

1 **"Type II Gonadotropin-releasing Hormone Receptor**
2 **and Polynucleotides Encoding Therefor"**

3
4 The present invention relates to a novel Type II
5 gonadotropin-releasing hormone receptor (Type II
6 GnRH-R), to genetically engineered host cells able
7 to express the Type II GnRH-R, and the ligands and
8 antibodies therefor.

9
10 Type I gonadotropin-releasing hormone (GnRH) is a
11 decapeptide released from the hypothalamus, and
12 acts through receptors to regulate the secretion of
13 gonadotropins required for reproductive function
14 (see Fink et al., "Gonadotrophin secretion and its
15 control", The Physiology of Reproduction, E Knobil
16 and I Neill, New York, Raven Press, pages 1349-
17 1377, 1988).

18
19 Receptors for Type I GnRH (ie Type I GnRH-R) are
20 members of the large G-protein-coupled receptor
21 family and are preferentially coupled to

1 phosphoinositidase C via the G_q/G_{11} family of G
2 proteins. Typically Type I GnRH-Rs are located in
3 the gonadotroph cells of the anterior pituitary
4 gland (where binding of Type I GnRH leads to
5 release of the gonadotropins luteinising hormone
6 and follicle-stimulating hormone), as well as on
7 the central and peripheral nervous systems, gonads,
8 placenta and on certain tumours, such as breast and
9 prostate. Type I GnRH receptors may display both
10 up and down regulation and Type I GnRH agonists
11 have been used in management of prostate and breast
12 cancer, as well as to stimulate gonadotropin
13 secretion in the treatment of infertility.

14
15 Expression of mouse and rat Type I GnRH-R was first
16 achieved by injecting poly(A)+ mRNA from a suitable
17 source (eg from the pituitary gland) into *Xenopus*
18 oocytes (see, for example, Eidne et al., J. Mol.
19 Endocr. Vol 1, pages R9-R12, 1988; Yoshida et al.,
20 Molecular Endocrinology, Vol 3, pages 1953-1960,
21 1989; and Sealfon et al., Molecular Endocrinology,
22 Vol 4, pages 119-124, 1990). This system allowed
23 some characterisation of the pharmacology of the
24 Type I GnRH-R.

25
26 The protein-encoding nucleotide sequence of the
27 murine Type I GnRH-R was first published by
28 Tsutsumi et al., (Molecular Endocrinology, Vol 6,
29 pages 1163-1169, 1992) together with the deduced
30 amino acid sequence for murine Type I GnRH-R.

1 Eleven different forms of GnRH in vertebrates have
2 been identified to date (see King and Millar, "Co-
3 ordinated evolution of GnRHs and their receptors",
4 in GnRH Neurons: Gene to Behavior, Eds. I.S. Parhar
5 and Y. Sakuma, Brain Shuppan, Tokyo, pages 51-77,
6 1997; see Sealfon et al., Endocr. Rev. 18:180-205,
7 1997; Millar et al., "Plasticity in the structural
8 and functional evolution of GnRH: A peptide for all
9 seasons", in Proceedings of the XIIIth
10 International Conference of Comparative
11 Endocrinology, Eds. S. Kawashima and S. Kikuyama,
12 Moduzzi Editore, Italy, pages 15-27, 1997; and see
13 Sherwood et al., General and Comparative
14 Endocrinology, 112, 1998). Many of these different
15 GnRH forms are in fact variants of GnRH Type I.
16 However, distinct Type II and Type III GnRHs have
17 been identified.

18
19 Type II GnRH was originally isolated from chicken
20 brain (see Millar and King, News Physiological
21 Science, 3:49-53, 1988) and was initially termed
22 "chicken GnRH II" or "cGnRH II". Subsequent
23 investigations have revealed that this isoform is
24 present in most vertebrate species, and of all the
25 GnRH isoforms GnRH II is the most ubiquitous. The
26 wide distribution of GnRH II suggests an important
27 function and it is postulated to have a
28 neuromodulatory, and possibly a neuroendocrine,
29 role in the central and peripheral nervous systems
30 (see Millar and King, 1988, supra). GnRH II has
31 been shown to regulate M currents (K^+ channels) in

1 the sympathetic ganglion (Bosma et al., in G
2 proteins and Signal Transduction, The Rockefeller
3 University Press, pages 43-59, 1990) and stimulates
4 reproductive behaviour (see King et al., in GnRH
5 Neurones : Gene to Behavior, eds. Parhar (Brain
6 Shuppan), Tokyo, pages 51-77, 1997. It has also
7 been postulated that GnRH II acts as a specific
8 FSH-releasing agent (Millar et al. Ref No. 33).

9
10 Type II GnRH is highly expressed in kidney, bone
11 marrow and prostate tissues as well as the
12 extrahypothalamic brain (White et al. Ref No. 15).

13
14 To date only partial sequence information has been
15 available for a Type II GnRH-R. Specifically,
16 Millar et al. (in Journal of Endocrinology,
17 162:117-126, 1999) reported a continuous nucleotide
18 sequence of 1642 nucleotides of the human gene,
19 obtained by screening the human genome EST
20 (expressed sequence tag) database. The EST
21 sequences were confirmed in the cloned human gene
22 and in PCR products of cDNA from several tissues.
23 All the EST transcripts detected were in the
24 antisense orientation with respect to the novel
25 GnRH receptor sequences herein described and were
26 highly expressed in a wide range of human brain and
27 peripheral tissues.

28
29 PCR analysis of the cDNA partial sequence obtained
30 by Millar et al., revealed that an intronic
31 sequence equivalent to intron 2 of human Type I

1 GnRH-R was retained. The intron itself was not
2 spliced out in the transcript, but this was
3 expected for anti-sense transcripts, as candidate
4 donor and acceptor sites were only present in the
5 gene when transcribed in the orientation encoding
6 the GnRH receptor homolog. Despite extensive 5'
7 RACE studies Millar et al. did not obtain any
8 transcript 5' to the sequence corresponding to
9 intron 2 of human Type I GnRH-R, and the antisense
10 transcripts terminated in poly A due to the
11 presence of a polyadenylation signal sequence in
12 the putative intron 2 when transcribed in the
13 antisense orientation (see Fig. 1).

14
15 None of the sequence revealed any contiguous open-
16 reading frame which would translate a functional
17 protein. Millar et al. concluded that the putative
18 receptor was probably a pseudogene representing a
19 receptor which had become redundant and further
20 investigations have revealed that the full-length
21 antisense transcript encodes a novel
22 ribonucleoprotein (RNP) which localised to
23 chromosome 14 (Ref 26). Subsequently it was found
24 that the gene was a pseudogene for both RNP and
25 Type II GnRH-R.

26
27 The presence of the RNP explains the widespread
28 tissue expression observed and it is significant
29 that only the 3' untranslated sequences of the RNP
30 cDNA overlap the putative GnRH Type II receptor

1 sequences encoding the equivalent of exon 1 and
2 exon 2.

3

4 Despite the findings of Millar et al. which suggest
5 that an operative version of GnRH-R Type II does
6 not exist in mammals, we have now obtained the full
7 nucleotide sequence encoding the Type II GnRH-R
8 from marmoset and also the complete nucleotide
9 sequence (including exon I) encoding the human Type
10 II GnRH-R. The human Type II GnRH-R herein
11 reported has been localised to chromosome 1 (1q12-
12 21).

13

14 The present invention thus provides a
15 polynucleotide encoding a functional Type II
16 gonadotropin-releasing hormone receptor (Type II
17 GnRH-R) peptide.

18

19 The term "peptide" is used herein to refer to any
20 peptidal compound without connotation of size, and
21 includes therefore larger molecules which elsewhere
22 may alternatively be termed "polypeptides" or
23 "proteins" also fall within this definition.

24

25 In one embodiment the peptide encoded comprises at
26 least a portion of exon I. By "exon I" we refer to
27 that portion of Type II GnRH-R which corresponds to
28 and exhibits substantial homology with exon I of
29 Type I GnRH-R. In particular we refer to a peptide
30 which includes over 90% of the amino acid sequence
31 1 to 170 in the marmoset Type II GnRH-R sequence of

1 SEQ ID No 2 or to a peptide which includes the
2 equivalent amino acids of the human Type II GnRH-R
3 sequence. Thus, for example, exon I refers to
4 amino acid nos. 1 to 168 of splicing alternative 1
5 as set out in SEQ ID No 5 (see Fig. 3). The above
6 terminology has been adopted for clarity; the short
7 splice referred to below is herein designated
8 intron 1' to distinguish it from intron 1.
9 Consequently the short exon (exon 1') comprising
10 amino acids nos. 1 to 9 of the human Type II GnRH-R
11 peptide is incorporated into exon I as herein
12 defined.

13
14 Preferred embodiments of the present invention
15 include polynucleotides having a nucleotide
16 sequence as set out in SEQ ID No 1 (marmoset) or
17 SEQ ID No 3 (human) or polynucleotides which encode
18 a polypeptide having an amino acid sequence as set
19 out in SEQ ID No 2 (marmoset) or SEQ ID Nos 4, 6, 8
20 or 10 (human).

21
22 As shown in Fig. 3 the marmoset amino acid sequence
23 of Type II GnRH-R has over 90% homology with the
24 corresponding human sequence. Accordingly, the
25 present invention incorporates any polypeptide
26 having at least 90% homology with the amino acid
27 sequence of SEQ ID Nos. 1 or 3.

28
29 When the human nucleotide sequence is compared to
30 that of the marmoset, there is an apparent single-
31 base deletion at position 30 of the coding

1 sequence. The earlier EST sequence (Genbank
 2 BG036291) matches the 5' end of the sequence, but
 3 the match ends at nucleotide 29 of the coding
 4 sequence and continues from nucleotide 290 of the
 5 coding sequence, indicating that this is a splice
 6 site. In the chicken receptor an intron was also
 7 located in a similar position. Excision of a very
 8 short intron (intron 1') at this position would
 9 account for the frame shift; very short introns
 10 have been noted in other genes, for example the
 11 mouse alpha-7 integrin gene has an intron of only
 12 16 bases (Genbank L23422). There are three
 13 alternative splicing possibilities, involving 5-,
 14 8- or 38-base deletions, each of which would
 15 restore the open reading frame of the human Type II
 16 GnRH-R.

17
 18 The start of the human Type II GnRH-R amino acid
 19 sequence following each of the three splicing
 20 alternatives is set out below:

21
 22 **Splicing alternative 1:**
 23 (5 bases deleted from position 29 on)

24
 25 Met Ser Ala Gly Asn Gly Thr Pro Trp 9
 26 1 ATG TCT GCA GGC AAC GGC ACC CCT TGG
 27
 28
 29 Ala Ala Gly Glu Glu Val Trp Ala 17
 30 28 GCA GCG GGG GAG GAG GTC TGG GCT
 31

1 **Splicing alternative 2:**

2 (8 bases deleted from position 29 on)

3

4 Met Ser Ala Gly Asn Gly Thr Pro Trp 9

5 1 ATG TCT GCA GGC AAC GGC ACC CCT TGG

6

7

8 Ala Gly Glu Glu Val Trp Ala Gly 17

9 28 GCG GGG GAG GAG GTC TGG GCT GGA

10

11 **Splicing alternative 3:**

12 (38 bases deleted from position 29 on)

13

14 Met Ser Ala Gly Asn Gly Thr Pro Trp 9

15 1 ATG TCT GCA GGC AAC GGC ACC CCT TGG

16

17 Val Glu Val Glu Gly Ser Glu Leu 17

18 28 GTG GAG GTG GAG GGC TCA GAG CTG

19

20

21 The full sequences for human Type II GnRH-R
 22 according to the first splicing alternative are set
 23 out in SEQ ID Nos 5 and 6, according to the second
 24 splicing alternative in SEQ ID Nos 7 and 8, and
 25 according to the third splicing alternative in SEQ
 26 ID Nos 9 and 10.

27

28 As shown in SEQ ID No. 3, the human nucleotide
 29 sequence includes an apparent stop codon in the
 30 first part of exon 2. In the marmoset the
 31 equivalent codon (shown at nucleotides 449-452 of

1 SEQ ID No 1) represents an arginine. A modified
2 version of SEQ ID No 3 whereby the stop codon is
3 engineered to represent an amino acid (for example
4 arginine) is hereby incorporated. However, we
5 believe that the codon TGA does not function as
6 stop codon, but is instead translated as
7 selenocysteine. In eukaryotes decoding of TGA as
8 selenocysteine requires the selenocysteine
9 insertion element (SECIS) in the 3' UTR of the mRNA
10 (see Copeland et al., EMBO J, 19(2): 306-14, 2000;
11 and Fagegaltier et al., Nucleic Acids Research,
12 29(14): 2679-80, 1995). The putative UTR of the
13 sequence (see SEQ ID No. 3) was found to contain
14 the SECIS pattern
15 RTGAN{13,15}AARN{23,26}GA. Thus, selenocysteine
16 insertion at that position of the protein (amino
17 acid no 177 in SEQ ID No 6; amino acid no 176 in
18 SEQ ID No 8; amino acid no 166 in SEQ ID No 10) is
19 possible. In SEQ ID Nos. 3, 5, 7 and 9 the TGA in
20 shown as Xaa (and defined as TGA in the preamble to
21 the sequence) simply to maintain the coding
22 sequence as a whole in the PATENTIN program.

23
24 Desirably the polynucleotide according to the
25 present invention encodes a peptide which is able
26 to bind specifically to Type II GnRH and,
27 preferably, is able to function as a receptor
28 therefor.

29
30 Those skilled in the art will appreciate that the
31 nucleotide sequences of SEQ ID No 1 and Nos 3, 5, 7

1 and 9 correspond to one allele of the marmoset and
2 human gene respectively, and that allelic variation
3 is likely. Allelic variants can be cloned by
4 probing cDNA or genomic libraries from different
5 individuals according to standard procedures.
6 Allelic variants of the DNA sequences shown in the
7 sequence listing, including those containing silent
8 mutations and those in which mutations result in
9 amino acid sequence changes, are within the scope
10 of the present invention, as are proteins which are
11 allelic variants of SEQ ID No 2 and Nos 4, 6, 8 and
12 10.

13
14 The present invention also comprises
15 polynucleotides encoding homologous Type II GnRH-Rs
16 from other species, preferably other mammalian
17 species. Murine, porcine, ovine, bovine, canine,
18 feline, equine and primate Type II GnRH-Rs are of
19 particular interest. The sequence information
20 provided in SEQ ID Nos 1, 3, 5, 7 and 9 together
21 with the techniques described herein and the
22 standard conventional cloning techniques known in
23 the art are sufficient to obtain such homologous
24 polynucleotides. For example, there is a
25 substantial body of knowledge concerning the
26 techniques required for the art of genetic
27 engineering and reference is made to Maniatis et
28 al, Molecular Cloning, A Laboratory Manual, Cold
29 Spring Harbor Laboratory, Cold Spring Harbor, New
30 York 1982 and "Principles of Genetic Engineering",
31 Old and Primrose, fifth addition, 1994.

1 The present invention also includes modified
2 sequences retaining Type II GnRH-R function (i.e.
3 the ability to bind GnRH Type II) and having at
4 least 70% homology (preferably 80% homology,
5 especially preferably 85-90% homology and most
6 preferably over 90% homology) with the nucleotide
7 sequence in question. Functional equivalents of
8 such polynucleotides are also part of this
9 invention. In particular, we include nucleotide
10 substitutions which do not affect the amino acid
11 expressed. The term "functional equivalent" used
12 herein refers to any derivative in which
13 nucleotide(s) and/or amino acid(s) have been added,
14 deleted or replaced without a significantly adverse
15 effect on expression of the gene product or on
16 biological function thereof. Thus, for example,
17 amino acid Glu may be encoded by the codon gag or
18 by the codon gaa and each construct may be varied
19 in this way without affecting the sequence of the
20 expressed peptide. Additionally, most vertebrates
21 have three types of GnRH and it is postulated that
22 three cognate receptors exist. Together with
23 collaborators (Troskie, Kwon and Wangli) we have
24 identified Types I, II and III GnRH receptors in
25 Xenopus and bullfrog. The Type III GnRH-R is more
26 similar to Type II GnRH-R than Type I. We
27 therefore propose the existence of a Type III human
28 GnRH-R with homology to Type II. Thus, the Type
29 III GnRH-R is expected to have substantial homology
30 with the Type II GnRH-R described herein and hence

1 will be covered by the modified versions of the
2 sequences disclosed.

3
4 The polynucleotides may be in any form (for example
5 DNA or RNA, double or single stranded) but
6 generally double stranded DNA is the most
7 convenient. Likewise the polynucleotides according
8 to the present invention may be part of a
9 recombinant genetic construct, which itself may
10 include a vector (for example an expression vector)
11 and eukaryotic vectors (as well as prokaryotic
12 vectors) are of interest. Alternatively, the
13 construct may be incorporated into the genome of a
14 transgenic animal. Any vectors or transgenic
15 animals comprising a polynucleotide as described
16 above form a further aspect of the present
17 invention.

18
19 Viewed in a yet further aspect the present
20 invention provides a recombinant expression system
21 able to express the Type II GnRH-R described above.
22 DNA constructs (i.e. a standard vector
23 recombinantly combined with a polynucleotide
24 sequence coding for the Type II GnRH-R of interest)
25 and cells transformed with such constructs are also
26 encompassed by the present invention.

27
28 The term "expression system" is used herein to
29 refer to a genetic sequence which includes a
30 protein-encoding region and is operably linked to
31 all of the genetic signals necessary to achieve

1 expression of that region. Optionally, the
2 expression system may also include a regulatory
3 element, such as a promoter or enhancer, to
4 increase transcription and/or translation of the
5 protein encoding region or to provide a control
6 over expression. The regulatory element may be
7 located upstream or downstream of the protein
8 encoding region or within the protein encoding
9 region itself.

10

11 In addition to the Type II GnRH-R construct
12 described above, the present invention also
13 provides host cells transformed with such
14 constructs and which may express the biologically
15 active modified gene product.

16

17 In a further aspect, the present invention provides
18 a stable cell-line capable of expressing Type II
19 GnRH-R, preferably human Type II GnRH-R, as
20 described above. By "stable" we mean that the
21 cell-line retains its ability to express useful
22 quantities of Type II GnRH-R after several (e.g.
23 10) generations, with any decrease in the level of
24 Type II GnRH-R expression being sufficiently low
25 not to materially affect the utility of the cell-
26 line.

27

28 Desirably the host cell transformed with the
29 construct encoding the human Type II GnRH-R is of
30 mammalian origin, but other cell types may also be
31 useful. Examples include prokaryotic cells (such

1 as *E. coli*), non-mammalian derived eukaryotic cells
2 (such as insect, yeast or plant cells). Suitable
3 host cells include, for example, COS-1 cells, COS-7
4 cells, COSM6 cells, CHO cells, BHK cells, GH₃
5 cells, HEK293 cells and 293EBNA cells.

6

7 The present invention also provides isolated
8 peptides comprising a functional Type II
9 gonadotropin-releasing hormone receptor (Type II
10 GnRH-R) peptide. Preferably said peptide comprises
11 at least a portion of exon I.

12

13 Preferred embodiments of the peptides according to
14 the present invention include peptides having an
15 amino acid sequence corresponding to the sequence
16 of amino acid nos 1 to 170 of SEQ ID No 2
17 (preferably comprising substantially the full
18 sequence as set out in SEQ ID No 2) or
19 corresponding to the sequence of amino acid nos 1
20 to 168 of SEQ ID No 6, amino acid nos 1 to 165 of
21 SEQ ID No 8 or amino acid nos 1 to 155 of SEQ ID No
22 10 (preferably also comprising substantially the
23 full sequence of SEQ ID Nos 4, 6, 8 or 10).

24

25 Desirably, the peptide is able to bind to Type II
26 GnRH and, preferably, is a functional receptor
27 therefor.

28

29 The present invention also provides antibodies
30 specific to Type II GnRH-R peptides, preferably
31 antibodies which are specific to the extracellular

1 domains of Type II GnRH-R, for example EC3 in exon
2 3 thereof. The term "antibodies" as used herein
3 includes not only monoclonal and polyclonal
4 antibodies, but also antigen binding fragments
5 thereof, trimeric or tetrameric constructs and
6 recombinant or proteolytic antibody fragments.

7
8 Antibodies directed to EC1, EC2 and EC3 (especially
9 EC2) are of particular interest and may be of
10 therapeutic value for cancer treatment.

11 The Type II GnRH-R can be expressed and used to
12 screen agents of potential therapeutic interest.

13
14 Thus, the present invention further provides a
15 method of screening an agent for pharmacological
16 activity (i.e. to ascertain its utility in binding
17 to Type II GnRH-R), said method comprising:

- 18 a) providing a Type II GnRH-R peptide as
19 described above and exposing said Type II
20 GnRH-R peptide to the agent to be tested; and
21
22 b) ascertaining whether said agent interacts with
23 (for example binds specifically to) said Type
24 II GnRH-R peptide.

25
26 Labelled (eg. radio-labelled or fluorescent-
27 labelled) antibodies may be used to determine
28 whether the agent and Type II GnRH-R interact
29 together. Optionally a washing step may be
30 included to remove labels not bound to the agent or
31 GnRH-R.

1
2 An expression system able to produce the Type II
3 GnRH-R described above may be used in this method;
4 preferably the expression system will be a
5 transformed cell-line, the host cell usually being
6 of mammalian origin. Alternatively the expression
7 system may be a transgenic animal.

8
9 Optionally, the receptor expressed may be mutated
10 to produce constitutively active receptors which
11 can be used to screen for antagonists and agonists.

12
13 An expression system able to produce the Type II
14 GnRH-R described above may be used to screen agents
15 of potential therapeutic use (such as Type II GnRH
16 agonists or antagonists). Desirably, therefore the
17 expression system will imitate at least some
18 aspects of Type II GnRH-induced signal
19 transduction. Optionally, the expression system
20 may be a stable cell line, the host cell usually
21 being of mammalian origin. Alternatively the
22 expression system may be a transgenic animal.

23
24 In another embodiment of the invention, the Type II
25 GnRH-R itself or extracellular domain thereof (e.g.
26 the EC2 or EC3 loop) could be administered *in vivo*.
27 The free GnRH-R or extracellular domains (which
28 could be synthetic peptides) could competitively
29 bind to GnRH and inhibit its reaction with the
30 native receptor *in vivo*.

31

1 Alternatively, the Type II GnRH-R or an
2 extracellular domain thereof may be used as a means
3 of contraception. For example, a patient may be
4 immunised by injection with the Type II GnRH-R.
5 This will induce antibody production to the Type II
6 GnRH-R and the antibodies so produced will also
7 interact with native Type II GnRH-R affecting
8 reproductive ability. Alternatively, the exogenous
9 Type II GnRhH-R may bind competitively for
10 endogenous GnRH.

11

12 The present invention will now be further described
13 with reference to the following, non-limiting,
14 examples and Figures in which:

15

16 Fig. 1 is a schematic representation of the human
17 gene structure of Type I GnRH-R and of Type II
18 GnRH-R. The positions of the extracellular (EC)
19 and intracellular (IC) loop domains, transmembrane
20 (dark blocks) and carboxy terminal tail (C) are
21 indicated. The approximate positions of the "short
22 splice" (SS) for intron 1' and selenocysteine
23 (SeCys) in the human receptor is shown.

24

25 Fig. 2 shows the immunolocalisation of Type II GnRH
26 receptor and Luteinizing Hormone (LH) to sheep
27 pituitary:

28

29 a) immunolocalisation of Type II GnRH-R using
30 antibody against EC3;

31

- 1 b) immunolocalisation of LH to pituitary
2 gonadotropes;
3
4 c) co-localisation using antibodies a) and b) and
5 enzymatic detection;
6
7 d) co-localisation of Type II GnRH-R (black) and
8 LH (grey) by confocal microscopy; some cells
9 contain LH only but most expressing Type II
10 GnRH-R are LH positive.

11
12 Fig. 3 is a comparison of the amino acid sequences
13 of human Type II GnRH-R according to splicing
14 alternative 1, and marmoset Type II GnRH-R.

15
16 The numbering used is that of the human sequence.
17 Asterisks (*) indicate identity, and vertical
18 slashes (|) conserved substitution. Regions
19 predicted to be helical from homology modelling
20 with the rhodopsin crystal structure are indicated.
21 Exon boundaries, the position of the short splice
22 (intron 1') and the apparent stop codon in the
23 human sequence are also shown.

24
25 Fig. 4 shows the heterologous competitor binding of
26 ^{125}I -[His⁵, D-Tyr⁶]GnRH mediated by the Marmoset
27 Type II GnRH-R expressed in COS-7 cells in the
28 presence of increasing doses of the peptides (IC₅₀
29 values indicated in nM): mGnRH (mammalian GnRH, ■,
30 51.7± 1.9nM; GnRH II (GnRH Type II, ▲, 1.3±0.2nM);

1 and sGnRH (salmon GnRH, ▼, $9.8 \pm 1.4 \text{ nM}$). Error bars
2 represent s.e.m. ($n=2$).

3
4 Fig. 5 shows the total inositol phosphate
5 production mediated by the Marmoset Type II GnRH-R
6 expressed in COS-7 cells in response to
7 concentration of various peptides (EC_{50} values
8 indicated in nM) mGnRH (mammalian GnRH, ■, $33.2 \pm$
9 6.3 nM); GnRH II (GnRH Type II, ▲, $0.46 \pm 0.12 \text{ nM}$);
10 sGnRH (salmon GnRH, ▼, $8.7 \pm 0.5 \text{ nM}$); [D-Arg⁶] GnRH II
11 ([D-Arg⁶]GnRH Type II, ◆, $1.1 \pm 0.23 \text{ nM}$); and Ant 135-
12 18 (Antagonist 135-18, ●, $223 \pm 7 \text{ nM}$). Error bars
13 represent s.e.m. ($n=2$).

14
15 Fig. 6 shows receptor binding (a) and inositol
16 phosphate production (b) of mammalian GnRH I (○)
17 and GnRH II (●) in COS-7 cells transfected with
18 marmoset Type II receptor (left panel) and human
19 Type I receptor (right panel). Stimulation of
20 inositol phosphate by Type I receptor Antagonist
21 135-18 (□) at the Type II receptor is also shown.
22 Error bars represent s.e.m. of 3-6 separate
23 experiments.

24
25 Fig. 7 shows the Luteinizing hormone (LH) and
26 Follicle Stimulating Hormone (FSH) response to GnRH
27 I and II in sheep.

28 Fig. 8 shows activation of ERK2 and p38 α MAP
29 kinases by Type I (open bars) and Type II (closed
30 bars) GnRH receptors in COS-7 cells. Stimulation of

1 Type I (a) and II (b) GnRH receptors with mammalian
2 GnRH I, GnRH II and Antagonist 135-18 of
3 immunoprecipitated myc-ERK2. Inset panels depict
4 anti-phospho-ERK2 immunoblotting of anti-myc COS-7
5 cell immunoprecipitates. c, Left panel, selective
6 and time-dependent activation of p38 α by GnRH II
7 stimulation (100 nM) of Type II GnRH receptor.
8 Stimulation of Type I GnRH receptor (100 nM) failed
9 to activate p38 α MAP kinase. The right panel
10 demonstrates that both Type I and Type II GnRH
11 receptor stimulation induces a time-dependent
12 activation of ERK but that the GnRH II stimulation
13 of the Type II receptor is more prolonged. Data
14 represent mean $\pm$ s.e.m., $n \geq 3$.

15
16 Fig. 9 shows expression of Type II GnRH receptor in
17 marmoset and human tissues. (a) RT-PCR was carried
18 out with specific primers on cDNA prepared from
19 marmoset RNA isolated from various tissues. PCR
20 products were fractionated by size on agarose gels.
21 Type II GnRH receptor levels were normalised to
22 actin RNA and represented as the log of the RNA
23 expression relative to pituitary. Hatched bars
24 indicate marmoset brain tissues, solid bars
25 indicate marmoset reproductive tissues while open
26 bars indicate other marmoset tissues. (b, c)
27 Expression of the Type II GnRH receptor in human
28 tissues was examined in Northern blots of mRNA
29 (Clontech) by hybridisation with ^{32}P labelled human
30 exon 1; (b) mRNA from human cerebellum (1),
31 cerebral cortex (2), medulla (3), spinal cord (4),

1 occipital pole (5), frontal lobe (6), temporal lobe
2 (7) and putamen (8); (c) mRNA from heart (1), whole
3 brain (2), placenta (3), lung (4), liver (5),
4 skeletal muscle (6), kidney (7) and pancreas (8).
5 Another blot showed moderate expression in the
6 amygdala and low expression in caudate nucleus,
7 corpus callosum, hippocampus, substantia nigra,
8 subthalamic nucleus and thalamus (data not shown).

9

10 Examples

11

12 Following numerous failed attempts to locate exon I
13 from the sequence reported by Millar et al.
14 (Journal of Endocrinology (1999), Vol 162, pages
15 117-126) subsequent work demonstrated that the
16 antisense sequence of Millar et al. was the 3'
17 untranslated end of an RNP which localised to
18 chromosome 14.

19

20 It was recognised that as long as exon 2 or exon 3
21 sequence information was used in PCR we would
22 always detect the 3' untranslated end of the RNP
23 since this is highly expressed in all tissues. In
24 addition, the putative intron 2 of Type II receptor
25 would always be retained. Since this transcript
26 ends with a polyadenylation in the region of the
27 equivalent of intron 1 of the putative Type II
28 receptor (read in the opposite direction to RNP),
29 we would never obtain information on exon 1 of the
30 Type II receptor.

31

1 We concluded that three new elements were necessary
2 to find the putative Type II GnRH receptor.

3

4 a) Search the data bases for a nucleotide
5 sequence encoding part of exon 1.

6

7 b) Generate an antibody against a highly specific
8 domain which is responsible for ligand
9 selectivity (see Sealfon et al., Endocr.
10 Review 18:180-205 and Troskie et al., General
11 and Comparative Endocrinology 112:296-302
12 (1998)) in extracellular loop 3 of the human
13 Type II vis-à-vis Type I receptor to determine
14 in which tissue this rare receptor is
15 expressed.

16

17 c) Undertake chromosomal localisation to
18 determine if the coding sequence exists in a
19 pseudogene locale (with RNP) and in another
20 "real" gene location elsewhere.

21

22 **Materials and Methods**

23

24 **Cloning of the marmoset Type II GnRH receptor.** RNA
25 was isolated from marmoset pituitary and brain stem
26 using RNAsol B (Biogenesis). 2 µg of RNA was
27 incubated with 1 mM dNTPs, 2 µM random hexa-
28 polynucleotides (Promega), gene specific primers or
29 anchored oligo-dT primers at 80°C for 10 min. 1x
30 RT buffer (Sigma), 1 U/µl RNAsin (Promega) and 0.5
31 U/µl AMV reverse transcriptase (Sigma) were added

1 in a total volume of 20 μ l and incubated at 55°C
2 for 2 h, then 65°C for 10 min. Primers designed to
3 the Type II marmoset GnRH receptor exon sequences.
4 Primers were chosen to span putative introns, to
5 enable detection of processed RNA in the presence
6 of possible genomic DNA contamination and the RNP
7 antisense transcript. 50 ng of purified (Qiagen)
8 cDNA produced with random hexa-polynucleotides were
9 subjected to PCR using human sequences previously
10 described (17) and human genomic sequence encoding
11 exon 1 (Zymogenetics AL 160282, BG 636291, AA
12 954764). Round 1 PCR: 5 cycles at 65°C, 23 cycles
13 at 63°C using primers S1 and A1 (S1,
14 GATGCCACCTGGAATATCACTG (SEQ ID No 17); A1,
15 AGGCAGCAGAAGG (SEQ ID No 18). Round 2 PCR: 5
16 cycles at 63°C, 25 cycles at 61°C using 1 μ l of
17 products from Round 1 as template and primers S2
18 and A2 (S2, CAGCCTGGGGACTTAGTTTCCTG (SEQ ID No 19);
19 A2,GGTTATAGGTGGTCTCTTGC (SEQ ID No 20). Products
20 were size-purified (Qiagen), cloned into pGEM-T
21 (Promega) and sequenced. Sense and antisense
22 oligonucleotides were designed from the novel
23 marmoset sequences and used in 3' and 5' RACE. For
24 the remaining PCRs a three-step protocol was used
25 where the annealing temperature of the first 5
26 cycles was 2°C higher than the lower T_m of the two
27 primers. In the second step the annealing
28 temperature of 5 cycles was the same as the lower
29 T_m of the two primers. The third step was 20
30 cycles with annealing temperatures 2°C below the
31 lower T_m of the two primers. For the 5' RACE a

1 poly-A sequence was added to 50 ng marmoset
2 pituitary cDNA produced with gene specific primers.
3 Products were purified (Qiagen) and subjected to
4 PCR. 2 μ l of products of a first round PCR, using
5 primers S3 and A3, were used in a second round of
6 PCR using primers S4 and A4 (S3,
7 GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTV (SEQ ID No
8 21) (anchored RACE primer from Boehringer
9 Mannheim); A3, GAAGGGACTGGACCAGCTCG (SEQ ID No 22);
10 S4, GACCACGCGTATCGATGTCGAC (SEQ ID No 23); A4,
11 CAAGGCAAGCAGGAACTAAG (SEQ ID No 24)). For the 3'
12 RACE 50 ng marmoset pituitary cDNA was produced
13 with anchored oligo-dT primers was subjected to PCR
14 using primers S5 and S3 (S5, ACCTCTTCACCTTCTGCTGCCT
15 (SEQ ID No 25)). 2 μ l of products from this PCR
16 and primers S6 and S4 were used in a secondary PCR
17 (S6, CTCCTCAATGCTCCTTTGGATC (SEQ ID No 26)).
18 Products were size purified (Qiagen), cloned into
19 pGEM-T (Promega) and sequenced. Full length
20 marmoset Type II GnRH receptor was produced by PCR
21 using oligos of the 5' UTR (S7,
22 GAATTCGCTTCATACTCACACTTCATC (SEQ ID No 27); S8,
23 CGGAATTCTCACACTTCATCCTCCTATCTC (SEQ ID No 28)) and
24 the 3' sequence including the stop codon
25 (A5, GCTCTAGAGATCAGATTGATGTTATAGGAATG (SEQ ID No
26 29)). 50 ng of marmoset brain stem cDNA produced
27 with random hexa-polynucleotides was subjected to
28 PCR using primers S7 and A5. 2 μ l of products and
29 primers S8 and A5 were used in a secondary round of
30 PCR. Products of this PCR were purified (Qiagen),
31 cloned into pCDNA3.1+ (Invitrogen) and sequenced.

1 The resultant plasmid was used in expression
2 studies.

3
4 **Immunocytochemistry.** Tissues from human, mouse,
5 sheep, rhesus and cynomologus monkeys were
6 obtained. An antiserum to the human Type II GnRH
7 receptor was produced by immunisation of rabbits
8 with a synthetic peptide corresponding to EC3
9 (YSPTMLTEVPPC (SEQ ID No 30)) conjugated to keyhole
10 limpet haemocyanin via the Cys residue. This
11 peptide, a synthetic peptide to EC3 of the human
12 Type I receptor (DPEMLNRLSDPC (SEQ ID No 31)) and
13 haemocyanin were used for immunoneutralisation
14 specificity studies. For detection of mammalian
15 GnRH I specific antiserum GF6 was used (18).

16
17 Tissue sections (15 μ m) were subjected to the
18 peroxidase/diaminobenzidine visualisation technique
19 as previously described (18, 19). Fluorescent
20 labelling was accomplished using the same procedure
21 up to the step prior to ABC reaction, when the
22 fluorescein label (Rhodamine 600, avidin D or FITC)
23 was applied to the slides and incubated at room
24 temperature in the dark for 2-4 hours. For double
25 labelling, slides were incubated sequentially with
26 avidin D and biotin blocking solutions for 15 min
27 each, then re-incubated with the next primary
28 antibody, followed by the other fluorescent
29 labelling (Rhodamine or FITC). Controls, including
30 omission of primary antibodies and order of
31 exposure, were consistently negative.

1 Immunofluorescence was viewed at two wave lengths
2 by confocal microscopy.

3

4 **Cell culture and transfection.** COS-7 cells were
5 cultured as previously described (20, 21).
6 Transient transfections of COS-7 cells with human
7 Type I GnRH receptor or marmoset Type II GnRH
8 receptor, along with myc-tagged ERK2, JNK and p38 α
9 constructs were performed using Superfect (Qiagen)
10 according to the manufacturer's protocol. All
11 assays described were performed 48 h post
12 transfection.

13

14 **Receptor binding and inositol phosphate production.**
15 Receptor binding utilising ^{125}I -[His⁵-D-Tyr⁶]GnRH I
16 and inositol phosphate production by GnRH ligands
17 were studied as previously described (20-22).

18

19 **Phospho-MAP kinase assay.** Serum starved (12-16 h)
20 COS-7 cells, transfected with human Type I or
21 marmoset Type II GnRH receptors were treated with
22 either mammalian GnRH I, GnRH II or antagonist 135-
23 18 for the time and dose specified for Fig. 9 at
24 37°C. After ligand stimulation, COS-7 cells were
25 prepared for immunoprecipitation (23). Co-
26 transfected myc-tagged MAP kinase constructs were
27 immunoprecipitated from cell lysates by overnight
28 incubation with myc-agarose slurry (Santa Cruz),
29 washed, an equal volume of 2x Laemmli sample buffer
30 added, resolved by SDS-PAGE and electrotransferred
31 to PVDF membrane (NEN Life Sciences). Activated MAP

1 kinase was detected using anti-phospho-
2 ERK/JNK/p38a-kinase specific antisera (New England
3 Biolabs), visualised by enzyme-linked
4 chemifluorescence (Amersham-Pharmacia) and
5 quantified using a phosphorimager. The degree of
6 phosphorylated MAP kinase was normalised to the
7 amount of unphosphorylated MAP kinase detected with
8 specific antisera.

9
10 **Expression of Type II receptor mRNA in marmoset and**

11 **human tissues.** Total RNA was extracted from
12 various marmoset tissues using TRI reagent (Sigma),
13 and cDNA was produced using oligo dT primers
14 (Ambion). PCR was performed on the cDNA using
15 marmoset Type II GnRH receptor cDNA specific
16 primers spanning exons 1-3 (sense:
17 CTCGGCTGGAGGGAACCTG, antisense:
18 GGTGCCCTCTTCGGCAGC), and actin specific primers.
19 PCR products were run on an agarose gel and blotted
20 onto HybondN⁺ nylon membrane (Amersham). The
21 Southern blot was probed with random primed
22 marmoset Type II GnRH receptor cDNA or actin cDNA.
23 Southern blots were quantified using a
24 phosphorimager and marmoset Type II GnRH receptor
25 expression was normalised to the expression of
26 actin.

27

28 Expression in human tissues was examined by
29 Northern blots of mRNA with random primed ³²P
30 labelled human Type II GnRH receptor exon 1. The
31 human Type II GnRH receptor genomic sequence (P1

1 clone) was obtained by Genome Systems (St Louis,
2 MD) using PCR screen of P1 clones with
3 oligonucleotides to human sequences (17).
4 Oligonucleotides (antisense: CTGTCCTGCCCCGGTCCTGAG;
5 sense: TGCCCACCTTCTCGGCAGCA) to this sequence were
6 used with the P1 clone to produce a 460 bp
7 amplicon. Labelling was done with ^{32}P dCTP (6000
8 Ci/mMole) using supplier's specified conditions
9 (Stratagene). Hybridisation was performed using 2
10 $\times 10^7$ cpm at 65°C in $5 \times \text{SSC}/0.005\% \text{SDS}/5\times$
11 Denhardt's/2 mg/ml salmon sperm DNA, washing with
12 $0.1 \times \text{SSC}/0.5\% \text{SDS}$ at 55°C and the blots exposed to
13 X-ray film for 6 days.

14

15 **Stimulation of LH and FSH in sheep.** The relative
16 potency of mammalian GnRH I and GnRH II at inducing
17 FSH and LH secretion *in vivo* was tested using our
18 Soay ram sheep model (24). This was repeated
19 during both the sexually active (short days, SD)
20 and inactive phases (long days, LD) of the
21 photoperiod-induced reproductive cycle (24). The
22 same animals ($n = 8$) were used on the two
23 occasions. The GnRHs were administered at doses of
24 250 ng/ram and 10 μg /ram in a cross-over design
25 with a week between treatments to permit full
26 recovery. The peptides were dissolved in 1 ml of
27 0.9% saline and given as an intravenous bolus.
28 Blood samples were collected every 10 min, from 20
29 min before, until 2 h after the treatments and
30 assayed by specific radioimmunoassays (24). The
31 FSH and LH responses to the GnRH agonists were

1 calculated as delta responses (2 h mean hormone
2 concentration post treatment minus 20 min mean pre-
3 treatment hormone concentration). These values
4 were used to calculate the FSH : LH response ratio
5 for GnRH II and GnRH I, and thus the overall ratio
6 for the GnRH II stimulation compared with the
7 mammalian GnRH I stimulation. This was assessed
8 for each animal and then for the group (mean $\pm$ SEM,
9 $n = 8$), under both LD and SD.

10

11 **Results and Discussion**

12

13 **Cloning and primary structure of the Marmoset Type** 14 **II GnRH receptor.**

15

16 Amplification of EC3 from genomic DNA from a range
17 of vertebrate species revealed two distinct
18 sequences of receptors representing the known Type
19 I receptors and novel Type II receptors in an
20 amphibian and reptile (25). Searches of human EST
21 databases revealed homologous sequences to the
22 reptile EC3 (17). From EST contigs we constructed
23 a partial receptor sequence encoding the putative
24 exons 2 and 3 corresponding to these exons of the
25 Type I receptor (17). All ESTs were in the
26 antisense orientation and it transpired that these
27 were in the 3' untranslated region (UTR) of a novel
28 human ribonucleoprotein (RBM8) which was highly
29 expressed in all tissues examined (26). The
30 equivalent of exon 1 was absent from the RBM8 3'
31 UTR. It was therefore evident that the

1 identification of sequences homologous to exon 1
2 was essential to discover the Type II receptors.
3 Searches of human databases, using as a query exon
4 1 of the human Type I receptor, revealed several
5 ESTs and genomic sequences BG 036291, AA 954764; AL
6 160282.

7
8 Since the receptor was likely to be a rare
9 transcript expressed in discrete tissues, we
10 generated antisera to the EC3 domain of the human
11 Type II receptor and found by immunocytochemistry
12 strong reactivity in pituitary and brain of the
13 human, rhesus monkey, sheep and mouse (Fig. 2). We
14 then used oligonucleotides to the human exon 1 and
15 RBM8 3' UTR sequences to amplify cDNA from marmoset
16 pituitary and brain by PCR and 5' RACE procedures.
17 The full length cDNA encodes a 380 amino acid
18 protein with characteristic G protein-coupled
19 receptor (GPCR) structure SEQ ID No. 1. Although
20 it is more homologous with GnRH receptors than
21 other GPCRs, it has only 41% sequence identity with
22 the human Type I receptor suggesting an early
23 evolutionary gene duplication. It also possesses a
24 carboxy terminal tail which is important for rapid
25 desensitization and is uniquely absent from
26 mammalian Type I receptors (27-30). The receptor
27 also does not have the unusual Asn/Asp microdomain
28 of transmembrane helices 2 and 7 of the mammalian
29 Type I receptors which plays a role in receptor
30 activation (21). Instead it has the Asp/Asp motif
31 as in non-mammalian Type I GnRH receptors recently

1 cloned (7, 9). The *Drosophila* GnRH receptor
2 homologue has the usual Asp/Asn motif
3 characteristic of most GPCRs (31) indicating that
4 there was an initial mutation to Asp/Asp in the
5 ancient vertebrate GnRH receptor followed by
6 mutation to Asn/Asp in the mammalian Type I
7 receptors. The activation role of this microdomain
8 (21) may therefore be further elucidated by
9 experimentation with the Type II receptor. The
10 LSD/EP sequence of EC3 which is important for
11 ligand selectivity of mammalian Type I receptors
12 (9, 20) is replaced by VPPS which is also present
13 in reptile (VPPS) and amphibian (VPPV) Type II GnRH
14 receptors (25). This difference in sequence is
15 likely, therefore, to be a determinant of Type II
16 receptor selectivity for GnRH II as all other
17 binding sites (9) are conserved.

18

19 **Pharmacological characterization of the marmoset**
20 **Type II GnRH receptor.**

21 Fig. 4 shows the heterologous competition binding
22 of ^{125}I -[His⁵, D-Tyr⁶]GnRH mediated by the Marmoset
23 Type II GnRH receptor expressed in the transfected
24 COS-7 cells and shows that the Type II GnRH
25 receptor demonstrates high selectivity for GnRH
26 Type II.

27

28 The affinity and specificity of the Type II GnRH
29 receptor for the natural GnRHs and two GnRH
30 analogues is shown in Fig. 5. Further
31 characterisation of GnRH agonists and antagonists

1 can be determined with the routine binding assays.
2 The identification of the signalling pathway of the
3 receptor was examined by testing the ability of the
4 ligand-induced receptor to activate second
5 messenger generating systems.

6
7 Using the above described expression system, we
8 have determined that the Type II GnRH-R activates
9 the production of Inositol phosphate (see Fig. 5)
10 but does not stimulate cyclic AMP production.

11
12 Thus, expression of the Type II receptor in COS-7
13 cells revealed that it is highly selective for GnRH
14 II in receptor binding assays (Fig. 4) and in the
15 stimulation of inositol phosphate intracellular
16 messenger production (40-fold and 90-fold greater
17 activity relative to mammalian GnRH I) (Fig. 5)
18 (Table 1). This contrasts with the Type I receptor
19 in which GnRH II has only 10% and 9% activities of
20 mammalian GnRH I in these assays (Fig. 6). Overall
21 GnRH II has an affinity 24-fold greater for the
22 Type II receptor than for the Type I receptor. The
23 Type II receptor was also more selective for salmon
24 GnRH and [D-Arg⁶]GnRH II (Table 1).

25
26
27
28
29

Table 1 : Comparative ligand binding and inositol phosphate production properties of marmoset type II & human Type 1 GnRH receptors.

Peptides	Ligand binding (IC ₅₀)		InsP production (EC ₅₀)	
	Marmoset Type II	Human Type I	Marmoset Type II	Human Type I
GnRH II	1.07±0.04	26.1±4	0.45±0.05	7.41±1.55
GnRH I	42.6±3.19	2.81±0.17	40.5±4.43	0.63±0.08
sGnRH	9.48±2.17	244±23.6	5.99±0.91	9.62±3.5
[D-Arg ⁶] GnRH II	3.34±0.06	11.9±0.35	2.39±0.64	3.8±0.71
Antagonist 135-18	1650±478	10.6±1.4	276±45.5	Full antagonist

Data are expressed in nanomolar and represent the s.e.m. (n=3-6). Mammalian GnRH I (GnRH I) salmon GnRH (sGnRH: [Trp⁷, Leu⁸]GnRH I), GnRH II ([His⁵, Trp⁷, Tyr⁸]GnRH I)

- 1 Moreover, a Type I receptor GnRH antagonist behaved
- 2 as an agonist at the Type II receptor (Fig. 5). It
- 3 has recently been demonstrated that control of
- 4 gonadotropin biosynthesis and regulation of
- 5 gonadotrope function regulated by GnRH can be
- 6 mediated by the activation of mitogen-activated

1 protein kinases (MAP kinases). Therefore we
2 assessed the capacity of both the Type I human GnRH
3 receptor and the marmoset Type II GnRH receptor to
4 activate the three major MAP kinase prototypes,
5 ERK, JNK (a particular type of MAP kinases
6 available through Eisuke Nishida, Kyoto University)
7 and p38 α in COS-7 cells. At the Type I receptor,
8 mammalian GnRH I was considerably more potent than
9 GnRH II in activating ERK2 (Fig. 8a). In contrast,
10 at the Type II receptor the ligand specificity was
11 the inverse and antagonist 135-18 had significant
12 agonist activity compared with low activity at the
13 Type I receptor (Fig. 8 panels a and b). Agonist-
14 induced activation of JNK was not detected with
15 stimulation of either the Type I or Type II
16 receptor (data not shown). However, activation of
17 p38 α was detected upon stimulation of the Type II
18 receptor but not with stimulation of the Type I
19 receptor (Fig. 8c). The time course of p38 α
20 activation was also considerably more protracted
21 than that for Type I/II receptor activation of
22 ERK2. In addition we noted that ERK2 stimulation
23 via the Type I GnRH receptor is more transient than
24 that mediated by Type II GnRH receptor stimulation
25 (Fig. 8c). There are therefore distinct
26 differences in signalling by the two receptors.

27

28 **Tissue distribution and expression of the marmoset**
29 **Type II GnRH receptor.** To gain insight into the
30 potential functions of the Type II receptor, its
31 expression in human and marmoset tissues was

1 examined. PCR amplification of cDNA from marmoset
2 brain tissues revealed that it is expressed in the
3 pituitary, spinal cord, pons, cerebellum, putamen,
4 medulla, hypothalamus, preoptic area, midbrain,
5 occipital pole, frontal lobe and corpus callosum
6 (Fig. 9a). Expression was high in reproductive
7 tissues such as testis, prostate, mammary glands,
8 seminal vesicles and epididymis. Substantial
9 expression was detected in adrenal, thyroid, heart
10 and skeletal muscle but little or no expression was
11 found in other tissues such as liver, ovary and
12 bladder (Fig. 9a). Northern blots yielded a
13 similar expression pattern (data not shown).
14 Northern blots on human tissues probed with exon 1
15 showed highest expression in the cerebral cortex
16 and occipital pole, moderate expression in the
17 frontal lobe, temporal lobe and putamen, and low
18 expression in the cerebellum, medulla and spinal
19 cord (Fig. 9b). There was substantial expression
20 in the amygdala and low expression in the caudate
21 nucleus, corpus callosum, hippocampus, substantia
22 nigra, subthalamic nucleus and thalamus (data not
23 shown). There was also significant expression in
24 the heart and pancreas but little or no expression
25 in placenta, lung, liver, skeletal muscle and
26 kidney (Fig. 9c).

27

28 **Type II GnRH receptor function in the pituitary.**

29 A specific antiserum to EC3 of the human Type II
30 receptor was used to conduct immunocytochemistry
31 and demonstrated specific expression of the

1 receptor in human anterior pituitary (Fig. 2). In
2 view of the possibility that the novel Type II
3 receptor may regulate pituitary function, we
4 determined if it was expressed in the pituitary of
5 other mammals. Staining was also found in about
6 10% of cells, (the relative occurrence of
7 gonadotropes), in the anterior pituitary of the
8 mouse and sheep (Fig. 2). In the sheep anterior
9 pituitary double staining with Type II receptor and
10 LH antisera revealed that the Type II receptor
11 immunoreactivity is co-localised in 69% of LH
12 positive cells (Fig. 2). Only 12% of Type II
13 receptor positive cells were negative for LH.
14 Since mammalian GnRH I binding sites also co-
15 localise with LH in up to 90% of gonadotropes in
16 the rat pituitary at proestrus (32), it is likely
17 that the majority of gonadotropes express both Type
18 II and Type I receptors and suggests that these
19 receptors may co-ordinately regulate LH and FSH
20 biosynthesis and secretion. The presence of Type
21 II receptors in the majority of gonadotropes is, at
22 first consideration, unexpected as there is a
23 substantial literature suggesting that a single
24 GnRH (mammalian GnRH I) is sufficient to regulate
25 the secretion of gonadotropins, and that the
26 differential secretion of LH and FSH during the
27 mammalian ovarian cycle may be adequately accounted
28 for by modulatory effects of gonadal steroids
29 (androgen, estrogen and progesterone) and peptides
30 (activin, inhibin and follistatin) (14). However,
31 a substantial number of physiological studies

1 invoke the existence of an FSH-releasing peptide to
2 account for the differential secretion of
3 gonadotropins (13-16).

4

5 In the early studies on the GnRH II (previously
6 called chicken GnRH II), it was found to have
7 preferential FSH-releasing activity when compared
8 with chicken GnRH I (chicken Type I GnRH) (33).
9 Moreover, GnRH II has been localised to the
10 hypothalamic area in species of non-mammalian
11 vertebrates (see Refs. (7, 8) for review) and the
12 supraoptic, paraventricular, arcuate and pituitary
13 stalk regions of monkeys where it is thought to
14 play a role in gonadotropin secretion (19, 34). We
15 therefore conducted studies using a well-
16 established sheep model to determine the relative
17 effects of mammalian GnRH I and GnRH II on LH and
18 FSH secretion. The responses to a 250 ng bolus of
19 GnRHs was too low for comparison of relative LH and
20 FSH secretion. At the 10 µg dose all the rams
21 showed a robust response and every individual
22 exhibited a higher ratio of FSH to LH secretion
23 when treated with GnRH II compared with mammalian
24 GnRH I (Fig. 7). The mean ratio of FSH to LH
25 induced by GnRH II was 2.14 ± 0.29 and 2.02 ± 0.34
26 (mean $\pm$ SD) times higher than that induced by
27 mammalian GnRH I for sexually active and sexually
28 quiescent rams respectively. The FSH/LH ratios
29 generated in both sexually active and quiescent
30 rams upon GnRH II treatment were both significantly
31 greater ($p=0.03$, $p=0.002$ respectively, paired two-

1 tailed t-test) than with mammalian GnRH I
2 treatment.

3

4 When it is considered that GnRH II has an affinity
5 and potency of $\leq 20\%$ of mammalian GnRH I at the
6 Type I receptor (7, 9, 20), and the *in vivo*
7 secretion of the two peptides is likely to be
8 finely tuned in both concentration and phasing of
9 pulsatile release, our exogenous bolus
10 administration of the peptides is a relatively
11 crude approach. Thus, the relative differential
12 stimulation of FSH by endogenously secreted GnRH II
13 may be much greater *in vivo*.

14

15 The findings of GnRH II in the hypothalamus, the
16 presence of the Type II receptor immunoreactivity
17 in gonadotropes and differential release of
18 gonadotropins suggest that, contrary to existing
19 dogma, gonadotropins are regulated by two different
20 forms of GnRH acting through two separate cognate
21 gonadotrope receptors. In order to elicit
22 differences in relative LH and FSH secretion
23 mammalian GnRH I and GnRH II would have to have
24 different patterns of duration of release,
25 concentration and pulse frequency of secretion
26 and/or differential intracellular signalling
27 pathways. The differential, temporal or
28 qualitative, downstream signalling between the Type
29 II and Type I receptors shown here may provide the
30 means for preferential FSH secretion.

31

1 GnRH II activation of the Type II receptor in
2 bullfrog sympathetic ganglia potently inhibits M-
3 type K⁺ channels (11). A similar action in
4 gonadotropes would partially depolarise them thus
5 facilitating external excitatory inputs to the cell
6 or entry of extracellular Ca²⁺ through L type
7 channels, which occurs on stimulation of Type I
8 receptors by mammalian GnRH I (1-3). These two
9 GnRHs and GnRH receptor systems, along with
10 differences in signalling pathways, provide the
11 means for differential FSH and LH secretion and
12 open the possibility of developing new GnRH II
13 analogues for the treatment of diseases of the
14 reproductive system as well as contraceptives which
15 selectively inhibit FSH and gametogenesis without
16 affecting sex steroid hormone production.

17

18 **Type II GnRH receptor may have roles in neural**
19 **development and sexual arousal.** In view of its
20 wide distribution in the central and peripheral
21 nervous systems, GnRH II has been proposed to have
22 a neuromodulatory role (7, 8) as evidenced by K⁺
23 channel inhibition in bullfrog sympathetic ganglia
24 (11). Our demonstration of a GnRH II-selective
25 receptor expression in many brain regions (Figs. 2,
26 9) supports this. Type II GnRH receptor antisera
27 immunoreactive cells were widely seen in the
28 extrahypothalamic regions, such as medial septum,
29 bed nucleus of the stria terminalis, medial
30 preoptic area, substantia innominata, basal nucleus
31 of Meynert, claustrum, amygdala and putamen, and in

1 the hypothalamic regions, such as supraoptic
2 nucleus, periventricular area, ventromedial nucleus
3 and dorsomedial nucleus in rhesus monkeys at
4 embryonic days E58, E70 and E78 as well as in the
5 adult cynomologus monkey. In some of these areas
6 (e.g. midbrain and supraoptic nucleus) the GnRH II
7 ligand is also expressed (19, 34). The
8 distribution pattern of Type II GnRH receptor
9 positive cells in extrahypothalamic regions
10 overlapped with that of the early developing
11 mammalian GnRH I cells we have described (18).
12 Later in embryonic development the GnRH I cells
13 were not consistently immunopositive with Type II
14 GnRH receptor antiserum. This suggests a potential
15 role for the receptor in the development of
16 mammalian GnRH I neurones. An intriguing
17 observation was that neurones which express the
18 mammalian GnRH I gene in the preoptic area and
19 periventricular region of the hypothalamus (Ref.
20 34) were stained with the Type II GnRH receptor
21 antiserum in the rhesus monkey, suggesting that
22 GnRH II may regulate mammalian GnRH I neurones.
23 Mammalian GnRH I is known to have ultrashort
24 feedback on its own secretion (8, 35) but the co-
25 localisation of Type II receptor on mammalian GnRH
26 I neurones in the hypothalamic regions suggests
27 that some effects on mammalian GnRH I secretion may
28 be mediated via GnRH II.
29 GnRH has been shown to have direct effects on
30 reproductive behaviour and sexual arousal in
31 rodents independent of its stimulation of sex

1 hormone production (8, 36). Rapid changes in GnRH
2 content of brain areas, cell number and cell size
3 in response to visual, olfactory and other
4 stimulants of sexual behaviour have been observed
5 in species of fish, amphibians, reptiles and
6 mammals (see Refs. (7, 8, 10, 36, 37) for review).
7 Moreover, GnRH II is much more effective than
8 mammalian GnRH I in stimulating courtship and song
9 in ring doves (7) and song sparrows (12), and GnRH
10 II distribution shifts from midbrain cell bodies to
11 terminal regions following the initiation of
12 courtship in newts (37). There is remarkable
13 concurrence of the distribution of the Type II GnRH
14 receptor in the temporal lobe, putamen, amygdala,
15 medial preoptic area, ventromedial nucleus,
16 dorsomedial nucleus and periventricular nucleus of
17 the human or monkey brain with effects of lesions
18 and/or electrical stimulation of these areas on
19 reproductive behaviours such as sexual interest,
20 erection, intromission, thrusting and ejaculation
21 in rats, dogs, cats, monkeys and humans (36-38).
22 GnRH II has also been localised to these regions in
23 the rhesus monkey (19, 34).

24

25 The mechanism of action of GnRH II in the nervous
26 system of mammals is unknown but the peptide has
27 been identified in sympathetic ganglia of
28 amphibians where it binds to selective high
29 affinity receptors (22) and potently inhibits M-
30 type K^+ channels (11). Inhibition of these K^+
31 channels by GnRH II facilitates fast excitatory

1 transmission by conventional neurotransmitters, by
2 increasing input resistance of postsynaptic
3 neurones and by partial depolarization (11). This
4 may, therefore, provide a general neuromodulatory
5 mechanism for GnRH II effects in the nervous
6 system, and specifically in reproductive behaviour,
7 by facilitating signalling by neurotransmitters.

8

9 **Type II GnRH receptor in reproductive tissues.** The
10 marmoset Type II GnRH receptor expression and GnRH
11 II ligand expression (7, 8, 11) in non-neural
12 reproduction-related tissues such as the mammary
13 gland, prostate, gonads and adrenal cells may
14 resolve the long-standing enigma of the non-
15 concurrence of the binding pharmacology of
16 receptors in these tissues and in various tumours
17 (e.g. prostate, ovarian and mammary gland (1-3))
18 with that of the known pituitary Type I receptor
19 which is believed to be the receptor in these
20 tissues. For example, the paradox of similar
21 effects of both GnRH agonists and antagonists (1,
22 2) on proliferation of these tumour cell lines can
23 be rationalised if the Type II receptor is
24 mediating these effects, as we have shown that
25 certain mammalian GnRH I antagonists (e.g. 135-18)
26 behave as agonists with the Type II receptor (Fig.
27 8b). Moreover, the antiproliferative effects of
28 GnRH analogues on cell lines of these tumours is
29 consistent with the activation of p38 α by the Type
30 II receptor since this MAP kinase is known to be
31 antiproliferative (39).

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1 CLAIMS

2

3 1. A polynucleotide encoding a functional Type II
4 gonadotropin-releasing hormone receptor (Type II
5 GnRH-R) peptide.

6

7 2. A polynucleotide as claimed in Claim 1 encoding
8 at least a portion of exon I of Type II GnRH-R.

9

10 3. A polynucleotide as claimed in either one of
11 Claims 1 and 2 which encodes mammalian Type II
12 GnRH-R.

13

14 4. A polynucleotide as claimed in Claim 3 which
15 encodes a primate Type II GnRH-R.

16

17 5. A polynucleotide as claimed in Claim 4 which
18 encodes marmoset Type II GnRH-R.

19

20 6. A polynucleotide as claimed in Claim 5 having
21 the nucleotide sequence as set out in SEQ ID No.
22 1 or which has over 90% homology thereto.

23

24 7. A polynucleotide as claimed in Claim 4 which
25 encodes human Type II GnRH-R.

26

27 8. A polynucleotide as claimed in Claim 7 which
28 comprises a nucleotide sequence as set out in
29 any one of SEQ ID Nos. 3, 5, 7 or 9 or which has
30 over 90% homology thereto.

31

50

- 1 9. A polynucleotide is claimed in Claim 8 which
2 encodes an amino acid sequence as set out in any
3 one of SEQ ID Nos. 4, 6, 8 or 10 or which has
4 over 90% homology thereto.
5
- 6 10. A polynucleotide as claimed in any one of Claims
7 1 to 9 which encodes a peptide able to bind
8 specifically to Type II GnRH.
9
- 10 11. A polynucleotide as claimed in Claim 10 which
11 encodes a peptide able to act as a receptor for
12 Type II GnRH-R.
13
- 14 12. A recombinant genetic construct comprising a
15 polynucleotide as claimed in any one of Claims 1
16 to 11.
17
- 18 13. An expression vector comprising a polynucleotide
19 as claimed in any one of Claims 1 to 11 and able
20 to express functional Type II GnRH-R peptide.
21
- 22 14. A host cell transformed with a vector as claimed
23 in Claim 13.
24
- 25 15. A host cell as claimed in Claim 14 able to
26 express functional Type II GnRH-R.
27
- 28 16. A transgenic animal having a construct as
29 claimed in Claim 12 stably integrated into its
30 genome.
31

51

- 1 17. A peptide comprising at least a portion of exon
2 I of Type II gonadotropin-releasing hormone
3 receptor (Type II GnRH-R).
4
- 5 18. An isolated functional Type II gonadotropin-
6 releasing hormone receptor (Type II GnRH-R).
7
- 8 19. A peptide as claimed in either one of Claims 17
9 and 18 which comprises a portion of exon I of
10 mammalian Type II GnRH-R.
11
- 12 20. A peptide as claimed in Claim 19 which comprises
13 a portion of exon I of primate Type II GnRH-R.
14
- 15 21. A peptide as claimed in any one of Claims 17 to
16 20 having an amino acid sequence as set out in
17 SEQ ID No. 2 which has over 90% homology
18 thereto.
19
- 20 22. A peptide as claimed in any one of Claims 17 to
21 20 having an amino acid sequence as set out in
22 any one of SEQ ID Nos. 4, 6, 8 or 10, or which
23 has over 90% homology thereto.
24
- 25 23. A peptide as claimed in any one of Claims 17 to
26 22 which is able to bind Type II GnRH
27 specifically.
28
- 29 24. A peptide as claimed in Claim 23 which is a
30 functional receptor for Type II GnRH.
31

- 1 25. An antibody able to bind specifically to Type II
2 GnRH-R.
3
- 4 26. An antibody as claimed in Claim 25 which is
5 specific to an extracellular domain EC1, EC2 or
6 EC3 of Type II GnRH-R.
7
- 8 27. A method of screening an agent for
9 pharmacological activity, said method
10 comprising:
11 a) providing functional Type II GnRH-R peptide
12 and exposing said peptide to the agent; and
13 b) ascertaining whether said agent interacts
14 with said Type II GnRH-R peptide.
15
- 16 28. The method as claimed in Claim 27 wherein said
17 Type II GnRH-R is expressed by a host cell as
18 claimed in Claim 15.
19
- 20 29. The method as claimed in Claim 27 wherein said
21 Type II GnRH-R is expressed by a host cell
22 transformed with a polynucleotide as claimed in
23 Claim 11 wherein said host cell imitates Type
24 II GnRH-R signal transduction at least
25 partially and wherein exposure of said host
26 cell to said agent results in Type II GnRH-R
27 signal transduction when said agent binds
28 successfully to said Type II GnRH-R peptide.
29
- 30 30. A method of inhibiting binding of GnRH to its
31 native receptor *in vivo*, said method comprising

1 administering Type II GnRH-R or an
2 extracellular domain thereof.

3

4 31. The method of Claim 30 wherein the EC2 loop of
5 Type II GnRH-R is administered.

6

7 32. A method of contraception, said method
8 comprising administering exogenous Type II
9 GnRH-R or an extracellular domain thereof to a
10 patient in quantities sufficient to
11 substantially diminish binding of endogenous
12 Type II GnRH to endogenous Type II GnRH-R.

13

14 33. Use of Type II GnRH-R or an extracellular
15 domain thereof to inhibit endogenous Type II
16 GnRH binding to its native receptor *in vivo*.

17

18 34. Use of Type II GnRH-R as a contraceptive.

19

20 35. The use as claimed in either one of Claims 33
21 and 34 wherein said Type II GnRH-R is expressed
22 from a recombinant genetic construct.