Parallel Regulation of Membrane Trafficking and Dominant-negative Effects by Misrouted Gonadotropin-releasing Hormone Receptor Mutants*

Paul E. Knollman‡, Jo Ann Janovick‡, Shaun P. Brothers‡§, and P. Michael Conn‡§¶

From the ¶Divisions of Neuroscience and Reproductive Biology, Oregon National Primate Research Center and §Department of Physiology and Pharmacology and ¶Cell and Developmental Biology, Oregon Health and Science University, Beaverton, Oregon 97006

Gonadotropin-releasing hormone (GnRH) receptor mutants from patients with hypogonadotropic hypogonadism are frequently misrouted proteins that exert a dominant-negative (DN) effect on human (h) wild-type (WT) receptor, due to oligomerization and retention in the endoplasmic reticulum. Pharmacologic chaperones restore correct folding, rescuing mutants and WT receptor from this oligomer. Rat WT retains the ability to oligomerize (since human and mouse mutants exert a DN effect on rat (r) WT sequence) but, unlike human or mouse, escapes the DN effect of GnRH receptor (GnRHR) mutants because rGnRHR mutants route to the plasma membrane with higher efficiency than mouse or human mutants. These distinct behaviors of mouse and rat GnRHRs (distinguished by only four semi- or non-conservative amino acid differences) led us to assess the role of each amino acid. The difference in both routing and the DN effect appears mediated primarily by Ser216 in the rGnRHR. The homologous amino acid in the hGnRHR is also Ser and is compensated for by the primate unique insertion of Lys191 that, alone, dramatically decreases routing of the receptor. These studies establish the relation between the DN effect and altered receptor trafficking and explain why hGnRHR is more susceptible to defective trafficking by disease-related point mutations than rodent counterparts.

The gonadotropin-releasing hormone (GnRH)1 receptor (GnRHR) is a heptahelical G protein-coupled receptor found in the plasma membrane of pituitary gonadotropes (1, 2). GnRHR mutants isolated from patients with hypogonadotropic hypogonadism (HH) are frequently misfolded (and consequently mislocalized) proteins, restored to function by pharmacological chaperones (“pharmacoperones,” low molecular weight peptidomimetics of several chemical classes that bind the GnRHR) (3–7). These serve as templates (8, 9) that reshape mutants so that they pass the quality control apparatus of the cell. As a result, originally misfolded mutants are then correctly routed to the plasma membrane and become functional (3–11).

GnRHR mutants (12–23) obtained from such patients also inhibit ligand binding and ligand-activated second messenger production by wild-type (WT) receptors when co-expressed in vitro, a dominant-negative (DN) effect (24). The cellular etiology of this effect is a stable physical interaction between the mutant and wild-type GnRHR, producing an oligomer that is recognized by the cellular quality control apparatus as a defective complex (25). This oligomer is retained by the endoplasmic reticulum then presumably degraded (or recycled) (11, 25); plasma membrane expression of the WT receptor is thereby diminished.

Not surprisingly, pharmacoperones also reverse the DN effect (24, 25) of this mutant on the wild-type receptor, since the rescued oligomer of the WT and mutant pass the cellular quality control apparatus.

In the present study, unexpected and dramatic differences in both the DN effect and plasma membrane routing of rat and mouse GnRHR mutants were observed; rat, but not mouse, mutants failed to show a DN effect when co-expressed with WT receptor. This was surprising in light of the modest differences (four semi- or non-conservative changes) between the rat and mouse sequences. To determine the residue(s) responsible for the loss of dominant negativity by the rWT GnRHR, mouse, and rat homologs of the human mutant Glu90 → Lys (E90K) were prepared along with WT and other HH mutants in which combinations of the four semi- or non-conservative changes were made (mouseXrat: P11Q, I24T, I160T, and G216S, where X is the amino acid sequence position). The mutants were shown to be misrouted proteins but when rescued with pharmacoperones became fully functional “receptors” able to bind ligand and couple to the effector system.

Additionally, the role of a primate-specific amino acid, Lys191, that is inserted in the human receptor sequence (328 amino acids) (20) was examined. The presence of Lys191 in the human GnRHR is associated with a decrease in the proportion of plasma membrane localized receptors. Like defective routing of mutants, this effect is superseded by pharmacoperones that rescue the mutant GnRHRs. Rodent GnRHRs (327 amino acids), which are normally transferred to the plasma membrane with higher efficiency than primate GnRHRs, lack this amino acid insertion. This observation suggests that the human sequence is normally trafficked to the plasma membrane with less than 100% efficiency. Removal of Lys191 from the human GnRHR(E90K) sequence rescues this mutant and increases the efficiency of hWT GnRHR routing the plasma membrane. The
role of this primate-specific modification was examined with regard to the amino acid that governs the highly efficient transfer of rat WT GnRHR to the plasma membrane and loss of the DN effect of its mutants.

**EXPERIMENTAL PROCEDURES**

Material—pcDNA3.1+ (Invitrogen), the GnRH analog, N-terminal butylamide-GnRH (Buserelin; Hoechst-Roussel Pharmaceuticals, Somerville, NJ), (2S)-2-[5-[(2-azaazacyclo[2.2.2]oct-2-yl]-1-dimethyl-2-oxoethyl]-2-[3,5-dimethylphenyl]-1H-indol-3-yl]-N'-(2-pyridin-4-yl)propan-1-amine (IN3; Merck), myo-[3H]-Inositol (New England Nuclear, Boston, MA; NET-114A), DMEM, Opti-MEM, Lipofectamine, phosphate-buffered saline (Invitrogen), competent cells (Promega, Madison, WI), and endofree plasmid maxi-prep kits (Qiagen, Valencia, CA) were obtained as indicated. Other reagents were obtained from commercial sources. Rodent and human WT and mutant GnRH cDNA were prepared as reported (3, 26).

**Transient Transfection and Co-transfection**—Cells were cultured and plated in growth medium (DMEM, 10% fetal calf serum, 20 μg/ml gentamicin), and growth conditions were 37 °C and 5% CO2 in a humidified atmosphere; all medium added to the cells was warmed to 37 °C prior to adding to the cells, unless otherwise noted. For transfection of WT or mutant receptors into COS-7 cells, 5 × 10^6 cells were plated in 2.5 ml of growth medium in 60-mm Costar cell culture plates. Twenty-four h after plating, the cells were washed once with 0.5 ml of Opti-MEM and then transfected with 100 ng of total cDNA (pcDNA3.1+) into each well of 6-well Costar cell culture plates. Twenty-four h after plating, the cells were washed once with 0.5 ml of Opti-MEM and then transfected with 100 ng of total cDNA (pcDNA3.1+) without insert “empty vector” was included to bring the total cDNA to 100 ng/well, unless otherwise indicated) and 1 μl of Lipofectamine in 0.125 ml of Opti-MEM (room temperature), according to manufacturer’s instructions. For co-transfection experiments the cells were co-transfected with WT GnRHR (5 ng/well) and empty vector or mutant GnRHR (95 ng/well). Five h after transfection, 0.125 ml of DMEM with 20% fetal calf serum and 20 μg/ml gentamicin was added to the wells. Twenty-three h after transfection, the medium was removed and replaced with 0.25 ml of fresh growth medium. Where indicated, 1 μg/ml IN3 in 0.1% final dimethyl sulfoxide (vehicle) was added in respective media to the cells and 18 h before agonist treatment, as described elsewhere (3, 5, 24). Data are presented as the means ± S.E., calculated from at least three independent experiments, each performed in replicates of six.

**Inositol Phosphate (IP) Assays**—Twenty-seven h after transfection, cells were washed twice with 0.5 ml of DMEM containing 0.1% bovine serum albumin and 20 μg/ml gentamicin and then “preloaded” for 18 h with 0.25 ml of 4 μg/ml myo-[3H]-Inositol in DMEM (prepared without out inositol). After preloading, cells were washed twice with 0.3 ml of DMEM containing 5 mm LiCl (without inositol), then treated for 2 h in 0.25 ml of a concentration of Buserelin (10^{-7} M Buserelin, unless otherwise indicated) in the same medium (LiCl prevents IP degradation). The media were removed, and the cells were frozen and thawed in the presence of 0.5 ml of 0.1 M formic acid (to rupture cells), and total IPs were determined (described previously by) by liquid scintillation counting. Data were normalized to correct for differences in counting efficiency and for differences in specific activity of tritiated inositol lots.

**Statistics**—The Student’s paired t test (SigmaStat 3.1) was used to determine significance, with p values < 0.05 considered significant. One-way analysis of variance was used to determine interexperimental variance between data sets.

**RESULTS**

**Structural Features of the GnRHR**—Fig. 1A is a graphic of the rat WT GnRHR sequence, indicating the four positions of semi- or non-conservative mutations between the rat and mouse WT sequences (shaded boxes: mouse Xrat: P110Q, T124T, I160T, and G216S, where “X” is the amino acid position). The amino acid at each of the four positions in the human sequence is also shown for comparison (shaded boxes). Conservative substitutions compared with the mouse sequence are shown in the small unshaded boxes. The human WT GnRHR also contains an “extra” amino acid, Lys191 (small circle, site of insertion shown by a black arrow); this amino acid is absent in all pre-primate species sequenced to date. The site of amino acid E90K, a mutant examined in this study, is shown (small circle, location shown with a black arrow) in the second transmembrane portion of the molecule. The comparative sequences for the fifth transmembrane domain of the three species are shown.

**Substantial Evolutionary Homology between the Rodent and Human GnRHR**—Fig. 1B (top right) also shows a phylogram tree constructed from the reported sequences (2) of mammalian GnRH receptors and specifically the relationship between rat, mouse, and human receptors studied in the present work, along with others. The mGnRHR sequence is slightly closer to the human sequence (89% homology) than the rGnRHR (88% homology), indicated by the shorter line length. The two rodent GnRHRs are 96% homologous to each other.

**GnRHR Mutants Exert a DN Effect on the Wild-type GnRHR**—Fig. 2A is an agonist dose response of IP production in cells transfected with 95 ng of human WT GnRHR. The absence of Buserelin produces a response only slightly above cells that did not contain GnRHR (note broken x axis in image). Cells lacking transfected GnRHR or mutant (i.e. cells containing empty vector only) do not respond to Buserelin (see legends to Figs. 6–10 for vector-only control values). 10^{-7} M of the GnRH agonist, Buserelin, produces the maximal response and for this reason that amount was used in subsequent studies. Fig. 2B shows the DN effect of the hGnRHR(E90K) mutant on function of the human WT GnRHR. Although the DN effect of human E90K on human WT is significant (p < 0.05) at a ratio of 3:1 (mutant:WT), a 19:1 ratio was used in the present study for a more pronounced effect. In vivo, each cell in a human heterozygote (mutant:WT) would likely express those genes equally, a circumstance that may not occur when equal amounts of vectors are transfected into cells in vitro. Furthermore, since heterozygotic patients expressing highly DN mutants would likely be infertile, such mutations would have been selected against and those that appear in the population would be among the least severe in this regard. Accordingly, higher ratios were used in the present study.

**Lys191, a Primate-specific Amino Acid Addition, Decreases Expression of the Human Sequence and Rescues Expression of the E90K Mutant**—Fig. 3 shows IP production data for rat, mouse, and human WT and the E90K mutant of the GnRH receptor. Each sequence was expressed as described under “Experimental Procedures” in COS-7 cells, and IP production was used as an indication of receptor-effector coupling. We have shown previously that IP production accurately reflects plasma membrane expression of these moieties (25). Fig. 3 also shows the effect of deleting the Lys191 residue from the human (WT or mutant E90K) GnRHR sequence. In addition, effects of the insertion of this amino acid into rat and mouse WT and E90K-derived sequences were examined. Removal of Lys191 from the human sequence increases expression of both the WT and mutant E90K forms of the receptor, since this amino acid normally precludes complete expression of the receptor at the plasma membrane. Interestingly, mouse and rat WT are apparently not affected by the Lys191 insertion. In both rodent sequences, expression of E90K mutants was reduced by the Lys191 insertion. The rat GnRHR(E90K) mutant expresses 15.06 ± 0.32% compared with the WT, followed by very modest expression of the mouse GnRHR(E90K) mutant (3.59 ± 0.20%) compared with the WT. The human E90K mutant is indistinguishable from a “vector only” control (vector only data in figure legend).

**Interspecific Specificity of the DN Effect**—The species specificity of the rat, mouse, and human GnRHRs modified at E90K to exert a DN effect on the WT receptor from each of the three species is shown in Fig. 4. This figure indicates that the rat E90K mutant pair does not show dominant negativity, although both mouse E90K and human E90K GnRHR mutants show DN activity when co-expressed with the rat WT. This
observation suggests that these human and mouse mutants are still able to interact with the rat WT, suggesting that the difference in the rat (compared with human or mouse) is not due to loss of the ability to oligomerize. Of interest, the human mutant is more effective as a DN regulator of the human WT and the mouse mutant is more effective in actions on both rodent WT receptors.

**Alteration of Amino Acid 216 in the Mouse GnRHR Mutant Creates a "Rat-like" Interspecific Structure and Increases Plasma Membrane Expression—** Next, each of the four semi- or non-conservative amino acid changes (between the rodent species) in the mouse GnRHR(E90K) sequence were modified (singly) to make it more rat-like (Fig. 5A). Individual modification of three of these residues was uneventful; however, the substitution mGnRHR(E90K/G216S) resulted in markedly increased plasma membrane expression of the mouse (double) mutant. This interspecific construct had about 75% of the activity of the rat GnRHR(E90K). Consistent with expectations, modification of the rat GnRHR(E90K) sequence to make it more "mouse-like" (S216G) is associated with a 2-fold loss of plasma membrane expression (Fig. 5B). Progressive modification of the remaining three semi- or non-conservative sites in the mouse GnRHR(E90K) sequence (with either G216S or Gly216) resulted in an unexpected loss of plasma membrane expression, suggesting a role for the conservative substitutions in the sequence (Fig. 5B). Because there are nine of these (Fig. 1, small boxes), further examination was not attempted in light of the earlier positive result.

**The Mutants in the Present Work Are Misrouted Proteins but Otherwise Fully Functional—** It is conceivable that the effects observed with the derivative mutants of GnRHR(E90K) might reflect altered levels of mRNA expression, alterations in the ligand binding site or the sites that interact with effectors. To address these concerns, it was demonstrated that these mutants could be rescued with the pharmacopeine (IN3) to near wild-type levels (Fig. 6). Accordingly, these mutants are actually misrouted but rescuable and fully functional once restored to the plasma membrane.

**The DN Effect Is Also Lost Whenever the G216S Substitution Is Present—** Fig. 7 shows the co-transfection of rat, mouse, and...
human WT GnRHR receptors with mouse GnRHR(E90K) mutants in which the four rat-specific amino acids were altered singly or in combination. DN action was assessed in COS-7 cells. When mouse GnRHR(E90K) sequences contain the G216S mutation, the result is a loss in the ability of the mutant mouse sequence to produce a significant DN effect on WT GnRHR. When other “rat” mutants are created that result in making the mouse GnRHR(E90K) sequence more rat-like, the DN effect is also lost whenever the G216S substitution is present.

**Functional Relation between Two Amino Acids**—The effect of inserting the Lys191 residue in rat and mouse WT GnRHR sequences, as well as in the G216S (mouse) and S216G (rat) or S217G (human) mutants (Fig. 8A), was studied. Both mouse and rat WT GnRHR (+Lys191) mutants show no altered IP production as compared with respective WT. However, when the rat sequence contains the S216G mutation, the insertion of Lys191 results in decreased IP production. Additionally, when the mouse G216S mutant contains the inserted Lys191, IP production is decreased. This suggests a possible unexplained relation between amino acids 191 and 216. As expected, both human WT GnRHR and human GnRHR(S217G) show increased IP production when the Lys191 residue is removed (des-Lys191). B–D show the result of co-transfection of the GnRH mutants in Fig. 8A with indicated WT (listed above graphs). No DN action was seen, but rather an additive effect was noticed in all cases. These observations suggest that these mutants do not oligomerize (as seen with the GnRHR(E90K) mutants) but rather express both WT and mutant GnRH at the plasma membrane.

**Reconstruction of Human Disease-causing Mutants in Rodent Sequences Shows That the Rodent Sequences Are Less Easily Destabilized by These Mutants**—Fig. 9 shows the effect of constructing a number of human mutations in the rodent GnRHR sequences (A and B). These point mutations were isolated from patients with hypogonadotropic hypogonadism. For comparison, the human data are shown (C). The pharma-
coperone, IN3, is used to restore proper folding and thereby rescue the misrouted mutant receptor. In the case of the rat GnRHR, the ability of these mutations to cause misrouting of the rat mutant is lost or greatly diminished (compared with their effects on hGnRHR), except in the mutations S216R, L265R, and Y283C. The rodent GnRHRs appear to be more tolerant of mutation than is the human GnRHR. Additionally, the mouse seems less forgiving than the rat to the HH substitutions, as the mouse mutations more greatly reduce plasma membrane expression of the mutant GnRHRs (compared with the rat). This observation validates the physiological significance of the small number of semi- and non-conservative differences between the mouse and rat GnRHRs.

Modification of Amino Acid 216 in the Wild-type Rodent Sequences Suggests That This Site Functions to Stabilize Partially Expressed Sequences but Has No Effect on Highly Expressed Rodent Wild-type GnRHR—

Fig. 5. Modification in the mouse GnRHR sequence at non-conservative sites, corresponding to the differences found in the rat sequence. A, the effect of individually mutating the four semi- or non-conservative site differences between mouse and rat WT receptors (amino acids 11, 24, 160, and 216) in the mouse GnRHR(E90K) sequence. For comparison the mouse and rat GnRHR(E90K) sequences (without further modification) are shown. B, once the importance of amino acid 216 was recognized, multiple non-conservative site mutants were created in the mouse GnRHR(E90K) sequence with the naturally occurring Gly216 sequence (black data bar) and the mutant G216S sequence (white and black data bar). It should be noted that the rat GnRHR(E90K) contains the native Ser216 and appears as the stacked black and white bar. The modification S216G in the rat GnRHR(E90K) sequence is shown as only the black bar on the same column. A/B, transfected COS-7 cells (95 ng of mutant or WT with 5 ng of vector) were stimulated with $10^{-7} \mu$M Buserelin, and IP production was measured. Data represent the mean ± S.E. of at least three independent experiments, each performed in replicates of six. *a, p < 0.05 compared with co-transfection of rWT GnRHR and vector. *b, p < 0.05 compared with co-transfection of mWT GnRHR and vector. *c, p < 0.05 compared with co-transfection of hWT GnRHR and vector.

Fig. 4. Specificity of rat, mouse, and human E90K mutants to exert a DN effect on WT receptors from these species. WT receptor (5 ng) from each of the three species (listed above each graph in a box) was co-transfected with vector or E90K (95 ng, 100 ng of total DNA) homolog corresponding to the rat, mouse, or human GnRHR sequence. IP production in response to $10^{-7} \mu$M Buserelin was assessed as described under "Experimental Procedures." The data are represented as the mean ± S.E. of at least five independent experiments, each performed in replicates of six. *a, p < 0.05 compared with co-transfection of rWT GnRHR and vector. *b, p < 0.05 compared with co-transfection of mWT GnRHR and vector. *c, p < 0.05 compared with co-transfection of hWT GnRHR and vector.
WT receptor at the plasma membrane. Consistent with these expression results, no DN effects were seen (B–D) with the mouse mutants on any of the three species WT GnRHR.

**DISCUSSION**

The present data indicate that a single amino acid, Ser216, in the rat GnRHR sequence increases the efficiency of routing to the plasma membrane of rat mutants that are otherwise misrouted. This same amino acid is responsible for loss of the DN effect of rat mutants when co-expressed with WT receptor. In the human sequence, also containing a Ser in the homologous position (Ser217) this effect is mitigated by the presence of an “extra” amino acid, Lys191, inserted in the primate sequence and not present in earlier species. Concerns for effects of mutation on altered levels of mRNA expression, ligand binding, or effector coupling have been eliminated by demonstration that the mutants used in the present study are rescuable by a pharmacoperone and are restored to nearly identical activity levels compared with the wild-type receptor. The finding that these mutants become fully functional proteins indicates that loss of activity is due to misrouting. The observation that the rat WT GnRHR retains the ability to oligomerize with mutants derived from human and mouse GnRHR sequences (listed above graphs) contain the naturally occurring amino acid 216 (Ser216 for rat and human and Gly216 for mouse). The dashed lines indicate the IP production level of the three species WT GnRHR when co-transfected with 95 ng of vector. The substitutions (listed above the top image in the legend) at position 216 were made to the rodent sequences co-transfected with WT GnRHR sequences. When comparing E90K mutant co-transfections note that the rat GnRHR(E90K) contains the Ser216 residue. Co-transfected COS-7 cells (5 ng of WT with 95 ng of vector or mutant DNA) were stimulated with 10^{-7} M Buserelin, and IP production was measured. Data represent the mean (n = 6) cpm ± S.E. of a representative experiment. 100 ng of empty vector (data not shown) produced 81.4 ± 2.9 cpm.

Pharmacological chaperones (3), suggesting that either such mutations have a dramatic effect on the conformation of the GnRHR or that this area is important for ligand binding or effector activation. Most models exclude the second possibility (25, 32). In addition, because the Ser216/217 is within the membrane (fifth transmembrane domain), it is not likely that this is a phosphorylation site; moreover it is not within a known consensus phosphorylation sequence (boxed, Fig. 1). It is not surprising that even a residue that will eventually end up in the plasma membrane can interact with the quality control apparatus of the cell in light of the observation that many naturally occurring mutants have this action (5).

It is interesting to consider the biochemical nature of the mutations that can lead to the HH disorder. At the time this

**FIG. 6.** Pharmacological rescue of mouse GnRHR(E90K)-derived mutants. Transfected COS-7 cells (95 ng of mutant or WT with 5 ng of vector) were stimulated with 10^{-7} M Buserelin, and IP production was measured. Data represent the mean (n = 6) cpm ± S.E. of a representative experiment. “Vehicle” data (dimethyl sulfoxide (DMSO)) shows IP production in the absence of the pharmacaperone. When the rescue pharmacaperone IN3 was present it was added at 1 μg/ml, shown previously (3) to result in optimum rescue. Transfection of 100 ng of empty vector (data not shown) produced a response in the absence of IN3 of 66.8 ± 2.1 cpm and 62.6 ± 2.1 cpm when IN3 was present.

**FIG. 7.** Co-transfection of rat, mouse, and human WT GnRHR receptor with double, triple, and quadruple mouse GnRHR(E90K) mutants. Rat, mouse, and human WT GnRHR sequences (listed above graphs) contain the naturally occurring amino acid 216 (Ser216 for rat and human and Gly216 for mouse). The dashed lines indicate the IP production level of the three species WT GnRHR when co-transfected with 95 ng of vector. The substitutions (listed above the top image in the legend) at position 216 were made to the rodent sequences co-transfected with WT GnRHR sequences. When comparing E90K mutant co-transfections note that the rat GnRHR(E90K) contains the Ser216 residue. Co-transfected COS-7 cells (5 ng of WT with 95 ng of vector or mutant DNA) were stimulated with 10^{-7} M Buserelin, and IP production was measured. Data represent the mean (n = 6) cpm ± S.E. of a representative experiment. 100 ng of empty vector (data not shown) produced 81.4 ± 2.9 cpm.

*a, p < 0.05 when compared with co-transfection of rWT GnRHR and vector.

*b, p < 0.05 when compared with co-transfection of mWT GnRHR and vector.
was being written, there are 18 mutants (5–6, 12–23) known to cause isolated HH. Of these, two are missing large sequences: one being a truncation of all amino acids between 314 and the carboxyl-terminal amino acid 328 (19) and the other a deletion mutant missing exon 2 (33, 34). It is reasonable to assume that such large sequence omissions would have a dramatic effect on the receptor structure. The remaining mutations are subtler, involving only a single amino acid. Of these, three involve loss (two occurrences) or gain (one occurrence) of a cysteine residue, an amino acid known to form bridges associated with the formation of third order protein structure. Disruption of required bridges or formation of inappropriate bridges would be significantly disruptive to the protein structure. One of the recently reported mutations (35) is the loss of a proline at position 320, which is replaced by a leucine. Because the peptide backbone of proline is constrained, occurrence of this imino acid is associated with a forced turn in the protein sequence. Although this is a hydrophobic for hydrophobic substitution, the turn is a likely requisite for receptor conformation involved in routing or activity and cannot be corrected by the pharmacoperones.

![Fig. 8. Effect of Lys<sup>191</sup> on plasma membrane expression and the DN effect of the rodent GnRHR(+Lys<sup>191</sup>) and human GnRHR(=Lys<sup>191</sup>) 216/217 double mutants. A, mutant and WT GnRHR sequences were transfected in COS-7 cells (95 ng of WT with 5 ng of vector DNA) and stimulated with 10<sup>−7</sup> M Buserelin, and IP production was measured. Data represent the mean (n = 6) cpm ± S.E. of a representative experiment. The indicated sequences were expressed in COS-7 to assess the effect of Lys<sup>191</sup> independently and in combination with glycine or serine at position 216 in the GnRH receptor sequence. B–D, the DN action of the mutant sequences from A (95 ng of DNA) on WT sequences (listed above graph in a box, 5 ng of DNA) was assessed as described under “Experimental Procedures.” WT GnRHR sequences were co-transfected in COS-7 cells (5 ng of WT with 95 ng of mutant or vector DNA) and stimulated with 10<sup>−7</sup> M Buserelin, and IP production was measured. Data are presented as the mean (n = 6) cpm ± S.E. of a representative experiment. For comparison, 5 ng of rat, mouse, or human WT with 95 ng of empty vector is shown on the left of B–D. 100-ng transfection of empty vector (data not shown) produced a response, in the absence of IN3, of 72.3 ± 2.9 cpm, and 69.6 ± 1.7 cpm when IN3 was present.

![Fig. 9. Mutations in the human GnRHR from patients with hypogonadotropic hypogonadism were reconstructed in the mouse and rat receptor sequences. Mutant and WT GnRHR sequences were transfected in COS-7 cells (95 ng of WT with 5 ng of vector DNA) and stimulated with 10<sup>−7</sup> M Buserelin, and IP production was measured. Data represent the mean (n = 6) cpm ± S.E. of one representative experiment. Vehicle only (dimethyl sulfoxide (DMSO)) data show IP production in the absence of the pharmacoperon. When the rescue pharmacoperon IN3 was present, it was added at 1 μg/ml, shown previously (3) to result in optimum rescue. 100-ng transfection of empty vector (data not shown) produced a response, in the absence of IN3, of 72.3 ± 2.9 cpm, and 69.6 ± 1.7 cpm when IN3 was present.](http://www.jbc.org/content/early/2018/05/20/jbc.M118.007658.full)
FIG. 10. The four semi- and non-conservative rodent differences were constructed in the mWT GnRHR receptor sequence. WT GnRHR sequences were transfected in COS-7 cells (95 ng of WT with 5 ng of vector DNA) and stimulated with 10⁻⁷ M Buserelin, and IP production was measured. Data represent the mean (n = 6) cpm ± S.E. of one representative experiment. A, expression of mouse WT GnRHR-derived mutants, whereas the bottom images (B–D) show co-transfection with indicated WT GnRHR (listed above graph). B, WT GnRHR sequences were co-transfected in COS-7 cells (5 ng of WT with 95 ng of mutant or vector DNA) and stimulated with 10⁻⁷ M Buserelin, and IP production was measured. Data are presented as the mean (n = 6) cpm ± S.E. of a representative experiment. The dashed lines indicate IP production of WT GnRHR co-transfected with vector. Vector alone (data not shown) produced IP responses of 71.6 ± 0.9 cpm (A) and 81.4 ± 2.9 cpm (B–D).

The remainder of the HH-associated mutants, fully 12 mutants, are caused by modest changes in a single charge. Ten of these 12 mutations involve lysine (three occurrences), arginine (six occurrences), or aspartic acid (one occurrence). Introduction of charge changes (even minor) appear sufficient to alter the structure. Of interest, none of the reported mutations are conservative in which, for example, alanine replaces a glycine or threonine replaces a serine; in each case adding a single carbon without modifying the net charge. Likewise, there are no examples of simple hydrophobic for hydrophobic exchanges (valine for alanine, for example), positive for positive (lysine for arginine) exchanges, or negative for negative (aspartic acid for glutamic acid) exchanges. Such exchanges may occur, of course, but may be clinically silent, or alternatively, the genotype may not be propagated into subsequent generations.

In the case of the mutant positions in human receptor associated with disease, the amino acid in the corresponding rodent WT is significantly conserved in mammals. The exceptions are Asn¹⁰ → Lys (for which aspartic acid is found in mouse and rat but asparagine in other mammals sequenced), Ser¹⁶₈ → Arg (for which isoleucine is found in mouse and rat but serine in other mammals sequenced), Ser²¹⁷ → Arg (mouse has glycine; guinea pig has isoleucine), Cys²⁰⁰ → Tyr (glycine in pig), Tyr²⁸⁴ → Cys (leucine in pig). The Arg¹³⁹ → His is part of the DRS motif (DRY in most mammalian receptors) in intracellular loop 2.

Nature likely relies on this remarkable charge sensitivity of the human GnRHR to diminish the efficiency of transfer of this protein to the plasma membrane by insertion of Lys¹⁹¹. Pharmacoperone rescue studies suggest that insertion of this charged amino acid decreases the expression of the human WT receptor by about 40% (31). The ability of pharmacoperones to elevate the plasma membrane expression of WT human GnRHR (WT contains Lys¹⁹¹), but not rodent GnRHR (WT does not contain Lys¹⁹¹), and the location in the receptor (second extracellular loop) suggests that the occurrence of this added amino acid is associated with regulation of routing in contrast to alteration in the coupling mechanism, for example. For reasons that are buried in the physically diffuse amino acid differences between the rodent and human sequence, the simple addition of Lys¹⁹¹ to WT rodent GnRHR does not result in the expected diminution of routing (31).

Even before mammals, there has been a progressive trend toward a decrease in the percentage of the synthesized GnRHR that is expressed at the plasma membrane. In fish and avians, the GnRHR homolog contains a long carboxyl-terminal tail associated with increased expression (36). Human or rodent chimeras containing this “C-tail” sequence route more efficiently to the plasma membrane than do the WT molecule lacking it (37, 38).

While the reason for this progressive loss of efficiency of trafficking to the plasma membrane is not clear, it may be associated with the added complexity and cyclicality of the reproductive process in mammals and, subsequently, in primates (39–42). In rodents and primates, the concentration of the GnRHR on the plasma membrane cycles, and a store of synthesized receptor may be valuable to call upon promptly and without the delay required for further transcription or translation. The added reliance of the human receptor on endoplasmic reticulum-resident protein chaperones may provide an important site of regulation.

These studies establish the relationship between the DN effect and altered receptor trafficking and explain why the delicately balanced plasma membrane expression of the human GnRHR is more susceptible to defective trafficking as a result of point mutations than its rat and mouse counterparts. The important role of protein routing is well established in biological systems (27–29, 43). The present work indicates a progressive evolution toward the regulation of trafficking of a particular protein to the plasma membrane and presents the
possibility that this site may be regulated as part of the control process of mammalian and primate reproduction.

REFERENCES
1. Ulloa-Aguirre, A., and Conn, P. M. (1998) Handbook of Physiology-Endocrinology, pp. 87–124, Vol. 7, Oxford University Press, New York
2. Millar, R. P., Lu, Z.-L., Pawson, A. J., Flanagan, C. A., Morgan, K., and Maudsley, S. R. (2004) Endocr. Rev. 25, 235–275
3. Janovick, J. A., Maya-Nunez, G., and Conn, P. M. (2002) J. Clin. Endocrinol. Metab. 87, 3255–3262
4. Maya-Nunez, G., Janovick, J. A., Ulloa-Aguirre, A., Soonderlund, D., Conn, P. M., and Mendez, J. P. (2003) J. Clin. Endocrinol. Metab. 87, 2144–2149
5. Leaños-Miranda, A., Janovick, J. A., and Conn, P. M. (2002) J. Clin. Endocrinol. Metab. 87, 4825–4828
6. Ulloa-Aguirre, A., Janovick, J. A., Leaños-Miranda, A., Conn, P. M. (2003) Expert Opin. Ther. Targets 7, 175–185
7. Conn, P. M., Leaños-Miranda, A., and Janovick, J. A. (2002) Mol. Interv. 5, 308–316
8. Ulloa-Aguirre, A., Janovick, J. A., Brothers, S. P., and Conn, P. M. (2004) Traffic 5, 821–837
9. Janovick, J. A., Goulet, M., Bush, E., Greer, J., Wettlaufer, D. G., and Conn, P. M. (2003) J. Pharmacol. Exp. Ther. 305, 608–614
10. Ulloa-Aguirre, A., Janovick, J. A., Leaños-Miranda, A., and Conn, P. M. (2004) Hum. Reprod. Update 10, 177–192
11. Castro-Fernandez, C., Maya-Nunez, G., and Conn, P. M. (2005) Endocr. Rev. 26, 479–503
12. Karges, B., Karges, W., and de Roux, N. (2003) Mol. Endocrinol. 17, 192–203
13. de Roux, N., Young, J., Misrahi, M., Genet, R., Chanson, P., Schaison, G., and Milgrom, E. (1997) N. Engl. J. Med. 337, 1597–1602
14. Beranova, M., Oliveira, L. M. B., Bédécarrats, G. Y., Chippani, E., Vallee, M., Ammini, A. C., Quintos, J. B., Hall, J. E., Martin, K. A., Hayes, F. J., Pitteloud, N., Kaiser, U. B., Crowley, W. F., Jr., and Seminara, S. B. (2001) J. Clin. Endocrinol. Metab. 86, 1580–1588
15. Seminara, S. B., Beranova, M., Oliveira, L. M. B., Martin, K. A., Crowley, W. F., and Hall, J. E. (2000) J. Clin. Endocrinol. Metab. 85, 556–562
16. Layman, L. C., McDonough, P. G., Cohen, D. P., Maddox, M., Tho, S. P. T., and Reindollar, R. H. (2001) Fertil. Steril. 75, 1148–1155
17. de Roux, N., Young, J., Barilly-Tabard, S., Misrahi, M., Milgrom, E., and Schaison, G. (1999) J. Clin. Endocrinol. Metab. 84, 567–572
18. Caron, P., Chauvin, S., Christin-Maire, S., Bennet, A., Lahou, N., Cousin, R., Bouchard, P., and Kottler, M. L. (1999) J. Clin. Endocrinol. Metab. 84, 910–916
19. Kottler, M. L., Chauvin, S., Lahou, N., Harris, C. E., Johnston, C. J., Lagarde, J. P., Bouchard, P., Farid, N. R., and Cousin, R. (2000) J. Clin. Endocrinol. Metab. 85, 3002–3008
20. Costa, E. M. F., Bédécarrats, G. Y., Mendonca, B. B., Arnhold, I. J. P., Kaiser, U. B., and Latronico, A. C. (2001) J. Clin. Endocrinol. Metab. 86, 2680–2686
21. Karges, B., Karges, W., Mine, M., Ludwig, L., Kühne, R., Milgrom, M., and de Roux, N. (2003) J. Clin. Endocrinol. Metab. 88, 1873–1879
22. Kottler, M. L., Cousin, R., and Bouchard, P. (1999) Arch. Med. Res. 30, 481–485
23. Bédécarrats, G. Y., Linher, K. D., and Kaiser, U. B. (2003) J. Clin. Endocrinol. Metab. 88, 834–843
24. Leaños-Miranda, A., Ulloa-Aguirre, A., Ji, T. H., Janovick, J. A., and Conn, P. M. (2003) J. Clin. Endocrinol. Metab. 88, 3360–3367
25. Brothers, S. P., Cornea, A., Janovick, J. A., and Conn, P. M. (2004) Mol. Endocrinol. 18, 1787–1797
26. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z., and Pease, L. R. (1993) Methods Enzymol. 217, 270–279
27. Morello, J.-P., Salaphour, A., Laperrière, A., Bernard, Y., Arthus, M. F., Lengler, M., Petoja-Ropo, U., Angers, S., Morin, D., Bichet, D. G., and Bouvier, M. (2000) J. Clin. Invest. 105, 887–895
28. Carrel, R. W., and Lomas, D. A. (1997) Lancet 350, 134–138
29. Colley, N., Cassill, J., Baker, E., and Zuker, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3070–3074
30. Huckle, W., and Conn, P. M. (1987) Methods Enzymol. 141, 149–155
31. Janovick, J. A., Ulloa-Aguirre, A., and Conn, P. M. (2003) Endocrine 22, 317–327
32. Reinhart, G. J., Xie, Q., Liu, X. J., Zhu, Y. F., Fan, J., Chen, C., and Struthers, R. S. (2004) J. Biol. Chem. 279, 34115–34122
33. Silveira, L. F., Stewart, P. M., Thomas, M., Clark, D. A., Bouloux, P. M., and MacColl, G. S. (2002) J. Clin. Endocrinol. Metab. 87, 2973–2977
34. Silveira, L. F., MacColl, G. S., and Bouloux, P. M. (2002) Semin. Reprod. Med. 4, 327–338
35. Meysing, A. U., Kanasaki, H., Bédécarrats, G. Y., Acierno, J. S., Jr., Conn, P. M., Martin, K. A., Seminara, S. B., and Kaiser, U. B. (2004) J. Clin. Endocrinol. Metab. 7, 3189–3198
36. Lin, X., Janovick, J. A., Brothers, S. P., Blemenröhr, J., Boger, J., and Conn, P. M. (1998) Mol. Endocrinol. 12, 161–171
37. Lin, X., Cornea, A., Janovick, J. A., and Conn, P. M. (1998) Mol. Cell. Endocrinol. 146, 27–37
38. Brothers, S. P., Janovick, J. A., and Conn, P. M. (2003) J. Clin. Endocrinol. Metab. 88, 6107–6112
39. Knobil, E. (1980) J. Biol. Chem. 255, 25710–25718
40. Kottler, E. (1980) Recent Prog. Horm. Res. 36, 53–88
41. Knobil, E. (1974) Recent Prog. Horm. Res. 30, 1–46
42. Knobil, E. (1988) Hum. Reprod. 40, 469–472
43. Katt, J. A., Duncan, J. A., Herbon, L., Barkan, A., and Marshall, J. C. (1985) Endocrinology 116, 2113–2115
44. Ward, C. L., and Kopito, R. R. (1994) J. Biol. Chem. 269, 25710–25718
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Paul E. Knollman, Jo Ann Janovick, Shaun P. Brothers and P. Michael Conn

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