Type II gonadotrophin-releasing hormone (GnRH-II) in reproductive biology

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Humans may be particularly unusual with respect to the gonadotrophin-releasing hormone (GnRH) control of their reproductive axis in that they possess two distinct GnRH precursor genes, on chromosomes 8p11–p21 and 20p13, but only one conventional GnRH receptor subtype (type I GnRH receptor) encoded within the genome, on chromosome 4. A disrupted human type II GnRH receptor gene homologue is present on chromosome 1q12. The genes encoding GnRH ligand precursors and GnRH receptors have now been characterized in a broad range of vertebrate species, including fish, amphibians and mammals. Ligand precursors and receptors can be categorized into three phylogenetic families. Members of each family exist in primitive vertebrates, whereas mammals exhibit selective loss of ligand precursor and receptor genes. One interpretation of these findings is that each ligand–cognate receptor family may have evolved to fulfil a separate function in reproductive physiology and that species-specific gene inactivation, modification or loss may have occurred during evolution when particular roles have become obsolete or subject to regulation by a different biochemical pathway. Evidence in support of this concept is available following the characterization of the chromosomal loci encoding the human type II GnRH receptor homologue, a rat type II GnRH receptor gene remnant (on rat chromosome 18) and a mouse type II GnRH ligand precursor gene remnant (on mouse chromosome 2). Whether type I GnRH and type II GnRH peptides elicit different signalling responses in humans by activation of the type I GnRH receptor in a cell type-specific fashion remains to be shown. Recent structure–function studies of GnRH ligands and GnRH receptors and their expression patterns in different tissues add further intrigue to this hypothesis by indicating novel roles for GnRH such as neuromodulation of reproductive function and direct regulation of peripheral reproductive tissues. Surprises concerning the complexities of GnRH ligand and receptor function in reproductive endocrinology should continue to emerge in the future.

Gonadotrophin-releasing hormone (GnRH) plays a pivotal role in the regulation of reproduction by stimulating release of LH and FSH from the anterior pituitary. The GnRH system has been analysed in a large number of different species with a view towards developing therapies for pathological conditions and for methods to assist reproduction. In excess of 1000 scientific publications per year indicate that the comprehensive role of GnRH in reproductive biology, although incompletely understood, remains of considerable therapeutic interest. Information is accumulating in several fields of investigation, including molecular genetics, gene expression, receptor structure–function and reproductive pathophysiology.

Recent advances are summarized in the following sections.

The prepro-GnRH-II and type II GnRH receptor genes

The prepro-GnRH-II gene consists of a 5'-untranslated exon and three coding exons, with the mature peptide encoded within coding exon 1 (White *et al.*, 1998). This gene lies in close proximity to two flanking genes. A recent study examining human prepro-GnRH-II gene regulation identified multiple regulatory elements within the untranslated exon 1 of the gene, including two putative E-box binding sites which function co-operatively to stimulate the basal transcription of the gene. It was also demonstrated that the transcription factor AP-4 is an enhancer protein for the GnRH-II

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Fig. 1. Current status of type II gonadotrophin-releasing hormone (GnRH) receptor gene characterization in a range of species. Solid lines indicate DNA strands and boxes indicate positions of coding exons. (GenBank accession numbers: human genomic DNA AL160282, chimp genomic DNA AG122659, marmoset cDNA AF368286, rhesus monkey cDNA AF353987, green monkey cDNA AF353988, pig genomic DNA BF702918, cow genomic DNA AV604131, mouse genomic DNA AC122037, rat genomic DNA AC106482 and bullfrog cDNA AF144062.) Remnants of coding sequences are depicted as slashed boxes. Distribution of selected repetitive sequence elements (human Alu and mouse B2) is indicated (arrows denote orientation). n.d.: Not determined; Pex11-beta: gene for peroxisomal protein 11 β (GenBank accession number AB018080); RBM8A: RNA binding motif protein 8A (GenBank accession number AF231511); SECIS: selenocysteine insertion sequence.

promoter (Cheng *et al.*, 2003). The function of the carboxyl-terminal domain of the precursor molecule remains unknown but it is possibly involved in packaging the hormone into storage vesicles or it may produce processed peptides with unknown regulatory functions such as that postulated for the GnRH-associated peptide (GAP) of the prepro-GnRH-I gene (Chavali *et al.*, 1997). The mammalian type II GnRH receptor gene consists of three coding exons spanning 7.5 kb. The human gene contains a frameshift in coding exon 1 and a premature stop codon in exon 2. The gene also overlaps two flanking genes but remains transcriptionally active and displays alternative splicing (Morgan *et al.*, 2003).

The cDNA and genomic DNA sequences encoding GnRH-II precursors and type II GnRH receptors have been characterized for an increasing number of vertebrate species (Millar *et al.*, 2001; Wang *et al.*, 2001; Bogerd *et al.*, 2002; Okubo *et al.*, 2002a,b). This information enables classification of ligand-precursor and

receptor isoforms according to their primary structures, rather than their pharmacological properties (Millar *et al.*, 2003).

Progress in genome sequencing projects and conservation of the arrangement of genes flanking those of the GnRH-system means that some of the latter can now be described in the context of the chromosomal loci that they occupy (Figs 1 and 2a). This information can be used to identify remnants of GnRH-II precursor and type II GnRH receptor coding sequences at homologous loci in the mouse and rat genomes, respectively (Fig. 2b).

Silencing of GnRH-II and type II GnRH receptor genes in certain mammalian species

Information regarding the status of prepro-GnRH-II and type II GnRH receptor genes in mammalian species is rapidly becoming more available as a result of advances in genomic informatics. The GnRH-II ligand structure

Fig. 2. (a) Current status of prepro-GnRH-II gene characterization in human, mouse, rat and medaka. Solid lines indicate DNA strands and boxes indicate positions of coding exons. Remnants of coding sequences are depicted as slashed boxes. (GenBank accession numbers: human genomic DNA AF036329, mouse genomic DNA AL772162, rat genomic DNA AC131863, medaka genomic DNA AB074500.) PTP alpha: protein tyrosine phosphatase receptor type alpha (GenBank accession number NM_002836); S-26: mitochondrial ribosome protein S-26 (GenBank accession number BC013018); oxytocin-neurophysin I (GenBank accession number NM_000915). (b) Alignment of remnant coding sequences for mouse GnRH-II and rat type II GnRH receptor with sequences from corresponding functional genes. + represents a conserved residue; * represents an in-frame stop codon. The mouse GnRH-II precursor remnant encompasses the mature GnRH-II peptide-coding region. The rat type II GnRH receptor remnant encompasses transmembrane domains 2 and 3 and extracellular loop 1.

is conserved from bony fish to humans, indicating that this is probably the earliest evolved form of GnRH and thus has critical and specific functions. This complete conservation of structure over 500 million years indicates that GnRH-II has an important function and a discriminating receptor (or receptors) which has selected against any structural change in the ligand. Nevertheless, it now appears that the GnRH-II gene has been silenced in the mouse (Fig. 2b), leaving only one functional

GnRH gene, the gene encoding GnRH-I. This situation is in contrast to earlier life forms, in particular fish species, which have retained three functional GnRH ligand subtype genes (Millar *et al.*, 2003). Disruption or silencing of the type II receptor gene has been noted in the human and chimp (Morgan *et al.*, 2003), cow (Millar *et al.*, 2003), sheep (Gault *et al.*, 2003) and rat (Fig. 2b) genomes. In contrast, marmosets (Millar *et al.*, 2001), african green and rhesus monkeys (Neill *et al.*, 2001) and

Table 1. Ligand selectivity and signalling of cognate ligands at marmoset type II and human type I gonadotrophin-releasing hormone (GnRH) receptors

Binding and coupling	Marmoset type II receptor	Human type I receptor
Binding		
GnRH-II	1.0 ^a	0.1 ^a
GnRH-I	0.02 ^a	1.0
GnRH-III	0.1 ^a	0.1 ^a
Antagonist 135-18	Full agonist	Full antagonist
Coupling		
Gq/11	+	+
Ca ²⁺	+	+
PKC	+	+
ERK	+ (transient)	+ (protracted)
p38	+	+
JNK	Nil	+
c-Src	Nil	+
Receptor internalization	Rapid	Slow
Receptor desensitization	+	Nil

Type I and type II GnRH receptors exhibit distinct functional differences. Whereas the type II GnRH receptor is highly selective for binding GnRH-II, type I binds both GnRHs reasonably well. The type II receptor also recognizes certain type I receptor antagonists as agonists. Although cognate ligands at both receptors activate the Gq/11–inositol phosphate–Ca²⁺–diacylglycerol–protein kinase C (PKC) pathways, they exhibit distinct differences in extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and Src activation, and in desensitization and the rate of receptor internalization.

JNK: c-Jun N-terminal kinase; c-Src: mammalian homologue v-Src of Rous sarcoma virus.

^aActivities are comparative binding affinities relative to cognate ligands GnRH-II and GnRH-I, respectively.

pigs (Morgan *et al.*, 2003) have retained the presence of a functional type II GnRH receptor. Furthermore, only fish and amphibian species have retained a functional type III GnRH receptor (Millar *et al.*, 2003). The emerging picture is that the more evolved or structurally complex the species, the fewer subtypes of receptor and ligand there appear to be.

Evolutionary hypotheses

Genes encoding G protein-coupled receptors (GPCRs) are thought to have evolved by sequential gene duplication events in ancestral eukaryotic cells (Taylor and Agarwal, 1993). Retention of closely related pairs of GPCRs within the genome may have been driven by acquisition of advantageous ligand binding or signalling properties due to evolutionary drift in the coding sequences. Overlapping and distinctive properties of type I and type II GnRH receptors are listed in Table 1. Duplication and sequence divergence of genes encoding ligand precursors would also be expected to exert a significant effect on the evolutionary fate of closely related ancestral GPCRs. It is interesting to note that GnRH-I

is more positively charged (and slightly less bulky) than GnRH-II. GnRH-I is also thought to be more conformationally labile compared with the preformed β -II turn-containing GnRH-II (Pfleger *et al.*, 2002; Millar *et al.*, 2003), and perhaps these differences affecting peptide conformation have influenced GnRH receptor evolution (Fig. 3).

Type II GnRH receptor structure–function

The primary amino acid sequence of the marmoset type II GnRH receptor has 39% identity (68% conservation) with the type I GnRH receptor. The most striking difference between the receptor subtypes is the retention of a 56 residue cytoplasmic tail domain at the carboxyl-terminus of the type II GnRH receptor compared with its absence in the type I receptor. The tail sequence is not conserved between mammalian and amphibian homologues. Perhaps the tail domain, encoded in exon 3 in an ancestral form of the type I receptor gene, was disrupted before a deletion event that led to the subsequent loss of the tail domain altogether. Other important sequence differences between receptor subtypes occur in the extracellular, transmembrane and cytoplasmic loop domains. The type I and type II receptor subtypes seem to differ significantly with respect to the charge distribution observed at their respective extracellular domains (Fig. 3). In type II GnRH receptors, the N-terminal domain is two residues longer and more negatively charged than that in type I GnRH receptors. Extracellular loop 1 (ECL-1) exhibits similar charge properties in the two receptor subtypes, but ECL-2 is relatively more positively charged in type II receptors. ECL-3 is less negatively charged in the type II receptor compared with the type I receptor. Furthermore, ECL-2 and ECL-3 are shorter in the type II GnRH receptor relative to the type I receptor. The premature stop codon in the human type II receptor gene knocks out a positively charged arginine (R) residue in ECL-2.

The presence of a cytoplasmic tail domain is expected to alter receptor trafficking dynamics. Indeed, a recent study demonstrated that the marmoset type II GnRH receptor undergoes a more rapid agonist-induced internalization compared with the human type I receptor, and the important residues within the cytoplasmic tail that are responsible for this process have been identified (K. Ronacher, C. A. Flanagan, R. P. Millar and A. A. Katz, unpublished). Furthermore, the importance of the cytoplasmic tail for rapid agonist-induced internalization has been shown for the chicken GnRH receptor (Pawson *et al.*, 1998, 2003) and a number of other non-mammalian GnRH receptors (Blomenrohr *et al.*, 1997; Lin *et al.*, 1998; Blomenrohr *et al.*, 1999; Heding *et al.*, 2000; Hislop *et al.*, 2000, 2001). The three bullfrog GnRH receptor subtypes display different internalization kinetics, which may be related to sequence or structural

Fig. 3. Comparison of distribution of charged amino acid residues in gonadotrophin releasing hormone (GnRH)-I and GnRH-II peptides and in extracellular hydrophilic domains of type I and type II GnRH receptors. GnRH-I and GnRH-II peptides are depicted here with similar conformations. Charged extracellular amino acids in the two receptor subtypes are indicated as coloured circles (yellow for negatively charged aspartate or glutamate residues, and red for positively charged arginine and lysine residues). Potential differences in the docking of the relatively conformationally labile GnRH-I ligand and the pre-conformed GnRH-II ligand to their respective cognate receptors is emphasized in simplistic cartoon form.

motif differences in the cytoplasmic tails of these receptors (Acharjee *et al.*, 2002).

GnRH-II in reproductive pathophysiology

GnRH-II occurs in the brain, pituitary gland, reproductive tissues and the immune system. Autocrine and paracrine roles have been hypothesized for GnRH in reproductive tissues but most studies have not progressed beyond preliminary characterization of gene expression (Chen *et al.*, 1999; Cheon *et al.*, 2001; Kang *et al.*, 2001; Siler-Khodr and Grayson, 2001). Expression of GnRH receptor gene and cell-surface GnRH binding are very low in most extra-pituitary tissues. Furthermore, it is unclear whether GnRH-II elicits responses in peripheral tissues that differ significantly from those caused by GnRH-I. In relation to this, the actions of GnRH-II on human placenta and baboon ovary have been investigated (Siler-Khodr and Grayson, 2001; Siler-Khodr *et al.*, 2003). These studies proposed that GnRH-II is a potent regulator of ovarian function and hormone regulation during pregnancy. The preformed β -II turn conformation of GnRH-II (Millar *et al.*, 2003) makes this ligand

more stable than GnRH-I. GnRH-II is therefore thought to be less susceptible to peptidase degradation and would therefore differ from GnRH-I in its pharmacological bioavailability in a tissue environment (Siler-Khodr *et al.*, 2003). Such a scenario has important implications for the role of GnRH-II, in particular for steroidogenesis and hormone regulation in reproductive tissues, and may lead to the elucidation of novel paracrine roles for GnRH-II in reproductive physiology. Complex autocrine and paracrine interactions involving neurones, epithelial cells, stromal cells, stem cells and infiltrating lymphocytes can certainly be envisaged for GnRH in disorders of puberty, ovarian cycle or menstrual cycle dysfunction, idiopathic female infertility, disorders of testis and prostate gland and in neoplasia of reproductive tissues.

Anti-proliferative or proliferative effects of GnRH

The effect of GnRH-II on human tumour cell proliferation is currently the subject of concerted investigation. It is well known that type I GnRH receptors are expressed in cancer cells, and that GnRH analogues can inhibit the proliferation of these cells. It appears that GnRH

analogues are set to emerge as novel therapeutics in cancers expressing the type I GnRH receptor. Two recent studies have examined the signalling and anti-proliferative effects mediated by GnRH receptors exogenously expressed in breast and prostate cancer cells. It was shown that the exogenous expression of type I GnRH receptors in these cells markedly enhances the anti-proliferative effect of GnRH analogues (Everest *et al.*, 2001; Franklin *et al.*, 2003). These studies demonstrated that the signalling properties of the exogenously expressed receptors were functionally indistinguishable from GnRH receptors in pituitary gonadotropes. This result was unexpected as it is widely accepted that GPCRs in cancer cells couple predominantly through G_i rather than their classical coupling G-protein (that is $G_{q/11}$ for pituitary type I GnRH receptors), and that the pharmacology of GnRH analogues at tumour GnRH receptors is quite different from that seen at the pituitary receptor. Although the above studies focused on signalling outputs of inositol phosphate production and extracellular signal-regulated kinase 2 (ERK2) phosphorylation, it is possible that a more in-depth examination of signalling events in these transfected cancer cells may reveal distinct differences. Evidence is emerging that G-protein coupling can be GnRH-concentration dependent (Krsmanovic *et al.*, 2003) and that downstream signalling outputs are important (Willars *et al.*, 2001).

Why is the type I mammalian GnRH receptor so effective in inhibiting the proliferation of tumour cells? The clues to answer this question are found in studies that show that the type I GnRH receptor is resistant to desensitization, a common and rapid event in the GPCR superfamily (Davidson *et al.*, 1994; Forrest-Owen *et al.*, 1999), and undergoes very slow internalization compared with non-mammalian (Blomenrohr *et al.*, 1997, 1999; Lin *et al.*, 1998; Pawson *et al.*, 1998, 2003; Heding *et al.*, 2000; Hislop *et al.*, 2000, 2001) and mammalian type II GnRH receptors (K. Ronacher, C. A. Flanagan, R. P. Millar and A. A. Katz, unpublished; Acharjee *et al.*, 2002). Thus, the type I GnRH receptor remains active at the cell surface for longer than most other GPCRs. GnRH analogues are therefore able to bind to these receptors, leading to a protracted and eventually detrimental activation of the various signalling pathways, and the inhibition of cell proliferation. A recent study investigated the anti-proliferative effects mediated by GnRH receptors expressed in breast cancer cells (Finch *et al.*, 2003). It was demonstrated that GnRH was able efficiently to inhibit proliferation via the sheep GnRH receptor, whereas the anti-proliferative effect was not mediated by the *Xenopus* type II GnRH receptor, indicating that receptor desensitization and internalization may influence the anti-proliferative effect of GnRH receptors (Finch *et al.*, 2003).

The anti-proliferative effects of GnRH-II have been demonstrated in human ovarian surface epithelial cells and ovarian tumours (Choi *et al.*, 2001). The finding that

the human type II GnRH receptor gene is disrupted by a frame-shift and premature stop codon indicates that a conventional type II GnRH receptor system is absent in humans (Morgan *et al.*, 2003). Recent studies have addressed whether type II GnRH receptors are expressed in human breast, endometrial and ovarian cancer cells. The effects of GnRH-II on the proliferation of these cells were also investigated (Grundker *et al.*, 2002; Emons *et al.*, 2003). It was demonstrated that type II GnRH receptor mRNA is expressed in these cells and that the anti-proliferative effect of GnRH-II is more potent than that of GnRH-I. Furthermore, an ovarian cancer cell line shown to be type II receptor mRNA-positive, but type I receptor-mRNA-negative, responded to GnRH-II and not GnRH-I. Such studies indicate that these effects are mediated by a GnRH-II-specific receptor. Perhaps the disrupted human type II GnRH receptor gene is able to encode a functional receptor by some as yet unknown mechanism. Certainly, a number of possible mechanisms have been suggested and thoroughly investigated (Morgan *et al.*, 2003). Alternatively, the effects of GnRH-II may be mediated by a distinct cell-surface receptor. Future research will no doubt provide answers to explain these findings. Clearly, the intracellular mechanisms mediating the anti-proliferative effects of GnRH analogues on tumour cells are not fully understood. Chen *et al.* (2002a) demonstrated that GnRH-I and GnRH-II are over-expressed in cancer cells and inhibit the expression of mRNA encoding the 60S ribosomal phosphoproteins P1 and P2, which play a key role in the protein translation process. This finding provides a putative mechanism for the direct anti-proliferative effects of GnRH on cancer cells, whereby reduced expression of P1 and P2 affects the rate of protein translation, thereby decreasing their rate of proliferation.

Effects of GnRH-II on neurones

The wide distribution of GnRH-II in the central and peripheral nervous systems indicates a neurotransmitter-neuromodulatory role. Such a role has been proposed (Millar *et al.*, 2001) based on the occurrence of GnRH-II in frog sympathetic ganglia where it binds high-affinity receptors (Troskie *et al.*, 1997) and potentially inhibits M-type K^+ channels (Jones, 1987; Bosma *et al.*, 1990). GnRH-I and -II genes are co-expressed in human neuroblastoma cell lines (Chen *et al.*, 2001), whereas the immortalized mouse neuronal cell line GT1-7 expresses GnRH-I, but whether and in what context the peptides are functional as synaptic neurotransmitters in each of these cells is unclear.

GnRH-responsive genes

The effects of GnRH-II on gene expression in certain types of cell have been addressed using gene array screening technology (Chen *et al.*, 2002b). It has been

suggested that GnRH-II may interact directly with human and mouse T cells to trigger gene transcription, and in particular transcription and cell-surface expression of laminin receptor, a 67 kDa protein involved in cell adhesion and migration and in tumour invasion and metastasis (Chen *et al.*, 2002b). More interestingly, it has been demonstrated that a specific type I GnRH receptor antagonist (Cetrorelix) blocked GnRH-I- but not GnRH-II-induced effects (Chen *et al.*, 2002b). This finding certainly indicates that the observed effects might be mediated via a GnRH-II-specific receptor. Alternatively, such a result may also be explained by the fact that many antagonists bind to receptors at sites that are distinct from those occupied by agonists. In this scenario, the binding of GnRH-I, known to be an unconformed ligand (Pfleger *et al.*, 2002), would be blocked by antagonist occupation at the type I receptor, but GnRH-II binding, known to be preformed (Pfleger *et al.*, 2002), would not, as it can occupy a different site on the type I receptor.

GnRH and tissue remodelling

The invasive ability of trophoblastic cells is known to be modulated by the regulation of their urokinase type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1) expression (Lala *et al.*, 2002). It has been reported that GnRH-I and -II facilitate the invasion by trophoblasts into the placenta by simultaneously upregulating uPA expression and downregulating PAI-1 expression (Chou *et al.*, 2002). In relation to this, GnRH regulation of the decidualization process, involving the remodelling of the extracellular matrix of stromal cells by the actions of matrix metalloproteinases (MMPs) and their inhibitors, has also been demonstrated (Chou *et al.*, 2003). In particular, the expression of MMP-2 and MMP-9 and their tissue-specific inhibitors, which play important roles in trophoblast invasion, were shown to be modulated by GnRH (Chou *et al.*, 2003).

Further studies of GnRH-II function

The DNA sequences of prepro-GnRH-II and type II GnRH receptor genes and their chromosomal loci enable studies of the patterns of their expression and a preliminary understanding of their evolutionary history. Determining how GnRH-I and GnRH-II bind to and activate GnRH receptors, and how antagonists such as Cetrorelix modulate peptide binding should be the goals of further structure–function investigations. Attempts to correlate these studies with reproductive physiology or diseases affecting reproductive tissues in mammals are ongoing. Perhaps mouse strains possessing targeted expression of human prepro-GnRH-II or functional type II GnRH receptor transgenes might be particularly informative in this respect.

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