A novel mammalian receptor for the evolutionarily conserved type II GnRH

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Mammalian gonadotropin-releasing hormone (GnRH I: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) stimulates pituitary gonadotropin secretion, which in turn stimulates the gonads. Whereas a hypothalamic form of GnRH of variable structure (designated type I) had been shown to regulate reproduction through a cognate type I receptor, it has recently become evident that most vertebrates have one or two other forms of GnRH. One of these, designated type II GnRH (GnRH II: pGlu-His-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2), is conserved from fish to man and is widely distributed in the brain, suggesting important neuromodulatory functions such as regulating K^+ channels and stimulating sexual arousal. We now report the cloning of a type II GnRH receptor from marmoset cDNA. The receptor has only 41% identity with the type I receptor and, unlike the type I receptor, has a carboxyl-terminal tail. The receptor is highly selective for GnRH II. As with the type I receptor, it couples to $G_{\alpha q/11}$ and also activates extracellular signal-regulated kinase (ERK1/2) but differs in activating p38 mitogen activated protein (MAP) kinase. The type II receptor is more widely distributed than the type I receptor and is expressed throughout the brain, including areas associated with sexual arousal, and in diverse non-neural and reproductive tissues, suggesting a variety of functions. Surprisingly, the type II receptor is expressed in the majority of gonadotropes. The presence of two GnRH receptors in gonadotropes, together with the differences in their signaling, suggests different roles in gonadotrope functioning.

The hypothalamic peptide, gonadotropin-releasing hormone (GnRH) (1) (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), stimulates pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulate the gonads (1–3). GnRH analogues have found wide application in the treatment of sex hormone-dependent diseases (e.g., prostatic and breast cancer) and in the treatment of infertility, and show promise as new contraceptive agents for both men and women (1–5). The first demonstration of GnRH structural variants in vertebrates (6) led to the structural elucidation of 14 novel peptides (7–9). It was also apparent that more than one form of GnRH was present in individual species of most vertebrates studied. Among these, a form originally isolated from chicken brain (chicken GnRH II) was found to be universally present and uniquely conserved between taxa from boney fish to *homo sapiens* (7–10). From studies on the structure of GnRH genes, it is apparent that this peptide is the result of an early gene duplication. In view of its universal occurrence, it has been designated GnRH II (9, 10). The evolutionary conservation of this peptide and its wide distribution in tissues suggest an important function. For example, it has been shown to regulate M currents (K^+ channels) in the sympathetic ganglion (11), and it stimulates reproductive behavior (7, 8, 12). Another possible function is in specific stimulation of FSH. Although GnRH stimulates both LH and FSH secretion, there is considerable evidence for the existence of a specific FSH-releasing factor (13, 14), and recent studies have reported preferential FSH release by certain GnRHs, including GnRH II and lamprey GnRH III (15, 16). In the present study, we have cloned and characterized a mammalian type II GnRH receptor from marmoset (*Callithrix jacchus*) that is highly selective for the evolutionarily conserved GnRH II ligand.

Materials and Methods

Cloning of the Marmoset Type II GnRH Receptor. RNA was isolated from marmoset pituitary and brainstem by using RNAsol B (Biogenesis, Bournemouth, U.K.). cDNA was generated by reverse transcription (RT)-PCR using the Enhanced Avian RT-PCR kit (Sigma). cDNA (50 ng) produced with random hexa-polynucleotides were subjected to PCR using human sequences to exons 2 and 3 previously described (17, 18) and using human sequence encoding exon 1 from sequencing genomic clones (see accession AL160282 and expressed sequence tags (ESTs) BG036291 and AA954764). Products (1 μl) from a first round PCR, using primers S1 (extracellular end of TMD-II; GATGCCACCTGGAATATCACTG) and A1 (TMD-V; AGGCAGCAGAAGG) were used in a second round PCR using primers S2 (TMD-IV; CAGCCTGGGGACTTAGTTTCCTG) and A2 (extracellular end of TMD-V; GGTTATAGGTGGTCTCTTGC). Products were cloned into pGEM-T (Promega), and sequenced. Sense and antisense oligonucleotides were designed from the novel marmoset sequences obtained and used in 3′ and 5′ rapid amplification of cDNA ends (RACE). For the 5′ RACE, a poly(A) sequence was added to 50 ng of marmoset pituitary cDNA produced with gene-specific primers. Products (2 μl) of a first round PCR, using primers S3 (anchored RACE primer from Boehringer Mannheim; GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV) and A3 [EC2 (extracellular loop domain 2)]; GAAGGGACTGGACCAGCTCG) were used in a second round of PCR using primers S4 (anchor primer; GACCACGCGTATCGATGTCGAC) and A4 (TMD-IV; CAAGGCAAGCAGGAAACTAAG). For the 3′ RACE, 50 ng of marmoset pituitary cDNA, produced with anchored oligo(dT) primers, was subjected to PCR using primers S5 (TMD-V; ACCTCTTCACCTTCTGCTGCCT) and S3. Products (2 μl) from this PCR and primers S6 (TMD-VII; CCTCCTCAATGCTCCTTTGGATC) and S4 were used in a secondary PCR. Products were cloned into pGEM-T (Promega), and sequenced. Full-length marmoset type II GnRH receptor was produced by PCR using oligos of the 5′ UTR

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Abbreviations: GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; TMD, transmembrane domain; EC, extracellular loop domain; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF368286).

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(S7, GAATTCGCTTCATACTCACACTTCATC; S8, CGGAATTCTCACACTTCATCCTCCTATCTC) and the 3′ sequence including the stop codon (A5, GCTCTAGAGATCAGATTGATGTTATAGGAATG). Marmoset brainstem cDNA (50 ng) produced with random hexa-polynucleotides was subjected to PCR using primers S7 and A5. Products (2 μl) and primers S8 and A5 were used in a secondary round of PCR. Products of this PCR were cloned into pcDNA3.1+ (Invitrogen) and sequenced. The resultant plasmid was used in expression studies.

Immunocytochemistry. Tissues from human, mouse, sheep, rhesus, and cynomologus monkeys were obtained after Ethical Committee approval from the University of Edinburgh and the University of Wisconsin. An antiserum to the human type II GnRH receptor was produced by immunization of rabbits with a synthetic peptide corresponding to EC3 (YSPTMLTEVPPC) conjugated to keyhole limpet hemocyanin via the Cys residue. A search of human genome databases showed the absence of a similar sequence in all other genes. This peptide, a synthetic peptide to EC3 of the human type I receptor (DPEMLNRLSDPC), and hemocyanin were used for immunoneutralization specificity studies. For detection of mammalian GnRH I specific antiserum, GF6 was used (19).

Tissue sections (15 μm) were subjected to the peroxidase/diaminobenzidine visualization technique as described (19, 20). Fluorescent labeling was accomplished by using the same procedure up to the step before avidin-biotin-complex reaction, when the fluorescein label (Rhodamine 600, avidin D, or FITC) was applied to the slides and incubated at room temperature in the dark for 2–4 h. For double labeling, slides were incubated sequentially with avidin D and biotin blocking solutions for 15 min each, then reincubated with the next primary antibody, followed by the other fluorescent labeling (Rhodamine or FITC). Controls, including omission of primary antibodies and order of exposure, were consistently negative. Immunofluorescence was viewed by confocal microscopy.

Cell Culture and Transfection. COS-7 cells were cultured as described (21, 22). Transient transfections with constructs were performed by using Superfect (Qiagen) according to the manufacturer's protocol.

Receptor Binding and Inositol Phosphate Production. Receptor binding assay utilized [His^5, D-^{125}I-Tyr^6]GnRH I (23) because [His^5]GnRH analogues were found to bind more effectively to the *Xenopus* sympathetic ganglion type II GnRH receptor (24). Whole cell binding studies and GnRH stimulation of inositol phosphate production were as described (22).

Phospho–Mitogen-Activated Protein (MAP) Kinase Assay. COS-7 cells transiently transfected with receptors and myc-tagged MAP kinases (ERK, JNK, and p38), were serum starved (12–16 h). After ligand stimulation, myc-tagged MAP kinases were immunoprecipitated (25) from cell lysates by overnight incubation with myc-agarose slurry (Santa Cruz Biotechnology) and washed. The immunoprecipitates were resolved by SDS/PAGE and electrotransferred to poly(vinylidene difluoride) (PVDF) membrane (NEN). Activated MAP kinase was detected by using anti-phospho-ERK/JNK/p38α kinase-specific antisera (New England Biolabs), visualized by enzyme-linked chemifluorescence (Amersham Pharmacia Biotech), and quantified by using a PhosphorImager (Molecular Dynamics). The degree of phosphorylated MAP kinase was normalized to the amount of unphosphorylated MAP kinase detected with specific antisera.

Expression of Type II Receptor mRNA in Marmoset and Human Tissues. Total RNA was extracted from various marmoset tissues by using TRI reagent (Sigma), and cDNA was produced by using oligo(dT) primers (Ambion, Austin, TX). PCR was performed on the cDNA using marmoset type II GnRH receptor cDNA-specific primers spanning all three exons (sense, CTTCGGCTGGAGGGAACCTG; antisense, GGTGCCCTCTTCGGCAGC) and actin-specific primers. PCR products were run on an agarose gel and blotted onto HybondN$^+$ nylon membrane (Amersham Pharmacia Biotech). The Southern blot was probed with random primed marmoset type II GnRH receptor cDNA or actin cDNA and quantified by using a PhosphorImager. Marmoset type II GnRH receptor expression was normalized to the expression of actin.

Expression in human tissues was examined by Northern blots of mRNA with random primed ^{32}P-labeled human type II GnRH receptor exon 1. The human type II GnRH receptor genomic sequence (P1 clone) was obtained by Genome Systems (St Louis) by using PCR screen of P1 clones with oligonucleotides to human sequences (17, 18). The presence of exon 1 was confirmed by sequencing. Oligonucleotides (sense, TGCCCACCTTCTCGGCAGCA; antisense, CTGTCCTGCCCGGTCCTGAG) to exon 1 were used with the P1 clone to produce a 460-bp amplicon. Labeling was done with [^{32}P]dCTP (6,000 Ci/mmol) using the supplier's specified conditions (Stratagene). Hybridization was performed by using 2×10^7 cpm at 65°C in 5× SSC/0.005% SDS/5× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% BSA)/2 mg/ml salmon sperm DNA and washing with 0.1× SSC/0.5% SDS at 55°C, and the blots were exposed to x-ray film for 6 days.

Stimulation of LH and FSH in Sheep. The relative potency of mammalian GnRH I and GnRH II at inducing FSH and LH secretion *in vivo* was tested by using our Soay ram sheep model (26) during both the sexually active (short days, SD) and inactive (long days, LD) phases of the photoperiod-induced reproductive cycle (26). The same animals ($n = 8$) were used on the two occasions. The GnRHs were administered as an i.v. bolus in 1 ml of 0.9% saline at doses of 250 ng per ram and 10 μg per ram in a crossover design, with a week between treatments. Blood samples were collected every 10 min, from 20 min before until 2 h after the treatments, and were assayed for LH and FSH (26). The responses were calculated as delta responses (2 h mean hormone concentration posttreatment minus 20 min mean pretreatment hormone concentration). These values were used to calculate the FSH:LH response ratio for GnRH II and GnRH I, and thus the overall ratio for the GnRH II stimulation, compared with the mammalian GnRH I stimulation. This ratio was assessed for each animal and then for the group (mean ± SEM, $n = 8$), under both LD and SD.

Results and Discussion

Cloning and Primary Structure of the Marmoset Type II GnRH Receptor. Our search for novel GnRH receptors was based on four concepts: first, the selectivity of mammalian GnRH receptors is determined by extracellular loop three (EC3) of the receptor (9, 21); second, this domain of these rare receptors could be amplified by PCR of genomic DNA from vertebrate species using degenerate oligonucleotides encoding the conserved flanking transmembrane domains (27); third, novel sequences obtained for EC3 could be used to identify homologues in the human genome databases; and fourth, antibodies generated against the EC3 of the human sequence would facilitate the identification of appropriate tissues expressing the receptor for cDNA production.

Amplification of EC3 from genomic DNA from a range of vertebrate species revealed two distinct sequences of receptors representing the known type I receptors and novel type II receptors in an amphibian and reptile (27). Searches of human EST databases revealed homologous sequences to the reptile EC3 (17, 18). From EST contigs, we constructed a partial receptor sequence encoding the putative exons 2 and 3 corresponding to these exons of the type I receptor (17, 18). All ESTs were in the antisense orientation, and it transpired that these were in the 3′ untranslated region (UTR) of a novel human ribonucleoprotein (RBM8) (17, 18). The equivalent of exon 1 was absent from the RBM8 3′ UTR. It was therefore

evident that the identification of sequences homologous to exon 1 was essential to discover the type II receptors. Searches of human databases, using as a query exon 1 of the human type I receptor, revealed homologous sequences. This exon localized to chromosome 1q (data not shown). Because the receptor was likely to be a rare transcript expressed in discrete tissues, we generated antisera to the EC3 domain of the human type II receptor and found, by immunocytochemistry, strong reactivity in pituitary and brain of the human, monkey, sheep, and mouse (Fig. 1). We then used oligonucleotides to the human exons 1–3 to amplify cDNA from marmoset pituitary and brain by PCR and 5′ and 3′ RACE procedures. The full-length cDNA encodes a 380-aa protein with a characteristic G protein-coupled receptor (GPCR) structure (Fig. 2). Although it is more homologous with GnRH receptors than other GPCRs, it has only 41% sequence identity with the human and marmoset type I receptors, suggesting an early evolutionary gene duplication. It also possesses a carboxyl-terminal tail, which is important for rapid desensitization and is uniquely absent from mammalian type I receptors (28–31). The receptor also does not have the unusual Asn/Asp microdomain of transmembrane helices 2 and 7 of the mammalian type I receptors, which plays a role in receptor activation (22). Instead, it has the Asp/Asp motif as in non-mammalian type I GnRH receptors recently cloned (28–32). The LSD/EP sequence of EC3, which is important for ligand selectivity of mammalian type I receptors (9, 21), is replaced by VPPS, which is also present in reptile (VPPS) and amphibian (VPPV) type II GnRH receptors (27). This difference in sequence is likely, therefore, to be a determinant of type II receptor selectivity for GnRH II because all other known binding sites (9) are conserved. Interestingly the marmoset type II receptor amino acid sequence is 80% identical to the partial human type II receptor sequence we have previously reported (17, 18), suggesting that the chromosome 1 to which exon 1 localized may represent a functional human type II receptor.

Pharmacological Characterization of the Marmoset Type II GnRH Receptor. Expression of the type II receptor in COS-7 cells revealed that it is highly selective for GnRH II in receptor binding assays (Fig. 3) and in the stimulation of inositol phosphate intracellular messenger production (40-fold and 90-fold greater activity relative to mammalian GnRH I; Table 1). These data contrast with the type I receptor in which GnRH II has only 10% and 9% activities of mammalian GnRH I in these assays. Overall, GnRH II has an affinity 24-fold greater for the type II receptor than for the type I receptor. The type II receptor was also more selective for salmon GnRH and [D-Arg^6]GnRH II (Table 1). Moreover, a type I receptor GnRH antagonist behaved as an agonist at the type II receptor (Fig. 3*b*). It has been demonstrated that control of gonadotropin biosynthesis and secretion by GnRH can be mediated by the activation of MAP kinases. Therefore, we assessed the capacity of both the type I human GnRH receptor and the marmoset type II GnRH receptor to activate the three major MAP kinase prototypes (ERK, JNK, and p38) in COS-7 cells. At the type I receptor, mammalian GnRH I was considerably more potent than GnRH II in activating ERK2 (Fig. 4*a*). In contrast, at the type II receptor (Fig. 4*b*), GnRH II was markedly more potent than GnRH I. In COS-7 cells expressing type II receptor, antagonist 135-18 (50 nM) demonstrated a similar agonist activity to GnRH II whereas, in cells expressing type I receptor, 1 nM GnRH I was significantly ($P < 0.05$; Student's *t* test) more effective at phosphorylating ERK2 than a 50-nM antagonist 135-18 dose (Fig. 4 *a* and *b*). Agonist-induced activation of JNK was not detected with stimulation of either the type I or type II receptor (data not shown). However, activation of p38α was detected on stimulation of the type II receptor with GnRH II but not with stimulation of the type I receptor with GnRH I (Fig. 4*c*). The time course of p38α activation was also considerably more protracted than that for type I/II receptor activation of ERK2. There are therefore distinct differences in signaling by the two receptors.

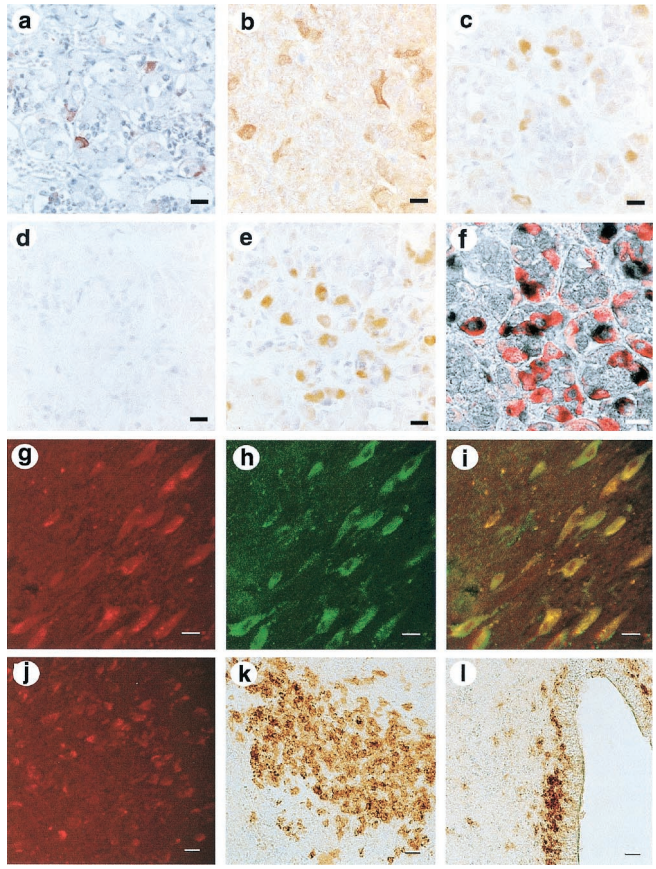

Fig. 1. Expression of type II GnRH receptor in pituitary and brain. Staining is shown in human (*a*), mouse (*b*), and sheep (*c–f*) pituitary. Sheep pituitary staining was neutralized by type II receptor peptide (*d*) but not by type I receptor peptide (*e*). The specificity of the staining was also indicated by the demonstration of similar staining by antisera raised in three other rabbits to the type II receptor peptide and an absence of staining by preimmune serum from all of these rabbits. Moreover, the hemocyanin carrier protein failed to neutralize staining. No staining was present with preimmune serum (not shown). Dual staining with type II receptor antiserum (black) and LH antiserum (red) shows colocalization (*f*). Type II GnRH receptor expressing neurones in the non-human primate brain by immunocytochemical staining is shown in panels *g–l*. Type II GnRH receptor-positive neurones are seen in the basal nucleus of Meynert (*g*), in the medial preoptic area (*j*) of an adult cynomologus monkey (*Macaca fascicularis*), and the amygdala (*k*) and periventricular region of the hypothalamus (*l*) of a fetal rhesus monkey (*Macaca mulatta*) at embryonic day 70. Note that the confocal photomicrographs with immunofluorescent staining show that the type II GnRH receptor-positive neurones (*g*, red-Rhodamine) in the basal nucleus of Meynert were also positive to mammalian GnRH I ligand (*h*, green-FITC). Colocalization is seen as yellow (*i*). In contrast, type II GnRH receptor-positive neurones in the medial preoptic area (*j*, red-Rhodamine), amygdala (*k*), and periventricular area (*l*) were negative for mammalian GnRH I ligand (data not shown). In *k* and *l*, immunoreactive products were visualized with DAB (brown). Scale bar: 10 μm for *a–i*; 20 μm for *j–l*. These brain areas also stained positive with antiserum raised against the carboxyl-terminal tail of the type II GnRH receptor (data not shown). Staining of type II GnRH receptor was neutralized in all tissues by incubation with type II peptide immunogen but not by type I peptide. Type II GnRH receptor-positive neurones were also seen in extrahypothalamic regions, such as medial septum, bed nucleus of the stria terminalis, substantia innominata, claustrum, amygdala, and putamen, and in the hypothalamic regions, such as supraoptic nucleus, ventromedial nucleus, and dorsomedial nucleus (data not shown).

Tissue Distribution and Expression of the Marmoset Type II GnRH Receptor. PCR amplification of cDNA from marmoset brain tissues revealed that it is expressed in the pituitary, spinal cord, pons,

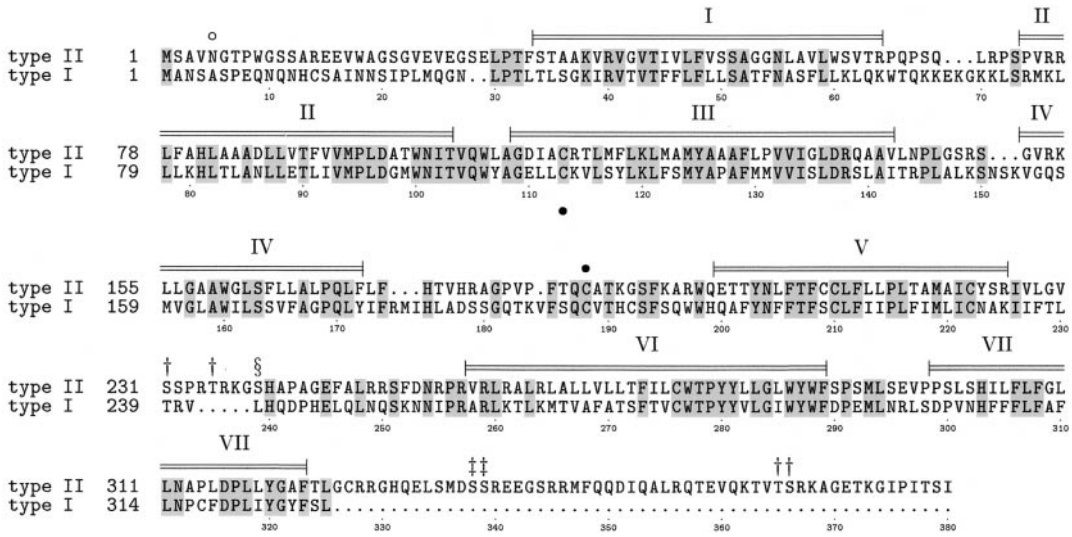

Fig. 2. Alignment of the amino acid sequences of the marmoset type II and human type I GnRH receptors. Conserved residues are shaded. α-helical regions predicted by homology modeling with the rhodopsin crystal structure are indicated. These helices will encompass the membrane spanning regions. A putative glycosylation site (○) and disulphide bridge (●) are indicated. Ser or Thr residues occurring in putative protein kinase C (†), casein kinase II (‡), and cAMP-/cGMP-dependent kinase (§) phosphorylation sites are indicated. Numerical residue annotation refers to marmoset type II sequence.

cerebellum, putamen, medulla, hypothalamus, preoptic area, midbrain, occipital pole, frontal lobe, and corpus callosum (Fig. 5*a*). Expression was high in reproductive tissues such as testis, prostate, mammary glands, seminal vesicles, and epididymis. Substantial expression was detected in adrenal, thyroid, heart, and skeletal muscle, but little or no expression was found in other tissues, such as liver, ovary, and bladder (Fig. 5*a*). Northern blots yielded a similar expression pattern (data not shown). Northern blots on human tissues probed with exon 1 showed a 2.4-kb transcript, with highest expression in the cerebral cortex and occipital pole; moderate expression in the frontal lobe, temporal lobe, and putamen; and low expression in the cerebellum, medulla, and spinal cord (Fig. 5*b*). There was substantial expression in the amygdala and low expression in the caudate nucleus, corpus callosum, hippocampus, substantia nigra, subthalamic nucleus, and thalamus (data not shown). There was significant expression in the heart and pancreas but little or no expression in placenta, lung, liver, skeletal muscle, and kidney (Fig. 5*c*). The exon 1 human type II receptor probe does not detect the antisense strand encoding the ribonucleoprotein (RBM8) because its transcript does not extend to exon 1 in the 3′ UTR (17, 18). Although there is general agreement of high expression in brain regions of both species and low expression in some peripheral tissues, such as lung, kidney, and liver, there appear to be some differences, which may arise from discrepancies in dissection and RNA recovery.

Marmoset Type II GnRH Receptor Function in the Pituitary. A specific antiserum to EC3 of the human type II receptor demonstrated specific expression of the receptor in human anterior pituitary (Fig. 1*a*). Staining was also found in about 10% of cells (the relative occurrence of gonadotropes) of the anterior pituitary of the mouse and sheep (Fig. 1 *b* and *c*). In the sheep anterior pituitary, double staining with type II receptor and LH antisera revealed that the type II receptor immunoreactivity is colocalized in 69% of LH-positive cells (Fig. 1*f*). Only 12% of type II receptor-positive cells were negative for LH. Because mammalian GnRH I binding sites also colocalize with LH in up to 90% of gonadotropes in the rat pituitary at proestrus (34), it is likely that the majority of gonadotropes express both type II and type I receptors and suggests that these receptors may coordinately regulate LH and FSH biosynthesis and secretion. The presence of type II receptors in the majority of gonadotropes is, at first consideration, unexpected because there is a substantial literature suggesting that a single GnRH (mammalian GnRH I) is sufficient to regulate the secretion of gonadotropins, and that the differential secretion of LH and FSH during the mammalian ovarian cycle may be adequately accounted for by changes in frequency of GnRH pulses and modulatory effects of gonadal steroids (androgen, estrogen, and progesterone) and peptides (activin, inhibin, and follistatin; ref. 14). However, a substantial number of physiological studies invoke the existence of an FSH-releasing peptide to account for the differential secretion of gonadotropins (13–16). Is the GnRH II possibly an FSH-releasing peptide? In the early studies on the GnRH II (previously called chicken GnRH II), it was found to have preferential FSH-releasing activity when compared with chicken GnRH I (chicken type I GnRH) (35). Moreover, GnRH II has been localized to the hypothalamic area in species of non-mammalian vertebrates (see refs. 7 and 8 for review) and the supraoptic, paraventricular, arcuate, and pituitary stalk regions of monkeys, where it is thought to play a role in gonadotropin secretion (20, 36). We therefore conducted studies by using a well-established sheep model to determine the relative effects of mammalian GnRH I and GnRH II on LH and FSH secretion. The responses to a 250-ng bolus of GnRHs was too low for comparison of relative LH and FSH secretion. At the 10-μg dose, all of the rams showed a robust response. Although GnRH I was more potent than GnRH II in releasing both LH and FSH, every individual ram exhibited a higher ratio of FSH to LH secretion when treated with GnRH II, compared with mammalian GnRH I. The mean ratio of FSH to LH induced by GnRH II was 2.14 ± 0.29 and 2.02 ± 0.34 (mean ± SD) times higher than that induced by mammalian GnRH I for sexually active and sexually quiescent rams, respectively ($P = 0.03$ and $P = 0.002$, respectively, paired two-tailed t test). When it is considered that GnRH II has an affinity and potency of ≤20% of mammalian GnRH I at the type I receptor (7, 9, 21), and the *in vivo* secretion of the two peptides is likely to be finely tuned in both concentration and phasing of pulsatile release, our exogenous bolus administration of the peptides is a relatively crude approach. More extensive experiments monitoring changes in expression of the two receptors in gonadotropes in relation to physiological changes and studies with pulsatile delivery of GnRH II and mammalian GnRH I in sheep with the hypothalamus disconnected are also needed.

GnRH II activation of the type II receptor in bullfrog sympathetic ganglia potently inhibits M-type K^+ channels (11). A similar

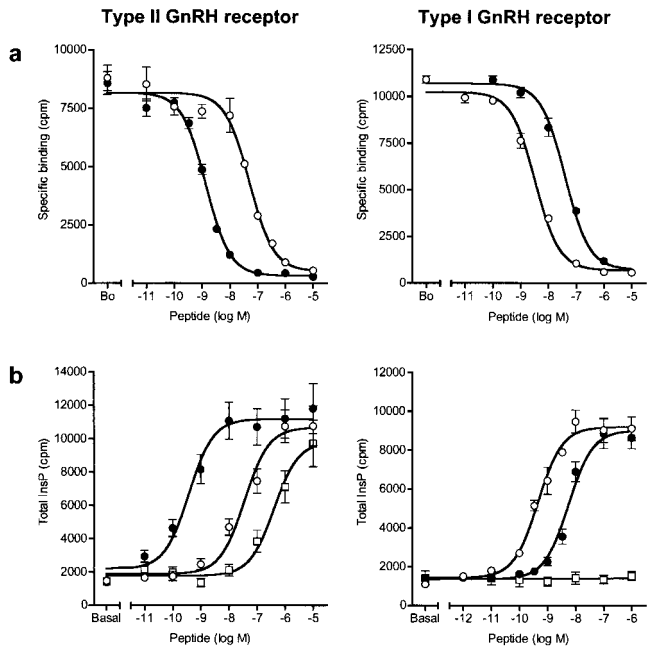

Fig. 3. Receptor binding (*a*) and inositol phosphate production (*b*) of mammalian GnRH I (○) and GnRH II (●) in COS-7 cells transfected with marmoset type II receptor (*Left*) and human type I receptor (*Right*). Stimulation of inositol phosphate by type I receptor antagonist 135-18 (□) at the type II receptor is also shown. Error bars represent SEM of three to six separate experiments.

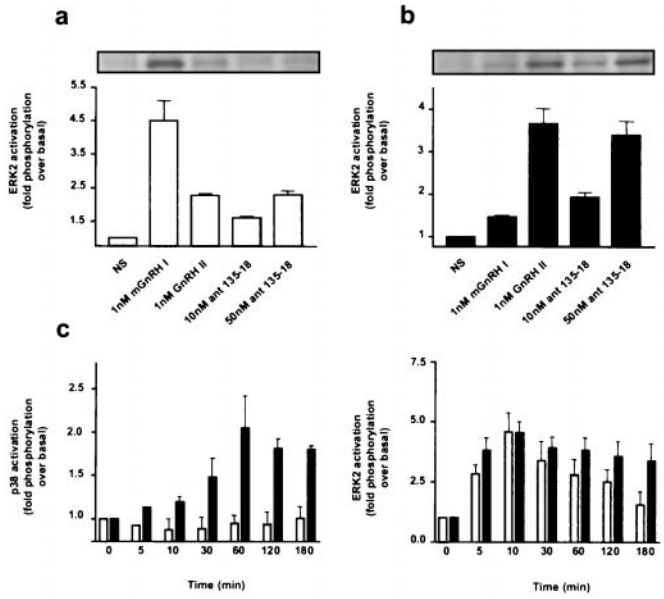

Fig. 4. Activation of ERK2 and p38α MAP kinases by type I (open bars) and type II (filled bars) GnRH receptors in COS-7 cells. Stimulation of type I (*a*) and II (*b*) GnRH receptors with mammalian GnRH I, GnRH II, and antagonist 135-18. *Inset* panels depict anti-phospho-ERK2 immunoblotting of anti-myc COS-7 cell immunoprecipitates. (*c Left*) selective and time-dependent activation of p38α by GnRH II stimulation (100 nM) of type II GnRH receptor. Each bar represents the mean ± SEM for fold p38α stimulation measured in the cells for the specified time of ligand stimulation. Open bars represent p38α stimulation by treatment of COS-7 transfected with type I receptor and stimulated with GnRH I. Filled bars represent p38α stimulation by GnRH II treatment of COS-7 cells transfected with type II GnRH receptor. The *Right* panel demonstrates analogous data, but each bar (open, type I receptor stimulated by GnRH I; filled, type II receptor stimulated by GnRH II) represents mean ± SEM for phosphorylation of ERK2.

action in gonadotropes would partially depolarize them, thus facilitating external excitatory inputs to the cell or entry of extracellular Ca^{2+}, which occurs on stimulation of type I receptors by mammalian GnRH I (1–3). These two GnRHs and GnRH receptor systems, along with differences in signaling pathways, provide the potential for differential FSH and LH secretion.

Marmoset Type II GnRH Receptor May Have Roles in Neural Development and Sexual Arousal. GnRH II has been proposed to have a neuromodulatory role (7, 8), as evidenced by K^+ channel inhibition in bullfrog sympathetic ganglia (11). Our demonstration of a GnRH II-selective receptor expression in many brain regions (Figs. 1 and 5) supports this hypothesis. Type II GnRH receptor antisera immunoreactive cells were widely seen in the hypothalamic and extrahypothalamic regions in embryonic development and adult (see Fig. 1 legend). In some of these areas (e.g., midbrain and supraoptic nucleus), the GnRH II ligand is also expressed (20, 36). The distribution pattern of type II GnRH receptor-positive cells in extrahypothalamic regions overlapped with that of the early developing mammalian GnRH I cells we have described (19). This suggests a potential role for the receptor in the development of mammalian GnRH I neurones. An intriguing observation was that neurones that express the mammalian GnRH I gene in the preoptic area and periventricular region of the hypothalamus (Fig. 1; ref. 36) were stained with the type II GnRH receptor antiserum (Fig. 1), suggesting that GnRH II may regulate mammalian GnRH I neurones.

GnRH has been shown to have direct effects on reproductive behavior and sexual arousal in rodents independent of its stimulation of sex hormone production (8, 38–40). Rapid changes in GnRH content of brain areas and cell number and cell size in response to visual, olfactory, and other stimulants of sexual behavior have been observed in species of fish, amphibians, reptiles, and mammals (see refs. 7, 8, 10, and 40 for review).** Moreover, GnRH II is much more effective than mammalian GnRH I in stimulating courtship and song in ring doves (7) and song sparrows (12), and GnRH II distribution shifts from midbrain cell bodies to terminal regions after the initiation of courtship in newts.** There is remarkable concurrence of the distribution of the type II GnRH receptor in the temporal lobe, putamen, amygdala, medial preoptic area, ventromedial nucleus, dorsomedial nucleus, and periventricular nucleus of the human or monkey brain, with effects of lesions and/or electrical stimulation of these areas on reproductive behaviors such as sexual interest, erection, intromission, thrusting, and ejaculation in rats, dogs, cats, monkeys, and humans (40, 41).**

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

	Ligand binding (IC_{50})		InsP production (EC_{50})	
Peptides	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	1,650 ± 478	10.6 ± 1.4	276 ± 45.5	Not detected

Data are expressed in nanomolar and represent the SEM (n = 3–6). GnRH II ([His^5, Trp^7, Tyr^8]GnRH I), mammalian GnRH I (GnRH I), salmon GnRH (sGnRH: [Trp^7, Leu^8]GnRH I).

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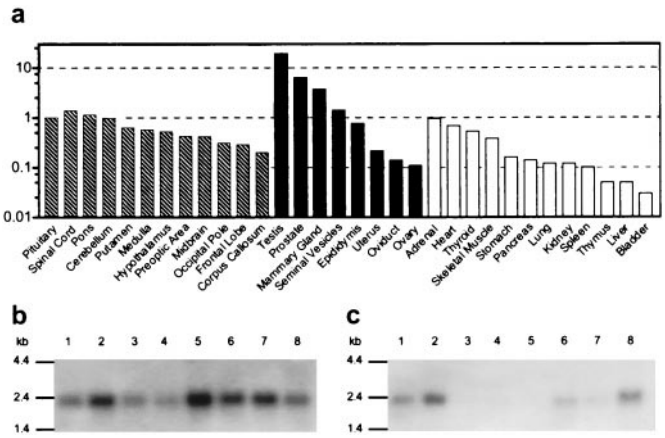

Fig. 5. Expression of type II GnRH receptor in marmoset and human tissues. (*a*) RT-PCR was carried out with specific primers on cDNA prepared from marmoset RNA isolated from various tissues. PCR products were fractionated by size on agarose gels. Type II GnRH receptor levels were normalized to actin RNA and represented as the log of the RNA expression relative to pituitary. Hatched bars indicate marmoset brain tissues, solid bars indicate marmoset reproductive tissues, whereas open bars indicate other marmoset tissues. (*b* and *c*) Expression of the type II GnRH receptor in human tissues was examined in Northern blots of mRNA (CLONTECH) by hybridization with ^{32}P-labeled human exon 1; (*b*) mRNA from human cerebellum (lane 1), cerebral cortex (lane 2), medulla (lane 3), spinal cord (lane 4), occipital pole (lane 5), frontal lobe (lane 6), temporal lobe (lane 7), and putamen (lane 8); (*c*) mRNA from heart (lane 1), whole brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). Another blot showed moderate expression in the amygdala and low expression in caudate nucleus, corpus callosum, hippocampus, substantia nigra, subthalamic nucleus, and thalamus (data not shown). The exon I probe is specific for the type II GnRH receptor and will not detect the ribonucleoprotein transcribed on the opposite strand (17, 18) because its transcript terminates with polyadenylation before exon I.

GnRH II has also been localized to these regions in the rhesus monkey (20, 36).

GnRH II has been identified in sympathetic ganglia of amphibians, where it binds to selective high affinity receptors (24) and potently inhibits M-type K^+ channels (11). Inhibition of these K^+ channels by GnRH II facilitates fast excitatory transmission by conventional neurotransmitters (11). This phenomenon may, therefore, provide a general neuromodulatory mechanism for GnRH II effects in the nervous system, and specifically in reproductive behavior, by facilitating neurotransmitter signaling. A frog sympathetic ganglion type II GnRH receptor has been recently cloned and demonstrates greatest similarity to the marmoset type II receptor described here (B.T., N. Illing, and R.M., unpublished results).

Marmoset Type II GnRH Receptor in Reproductive Tissues. The marmoset type II GnRH receptor expression (this study) and GnRH II ligand expression (7, 8, 11) in non-neural reproduction-related tissues (such as the mammary gland, prostate, gonads) may resolve the long-standing enigma of the non-concurrence of the binding pharmacology of receptors in these tissues and in various tumors [e.g., prostate, ovarian, and mammary gland (1–3)] with that of the known pituitary type I receptor, which is believed to be the receptor in these tissues. For example, the paradox of similar effects of both GnRH agonists and antagonists (1, 2) on proliferation of these tumor cell lines can be rationalized if the type II receptor is mediating these effects, because we have shown that certain mammalian GnRH I antagonists (e.g., 135-18) behave as agonists with the type II receptor (Fig. 3*b*). Moreover, the antiproliferative effects of GnRH analogues on cell lines of these tumors is consistent with the activation of p38α by the type II receptor because this MAP kinase is known to be antiproliferative (42).

The universal occurrence and conservation of structure of the GnRH II in taxa representing 500 million years of evolution and its apparent role in diverse reproductive tissues, including regulation of gonadotropin secretion and reproductive behavior and gonadal function, suggests that GnRH II might be the earliest evolved GnRH peptide with coordinated regulation of reproduction. Our cloning of the type II GnRH receptor provides the opportunity to elucidate the effects and functions of the ligand in detail and for the development of selective GnRH II therapeutics.

Note. This work was presented at the U.S. Endocrine Society, Toronto, June 21–24, 2000 (abstr. 98, p. 24). A paper reporting the cloning of a monkey type II GnRH receptor has been published since this paper was submitted for review (42).

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