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## GnRHs and GnRH receptors<sup>☆</sup>

Robert P. Millar\*

*MRC Human Reproductive Sciences Unit, The Chancellor's Building, 49 Little France Crescent,  
Edinburgh EH16 4SB, Scotland, UK*

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### Abstract

GnRH is the pivotal hypothalamic hormone regulating reproduction. Over 20 forms of the decapeptide have been identified in which the NH<sub>2</sub>- and COOH-terminal sequences, which are essential for receptor binding and activation, are conserved. In mammals, there are two forms, GnRH I which regulates gonadotropin and GnRH II which appears to be a neuromodulator and stimulates sexual behaviour. GnRHs also occur in reproductive tissues and tumours in which a paracrine/autocrine role is postulated. GnRH agonists and antagonists are now extensively used to treat hormone-dependent diseases, in assisted conception and have promise as novel contraceptives. Non-peptide orally-active GnRH antagonists have been recently developed and may increase the flexibility and range of utility. As with GnRH, GnRH receptors have undergone co-ordinated gene duplications such that cognate receptor subtypes for respective ligands exist in most vertebrates. Interestingly, in man and some other mammals (e.g. chimp, sheep and bovine) the Type II GnRH receptor has been silenced. However, GnRH I and GnRH II still appear to have distinct roles in signalling differentially through the Type I receptor (ligand-selective-signalling) to have different downstream effects. The ligand–receptor interactions and receptor conformational changes involved in receptor activation have been partly delineated. Together, these findings are setting the scene for generating novel selective GnRH analogues with potential for wider and more specific application.

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*Keywords:* GnRH I; GnRH II; Agonists; Antagonists; Analogues; GnRH receptors

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\* Tel.: +44 131 242 6227; fax: +44 131 242 6231.

*E-mail address:* r.millar@hrsu.mrc.ac.uk.

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## 1. Introduction

Gonadotropin-releasing hormone (GnRH), the central initiator of the reproductive hormonal cascade, was first isolated from mammalian hypothalami as the decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH<sub>2</sub>) (Schally et al., 1971; Matsuo et al., 1971; Baba et al., 1971). GnRH is processed in specialised neurones of the hypothalamus from a precursor polypeptide by enzymic processing and packaged in storage granules that are transported down axons to the external zone of the median eminence (Fink, 1988; Seeburg et al., 1987). The hormone is released in synchronised pulses from the nerve endings of about 1000 neurones into the hypophyseal portal system every 30–120 min to stimulate the biosynthesis and secretion of LH and FSH from pituitary gonadotropes (Fink, 1988). Each GnRH pulse stimulates a pulse of LH but FSH pulses are less clear. 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 GnRH, and differences in the half-lives of the two hormones. Moreover, while LH is stored and largely dependent on GnRH for secretion, FSH tends to be constitutively secreted and more dependent on biosynthesis for secretion.

## 2. Current concepts and applications of GnRH analogues

Low doses of GnRH delivered in a pulsatile fashion equivalent to that found in the portal vessels (pg/ml) restore fertility in hypogonadal men and women, and are also effective in the treatment of delayed puberty (Millar et al., 1987; Casper, 1991; Conn and Crowley, 1991; Barbieri, 1992; Moghissi, 1992; Conn and Crowley, 1994; Filicori, 1994). However, high doses of natural GnRH or agonist analogues desensitise the gonadotrope with resultant decrease in LH and FSH and a decline in ovarian and testicular function (Millar et al., 1987; Casper, 1991; Conn and Crowley, 1991; Barbieri, 1992; Moghissi, 1992; Conn and Crowley, 1994; Filicori, 1994; Emons and Schally, 1994). This desensitisation phenomenon is extensively applied in clinical medicine for the treatment of a wide range of disease (Millar et al., 1987; Casper, 1991; Conn and Crowley, 1991, 1994; Barbieri, 1992; Moghissi, 1992; Filicori, 1994; Emons and Schally, 1994) (Table 1). GnRH peptide antagonists also inhibit the reproductive system through competition with endogenous GnRH but the doses required are higher than the desensitising agonist doses and antagonists are currently less extensively employed (see Chapter 2). The development of non-peptide orally-active GnRH antagonists (Millar et al., 2000) are likely to replace agonists as they avoid the undesirable stimulation that precedes desensitisation. In addition to the therapeutic applications, GnRH analogues have promise as new generation male and female contraceptives in conjunction with steroid hormone replacement (Nieschlag et al., 1992; Fraser, 1993).

The extensive clinical applications of GnRH analogues have attracted detailed studies of the physiological, cell biological and molecular function of the hormone to enhance our understanding of the entire system and for the optimal application of analogue therapies.

Table 1  
Clinical applications of GnRH and GnRH analogues

Pulsatile GnRH (stimulation)	
Infertility	Stimulates gamete and hormone production
Cryptorchidism	Descent of testes
Delayed puberty	Advances puberty
GnRH agonists and antagonists (inhibition)	
Contraception	Inhibition of ovulation and spermatogenesis with add-back sex steroid hormones
Hormone-dependent diseases	Prostatic cancer
	Benign prostatic hypertrophy
	Breast cancer
	Endometriosis
	Uterine fibroids
	Premenstrual syndrome
	Polycystic ovarian syndrome
	Hirsutism
	Acne vulgaris
	Precocious puberty
Infertility	Inhibition of endogenous gonadotropin together with controlled administration of exogenous gonadotropin, especially in induction of ovulation in assisted reproduction techniques

This overview describes the structure of various forms of GnRH and their evolution, their tissue distribution and putative functions, the essential structural features and actions of GnRH analogues and their interaction with cognate receptors.

### 3. Primary structures of gonadotropin-releasing hormones

Although the isolated mammalian hypothalamic GnRH was thought to be a unique structure with a primary role in regulating LH and FSH, it became apparent that diverse forms exist in vertebrates (King and Millar, 1980). This led to the structural identification of 23 different forms (Millar et al., 1987, 1997; Millar and King, 1988; King and Millar, 1992, 1995, 1997a,b; Sherwood, 1987; Sherwood and Lovejoy, 1989; Sherwood et al., 1993; Jimenez-Linan et al., 1997; Yoo et al., 2000; Okubo et al., 2000a,b; Adams et al., 2003) (Fig. 1). In most vertebrates there are at least two, and usually three, forms of GnRH (Millar et al., 1987, 1997; Millar and King, 1988; King and Millar, 1992, 1995, 1997a,b; Sherwood, 1987; Sherwood and Lovejoy, 1989; Sherwood et al., 1993; Sealfon et al., 1997; Troskie et al., 1998). The most ubiquitous is chicken GnRH II (cGnRH II) which was first isolated from chicken brain (Miyamoto et al., 1984). Since the cGnRH II structure is totally conserved from bony fish to men, this is probably the earliest evolved form and has critical functions (Millar and King, 1987). We have designated this form Type II GnRH II (GnRH) while the hypophysiotropic form is designated GnRH I (Sealfon et al., 1997). In many vertebrate species a third form of GnRH (salmon GnRH, Fig. 1) is present and is designated GnRH III (White et al., 1994). Analysis of the genes encoding the GnRHs supports this general classification (White et al., 1994).

	1	2	3	4	5	6	7	8	9	10	
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH <sub>2</sub>
Guinea Pig	pGlu	Tyr	Tyr	Ser	Tyr	Gly	Val	Arg	Pro	Gly	NH <sub>2</sub>
Chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH <sub>2</sub>
Rana d.	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly	NH <sub>2</sub>
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH <sub>2</sub>
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH <sub>2</sub>
Medaka	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly	NH <sub>2</sub>
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH <sub>2</sub>
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH <sub>2</sub>
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH <sub>2</sub>
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH <sub>2</sub>
Lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly	NH <sub>2</sub>
Lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly	NH <sub>2</sub>
Chelyosoma I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH <sub>2</sub>
Chelyosoma II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly	NH <sub>2</sub>
Ciona I	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly	NH <sub>2</sub>
Ciona II	pGlu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly	NH <sub>2</sub>
Ciona III	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly	NH <sub>2</sub>
Ciona IV	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly	NH <sub>2</sub>
Ciona V	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly	NH <sub>2</sub>
Ciona VI	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly	NH <sub>2</sub>
Ciona VII	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly	NH <sub>2</sub>
Octopus	pGlu	Asn	Tyr	Ser	Phe	Ser	Trp	His	Pro	Gly	NH <sub>2</sub>

Ser
   
 / \
 Asn Gly

Fig. 1. Primary amino acid sequences of naturally occurring GnRHs spanning approximately 600 million years of evolution. The boxed regions show the conserved NH<sub>2</sub> and COOH terminal residues which play important functional roles. Non-conserved residues are either unimportant or convey ligand-selectivity for a particular GnRH receptor. Note that 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 also present in amphibian and primitive bony fish species, and chicken GnRH II is present in most vertebrate species, including man.

#### 4. Functional domains and conformation of GnRH and analogues

The 23 GnRHs from vertebrate and protochordate species reveal features which have been conserved for more than 500 million years of evolution (Fig. 1). The NH<sub>2</sub>-terminus

(pGlu-His-Trp-Ser) and COOH-terminus (Pro-Gly NH<sub>2</sub>) sequences are conserved, indicating that these features are critically important for receptor binding and activation (Fig. 2). This is confirmed by structure/activity data from thousands of analogues which were developed largely on an empirical basis. Position eight is the most variable amino acid, followed by positions six, five and seven. The considerable variation in position eight suggests that virtually any residue is tolerated in this position. However, this has been known for some time to not be the case for the mammalian pituitary Type I receptor (Sealfon et al., 1997; Karten and Rivier, 1986) which requires Arg in position eight, and recent work on cloned non-mammalian receptors also indicates certain specificities for the amino acid in position eight (Millar et al., 1989, 1997; Illing et al., 1999). Thus, this residue seems to play an important role in ligand-selectivity of the different GnRH receptors.

The mammalian pituitary GnRH receptor appears to be more stringent in its requirements of ligand conformation (Millar et al., 1987, 1989, 1997; King and Millar, 1995; Sealfon et al., 1997; Millar, 2002). Studies have indicated that the conserved NH<sub>2</sub>- and COOH-terminal domains of GnRH are closely opposed when mammalian GnRH binds its receptor resulting from a  $\beta$ -II type turn involving residues 5–8 (Sealfon et al., 1997; Karten and Rivier, 1986) (Fig. 2). This is partly due to intramolecular interactions with side chain of Arg<sup>8</sup>, as various studies, including Trp fluorescence (Milton et al., 1983) computer simulations using the technique of conformational memories (Guarnieri and Weinstein, 1996) and NMR (Maliekal et al., 1997) have shown that substitution of Arg<sup>8</sup> (e.g. with Gln<sup>8</sup> as in chicken GnRH I) results in a more extended structure with a loss of predominance of the folded conformers and a low biological activity. Yet these extended forms (e.g. Gln<sup>8</sup>GnRH) have high activity in many non-mammalian GnRH receptors (Sealfon et al., 1997; Illing et al., 1999; Millar et al., 1989; Tensen et al., 1997) in spite of their low activity at mammalian

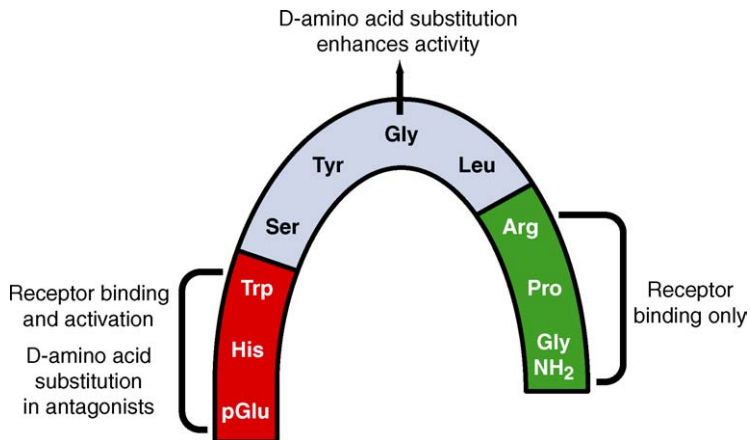


Fig. 2. Schematic representation of mammalian GnRH in the folded conformation in which it is bound to the GnRH pituitary receptor. The molecule is bent around the flexible glycine in position six. Substitution with D-amino acids in this position stabilises the folded conformation and increases binding affinity and decreases metabolic clearance. This feature is incorporated in all agonist and antagonist analogues. The NH<sub>2</sub> (red) and COOH (green) termini are involved in receptor binding. The NH<sub>2</sub> terminus alone is involved in receptor activation and substitutions in this region produce antagonists (see Millar, 2002).

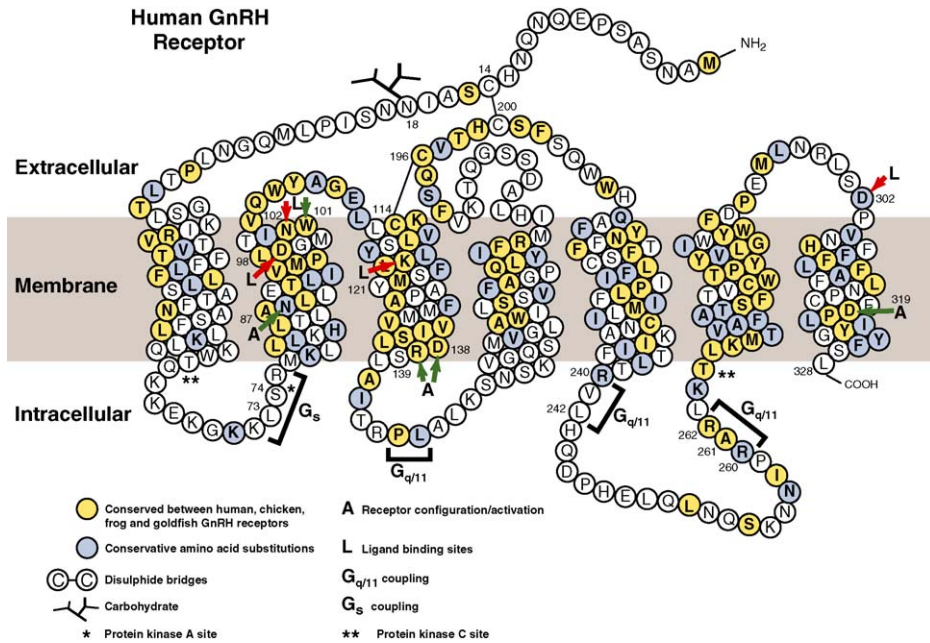


Fig. 3. Two-dimensional representation of the human GnRH receptor showing amino acids conserved between cloned vertebrate GnRH receptors (yellow) and conservative substitutions (blue). Putative ligand binding sites and residues important in receptor configuration, activation and G-protein coupling are indicated. Note the conservation of residues on single faces of TMs suggests those whose side chains probably face into the hydrophilic interior of the pocket formed by the helices (Fig. 6). Glycosylation, phosphorylation and disulphide bridge sites are also shown (see Millar, 2002).

receptors (Flanagan et al., 1994; Fromme et al., 2001). The  $\beta$ -II type turn conformation of GnRH also appears to be induced in part by the interaction of Arg<sup>8</sup> with an acidic residue (Asp<sup>302</sup>) in extracellular loop 3 (EC3) of the mammalian receptor (Sealfon et al., 1997; Flanagan et al., 1994) (Fig. 3). Substitution of a D-amino acid for Gly<sup>6</sup> enhances the  $\beta$ -II type conformation and increases the activity of Arg<sup>8</sup> GnRH about 10-fold in mammals. The D-amino acid substitution overcomes the deleterious effects of Arg<sup>8</sup> substitution (e.g. with Gln<sup>8</sup>) such that binding affinity for the mammalian receptor is increased almost 1000-fold (Millar et al., 1989; Flanagan et al., 1994).

The amino-terminal residues of GnRH are involved in receptor activation and modification of these residues in GnRH produces analogues with antagonistic properties (Sealfon et al., 1997; Karten and Rivier, 1986) (Fig. 2). As in agonists, substitution of Gly<sup>6</sup> with a D-amino acid enhances the activity of the antagonists.

**5. Diverse functions of gonadotropin-releasing hormones**

As well as its function as a stimulator of gonadotropin release GnRH has diverse functions in vertebrates and protochordates. These include neuroendocrine (e.g. growth

hormone release in certain fish species), paracrine (e.g. in placenta and gonads), autocrine (e.g. GnRH neurones, immune cells, breast and prostatic cancer cells) and neurotransmitter/neuromodulatory roles in the central and peripheral nervous system (e.g. sympathetic ganglion, mid-brain) (Millar et al., 1987, 1997; Emons and Schally, 1994; Millar and King, 1987) (Millar and King, 1988; King and Millar, 1992, 1995, 1997a,b; Sherwood, 1987; Sherwood and Lovejoy, 1989; Sherwood et al., 1993; Hsueh and Schaeffer, 1985; Jenness and Conn, 1994; Sealfon et al., 1997).

The isolation of several forms of GnRH in neural tissue of tunicates and their activation of the gonads (Powell et al., 1996; Adams et al., 2003) suggests that direct regulation of the gonads was an early evolved function and that the neuroendocrine role in regulating the pituitary was a later evolutionary development. The presence of GnRH and GnRH receptors in the gonads of various vertebrate species, including mammals, may reflect this early function. Neurones are probably one of the earliest cells in evolution to elaborate GnRH peptides.

GnRH peptides may have been originally involved in cellular communication in sexual reproduction of simple unicellular and multicellular organisms. Later they were recruited for expression in nerve cells to translate external and internal signals into activation of reproduction, initially by acting directly on germ cells, and subsequently via pituitary gonadotrope activation. Additional elaboration of GnRH utilisation was in sensitising the olfactory neuroepithelium to pheromones in bony fish and in activating neurones in the CNS to affect sexual behaviour. While the peptide has been co-opted as a regulator at a number of levels in the reproductive system (hypothalamus, gonad, breast, uterus, placenta) there is apparently considerable plasticity in also recruiting it as a regulator in non-reproductive tissues (e.g. adrenal, extrahypothalamic brain, the immune system, retina, pancreas) (Millar et al., 1987; Millar and King, 1988; King and Millar, 1992, 1995; Hsueh and Schaeffer, 1985; Jenness and Conn, 1994).

## 6. Putative functions of the evolutionarily conserved Type II GnRH

The second form of GnRH identified from chicken brain (chicken GnRH II, GnRH II) (Fig. 1) is ubiquitous in vertebrates from primitive bony fish to man (Millar et al., 1987, 1997; Millar and King, 1988; King and Millar, 1992, 1995, 1997a,b; Sherwood, 1987; Sherwood and Lovejoy, 1989; Sherwood et al., 1993; White et al., 1994; Lescheid et al., 1997). This complete conservation of structure for more than 500 million years suggests that GnRH II has an important function and a discriminating receptor (or receptors) which has selected against any structural change in the ligand. The wide distribution of GnRH II in the central and peripheral nervous systems suggests a neurotransmitter/neuromodulatory role. The most thorough studies have demonstrated the inhibition of M currents in the bullfrog sympathetic ganglion which sensitises neurones to depolarisation (Jones, 1987). The mediating receptor in the amphibian sympathetic ganglia is highly selective for GnRH II (Troskie et al., 1997).

Since GnRH had been shown to have direct effects on sexual arousal in rodents (Moss, 1979; Muske, 1993; Rissman et al., 1997) and GnRH II is localised in brain areas associated with reproductive behaviour, it was suggested that this may be a role for the peptide (Moss,

1979; Muske, 1993; Rissman et al., 1997; Millar, 2003). GnRH II and an analogue were found to be potent stimulators of reproductive behaviour in ring doves (King and Millar, 1995, 1997a,b) and song sparrows (Maney et al., 1997). Recently the cognate receptor for GnRH II was cloned from the marmoset and found to be distributed in those areas of primate brain associated with reproductive behaviours (Millar et al., 2001). In nutritionally compromised musk shrews GnRH II stimulated reproductive behaviour while GnRH I is inactive (Temple et al., 2003) and most recently we have shown stimulation of sexual behaviour by GnRH II in female marmosets (unpublished).

In addition to its apparent role as a neuromodulator in the nervous system, GnRH II (White et al., 1994) and a Type II receptor transcript (Millar et al., 2001; Morgan et al., 2003) are present in non-neural reproductive tissues such as the prostate. GnRH binding sites and antiproliferative effects of GnRH analogues have been described in reproductive tissue tumours and their cell lines (Millar et al., 1987; Conn and Crowley, 1991, 1994; Emons and Schally, 1994). Interestingly the GnRH binding sites, signalling and pharmacological effects of analogues were not in accordance with these being via Type I GnRH receptors but were more similar to Type II GnRH receptors (Emons and Schally, 1994; Millar et al., 2001). However, a full-length Type II GnRH receptor is absent from man, chimpanzee, cow, horse, sheep, rat and mouse in spite of its presence in marmoset (Fig. 4), green monkey, rhesus monkey and pig (Morgan et al., 2003) (and unpublished data). We have now been able to rationalise that silencing of the Type II receptor can be accommodated by GnRH II signalling through the Type I receptor with a distinctly different pharmacological profile (Maudsley et al., 2004). We have called this phenomenon ligand-induced-selective-signally (LISS) (Millar and Pawson, 2004).

## 7. GnRH receptors

### 7.1. Primary structures

The amino acid sequence of the GnRH receptor was first deduced for the mouse receptor cloned from the pituitary  $\alpha$ T3 gonadotrope cell line (Tsutsumi et al., 1992). This sequence was confirmed (Reinhart et al., 1992) and provided the basis for the cloning of GnRH pituitary receptors from the rat (Kaiser et al., 1992; Perrin et al., 1993; Eidne et al., 1992), human (Chi et al., 1993; Kakar et al., 1992) (Fig. 3), sheep (Illing et al., 1993; Brooks et al., 1993), cow (Kakar et al., 1993) and pig (Weesner and Matteri, 1994) which share over 80% amino acid identity. Homologues of the mammalian GnRH receptors have also been cloned from a marsupial (possum) (King et al., 2000), catfish (Tensen et al., 1997) two forms from the goldfish (Illing et al., 1999), *Rana* (Wang et al., 2001a,b), *Xenopus* (Troskie et al., 2000), chicken (Sun et al., 2000), Medaka (Okubo et al., 2001), striped bass (Alok et al., 2000), trout (Madigou et al., 2000), cichlid ((Robison et al., 2001), Japanese eel (Okubo et al., 2000a,b), amberjack (CAB 65407), rubber eel (AD 49750) and more recently many other fish species (Fig. 4). The non-mammalian receptors with greatest homology to the mammalian pituitary receptors have 42–47% amino acid identity with the mammalian receptors but 58–67% identity amongst each other. We have designated all of these as Type I GnRH receptors (Fig. 4). It is not yet altogether clear from homology comparisons that this classification

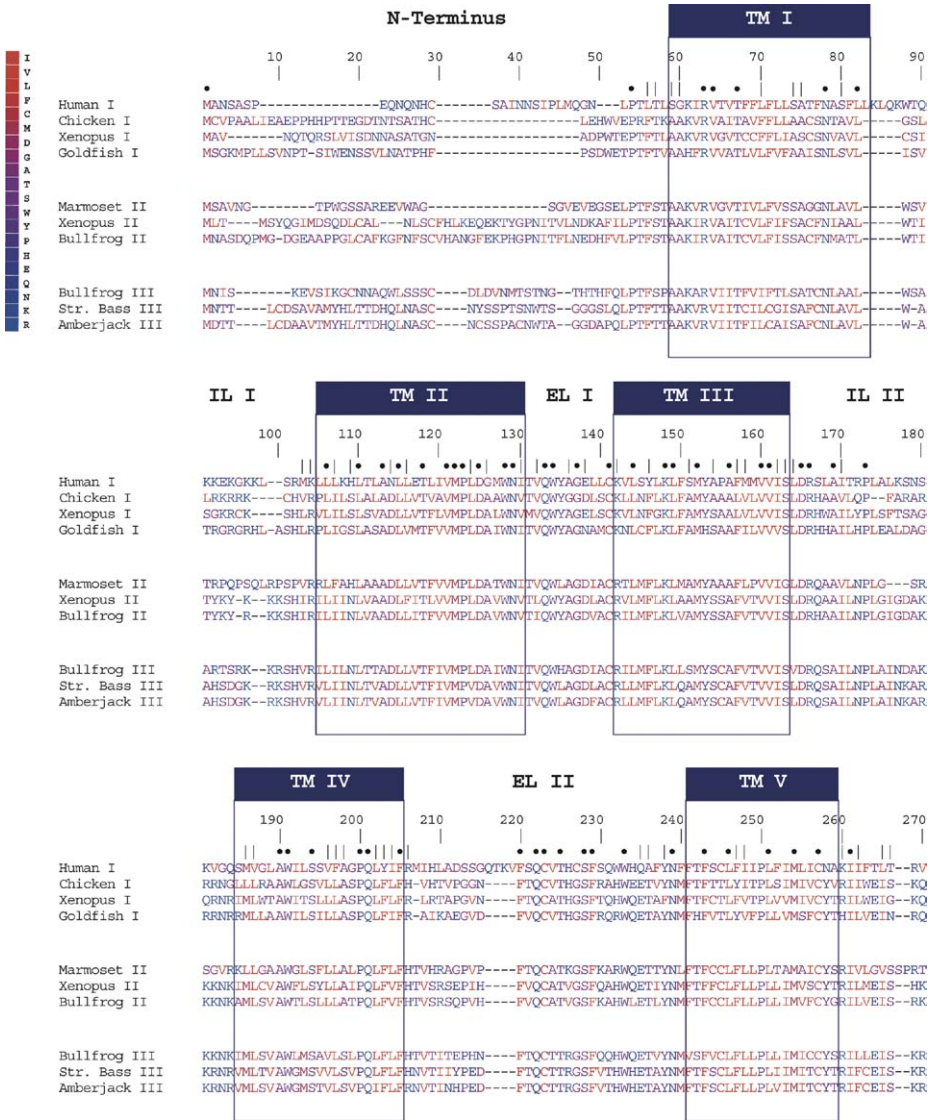


Fig. 4. Alignment of representative GnRH Types I, II and III receptors from selected species. The transmembrane domains and the intracellular loops (IL) and extracellular loops (EL) indicated. The overall identity (●) or homology (|) is indicated above. The consensus for the most characteristic domain (EL III going into TM VII) of the three receptor types is shown. This domain was used to clone the three receptor types. The colours of the amino acids are graded from red (most hydrophobic) through to blue (most hydrophilic). Note that TM domains are predominantly hydrophobic and loop domains hydrophilic (see Millar, 2002).



*Rana* (Wang et al., 2001a,b) (Fig. 4). The findings suggest an early evolution of the three GnRH receptor subtypes in vertebrates which parallels that of the GnRH ligands. GnRH receptor orthologues have been identified in *Drosophila melanogaster* (Hauser et al., 1998) and *C. elegans* (Swanson et al., unpublished) indicating a very early evolutionary origin.

GnRH receptors have the characteristic features of G-protein coupled receptors (GPCRs) (Figs. 3 and 4). The NH<sub>2</sub>-terminal domain is followed by seven putative  $\alpha$ -helical transmembrane (TM) domains connected by three extracellular loop domains (EL) and three intracellular loop domains (IL) (Figs. 3 and 4). The extracellular domains and superficial regions of the TMs are usually involved in binding of peptide hormones like GnRH, the TMs are believed to be involved in conformational change associated with signal propagation (receptor activation), while the intracellular domains are involved in interacting with G-proteins and other proteins for intracellular signal transduction.

A unique feature of the mammalian GnRH receptor is the absence of a carboxy terminal tail present in all other GPCRs and in all of the non-mammalian GnRH receptors. This suggests a recently evolved feature which presumably serves an important role in the functioning of the mammalian GnRH receptor (see below).

The conservation of amino acids during evolution from bony fish to mammals is likely to identify those residues which are crucial for GnRH receptor function (Millar et al., 1997; Sealfon et al., 1997). These are shown in Fig. 3 and include those involved in receptor activation and W (P<sup>223</sup>, P<sup>282</sup> and P<sup>320</sup> which are conserved in all of the rhodopsin family of GPCRs).

### 7.2. Tertiary structure of the mammalian Type I GnRH receptor

A knowledge of the three-dimensional structure of the GnRH receptor is essential for a complete understanding of its molecular functioning. To date it has only been possible to crystallise and obtain X-ray structural information for the rhodopsin GPCR (Palczewski et al., 2000). Other structural information on GPCRs is derived from low resolution electron microscopy of bacteriorhodopsin and rhodopsin (Sealfon et al., 1997; Sealfon and Millar, 1994; Flanagan et al., 1997; Schertler et al., 1993). Structural information for other GPCRs relies on molecular models (Sealfon et al., 1997; Naor et al., 1998; Baldwin, 1993; Lesk and Boswell, 1992; Donnelly et al., 1989; Ballesteros and Weinstein, 1992) which are based on the alignment with rhodopsin.

The development of a GnRH receptor molecular model is based on initial positioning of the TM helices as in rhodopsin and then refinement of the angles, kinking and side chain orientation of the TMs based on the specific amino acids comprising the GnRH receptor TMs (Sealfon et al., 1997). The validity of the model and proposed interactions of the TM side chains may be tested by site-directed mutagenesis. An example is our observation that two residues that are highly conserved in GPCRs, Asp in TM2 and Asn in TM7, appear to have undergone reciprocal mutation to Asn<sup>87</sup> and Asp<sup>318</sup> in the mouse GnRH receptor (Asp<sup>319</sup> in human) (Fig. 3). This suggests that the two residues interact with each other. Mutation of Asn<sup>87</sup> in TM2 to Asp abolished receptor function, but a second mutation in TM7, recreating the arrangement found in other GPCRs (Asp<sup>87</sup>Asn<sup>318</sup>), regenerated ligand binding (Zhou et al., 1994). This restoration of binding by reciprocal mutation demonstrates that the side chains of two residues in helices TM2 and 7 have complementary roles in

maintaining the structure of the receptor and occupy the same microenvironment within the receptor as indicated in the rhodopsin crystal structure (Palczewski et al., 2000). Although the structural integrity of the receptor is restored by these reciprocal mutations receptor activation machinery is impaired.

The seven TM helical domains are known to be arranged in a tight bundle enclosing a hydrophilic pocket and surrounded by the hydrophobic membrane environment (Sealfon et al., 1997; Palczewski et al., 2000; Flanagan et al., 1997; Schertler et al., 1993; Naor et al., 1998; Baldwin, 1993; Lesk and Boswell, 1992; Donnelly et al., 1989; Ballesteros and Weinstein, 1992). The evolutionary conservation of residues along a distinct face of the TM domains in the various GnRH receptors is clearly evident in Fig. 3. This suggests that the conserved, more hydrophilic faces are orientated towards the hydrophilic pocket formed by the helical bundle. This is supported by the studies on the TM2/TM7 interaction of Asn and Asp since Asn<sup>87</sup> is clearly part of the conserved hydrophilic face of TM2 (Fig. 3).

The relative positioning of TM3 and TM4 could be deduced by the demonstration that Cys<sup>114</sup> in EC1 and Cys<sup>196</sup> in EC2 form a disulphide bridge (Fig. 3). This was determined by a combination of photoaffinity labelling with photoactive GnRH followed by protease digestion, reduction of S–S bonds and separation of the receptor fragments by gel electrophoresis (Davidson et al., 1997). The study also indicated that Cys<sup>14</sup> in the NH<sub>2</sub> terminal domain and Cys<sup>200</sup> in EC2 form a second disulphide bridge thus further defining the position of NH<sub>2</sub> terminus and EC2 loop structures.

Glycosylation sites have been shown at Asn<sup>4</sup> and Asn<sup>18</sup> in the mouse and Asn<sup>18</sup> in the human receptors (Davidson et al., 1995, 1996a,b) (Fig. 3). Glycosylation does not influence receptor binding affinity of GnRH but increases the number of receptors on the cell membrane. Introduction of the additional mouse receptor glycosylation site in the human receptor increased receptor number (Davidson et al., 1996a,b).

Although significant progress has been made in establishing a molecular model of the transmembrane helix bundle of the GnRH receptor, our knowledge of the structure of the extracellular and intracellular loops is scant. Considerable effort has been put into establishing programmes to define loop structures. However these are not applicable to large loop sequences. Thus a future challenge is the determination of the structure of the loops in the GnRH receptors. Some progress has been made in establishing the structure of EC3 by NMR structural analysis of a synthetic peptide of EL3 anchored by cross links similar to the distance between the TM6 and TM7 (Petry et al., 2002).

## 8. Binding sites of GnRH at the mammalian Type I GnRH receptor

In the course of the targeted mutation of almost one third of all the amino acid residues of the GnRH receptor (Millar et al., 2004) considerable advances in identifying putative ligand contact sites in the mammalian GnRH receptor have been made (Figs. 3 and 5).

### 8.1. Aspartate<sup>302</sup> (D<sup>302</sup>)

The arginine in position eight of mammalian GnRH is essential for high affinity binding and selectivity of the mammalian GnRH receptor (Sealfon et al., 1997; Illing et al., 1999; Millar et al., 1989; Millar and King, 1983; Flanagan et al., 1994) Flanagan et al postulated

that acidic amino acids would be candidates through ionic interaction with the positive Arg side chain. Mutation of all extracellular acidic amino acids to their isosteric amides revealed that only mutation of Glu<sup>301</sup> to Gln<sup>301</sup> in EC3 of the mouse receptor resulted in an appropriate decrease in affinity of 100-fold and loss of selectivity between Arg<sup>8</sup> and Gln<sup>8</sup> GnRH and improved binding of Glu<sup>8</sup> GnRH. These data are consistent with an interaction of Arg<sup>8</sup> in native GnRH with Glu<sup>301</sup> in EL3. Due to the presence of an additional amino acid in EL2 of the human receptor, the equivalent residue is Asp<sup>302</sup> (Figs. 3 and 4) which has the same property as Glu<sup>301</sup> (Fromme et al., 2001; Hoffmann et al., 2000). These data therefore support the concept that Glu<sup>301</sup> (Asp<sup>302</sup> in the human) GnRH receptor determines selectively for Arg<sup>8</sup>. These residues are able to interact when GnRH is docked to the GnRH receptor model (Fig. 5). Since analogues which are constrained in the  $\beta$ II conformation bind with high affinity regardless of the amino acid in position eight and the Glu<sup>301</sup> in the mouse receptor, it appears that the role of Arg<sup>8</sup> interaction with the acidic residue in the receptor is to induce or select the  $\beta$ II conformation of the ligand (Fromme et al. (2001) which allows its binding to the other sites listed below.

### 8.2. Lysine<sup>121</sup> (K<sup>121</sup>)

The highly conserved Asp in TM3 of the biogenic amine receptors which interacts with the positively charged amine head group was postulated to play a similar role in the GnRH receptor. The equivalent residue is Lys<sup>121</sup> in the GnRH receptor and mutation to Asp, Ala or Leu leads to a major decrease in agonist binding while mutations to Gln results in a 1000-fold reduction in agonist binding affinity without affecting antagonist binding (Zhou et al., 1995). We proposed that the Lys<sup>121</sup> interacts with His<sup>2</sup> or pGlu<sup>1</sup> of GnRH (Figs. 3 and 5) by a charge-strengthened hydrogen bond. This was confirmed (Hoffmann et al., 2000) and shown to be feasible (particularly for pGlu<sup>1</sup>) in molecular models (Sealfon et al., 1997; Hoffmann et al., 2000; Millar et al., 2004).

### 8.3. Asparagine<sup>102</sup> (N<sup>102</sup>)

We postulated that Asn<sup>102</sup>, located near the extracellular surface of TM2, forms a hydrogen bond with the C=O moiety of Gly<sup>10</sup>NH<sub>2</sub> in GnRH (Figs. 3 and 5). Mutation to Ala results in a 100–1000-fold loss of potency in Gly<sup>10</sup>NH<sub>2</sub> GnRH analogues in stimulating phosphoinositol hydrolysis while having much less effect on the potency of NH–CH<sub>2</sub>–CH<sub>2</sub><sup>10</sup> analogues (Davidson et al., 1996a,b) which appear to make an alternative contact. Another study confirmed these findings (Hoffmann et al., 2000) and also showed that mutation of the adjacent Trp<sup>101</sup> also displayed a much greater decline in binding affinity of Gly<sup>10</sup>NH<sub>2</sub> analogues than for the NH–CH<sub>2</sub>–CH<sub>2</sub><sup>10</sup> analogues. However this group concluded that the effects of the Trp<sup>101</sup> mutation were indirect in distorting the binding pocket. Interestingly, antagonist binding was affected only slightly by the mutations indicating that the antagonist binding site differs (Hoffmann et al., 2000).

### 8.4. Aspartate<sup>98</sup> (D<sup>98</sup>)

In mutating all extracellular acidic residues as putative interacting sites for Arg<sup>8</sup> of GnRH, it was noted that mutation of Asp<sup>98</sup> to Asn resulted in a large decrease in inositol

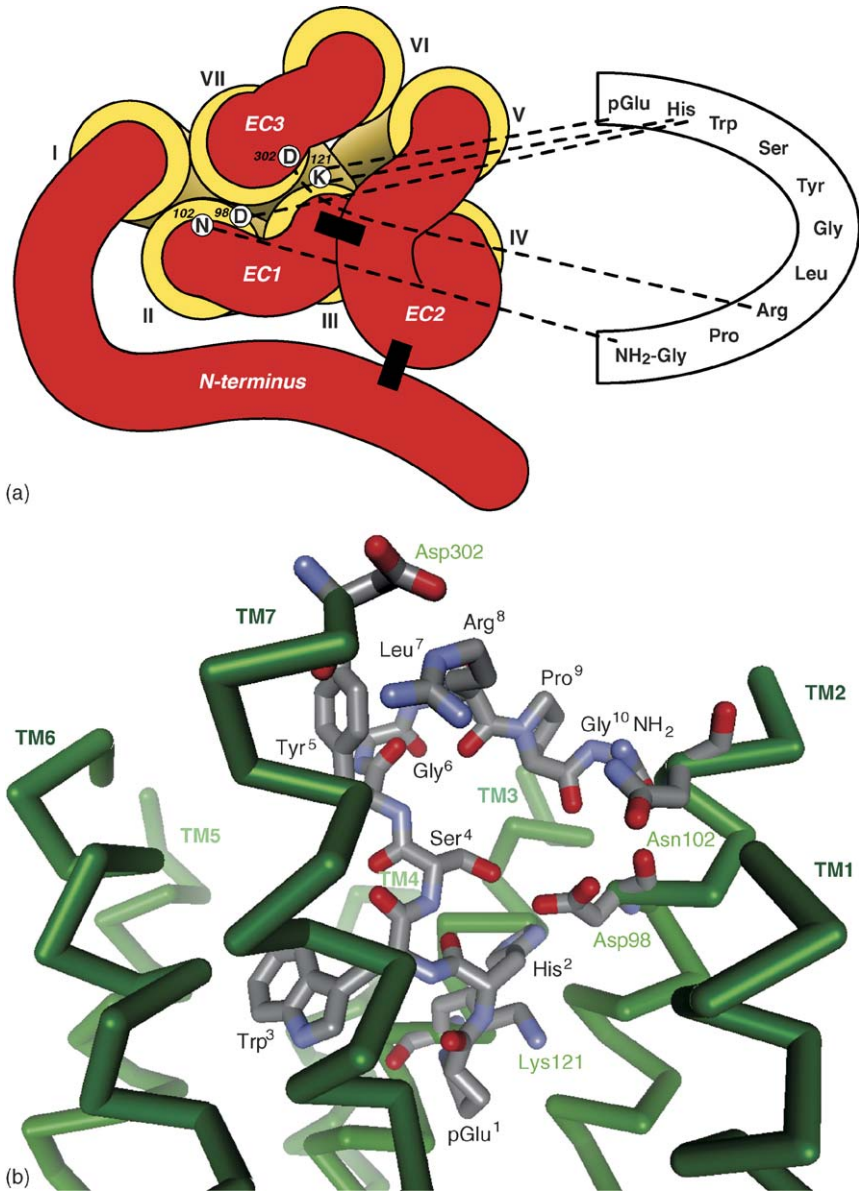


Fig. 5. GnRH binding to its receptor. (a) A schematic representation of GnRH interaction with the human GnRH receptor. The receptor is viewed from above and shows the transmembrane helices as a cluster of cylinders (yellow; going into the page) which encompass the hydrophilic pocket and are surrounded by the light hydrophobic membrane environment. The TM helices are connected by the extracellular loops (red). The dark bands represent the disulphide bridges stabilising extracellular domains. GnRH is shown in its folded conformation interacting via pGlu<sup>1</sup>, His<sup>2</sup>, Arg<sup>8</sup> and GlyNH<sub>2</sub><sup>10</sup> with cognate sites D<sup>98</sup>, K<sup>121</sup>, D<sup>302</sup> and N<sup>102</sup> in the receptor. (b) A molecular model of the human GnRH receptor in which only the essential elements are shown to reveal the interactions of GnRH in a folded conformation with the receptor residues described above (see Millar, 2002).

phosphate production (Flanagan et al., 1994). Further study has revealed that mutation of Asp<sup>98</sup> to Asn has little effect on ED<sub>50</sub> of Trp<sup>2</sup> GnRH while cause a large increase in ED<sub>50</sub> of the native ligand which has His<sup>2</sup> (Rodic et al., 1996). It appears that NH of Trp can substitute for the His NH in the wildtype receptor and is still accessible for interaction with Asn in the mutant while the His NH cannot interact due to the longer side chain. These findings suggest that Asp<sup>98</sup> may also interact with GnRH, possibly through His<sup>2</sup> (Fig. 5).

In summary, putative ligand interaction sites with the GnRH receptor (viz Asp<sup>302</sup>(Arg<sup>8</sup>), Lys<sup>121</sup>(pGlu<sup>1</sup>His<sup>2</sup>), Asn<sup>102</sup> (Gly<sup>10</sup>NH<sub>2</sub>) and Asp<sup>98</sup> (pGlu<sup>1</sup>His<sup>2</sup>)) have been identified. All of these sites have been conserved in vertebrate Type I GnRH receptors cloned from bony fish, amphibian and bird species (Figs. 3 and 4). It is interesting that the acidic residue in EL3 is conserved in the non-mammals as these receptors are not selective for Arg<sup>8</sup>. We have shown that the insertion of Pro before the acidic residue as apart to after it (Fig. 4) is responsible for this (Fromme et al., unpublished). Although a number of other natural (de Roux et al., 1997, 1999; Bertherat, 1998; Chauvin et al., 2000) and experimental mutations (Chauvin et al., 2000) affect GnRH binding, it is uncertain whether they represent ligand contact sites or are affecting configuration, expression or stability of the receptor.

## 9. Receptor activation

The molecular mechanisms of ligand-mediated receptor activation has been best delineated for rhodopsin and are only partially elucidated for other GPCRs.

The propagation of the hormone message by the receptor to activation of intracellular pathways within the cell involves a change in receptor conformation (Kenakin, 1993). For GPCRs, the active conformation is related to a ternary complex consisting of hormone, receptor and G-protein (De Lean et al., 1980). This model includes an initial binding step common to both agonists and antagonists, followed by a transition step, exclusive to agonists, which leads to formation of the ternary complex. The model also allows for spontaneous formation of a receptor–G-protein complex, which has a higher affinity for agonist ligands and is stabilised by binding of agonists. When GTP binds to the G-protein the receptor returns to the low-affinity conformation and the complex dissociates (De Lean et al., 1980). A revised model proposes that receptors fluctuate between an inactive R conformation and an active R\* conformation (Samama et al., 1993). Agonist binding shifts the equilibrium towards R\*. The R\* conformation has high affinity for agonists, and is the only form that can bind G-proteins. The models are similar in that they both require conformational change in the receptor; one ligand-induced (conformation induction) and the other ligand-stabilised (conformation selection). Studies on the GnRH receptor have provided insight into the mechanism of activation of this receptor. It has also recently become apparent that there are a number of different GnRH receptor active conformations that are selective for GnRH analogues and intracellular signalling pathways (Pawson and Millar, 2004).

### 9.1. Interaction of Asn/Asp in TM II/ VII

Mutation of Asn<sup>87</sup> and Asp<sup>318</sup> in the mouse GnRH receptor (Asn<sup>87</sup> and Asp<sup>319</sup> in the human) (Fig. 3) revealed that the Asp<sup>318</sup> is involved in receptor activation (signal propa-

gation) as the mutants Asn<sup>87</sup>Asn<sup>318</sup> and Asp<sup>87</sup>Asn<sup>318</sup> both retained good ligand binding but poor stimulation of inositol phosphate production (Zhou et al., 1994). These findings indicate that the unusual arrangement of Asp in TM7 in the GnRH receptor is an essential component of ligand-mediated receptor activation normally subserved by the conserved Asp in TM2 of other GPCRs and the non-mammalian receptors (Mitchell et al., 1998) which have Asp in both TM2 and TM7 (Fig. 4). This seems to be an intermediate arrangement as the *Drosophila* GnRH receptor homologue has the usual GPCR Asn in TM7 combined with Asp in TM2 (Hauser et al., 1998).

Interestingly, the presence of an Asp in TM7 of the GnRH receptor facilitates coupling to PLC via Gq but prevents coupling to phospholipase D (PLD) by the small monomeric G-protein (Flanagan et al., 1999). Mutation to Asn, as in the majority of GPCRs, recreates this coupling to phospholipase D. Thus the reverse arrangement of Asn and Asp in TMs 2 and 7 in the GnRH receptor appears to have been selected to allow PLC coupling and prevent PLD coupling (Mitchell et al., 1998). Further exploration by mutation of TM2 Asn and TM7 Asp to various amino acids has confirmed that the TM2 Asn is essential for configuring and expression of the receptor while the TM7 Asp is only essential for receptor activation (Flanagan et al., 1999).

### 9.2. Asp/Arg interaction in TM III

The highly conserved motif DRXXXI/V at the intracellular end of TM3 has also been implicated in receptor activation in the GnRH receptor (Ballesteros et al., 1998; Arora et al., 1997). In the molecular model Asp<sup>137</sup> and Arg<sup>138</sup> (DR) (Fig. 3) appear capable of a charge interaction (Ballesteros et al., 1998) and this has been confirmed in the crystal structure of rhodopsin (Palczewski et al., 2000). Disruption of this by mutating Asp to an uncharged residue conveys constitutive activity and increased coupling efficiency, presumably through the release of Arg to interact with other residues (Ballesteros et al., 1998). The Ile located one turn of an  $\alpha$  helix below the Arg appears to play a role in “caging” the Arg side chain for this interaction by sterically limiting its movement. Mutation to small residues (e.g. Ala) results in some uncoupling (Ballesteros et al., 1998). The Arg is crucial for coupling as mutation to Gln leads to very poor coupling efficiency (Ballesteros et al., 1998). It is proposed that the Arg side chain is involved in a triad interaction with the TM2 Asn and TM7 Asp in stabilising the active conformation of the receptor (Ballesteros et al., 1998) (Fig. 6). Thus an essential element of activation of the GnRH receptor and other GPCRs is the protonation of Asp<sup>132</sup> to release Arg<sup>138</sup> for interaction with the Asp/Asn residues in TMs 2 and 7. Since the conserved Asn in TM1 of rhodopsin interacts with Asp in TM2 in the inactive state this residue may also play a role in the transmembrane domain network involved in receptor activation.

## 10. New concepts in ligand–receptor mediated signal transduction and intracellular signalling

Our most recent work has indicated that the GnRH may assume a number of different active conformations. The classical activated form in the gonadotrope couples to Gq with

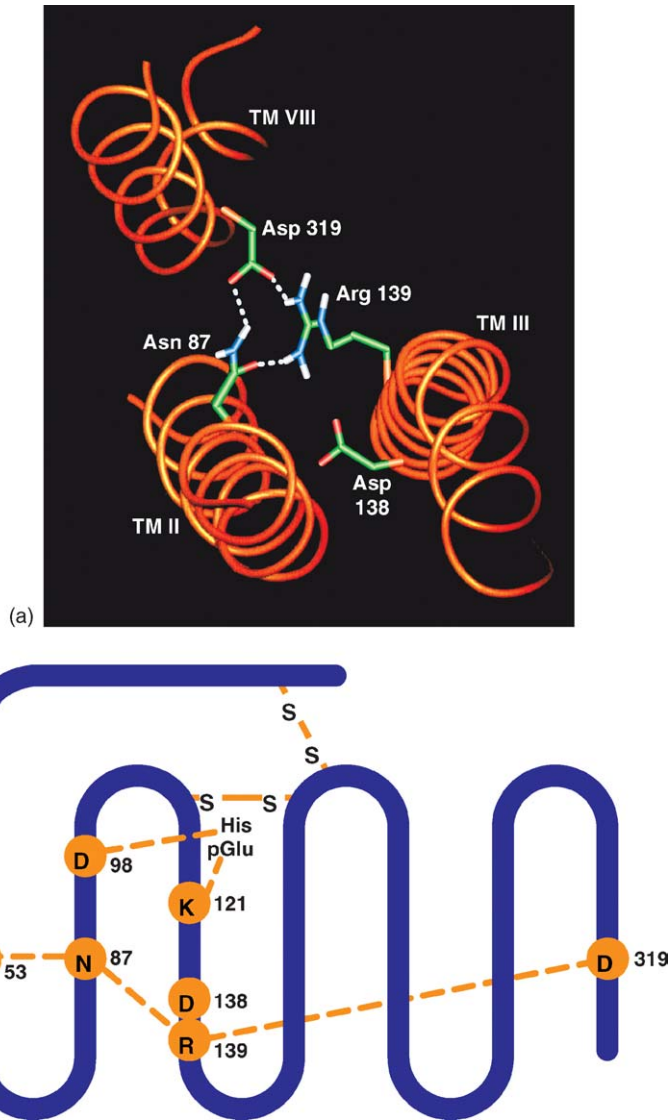


Fig. 6. Activation of the GnRH receptor. (a) A three-dimensional model of the transmembrane domains (TMII, TMIII and TMVII) shows how the protonation of Aspartate<sup>137</sup> breaks the ionic bond with Arginine<sup>138</sup> to allow it to form hydrogen bonds with Asparagine<sup>87</sup> and Aspartate<sup>319</sup> in the active conformation of the human GnRH receptor. (b) A schematic model showing the relationship between GnRH NH<sub>2</sub> terminal contact sites with the receptor and the residues involved in receptor activation. The interaction of the two GnRH residues known to be involved with receptor activation (pGlu His) with D<sup>98</sup> and K<sup>121</sup> are postulated to stabilise the active state of the receptor in which the ionic bond between D<sup>138</sup> and R<sup>139</sup> is broken to allow R<sup>139</sup> interaction with N<sup>87</sup> and D<sup>319</sup>. It is also proposed that N<sup>87</sup> may interact with the conserved N<sup>53</sup> in TMI. These interactions are thought to give rise to changes in the orientations of the transmembrane domains which translate into conformational changes in the intracellular loops to allow coupling to signalling proteins (e.g. Gq/11). The disulphide bridges appear to play a role in connecting the conformational changes between TMs (see Millar, 2002).

the activation of phospholipase C (PLC) and the production of IP<sub>3</sub> which results in Ca<sup>2+</sup> mobilisation, and diacylglycerol production and the activation of protein kinase C (see chapter 5). However, we have recently shown that in peripheral cells and tumour cells, Gi may be activated with resultant inhibition of tumour cell proliferation. The pharmacology of these effects is distinctly different from those in gonadotropes. For example, agonists such as GnRH II which have poor activation of Gq, and certain antagonists which are competitive for Gq activation, are potent activators of Gi. This indicates that these analogues stabilise the receptor in a different conformation that activates Gi. Thus the nature of the intracellular signal through GnRH and the Type I GnRH receptor is dependent on cell type. The signal is also affected by the nature of the ligand and we have called this “ligand-induced-selective-signalling”.

The sequelae of ligand-mediated receptor action are translated into conformational changes in the intracellular loops (Myburgh et al., 1998; Chi et al., 1994; Arora et al., 1998; Ulloa-Aguirre et al., 1998) which results in the activation of G-proteins and non-G-proteins and downstream signalling cascades. These ultimately result in the stimulation of gonadotropin gene expression and gonadotropin exocytosis. These elements are reviewed in Chapter 5.

## 11. Concluding remarks

The fundamental role of hypothalamic GnRH in the reproductive system through stimulating pituitary gonadotropin secretion has made it a prime drug target for treatment of infertility, sex hormone-dependent diseases and for novel contraception in man. At the same time increasing applications have emerged in domestic animals for experimental and practical purposes. It is now clear that GnRHs have been co-opted during evolution for other functions distinct from regulating gonadotropins. The identification of structural variants of GnRH, the discovery of their cognate GnRH receptor types in lower vertebrates and some mammals, and the influence of ligands and the intracellular milieu on signalling are providing considerable insight into novel physiological and pathophysiological roles of GnRHs in diverse processes. In man, higher apes, the laboratory mouse and some ungulates the GnRH Type II receptor is non-functional as a GPCR. Nevertheless, GnRH II is able to bind the Type I receptor and signal in a manner distinctly different from GnRH I. A detailed molecular delineation of the interaction of these GnRH variants and GnRH analogues with GnRH receptors in different cellular environments is contributing to the development of novel GnRH therapeutics including non-peptide antagonists with potential oral application and tissue-direction actions.

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