the origin of vertebrates. The octopus receptor is widely present in nervous and peripheral tissues and responds specifically to its native GnRH giving activation of  $G_{q/11}$ , resulting in steroidogenesis in reproductive tissues and muscle contractions (Kanda et al., 2006). These results show that the GnRH system in invertebrates shares some functional characteristics with that of the vertebrates, in addition to other potential roles. The determination of more invertebrate genomes enabled finding GnRH receptors, homologs to vertebrate receptors in sea urchin (*Stroglyocentrotus purpuratus*), marine worm (*Capitella teleta*) and limpet snail (*Lottia gigantea*), although they have not been cloned or assayed for ligand binding (Roch et al., 2011).

Another question is whether the families of peptides or receptors that are closely related to the GnRH family exist in vertebrates and in invertebrates. Is GnRH part of a superfamily? Until recently, GnRH appeared to be a stand-alone family unlike other hormones such as the insulin superfamily or secretin superfamily. The first clue to the possibility of a GnRH superfamily may be the identification of a receptor in *Drosophila* that was closely related to the GnRH receptor (Hauser et al., 1998). It was also shown that this receptor may bind a different hormone, adipokinetic hormone (AKH), as the ligand (Staubli et al., 2002). AKH is of similar length to GnRH and has a few key amino acids that match GnRH II. The same is true for several peptides related to AKH: corazonin, adipokinetic hormone/corazonin-related peptides (ACP), and pigment-concentrating hormone (RPCH) (Cazzamali et al., 2002; Park et al., 2002; Hansen et al., 2010; Martin et al., 2011). Corazonin is even closer than AKH in sequence to GnRH II as they share pGlu<sup>1</sup>, Ser<sup>4</sup>, Gly<sup>6</sup>, Trp<sup>7</sup>, and amidation. It is possible that they are members of a GnRH superfamily based on peptide structure. Receptors for these peptides have significant sequence

conservation with GnRH receptors. There is also a close relationship between the vasopressin/oxytocin (VP/OT) superfamily and the GnRH superfamily. It was reported that the mouse GnRH receptor was most closely related in structure to that of the VP/OT receptors (Reinhart et al., 1992). Subsequently, more VP/OT and GnRH receptors have been identified, supporting the close structural relationship. The GnRH and VP/OT peptides in these superfamilies are very different, however. To understand GnRH as a functional peptide, it is necessary to consider the evolution of the pituitary gland, which is the main target of GnRH in vertebrates. It is very possible that AKH and corazonin share a common ancestor with GnRH and after the duplication of this ancestral peptide, within the ecdysozoan lineage, AKH and corazonin evolved without GnRH (Roch et al., 2011). The functions of the invertebrate GnRH-like peptides are not necessarily related to reproduction. Roch et al. (2011) suggest that structurally related families of invertebrate peptides including corazonin and adipokinetic hormone (AKH) form a superfamily of neuropeptides with the GnRH family. The obtained data from both identified sequences and from precise predictions strongly support the conclusion that such a superfamily, which consists of GnRH, AKH and corazonin receptors, exists. Closely related to the GnRH-R superfamily is also the vasopressin/oxytocin superfamily of receptors (Roch et al., 2011). It is interesting that throughout vertebrate genomes there is the arrangement of GnRH II and VP/OT genes as adjacent neighbors on the chromosome (Gwee et al., 2009).

Recent evidence suggests that invertebrate AKH, ACP and corazonin receptors are phylogenetically closer to the GnRH receptors than the VP/OT receptors. The GnRH, AKH, ACP and corazonin receptors form a monophyletic clade suggesting that they should be grouped as one superfamily. In addition, the VP and OT receptors form their own superfamily, which is phylogenetically the most homologous to the GnRH receptor superfamily (Roch et al., 2011).

It is compelling that synteny analysis shows that GnRH II remained the nearest genomic neighbor to oxytocin throughout the whole evolution of vertebrates. Vasopressin is the other neighbor of oxytocin on the chromosome. This syntenic arrangement of GnRH II-OT-VP shows that each peptide is orthologous in vertebrates, justifying the names of vasopressin, oxytocin, and GnRH II to be used for all vertebrates (Larhammar et al., 2009). The synteny of these genes is disrupted, however, in tunicates and amphioxus (Gwee et al., 2009; Tello and Sherwood, 2009) and therefore it is not possible to identify the orthologs and paralogs beyond the vertebrates.

Finally, considering the relationship of the GnRH-R superfamily to the vasopressin/oxytocin receptor (VPR/OTR) superfamily, probably these two large superfamilies with deep roots in the invertebrates, share a common ancestor at the transition to bilaterians. Although a relationship cannot be determined between

the superfamily hormones by homology, their conserved synteny as genomic neighbors throughout the vertebrates indicates that the GnRH and VP/OT systems co-evolved for some time. The receptors in these superfamilies perform many physiological functions in vertebrates, but there is only a little overlap established between specific GnRH and VP/OT functions, one of them is the slight influence of oxytocin on LH release from pituitary gonadotrope (Roch et al., 2011).

## CONCLUSIONS

During 400 million years of evolution, the N- and C- terminals of GnRH were well conserved as functional domains for binding and activating of cognate receptors to accomplish physiological functions. About 400 millions years ago, a single substitution of the chiral amino acid in position 6 of GnRH in jawless fish by the achiral glycine facilitated formation of a type II'  $\beta$ -turn conformation of GnRH to allow close spatial interaction of these two functional elements.

Most substitutions of a d-amino acid in position 6 have a limited effect on binding affinity for GnRH II. This native GnRH ligand, unlike the other natural GnRHs, is preconfigured early in evolution in a bioactive configuration through intramolecular interactions, which account for its relatively high binding affinity and explain the total and unique conservation of its primary structure over the entire 400 million years of evolution. It should be stressed that the surprising total conservation of GnRH II's primary structure, from bony fish to man, appears to be a result of the excellent coordinated evolutionary selection of amino acids participating in binding, activation and configuration such that its structure cannot be improved by substitution with any natural amino acid at any position. The major determinant of bioactive conformation at mammalian GnRH type I receptors, the type II'  $\beta$ -turn involving residues 5-8, is also important for high-affinity binding at non-mammalian GnRH receptors.

The present knowledge concerning the evolution of the GnRH structure is mainly due to the research of Professor P. Millar and his expert and talented coworkers in different laboratories, and to other leading scientists in this field, but also to the great number of indefatigable researchers exploring the different aspects of GnRH, from the discovery of neurosecretion until the present time.

The discovery of the fact that one decapeptide molecule, among the GnRHs, was constructed perfectly at the beginning of 400 million years evolution and that it is not possible to improve its physiological potency using the any natural amino acid is, in my opinion, important, fascinating and beautiful.

Maria Skłodowska-Curie said: “*In my opinion science is something very beautiful and also very useful.*” It is well to mention that she also said: “*I was taught that the way to scientific success is neither simple nor easy*”.

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