Published in final edited form as:

Mol Cell Endocrinol. 2007 January 2; 260-262: 287–293.

Insights Learned from L457^(3.43)R, an Activating Mutant of the Human Lutropin Receptor

Ana Claudia Latronico[&] and Deborah L. Segaloff[#]

&Developmental Endocrinology Unit, Hormone and Molecular Genetics Laboratory, Clinical Hospital, Sao Paulo University Medical School, Sao Paulo, Brazil

#Department of Physiology and Biophysics, The Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA, USA

Abstract

The L457^(3.43)R mutation of the hLHR was initially identified in a Brazilian boy with gonadotropin-independent precocious puberty. As would be expected, L457^(3.43)R, when expressed in 293 cells, caused a marked elevation in basal cAMP levels. Interestingly, in spite of the fact that the elevated basal levels of cAMP elicited by L457R were not as great as those elicited by the wild-type hLHR when stimulated with hCG, L457^(3.43)R cells were unresponsive to further hormonal stimulation. We have since determined that the L457^(3.43)R mutant, as well as other constitutively active mutants of the hLHR, causes an increase in phosphodiesterase activity that attenuates the target cell to hormonal stimulation of the wild-type hLHR or other Gs-coupled GPCRs. We have also shown that the constitutive activity and lack of hormonal responsiveness of L457^(3.43)R are due to the formation of a salt bridge between the introduced arginine in the mid portion of helix 3 with D578^(6.44) in the mid portion of helix 6. The formation of this salt bridge results in the disruption of interactions between the cytoplasmic ends of helices 3 and 6 that are associated in general with activation of the hLHR. As such, this mutant has provided novel insights into the properties of target cells expressing activating hLHR mutants and into the structural basis for hLHR activation.

Keywords

lutropin receptor; LH receptor; G protein-coupled receptor; GPCR; constitutive activation; constitutively active mutant

Constitutive activation of the hLHR

Similar to other G protein-coupled receptors (GPCRs), the human lutropin receptor (hLHR) is hypothesized to exist in the plasma membrane in equilibrium between the resting state and one or more active states even in the absence of bound hormone. The revised ternary model for GPCR activation postulates that the binding of agonist stabilizes the active state of the receptor, thereby shifting the equilibrium to the active state (Kenakin, 2001; Kenakin, 2002; Samama et al., 1993). This model fits nicely with observations that the basal activity of the hLHR, as measured by basal cAMP levels, is detectable in the absence of bound LH or hCG and that the basal activity increases with cell surface hLHR density (Zhang et al., 2005).

Corresponding author: Deborah L. Segaloff, Ph.D. Department of Physiology and Biophysics The University of Iowa Iowa City, IA 52246 Tel: 319-335-7850 FAX: 319-335-7330 email deborah-segaloff@uiowa.edu.

These studies were supported by NIH grant HD22196 (D.L.S.) and CNPq grant 303192/2002-0 (A.C.L.).

In recent years it has become apparent that certain mutations can also cause an active state of a GPCR to be stabilized. When compared to cells expressing the same number of cell surface wild-type (wt) receptor, cells expressing mutants such as these exhibit increased levels of second messenger in the absence of bound agonist. As such, these mutations have been termed constitutively activating mutations. The first constitutively activating mutation of the *hLHR* gene was reported in 1993 by A. Shenker *et al.*, who described a young boy with gonadotropin independent precocious puberty, also termed testotoxicosis, heterozygous for a D578G mutation in the *hLHR* gene (Shenker *et al.*, 1993). In the last decade, several constitutively activating mutations in the serpentine region of the *hLHR* have been demonstrated in boys affected by sporadic or familial gonadotropin-independent precocious puberty (Latronico and Segaloff, 1999; Themmen and Huhtaniemi, 2000). Interestingly, related females heterozygous for activating *hLHR* mutations have not been found to exhibit any reproductive abnormalities (Latronico and Segaloff, 1999; Rosenthal *et al.*, 1996).

The activating mutations of the *hLHR* described thus far are defined as such because cells expressing the mutant exhibit elevated levels of basal cAMP as compared to cells expressing comparable levels of wt *hLHR*. The stimulation of basal cAMP by the mutants, however, is not as great as that observed for the wt *hLHR* when incubated with a maximally effective concentration of hCG. Therefore, it is likely that these mutations stabilize states of intermediary activation. Nonetheless, the *hLHR* constitutively active mutants have proven to be valuable tools for investigators to elucidate the structural basis for the activation of the *hLHR*.

From a clinical perspective, testotoxicosis is a clinically heterogeneous disorder caused by diverse activating mutations of the *hLHR* that might be associated with particular clinical features. For example, the D578Y mutation has been associated with an early and severe phenotype (Muller *et al.*, 1998) and the M398T mutation is reported to have incomplete penetrance (Evans *et al.*, 1996). The activating L457^(3.43)R mutation appears to be associated with extremely high levels of serum testosterone that resulted in rapidly advancing bone age and lack of physiologic central gonadotropin maturation. In addition, this mutation is associated with unresponsiveness to hCG stimulation *in vivo*.

The present review focuses on the L457^(3.43)R¹ activating mutation and the insights gained from its study.

Clinical features of a boy with sporadic gonadotropin-independent precocious puberty due to the constitutively activating L457^(3.43)R mutation

To date, a constitutively activating mutation L457^(3.43)R in the third transmembrane helix of the *hLHR* has been identified in only one Caucasian Brazilian boy with gonadotropin-independent precocious puberty (Latronico *et al.*, 1998). His biological parents each had normal *hLHR* gene sequences, establishing the sporadic nature of the L457^(3.43)R mutation (Latronico *et al.*, 1998). The patient was first seen at 2 yr and 6 months of age because of penile enlargement, frequent erections, accelerated growth, deepening voice and aggressive behavior (Latronico *et al.*, 1998). At the time of diagnosis, his height was 113.3 cm (+ 6.2 SD) and weight was 24.3 kg. Penile length was 10.3 cm (> 2.5 SD), testicular size was 2.0 × 1.4 cm, bilaterally, and pubic hair was Tanner stage IV. His bone age was significantly advanced before treatment (6 years).

¹The amino acid numbering in superscript is that proposed by Ballesteros and Weinstein Ballesteros, J. A., and Weinstein, H., 1995. Integrated methods for the construction of three-dimensional models of structure-function relations in G protein-coupled receptors. *Methods Neurosci.* 25, 366-428. In this nomenclature, the first number indicates the helix and the numbers thereafter indicate the position of the helical residue relative to the most highly conserved residue within that helix, which is denoted as 50.

The hormonal profile of this boy was characterized by pubertal basal serum testosterone levels and undetectable GnRH-stimulated serum LH and FSH levels. However, his testosterone levels were very high when assayed on several occasions, frequently reaching levels > 1000 ng/dL, which are rarely seen in boys with this same disorder (Latronico et al., 2000). He was first treated with ketoconazole, a P450 cytochrome inhibitor, which inhibits adrenal and testicular biosynthesis (Soriano-Guillen et al., 2005). However, the drug was discontinued due to a transient and modest increase in the serum transaminases. Subsequently, he was treated with the combination of an antiandrogen to antagonize androgen action at the receptor level (oral cyproterone acetate 70 mg/m²/day) and an aromatase inhibitor (anastrozole 1-2 mg/day) to block the conversion of androgen to estrogens. Both drugs have no effect on the serum testosterone level, and therefore, the efficacy must be assessed indirectly by monitoring growth, bone maturation, signs of virilization and sexual behavior. This drug regimen was well tolerated. Despite continuing treatment, bone age progressively advanced, increasing the difference between bone and chronological ages. The treatment was interrupted at 11 years when his bone age achieved 17 years. At 15 years old, his adult height was 162 cm (−2 SD), significantly lower than the target height (178 cm) and the predicted height before treatment (180 cm). These findings indicated a poor treatment response.

The secondary activation of the gonadotropic axis usually occurs in patients with familial male-limited precocious puberty (Bertelloni et al., 1997; Laue et al., 1993). At 7 years of age (bone age 13), a GnRH stimulation test evidenced suppressed LH and FSH levels in the boy with the L457^(3.43)R mutation. His basal testosterone levels were 1556 ng/dL, and did not increase after hCG stimulation (5000 U, im) (Latronico et al., 1998). A recent GnRH stimulation test was performed at 15 years old (adult bone age) and also showed suppressed LH and FSH levels. These findings indicate the lack of central physiological gonadotropin maturation with potential clinical implications in this patient.

Currently, he complains of severe facial and trunk acne and early-onset temporal alopecia. These signs are directly related to androgen actions. He has recently been treated with a competitive inhibitor of the human steroid 5- α -reductase-type II that catalyzes the conversion of testosterone to dihydrotestosterone (finasteride 1 mg/day, orally) to prevent the progression of the androgenic alopecia. The high testosterone levels in this boy can increase the risk of prostate hyperplasia and so we have followed his prostate size by ultrasound as well as the prostate serum antigen levels. Both parameters are normal so far. Another significant concern regarding the high testosterone levels that lead to permanent gonadotropin suppression is related to his fertility. Therefore, a sperm count was requested recently, the results of which indicate oligozoospermia.

Molecular basis for the activation of the hLHR by the L457^(3.43)R mutation

The L457^(3.43)R mutation of the *hLHR* gene results in the substitution of a leucine residue in the mid region of TM3 with arginine. This leucine is highly conserved in the Family A group of GPCRs (Mirzadegan et al., 2003).

The L457^(3.43)R mutation was introduced into recombinant hLHR and then HEK293 cells were transiently transfected with the wt or mutant hLHR were examined for cell surface binding expression and basal and hCG-stimulated cAMP production. This mutant is expressed at the cell surface at higher densities than the wt receptor. As shown in Figure 1, when functional assays are performed using cells transfected with amounts of plasmid adjusted so that the cell surface levels of wt and mutant receptors were similar, it is observed that the L457^(3.43)R mutation causes a marked elevation in basal cAMP levels (Latronico et al., 1998; Shinozaki et al., 2001). Although the L457^(3.43)R mutant causes a strong activation of the hLHR in the absence of hormone, the levels of basal cAMP are nonetheless not as high as those observed

for cells expressing comparable levels of wt hLHR that have been incubated with a saturating concentration of hCG (Figure 1 and (Latronico et al., 1998;Shinozaki et al., 2001;Zhang et al., 2005)). However, cells expressing L457^(3.43)R are unresponsive to hormonal stimulation (Figure 1 and (Latronico et al., 1998;Shinozaki et al., 2001;Zhang et al., 2005)). This lack of hormonal responsiveness is not due to an impairment in hormone binding activity because the L457^(3.43)R mutant binds hCG with the same affinity as the wt hLHR (Latronico et al., 1998).

Mutagenesis studies showed that the induction of constitutive activity of the hLHR by substitution of L457^(3.43) is dependent upon the introduction of a positively charged residue at that position (Shinozaki et al., 2001). In collaboration with Dr. Francesca Fanelli (University of Modena e Reggio and Emilia, Modena, Italy), computational studies were performed to predict the structural rearrangement of the hLHR transmembrane helices upon the introduction of the L457^(3.43) mutation. As discussed in more detail in a review by Dr. Fanelli in this issue of *Molecular and Cellular Endocrinology*, molecular dynamic (MD) simulations of the wt hLHR and constitutively active mutants of the hLHR predict that the activation of the hLHR is accompanied by the release of a salt bridge between the cytoplasmic ends of helices 3 and 6, resulting in a greater solvent accessible surface area (SAS value) over selected residues at the cytoplasmic ends of helices 3 and 6 (Angelova et al., 2002; Ascoli et al., 2002; Fanelli, 2000). These structural changes were noted in MD simulations of the L457^(3.43)R mutant (Ascoli et al., 2002; Zhang et al., 2005).

The environment surrounding the introduced Arg457^(3.43) in the L457^(3.43)R mutant was also examined in the MD simulations and is shown in Figure 2. As such, it is thought that the side chain of the introduced arginine would be positioned such that it would form a salt bridge with D578^(6.44), an aspartate residue in TM6 that is unique to the glycoprotein hormone receptors (Shinozaki et al., 2001;Zhang et al., 2005). To determine experimentally if such a salt bridge exists, disruptive and reciprocal mutagenesis studies were performed (Figure 3). These experiments were performed under conditions where basal cAMP levels were assayed over a wide range of receptor concentrations (as determined by cell surface hCG binding assays). At all concentrations of receptor, the L457^(3.43)R exhibited strong constitutive activity, but the activity was not linear with receptor concentration (Figure 3). To test the effects of disrupting a putative salt bridge between R457^(3.43) and D578^(6.44), the mutant L457^(3.43)R/D578^(6.44)N was created. This mutant represents the L457^(3.43)R mutant in which D578^(6.44) has been mutated to asparagine. The constitutive activity of the L457^(3.43)R/D578^(6.44)N mutant was severely attenuated as compared to the L457^(3.43)R mutant (Figure 3 and (Zhang et al., 2005)). It remained possible, however, that the loss of constitutive activity in the L457^(3.43)R/D578^(6.44)N mutant resulted from an allosteric effect of substitution of D578^(6.44) unrelated to disruption of a salt bridge. However, if the loss of constitutive activity was specifically due to the breakage of a salt bridge between the mid regions of helices 3 and 6, then it should be possible to restore constitutive activity if a salt bridge were reintroduced, but with a reciprocal ion pair. Therefore, the mutant L457^(3.43)D/D578^(6.44)R was created. Note that in this mutant there is still the potential for a salt bridge between residues 3.43 and 6.44, but instead of helix 3 contributing the arginine and helix 6 the aspartate, in the reciprocal mutant helix 3 would be contributing the aspartate and helix 6 the arginine. Remarkably, the L457^(3.43)D/D578^(6.44)R mutant displayed the same strong constitutive activity as the L457^(3.43)R mutant (Figure 3 and (Zhang et al., 2005)). In addition, the SAS values calculated for the L457^(3.43)R mutant or its reciprocal mutant L457^(3.43)D/D578^(6.44)R were consistent with strong constitutive activity and were similar to each other (Zhang et al., 2005).

Previous studies have shown that, in the resting state of the hLHR, D578^(6.44) interacts via hydrogen bonds with both N615^(7.45) and N619^(7.49) and that mutation of D568^(6.44) causes constitutive activity, but only if N615^(7.45) and N619^(7.49) are intact (Angelova et al., 2002;

Fanelli et al., 2004; Lin et al., 1997). Both mutagenesis and computational studies show that L457^(3.43)R similarly requires these two asparagines to be intact for constitutive activity (Zhang et al., 2005). This does not necessarily suggest that R457^(3.43) interacts directly with these two residues. Indeed, mutational and computational analyses argue against that scenario (Zhang et al., 2005). Rather, these two asparagines in TM7 appear to be necessary for the appropriate structural transfer of information to the cytosolic portions of helices 3 and 6. It was therefore considered that, by forming a salt bridge with D568^(6.44), L457^(3.43)R may cause constitutive activity by releasing interactions between D568^(6.44) and N615^(7.45) and/or N619^(7.49). Computational modeling argues against this scenario, though, as do mutational studies with the closely related FSH receptor (Tao et al., 2002; Zhang et al., 2005). Rather, it appears that it is the formation of the salt bridge *per se* between 3.43 and 6.44, and not a perturbation of interactions between D568^(6.44) and N615^(7.45) and/or N619^(7.49), that induces the constitutive activity.

Molecular dynamic simulations further predict that the L457^(3.43)R mutant and its reciprocal mutant L457^(3.43)D/D578^(6.44)R, both of which are constitutively active and both of which can form a salt bridge between residues 3.43 and 6.44, share the release of charge-reinforced H bonding between R464^(3.50) of the E/D-R-Y/W motif at the cytoplasmic end of TM3 and both the adjacent E463^(3.49) as well as D564^(6.30) at the cytoplasmic end of TM6 (Zhang et al., 2005). These studies suggest, therefore, that hormone-independent activation of the hLHR may include the formation of structural links between the transmembrane helices, such as the salt bridge formed between 3.43 and 6.44 in the L457^(3.43)R mutant, that ultimately cause the release of interactions in the cytosolic domains of the helices, the latter of which presumably results in the unmasking of sites necessary for G protein activation.

Molecular basis for lack of hormonal responsiveness of the L457^(3.43)R mutation

Cells transfected with L457^(3.43)R do not show an increase in cAMP levels with hCG (see Figure 1) even though this mutant is expressed well at the cell surface, it binds hCG with high affinity and the basal levels of cAMP, though elevated, as not as great as those of the wt hLHR incubated with a maximally effective concentration of hormone (Latronico et al., 1998; Shinozaki et al., 2001; Zhang et al., 2005). Interestingly, when membranes are isolated from cells expressing L457^(3.43)R, basal levels of adenylyl cyclase are elevated, consistent with the constitutive activity of this mutant, but the membranes do not respond any further with hCG stimulation (Shinozaki et al., 2001). These observations suggest that the hormonal unresponsiveness of the L457^(3.43)R mutant is inherent to a structural alteration of the receptor and not due to a cellular event such as increased receptor internalization, which would not be manifest in isolated membranes.

As discussed above, it was recently shown that the constitutive activity of the L457^(3.43)R mutant is due to its ability to contribute an arginine residue to form a salt bridge with D578^(6.44) and that this, in turn leads to the disruption of the salt bridge at the cytoplasmic ends of helices 3 and 6 that stabilize the receptor in a resting state (Zhang et al., 2005). Results from this study further suggest that the salt bridge introduced between 3.43 and 6.44 is also responsible for the lack of hormonal responsiveness of the L457^(3.43)R mutant (Zhang et al., 2005).

Similar to other boys with gonadotropin-independent precocious puberty, the boy with the L457^(3.43)R mutation is heterozygous for this mutation (Latronico et al., 1998). As discussed above, when this boy was administered exogenous hCG he did not respond with a further elevation in testosterone secretion. One might have expected to observe an increase in testosterone with hCG stimulation due to the presence of the wt hLHR allele. Although other

possible causes may underlie his lack of responsiveness to hCG (including his extremely high basal levels of testosterone), we thought it important to examine *in vitro* the effects of the L457^(3.43)R mutant when co-expressed with the wt hLHR as it would be in a heterozygous state *in vivo*.

When cells are co-expressed with submaximal levels of wt hLHR and L457^(3.43)R, both receptors are expressed at the cell surface and, as would be expected, the basal levels of cAMP are elevated as compared to cells expressing wt hLHR only (Figure 4 and (Shinozaki et al., 2003)). Because the L457^(3.43)R mutant is completely unresponsive to hCG, a hormonal challenge to cells co-expressing the wt and mutant receptor would reflect the hormonal responsiveness of the wt receptor when expressed in the presence of L457^(3.43)R. Under these conditions (i.e., the mutant and wt receptors co-expressed) the hCG-stimulated cAMP levels were attenuated as compared to cells expressing wt receptor only (Figure 4, Top Panel and (Shinozaki et al., 2003)). These results show that the ability of the wt hLHR to respond to hCG is compromised by co-expression of L457^(3.43)R. Notably, however, this attenuation was only observed if the incubation with hormone was performed in the absence of a phosphodiesterase inhibitor (Figure 4 and (Shinozaki et al., 2003)).

Further studies done in collaboration with Dr. Marco Conti (Stanford University, California) determined that L457^(3.43)R induced phosphodiesterase (PDE) activity, specifically that of PDE4D3, a cAMP-responsive PDE that is expressed in gonadal tissues as well as 293 cells (Shinozaki et al., 2003). The phosphorylation of PDE4D3, indicative of its activation, in cells expressing L457^(3.43)R is shown in Figure 5. PDE4D3 was also stimulated by the stimulation of wt hLHR expressing cells with hCG. The constitutive activation of this PDE in L457^(3.43)R cells under basal conditions suggests that the elevated basal levels of cAMP induced by L457^(3.43)R stimulate PDE activity. Importantly, the cAMP-mediated increase in PDE activity does not entirely blunt the cAMP response (either of the constitutively active L457^(3.43)R mutant or hCG-stimulated wt hLHR), since in either case one observes an increase in cAMP. Rather, the increased PDE activity most likely serves to prevent the elevated cAMP levels from becoming excessively high. Further studies showed that the induction of PDE activity is not restricted to the L457^(3.43)R mutant, but is observed with other constitutively activating mutants of the hLHR as well (Shinozaki et al., 2003).

The induction of PDE activity by L457^(3.43)R or other activating mutants of the hLHR attenuates the hormonal responsiveness of Gs-coupled receptors that are co-expressed with the activating mutant, including but not limited to, the wt hLHR (Shinozaki et al., 2003). However, observations of the reproductive status of women heterozygous for activating hLHR mutations and of boys heterozygous for activating mutants of the hLHR other than L457^(3.43)R, suggest that this attenuation does not have detectable physiological ramifications.

Closing remarks

The L457^(3.43)R mutation is an unusual activating mutant of the hLHR in that it exhibits particularly strong constitutive activity and it is unable to translate high affinity binding of hormone into further stimulation of Gs. These properties have been proven valuable in gleaning new insights into the clinical ramifications of activating hLHR mutations as well as the molecular and cellular basis for hLHR mutation and hormone-induced activation.

References

- Angelova K, Fanelli F, Puett D. A model for constitutive lutropin receptor activation based on molecular simulation and engineered mutations in transmembrane helices 6 and 7. *J. Biol. Chem* 2002;277:32202–32213. [PubMed: 12070159]

- Ascoli M, Fanelli F, Segaloff DL. The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocr. Rev* 2002;23:141–174. [PubMed: 11943741]
- Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models of structure-function relations in G protein-coupled receptors. *Methods Neurosci* 1995;25:366–428.
- Bertelloni S, Baroncelli GI, Lala R, Cappa M, Matarazzo P, De Sanctis C, Saggese G. Long-term outcome of male-limited gonadotropin-independent precocious puberty. *Horm. Res* 1997;48:235–239. [PubMed: 9362394]
- Evans BAJ, Bowen DJ, Smith PJ, Clayton PE, Gregory JW. A new point mutation in the luteinising hormone receptor gene in familial and sporadic male limited precocious puberty: genotype does not always correlate with phenotype. *J. Med. Genet* 1996;33:143–147. [PubMed: 8929952]
- Fanelli F. Theoretical study on mutation-induced activation of the luteinizing hormone receptor. *J. Mol. Biol* 2000;296:1333–1351. [PubMed: 10698637]
- Fanelli F, Verhoef-Post M, Timmerman M, Zeilemaker A, Martens JW, Themmen AP. Insight into mutation-induced activation of the luteinizing hormone receptor: molecular simulations predict the functional behavior of engineered mutants at M398. *Mol. Endocrinol* 2004;18:1499–1508. [PubMed: 15016840]
- Kenakin T. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* 2001;15:598–611. [PubMed: 11259378]
- Kenakin T. Efficacy at G-protein-coupled receptors. *Nat. Rev. Drug Discov* 2002;1:103–110. [PubMed: 12120091]
- Latronico A, Segaloff D. Naturally occurring mutations of the luteinizing hormone receptor: Lessons learned about reproductive physiology and G protein-coupled receptors. *Amer. J. Hum. Genet* 1999;65:949–958. [PubMed: 10486313]
- Latronico AC, Abell AN, Arnhold IJP, Liu X, Lins TSS, Brito VN, Billerbeck AE, Segaloff DL, Mendonca BB. A unique constitutively activating mutation in the third transmembrane helix of the luteinizing hormone receptor causes sporadic male gonadotropin independent precocious puberty. *J. Clin. Endocrinol. Metab* 1998;83:2435–2440. [PubMed: 9661624] Copyright © 2003 The Endocrine Society
- Latronico AC, Lins TS, Brito VN, Arnhold IJ, Mendonca BB. The effect of distinct activating mutations of the luteinizing hormone receptor gene on the pituitary-gonadal axis in both sexes. *Clin. Endocrinol* 2000;53:609–613.
- Laue L, Jones J, Barnes KM, Cutler GB Jr. Treatment of familial male precocious puberty with spironolactone, testolactone, and deslorelin. *J. Clin. Endocrinol. Metab* 1993;76:151–155. [PubMed: 8421081]
- Lin Z, Shenker A, Pearlstein R. A model of the lutropin/choriogonadotropin receptor: insights into the structural and functional effects of constitutively activating mutations. *Prot. Engineering* 1997;10:501–510.
- Mirzadegan T, Benko G, Filipek S, Palczewski K. Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. *Biochemistry* 2003;42:2759–2767. [PubMed: 12627940]
- Muller J, Gondos B, Kosugi S, Mori T, Shenker A. Severe testotoxicosis phenotype associated with Asp578-->Tyr mutation of the lutrophin/choriogonadotrophin receptor gene. *J. Med. Genet* 1998;35:340–341. [PubMed: 9598734]
- Rosenthal IM, Refetoff S, Rich B, Barnes RB, Sunthornthepvarakul T, Parma J, Rosenfield RL. Response to challenge with gonadotropin-releasing hormone agonist in a mother and her two sons with a constitutively activating mutation of the luteinizing hormone receptor-A clinical research center study. *J. Clin. Endocrinol. Metab* 1996;81:3802–3806. [PubMed: 8855841]
- Samama P, Cotecchia S, Costa T, Lefkowitz RJ. A mutation-induced activated state of the β_2 -adrenergic receptor: extending the ternary complex model. *J. Biol. Chem* 1993;268:4625–4636. [PubMed: 8095262]
- Shenker A, Laue L, Kosugi S, Merendino JJ Jr. Minegishi T, Cutler GB Jr. A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* 1993;365:652–654. [PubMed: 7692306]
- Shinozaki H, Butnev V, Tao YX, Ang KL, Conti M, Segaloff DL. Desensitization of Gs-coupled receptor signaling by constitutively active mutants of the human lutropin/choriogonadotropin receptor. *J. Clin.*

Endocrinol. Metab 2003;88:1194–1204. [PubMed: 12629106] Copyright © 2003 The Endocrine Society

Shinozaki H, Fanelli F, Liu X, Jaquette J, Nakamura K, Segaloff DL. Pleiotropic effects of substitutions of a highly conserved leucine in transmembrane helix III of the human lutropin/choriogonadotropin receptor with respect to constitutive activation and hormone responsiveness. Mol. Endocrinol 2001;15:972–984. [PubMed: 11376115]

Soriano-Guillen L, Lahlou N, Chauvet G, Roger M, Chaussain JL, Carel JC. Adult height after ketoconazole treatment in patients with familial male-limited precocious puberty. J. Clin. Endocrinol. Metab 2005;90:147–151. [PubMed: 15522928]

Tao Y-X, Mizrachi D, Segaloff DL. Chimeras of the rat and human FSH receptors (FSHRs) identify residues that permit or suppress transmembrane 6 mutation-induced constitutive activation of the FSHR via rearrangements of hydrophobic interactions between helices 6 and 7. Mol. Endocrinol 2002;16:1881–1892. [PubMed: 12145341]

Themmen APN, Huhtaniemi IT. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. Endocr. Rev 2000;21:551–583. [PubMed: 11041448]

Zhang M, Mizrachi D, Fanelli F, Segaloff DL. The formation of a salt bridge between helices 3 and 6 is responsible for the constitutive activity and lack of hormone responsiveness of the naturally occurring L457R mutation of the human lutropin receptor. J. Biol. Chem 2005;280:26169–26176. [PubMed: 15908694]

Figure 1. Constitutive activity and lack of hCG responsiveness of the L457^(3,43)R mutant. HEK293 cells were transiently transfected with wt or mutant hLHR cDNA plasmids under conditions where the cells expressed the same levels of cell surface receptors. Under these conditions, basal cAMP and hCG-stimulated cAMP were assayed. Results are presented as the fold-increase in cAMP relative to basal levels present in cells expressing wt hLHR and are shown as the mean of two independent experiments. Adapted with permission from (Latronico et al., 1998).

Figure 2.

Average minimized structures of the wt hLHR and L457^(3.43) R mutant. In the left hand side of each panel, the helix bundles are shown, represented by cylinders and seen from the intracellular side in a direction perpendicular to the membrane surface. The extracellular domains are not shown. Helices 1, 2, 3, 4, 5, 6 and 7 are colored in blue, orange, green, pink, yellow, cyan and violet, respectively, and the first, second and third intracellular loops are colored in light green, white and magenta, respectively. The solvent accessible surface area computed over R464^(3.50), T467^(3.53), I468^(3.54) and K563^(6.29) is represented by dots colored according to the amino acid location. In the right hand side of each of panel, details of the

interactions of the L457^(3.43) and D578^(6.44) mutants are shown. The amino acid side chains are colored according to their location. Adapted with permission from (Zhang et al., 2005).

Figure 3.

Disruptive and reciprocal mutagenesis support the formation of a charge-reinforced H-bond between positions 3.43 and 6.44 as a cause of constitutive activation of the hLHR. HEK293 cells were transiently transfected with increasing concentrations of cDNAs encoding the indicated hLHR constructs. In the same experiment, cell surface receptors (reflected by hCG binding assays to intact cells) and basal cAMP levels were determined. One experiment, representative of at least three is shown. Data show the mean $\pm$ SEM of triplicate determinations within the experiment. Reproduced with permission from (Zhang et al., 2005).

Figure 4.

Attenuation of hCG-stimulated cAMP production by the wt hLHR caused by the co-expression of L457^(3,43)R. HEK293 cells were transiently transfected to yield cells expressing submaximal but equal levels of either wt hLHR or L457^(3,43)R or a combination of both. Cells were incubated 2h at 37°C in the absence or the presence of a saturating concentration of hCG and in the absence (Top Panel) or presence (Bottom Panel) of the PDE4 inhibitor Rolipram (20 μ M). The media was aspirated and intracellular cAMP was extracted and measured by RIA. Results shown are the mean $\pm$ range of two independent experiments. Cells expressing hLHR (wt), hLHR(L457R), or hLHR(wt) plus hLHR(L457R) bound 2.25 ± 0.75 , 2.45 ± 0.65 , and

$5.10 \pm 0.07 \text{ ng } ^{125}\text{IhCG} / 10^6 \text{ cells}$, respectively. Reproduced with permission from (Shinozaki et al., 2003).

Figure 5. L457^(3,43)R causes the phosphorylation of PDE4D3 under basal conditions. HEK293 cells were transiently transfected to yield submaximal (Panel A) or maximal (Panel B) levels of wt hLHR or L457^(3,43)R. Cells were incubated with hCG (100 ng/ml final concentration) or forskolin (90 μ M) for 15 min at 37°C and then lysates were prepared and resolved by SDS-PAGE. Western blots were probed with antibodies specific to PDE4D. rPDE4D3 refers to recombinant PDE4D3 that was included as an internal control. In Panel A, hLHR(wt) and hLHR(L457R) cells bound 6.0 and 3.3 ng ¹²⁵I-hCG/10⁶ cells, respectively, and 110 μ g protein was applied per lane. In Panel B, hLHR(wt) and hLHR(L457R) cells bound 24.0 and 17.1 ng ¹²⁵I-hCG/10⁶ cells, respectively, and 55 μ g protein was applied per lane. Results shown are from one representative experiment and depict only the relevant portions of the gels. Similar results were obtained with two different PDE4D-specific antibodies. Reproduced with permission from (Shinozaki et al., 2003).