# Restoration of testis function in hypogonadotropic hypogonadal mice harboring a misfolded GnRHR mutant by pharmacoperone drug therapy

Jo Ann Janovick[a,b,c,1], M. David Stewart[d,e,1], Darla Jacob[f], L. D. Martin[f], Jian Min Deng[d], C. Allison Stewart[d], Ying Wang[d], Anda Cornea[a], Lakshmi Chavali[e], Suhujey Lopez[e], Shoukhrat Mitalipov[a], Eunju Kang[a], Hyo-Sang Lee[a,2], Pulak R. Manna[c], Douglas M. Stocco[c], Richard R. Behringer[d], and P. Michael Conn[a,b,c,g,3]

Divisions of [a]Reproductive and Developmental Science and Neuroscience and [f]Comparative Medicine, Oregon National Primate Research Center, Beaverton, OR 97006; Departments of [b]Internal Medicine and [c]Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430; [d]Department of Genetics, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030; [e]Department of Biology and Biochemistry, University of Houston, Houston, TX 77204; and [g]Departments of Physiology and Pharmacology, Cell Biology and Development, and Obstetrics and Gynecology, Oregon Health and Science University, Beaverton, OR 97006

Edited by William F. Crowley, Massachusetts General Hospital, Boston, MA, and accepted by the Editorial Board November 4, 2013 (received for review September 10, 2013)

**Mutations in receptors, ion channels, and enzymes are frequently recognized by the cellular quality control system as misfolded and retained in the endoplasmic reticulum (ER) or otherwise misrouted. Retention results in loss of function at the normal site of biological activity and disease. Pharmacoperones are target-specific small molecules that diffuse into cells and serve as folding templates that enable mutant proteins to pass the criteria of the quality control system and route to their physiologic site of action. Pharmacoperones of the gonadotropin releasing hormone receptor (GnRHR) have efficacy in cell culture systems, and their cellular and biochemical mechanisms of action are known. Here, we show the efficacy of a pharmacoperone drug in a small animal model, a knock-in mouse, expressing a mutant GnRHR. This recessive mutation (GnRHR $E^{90}K$) causes hypogonadotropic hypogonadism (failed puberty associated with low or apulsatile luteinizing hormone) in both humans and in the mouse model described. We find that pulsatile pharmacoperone therapy restores $E^{90}K$ from ER retention to the plasma membrane, concurrently with responsiveness to the endogenous natural ligand, gonadotropin releasing hormone, and an agonist that is specific for the mutant. Spermatogenesis, proteins associated with steroid transport and steroidogenesis, and androgen levels were restored in mutant male mice following pharmacoperone therapy. These results show the efficacy of pharmacoperone therapy in vivo by using physiological, molecular, genetic, endocrine and biochemical markers and optimization of pulsatile administration. We expect that this newly appreciated approach of protein rescue will benefit other disorders sharing pathologies based on misrouting of misfolded protein mutants.**

protein trafficking | protein misrouting | intracellular trafficking

Missense mutations can result in protein misfolding, an event that leads to retention in the endoplasmic reticulum (ER) and degradation by the cellular quality control system (QCS). As a consequence, loss-of-function diseases result (1, 2). G protein-coupled receptors (GPCRs) that are sensitive to these misfolding mutations have been identified in patients with a wide range of diseases (3) Drugs used to alleviate symptoms of these diseases do not correct the root problem, restoration of plasma membrane (PM) expression and function of the misfolded GPCR. In cell cultures, pharmacoperone drugs diffuse into cells and refold ER-retained mutants into a conformation that is acceptable to the QCS, enabling correct routing to the PM and restoration of function. The efficacy of this class of drugs for treating disease caused by misfolded proteins has yet to be established in vivo.

The gonadotropin releasing hormone (GnRH) receptor (GnRHR) is a GPCR expressed in pituitary gonadotropes. Its PM localization enables cells to respond to extracellular GnRH by production and release of the gonadotropins, luteinizing hormone (LH), and follicle stimulating hormone (FSH). These hormones enter the peripheral circulation and regulate gonadal growth, steroidogenesis, and gamete maturation. Many mutants of the GnRHR become misrouted (4, 5) and cannot respond to GnRH, resulting in low serum gonadotropin levels and loss of gonadal function, a disease termed hypogonadotropic hypogonadism (HH) in humans (6).

Our previous studies in cell cultures demonstrated the efficacy of GnRHR-specific pharmacoperones for rescuing misfolded GnRHR mutants from ER retention, leading to PM localization and normal signaling activity (4, 5). Here, we report a mouse model designed to test the therapeutic utility of pharmacoperones in vivo. These mice harbor the mutation GnRHR $E^{90}K$, which results in HH in humans and in this animal model. The GnRHR $E^{90}K$ protein is misrouted because the substitution of a positively charged $K^{90}$ breaks the $E^{90}$-$K^{121}$ salt bridge. This bridge, between transmembrane segments 1 and 2, creates a structural relation required for acceptability of the receptor to the cellular QCS (7). A pharmacoperone specific to GnRHR was able to restore testis function in *Gnrhr* $E^{90}K$ mutant mice. Importantly, once routed to the PM, the mutant GPCR functioned normally and the disease symptoms were attenuated. This study demonstrates the in vivo efficacy of pharmacoperone therapy for a disease caused by protein misfolding.

## Significance

**Many diseases result from genetic mutations that cause protein misfolding. Medical treatments often address the symptoms, but do not correct the underlying etiology. This study illustrates proof of principle that a disease caused by a misfolded cell surface receptor can be corrected with a pharmacoperone, a unique class of target-specific drugs that assist protein folding.**

Author contributions: J.A.J., M.D.S., R.R.B., and P.M.C. designed research; J.A.J., M.D.S., D.J., L.D.M., J.M.D., C.A.S., Y.W., A.C., L.C., S.L., S.M., E.K., H.-S.L., P.R.M., D.M.S., R.R.B., and P.M.C. performed research; J.A.J., M.D.S., R.R.B., and P.M.C. contributed new reagents/analytic tools; J.A.J., M.D.S., D.J., L.D.M., J.M.D., C.A.S., Y.W., A.C., L.C., S.L., S.M., E.K., H.-S.L., P.R.M., D.M.S., R.R.B., and P.M.C. analyzed data; and J.A.J., M.D.S., R.R.B., and P.M.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. W.C. is a guest editor invited by the Editorial Board.

[1]J.A.J. and M.D.S. contributed equally to this work.

[2]Present address: Laboratory Animal Center, Osong Medical Innovation Foundation, Chungbuk 363-951, Republic of Korea.

[3]To whom correspondence should be addressed. E-mail: michael.conn@ttuhsc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1315194110/-/DCSupplemental.

## Results

**Mutation GnRHR $E^{90}K$ Produces a Mouse with Hypogonadotropic Hypogonadism.** A single base-pair substitution that produces GnRHR $E^{90}K$ was introduced into exon 1 of the mouse *Gnrhr* locus by homologous recombination in mouse embryonic stem (ES) cells. The gene targeting strategy left a *loxP*-flanked neomycin resistance gene expression cassette (*neo*) in intron 1 in reverse orientation to the direction of *Gnrhr* transcription. Previously, we removed *neo* and characterized the $E^{90}K$ phenotype. $E^{90}K/E^{90}K$ males have slightly smaller testes compared with controls but are fertile. $E^{90}K/E^{90}K$ females generate antral follicles but do not ovulate (8). Interestingly, when *neo* was left in the locus, the HH phenotype was more severe, making it a better mouse model for the pharmacoperone trials reported here. As a control, we examined the wild-type (WT) littermates, both male and female mice homozygous for *neo* alone were fertile and exhibited normal testis and ovary histology (Fig. 1). Thus, *neo* alone is not sufficient to induce HH. The observation that $E^{90}Kneo$ exhibits a more severe HH phenotype than $E^{90}K$ alone suggests that *neo* reduces transcription of *Gnrhr*. The combination of reduced transcription and $E^{90}K$-induced protein misfolding produces the more severe HH phenotype in $E^{90}Kneo$ mice. $E^{90}Kneo/E^{90}Kneo$ males and females exhibit a strong HH phenotype, but one that is less severe than the previously isolated $L^{117}P$ allele, which is equivalent to a null allele (9). These results indicate that the $E^{90}Kneo$ allele is a strong hypomorph.

Males were chosen for pharmacoperone infusion experiments; however, $E^{90}Kneo$ homozygous females exhibited HH as well. $E^{90}Kneo$ heterozygous females were fertile, but homozygous mutants were infertile. At the gross and microscopic levels, the ovaries of $E^{90}Kneo$ heterozygous females were indistinguishable from WT. The ovaries of homozygous mutants were small and lacked follicular development past the secondary follicle stage (Fig. S1).

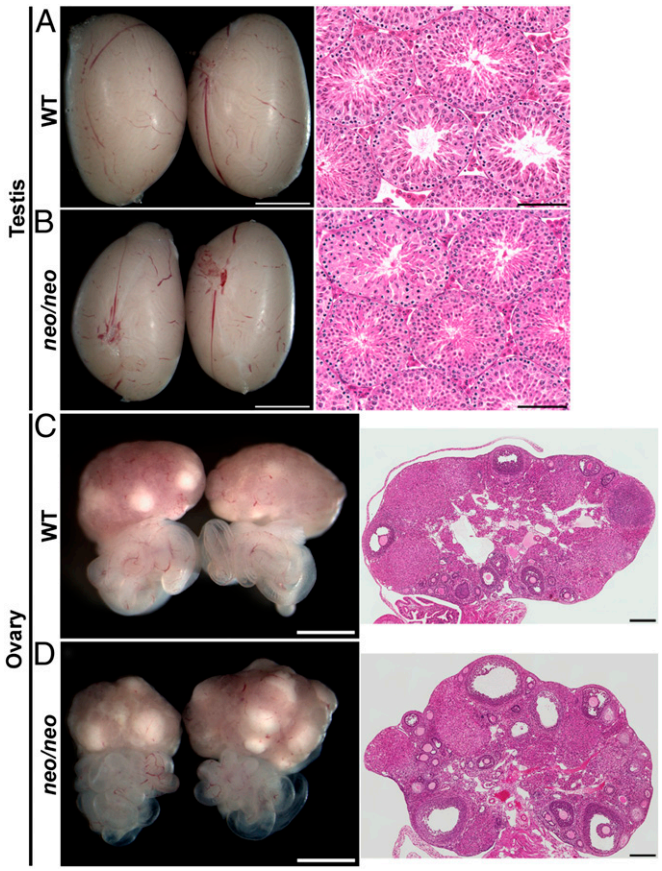


**Fig. 1.** The presence of the *neo* cassette alone does not produce hypogonadism. Mice were generated that harbor the *neo* expression cassette in the context of WT *Gnrhr* to determine the effect of *neo* alone on gonadal function. Testes or ovaries from mice of the indicated genotypes were collected at 90 d of age, imaged with a stereomicroscope, and then processed for H&E staining. Both male and female mice homozygous for *neo* were fertile and exhibited gross morphology and histology comparable to that of WT. The stereomicroscope image is shown in *Left* and the H&E-stained section is shown on *Right*. (Scale bars: *A–D Left*, 2 mm; *A* and *B*, *Right*, 0.1 mm; *C* and *D*, *Right*, 0.2 mm.)

**A Catheter Enables Pharmacoperone Infusion to the Pituitary Gonadotropes.** A catheter was inserted in the left carotid of 60-d-old male $E^{90}Kneo/E^{90}Kneo$ mice and fitted to a jacket system that enabled the mouse free-range with access to food and water ad libitum. The catheter positioning was confirmed at autopsy.

Pharmacoperone IN3 ((2S)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]-2-(3,5-dimethylphenyl)-$^1$H-indol-3-yl]-*N*-(2-pyridin-4-ylethyl)propan-1-amine) (Merck and Company) is an antagonist of the GnRHR. We hypothesized that LH release in response to a GnRHR agonist would be attenuated after IN3 infusion. We used this assumption to determine whether IN3 was being delivered to pituitary gonadotropes in our infusion model. To this end, we evaluated the concentration of serum LH 60 min after injection of 10 μg of Buserelin (a GnRHR agonist, administered s.c. in 50 μL of saline). Before being catheterized, animals responded to 10 μg of Buserelin with a serum LH of 8.2 ± 1.1 ng/mL LH, $n$ =3. They were then infused with saline-heparin (SH) for 6 d to allow recovery of pituitary LH and adaptation. IN3 (5 μg/mL, 25 μL/h) was then infused for 6 h (the last hour of which was concurrent with s.c. Buserelin administration) and selected to allow endogenous LH (released in response to endogenous GnRH) to be cleared. Ten micrograms of Buserelin was administered as described. Circulating LH levels were <0.2 ng/mL, $n$ = 3. These data show suppression of LH after IN3 infusion and support the notion that IN3 is efficiently reaching pituitary gonadotropes in our infusion model.

**Pulsatile Infusion of Pharmacoperone IN3 Rescues Testicular Weight.** The search for pharmacoperones of misfolded mutants of the GnRHR has relied on repurposing peptidomimetics that were initially identified as receptor antagonists. Because they are small and hydrophobic, these molecules diffuse into cells. Because they are antagonists, it is necessary to wash out the molecule after treatment to allow endogenous ligand binding to the refolded receptor. For this reason, we first optimized the pattern of administration so as to promote its action as a pharmacoperone and minimize antagonistic action.

Male $E^{90}Kneo/E^{90}Kneo$ animals displayed severely decreased gonadal size (0.04 g ± 0.005 SEM per 2 testes; $n$ = 19) compared with WT animals (0.19 g ± 0.004 per 2 testes; $n$ = 26) or heterozygotes (0.20 g ± 0.007 per 2 testes; $n$ = 16) at 60 d of age. Male animals were infused at a constant rate of 25 μL/h with SH or 5 μg/mL pharmacoperone IN3 in SH. We varied the frequency (pulses per day) and pulse duration (hours) of pharmacoperone administration in the model animal to identify conditions that allowed rescue of the mutant, followed by washout of the rescue agent. A selection of conditions is shown (Fig. 2*A*). The optimum drug infusion conditions produced, in 30 d, animals with a mean testis weight (0.11 g per 2 testes; $n \geq 3$). When 60-d-old animals were continuously infused with saline or with IN3 for 30 d additionally, testicular weights (sum of left and right) were 0.07 g ± 0.01 and 0.07 g ± 0.01 per 2 testes, respectively.

We next compared testis weights for WT, $E^{90}Kneo$ heterozygotes, $E^{90}Kneo$ homozygotes, and $E^{90}Kneo$ homozygotes after IN3 infusion. Testis weights were similar for WT and $E^{90}Kneo$ heterozygotes. $E^{90}Kneo$ homozygotes, which exhibit HH, showed a significant reduction in testis weight. IN3 treatment for 30 d increased testis weight in the homozygotes, but not to WT levels (Fig. 2*B*).

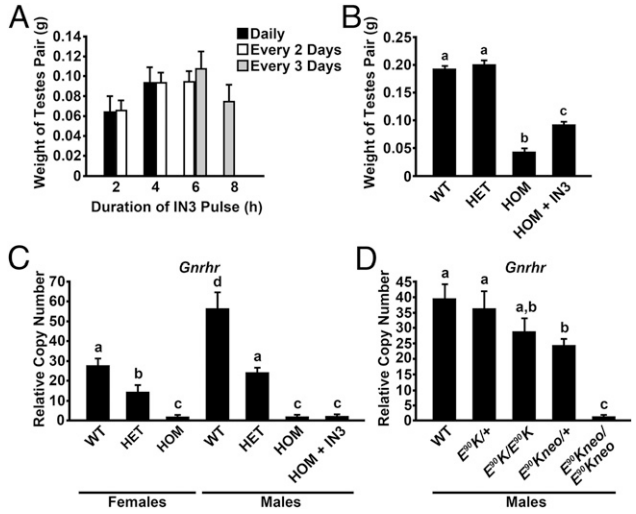


**Fig. 2.** (*A*) Testicular weight was determined in 60-d-old animals that were infused for 30 d with pharmacoperone IN3 each, 1, 2, or 3 d with pulse duration of 2, 4, 6, or 8 h ($n \geq 3$ per group). When animals were continuously infused with saline or with IN3 for 30 d, testicular weights (sum of left and right) were 0.07 ± 0.01 and 0.07 ± 0.01 g, respectively ($n \geq 3$ per group). (*B*) Comparison of testicular weights from WT, $E^{90}Kneo$ heterozygotes (HET), $E^{90}Kneo$ homozygotes (HOM), and $E^{90}Kneo$ homozygotes following a 30-d IN3 regimen (HOM+IN3). (*C*) *Gnrhr* qPCR: animals containing the *neo* cassette. Real-time PCR was performed by using total RNA extracted from the pituitary. Data were analyzed by the ΔΔCT method and reported as copy number relative to that of *Gapdh*. Females. *Gnrhr* expression was reduced in $E^{90}Kneo$ heterozygous females compared with WT. *Gnrhr* expression was further reduced in $E^{90}Kneo$ homozygotes, which exhibit the HH phenotype. For males, *Gnrhr* expression was reduced in $E^{90}Kneo$ heterozygous males compared with WT. *Gnrhr* expression was further reduced in $E^{90}Kneo$ homozygotes, which exhibit the HH phenotype. Pharmacoperone IN3 treatment did not affect *Gnrhr* mRNA levels. For females, for WT, $E^{90}Kneo/+$ and $E^{90}Kneo/E^{90}Kneo$ groups, $n = 8$, 3, and 6, respectively. For males, for WT, $E^{90}Kneo/+$, $E^{90}Kneo/E^{90}Kneo$, and IN3 groups, $n = 5$, 11, 8, and 4, respectively. (*D*) *Gnrhr* qPCR: Comparison between males harboring only the $E^{90}K$ mutation and those in which the *neo* cassette is retained. Real-time PCR was performed and data were analyzed as described in *A*. *Gnrhr* expression was lower in $E^{90}Kneo$ heterozygotes than in WT or either $E^{90}K$ genotype. $E^{90}Kneo$ homozygous males exhibited the lowest *Gnrhr* mRNA expression of any genotype. For WT, $E^{90}K/+$, $E^{90}K/E^{90}K$, $E^{90}Kneo/+$, and $E^{90}Kneo/E^{90}Kneo$ groups, $n = 6$, 8, 7, 13, and 9, respectively. Significant differences ($P < 0.05$) are denoted by the lowercase letters above each bar (a, b, c). Equivalent means have the same letter; different letters indicate statistically significant differences. Error bars show SEM.

**Serum LH Response to an Agonist of Mutant $E^{90}K$ Is Promoted by Pulsatile IN3 Infusion but Not a Constant Infusion of IN3.** We compared the LH response of pulsatile IN3-treated mutant animals (5 μg/mL IN3 for 6 h every third day for 30 d) to the response from animals infused with either a constant infusion of IN3 or constant SH for 30 d. After constant SH or IN3 or pulsatile IN3 treatments, animals were infused for 1 h with 100 μg/mL $Asp^2$-[GnRH], an agonist of mutant $E^{90}K$ that is not recognized by the WT receptor (10). Serum LH was then measured by radioimmunoassay. Animals receiving constant infusion of IN3 or SH showed <0.2 ng/mL serum LH ($n = 3$). Animals receiving pulses of IN3 showed serum levels of 9.7 ± 4.2 ng/mL (SEM) LH ($n = 3$) following $Asp^2$-GnRH. These data suggest that pulsatile IN3 rescued the $E^{90}K$ mutant, whereas constant IN3 was unable to do so. For this reason, the pulsatile IN3 regime was used for all further studies.

**Pharmacoperone IN3 Does Not Influence Transcription Levels of the *Gnrhr* Gene.** *Gnrhr* mRNA transcript levels in both males and females were reduced in $E^{90}Kneo$ heterozygotes compared with WT and further reduced in homozygous mutants, consistent with the view that *neo* reduces transcription of the *Gnrhr* gene. Pharmacoperone IN3 did not significantly influence transcript levels of *Gnrhr*, consonant with a role of the pharmacoperone solely in protein folding (Fig. 2*C*).

To test whether the reduction in *Gnrhr* mRNA expression was due to *neo* and not $E^{90}K$, we compared *Gnrhr* mRNA expression in pituitaries from male $E^{90}K$ mice lacking *neo* versus $E^{90}Kneo$ by real-time PCR. The results of this assay showed no effect of $E^{90}K$ alone but a significant reduction in *Gnrhr* mRNA levels in $E^{90}Kneo$ animals (Fig. 2*D*). These data are consistent with the idea that the reduction in *Gnrhr* mRNA expression is due to the presence of *neo* and not due to the $E^{90}K$ mutation. *Neo* may negatively affect mRNA expression by suppressing transcription, causing aberrant splicing or destabilizing *Gnrhr* transcripts.

**Pharmacoperone Infusion Improves Testis Function.** At the gross and microscopic levels, the testes of $E^{90}Kneo$ heterozygotes were indistinguishable from that of WT. The penetrance of the HH phenotype in $E^{90}Kneo$ homozygotes was variable. Whereas 100% of homozygotes exhibited reduced testis weight and infertility, ~50% exhibited reduced numbers of elongated spermatids in H&E-stained sections (Fig. 3*A* and Table S1). After 30 d of IN3 infusion, testis size had increased and histology looked equivalent to WT as evident by larger seminiferous tubules harboring elongated spermatids and eosin-enriched Leydig cells (Fig. 3*A*).

We quantified alterations in testis histology by measuring the diameter of the seminiferous tubules in H&E-stained sections. The mean seminiferous tubule diameter of $E^{90}Kneo$ heterozygotes was similar to that of WT animals. Mean seminiferous tubule diameter was reduced for $E^{90}Kneo$ homozygotes. IN3 treatment increased the mean seminiferous tubule diameter for $E^{90}Kneo$ homozygotes but did not restore it to the full WT diameter size (Fig. 3*B*).

**Pharmacoperone Infusion Rescues Sperm Morphology.** Because the mouse jacket interfered with mating studies, we isolated sperm from the epididymis of IN3-rescued $E^{90}Kneo/E^{90}Kneo$ males and performed sperm analysis and in vitro fertilization (IVF). Sperm from cauda epididymides were isolated for IVF and analysis of total count, viability, motility, forward progression, and general morphology. Sperm from $E^{90}Kneo$ homozygotes possessed defects, including reduced concentration, viability, and motility, and abnormal morphologies, including headlessness and looped tails. IN3 rescue resulted in increased sperm concentration, a higher percentage of normal sperm, and less looped tails (Table 1). Sperm from three IN3-treated animals were used for IVF and produced blastocysts that were implanted into a surrogate female and resulted in a mouse pup with the expected (heterozygous) genotype.

**Pituitaries of WT, Mutant, or Rescued Mutant Lack Hyperfunctioning Gonadotropes.** The pituitaries of $E^{90}Kneo/E^{90}Kneo$ males do not show castration cells (11), which form after gonadectomy in response to elevated stimulation by GnRH (Fig. 3*C*). The likely explanation for the absence of these cells is that $E^{90}Kneo/E^{90}Kneo$ males lack PM GnRHRs and cannot respond to stimulation by the elevated endogenous GnRH associated with loss of steroidal feedback. This result is similar to that observed following blockade of endogenous GnRH with anti-GnRH antibodies in castrate rodents (11).

**Pharmacoperone Infusion Increases StAR and CYP11A1 Protein Levels in the Testis.** Steroidogenic acute regulatory protein (StAR) mediates cholesterol transfer within the mitochondria, the rate-limiting step in the production of steroid hormones; its production in the testes is promoted by circulating levels of LH. We examined StAR protein levels in three testes each of WT, $E^{90}Kneo$ homozygotes, and IN3-treated $E^{90}Kneo$ homozygotes by Western blotting. Compared with WT, StAR protein was markedly decreased in $E^{90}Kneo$ homozygotes. The loss was substantially reversed by IN3 treatment (Fig. 4*A*).

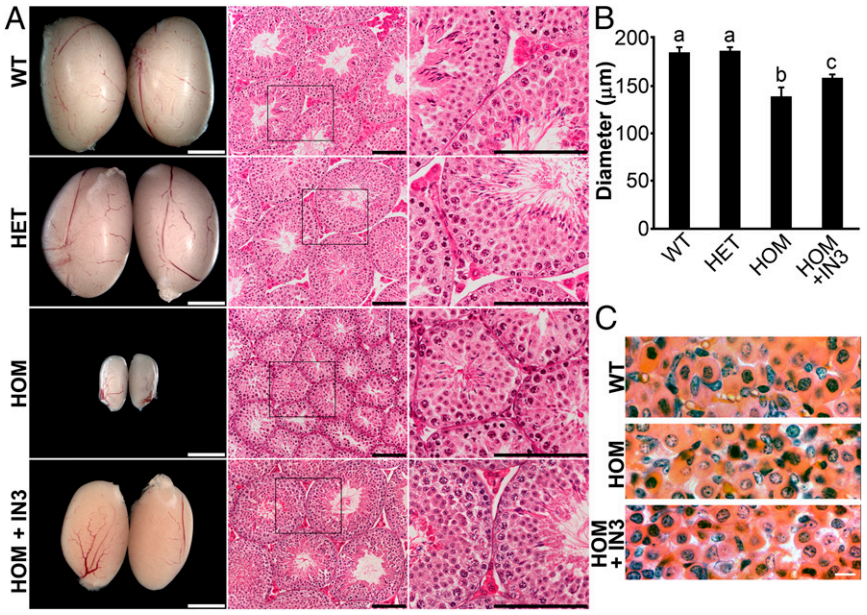


**Fig. 3.** (*A*) Male phenotype. Testes from the indicated genotypes were collected at 90 d of age, imaged with a stereomicroscope, and histology was examined by H&E staining. WT and $E^{90}Kneo$/+ (HET) testes were indistinguishable in size, appearance, abundance of Leydig cells, and presence of spermatozoa in the seminiferous tubules. The testes of $E^{90}Kneo/E^{90}Kneo$ (HOM) males were smaller and exhibited varying degrees of hypogonadism. There were few to no eosin-stained Leydig cells or elongated spermatids. Treatment of $E^{90}Kneo/E^{90}Kneo$ males with pharmacoperone IN3 for 30 d (HOM + IN3) resulted in increased testis size and restored spermatogenesis. The stereomicroscopic image is shown in *Left*, and the H&E-stained sections are shown on *Center* and *Right*. (*B*) Spermatogenic activity was assayed by measuring seminiferous tubule diameter (*SI Materials and Methods* and Table S1). Mean seminiferous tubule diameter was not different between WT and $E^{90}Kneo$ heterozygotes. Seminiferous tubule diameter was reduced in $E^{90}Kneo/E^{90}Kneo$ males. IN3 treatment increased the mean seminiferous tubule diameter of $E^{90}Kneo/E^{90}Kneo$ males, but did not restore it to WT levels. Significant differences ($P < 0.05$) are denoted by the lowercase letters above each bar. Equivalent means have the same letter; different letters indicate statistically significant differences (a, b, c). Error bars show SEM. For WT, $E^{90}Kneo$/+, $E^{90}Kneo/E^{90}Kneo$, and IN3 groups, $n = 6$, 12, 10, and 12, respectively. (*C*) Pituitary morphology. Images of the anterior pituitary from WT, $E^{90}Kneo/E^{90}Kneo$, and IN3-rescued $E^{90}Kneo/E^{90}Kneo$ males look similar. (Scale bars: *A Left*, 2 mm; *A Center* and *Right*, 0.1 mm; *C*, 10 μm.)

Side-chain cleavage cytochrome (P450scc or CYP11A1) is a mitochondrial enzyme that catalyzes conversion of cholesterol to pregnenolone, the first reaction in the process of steroidogenesis in all mammalian tissues that specialize in the production of various steroid hormones. Similar to StAR, expression of this enzyme was reduced in $E^{90}Kneo$ homozygotes as assessed by Western blotting (Fig. 4*A*). CYP11A1 protein expression appeared to increase following IN3 treatment, but our densitometric analysis showed no significant effect of IN3 (Fig. 4*A*). The more modest reduction in CYP11A1 protein levels may reflect the observation that this enzyme is always active, but its activity is limited by the supply of cholesterol in the inner mitochondrial membrane. The transfer of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in steroid production, a process mediated by StAR. Upon stimulation of a steroidogenic cell, the expression of StAR is rapidly increased and is thus available to transfer cholesterol to the inner membrane for conversion to pregnenolone.

**Pharmacoperone Infusion Improves Steroid Dehydrogenase 17β Gene Expression and Serum Testosterone.** Testes were evaluated by quantitative real-time PCR for mRNA levels of hydroxyl steroid dehydrogenase 17β3 (*Hsd17b3*) (Fig. 4*B*), an enzyme that catalyzes the conversion of the poorly bioactive 17-keto steroids to the highly bioactive 17β-hydroxy steroids including testosterone (12). Levels were similar between WT and heterozygotes. Levels in homozygotes were markedly reduced. IN3 treatment increased *Hsd17b3* expression in homozygotes, but not to WT levels. Despite the "subnormal" level of this rate-limiting enzyme, circulating levels of testosterone were improved in IN3-treated homozygous mutants (Fig. 4*C*).

## Discussion

**Comparison of *Gnrhr* Mouse Mutants Reveals the Consequences of Fine Alteration in GnRHR PM Expression.** We created the $Gnrhr^{E90K}$ allele in mice to mimic the human disease HH and to generate a small animal model to study the efficacy of pharmacoperone treatment in vivo. In humans, mutant $E^{90}K$ can produce HH when homozygous. It is completely retained by the QCS and acts as a dominant negative to WT GnRHR (13, 14). In cell culture, mouse $E^{90}K$ is also recognized as misfolded by the QCS and retained in the ER. For both human and mouse $E^{90}K$, PM localization and signal transduction can be restored with pharmacoperone IN3 (7). With this knowledge, we generated mice harboring $Gnrhr^{E90K}$ to test the ability of pharmacoperones to correct a disease resulting from GPCR misfolding in vivo. Knowing that human $E^{90}K$ is a dominant negative and can produce HH when recessive, we constructed a gene targeting vector with *neo* in reverse orientation to the transcriptional direction of *Gnrhr*. If mouse $E^{90}K$ acted as

**Table 1. Evaluation of sperm morphology and motility**

| Parameter | $E^{90}Kneo/E^{90}Kneo$ | $E^{90}Kneo/E^{90}Kneo$ + IN3 | WT (IVF laboratory average) |
|---|---|---|---|
| Sperm concentration, x $10^6$/mL | 1.1 ± 0.2 | 2.5 ± 0.2* | 3.0 |
| Viability, % | 65 ± 0.0 | 66.7 ± 1.7† | 75 |
| Total motile, % | 50 ± 0.0 | 51.7 ± 1.7† | 60 |
| Grade A motility, % | 8 ± 1.2 | 30 ± 0.0† | 30 |
| Normal sperm, % | 22.5 ± 4.8 | 56.3 ± 9.1* | 75 |
| Headless, % abnormal sperm | 8.0 ± 2.1 | 13.7 ± 3.3 | 0 |
| Thin head, % abnormal sperm | 10.8 ± 4.7 | 9.0 ± 1.5 | 15 |
| Looped tail, % abnormal sperm | 59.0 ± 3.9 | 21.0 ± 6.0* | 9 |

Comparisons were made between K90neo/K90neo and IN3 groups by *t* test. Data are presented as mean ± SEM. For K90neo/K90neo and IN3 groups, $n = 4$ and 3, respectively. Significant improvement, $*P < 0.05$.
†The *t* test could not be performed because variance = 0 for at least one group.

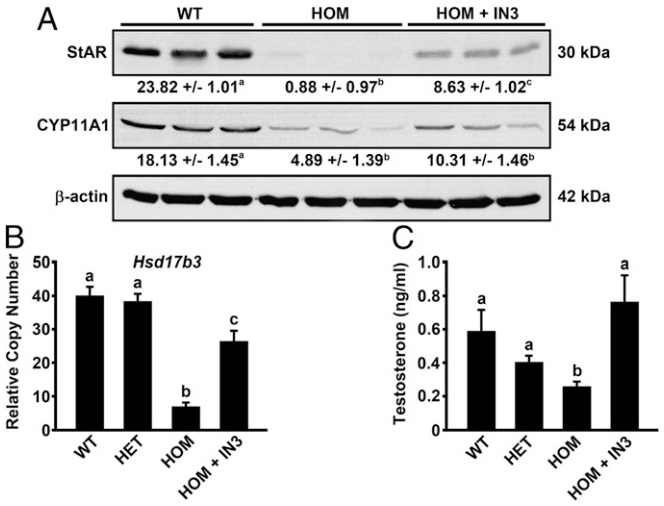


**Fig. 4.** (*A*) Comparison of StAR and CYP11A1 protein expression in the testes of WT, $E^{90}Kneo/E^{90}Kneo$ (HOM), and IN3-rescued $E^{90}Kneo/E^{90}Kneo$ males (HOM +IN3). Fifty micrograms of protein from each animal was analyzed by Western blot. Protein levels of β-actin were used as a loading control. Western results were quantitated by densitometry. The mean values obtained from densitometry ± SEM are reported below each blot. These data were subject to ANCOVA by using β-actin values as the covariable. Statistically different means ($P < 0.05$) are denoted by the superscript letter following each value. Equivalent means have the same letter; different letters indicate statistically significant differences (a,b,c). When animals received IN3, they received 6-h pulses every 3 d for 30 d. (*B*) *Hsd17b3* qPCR. Real-time PCR was performed by using total RNA extracted from the testis. Data were analyzed by the ΔΔCT method and reported as copy number relative to that of *Gapdh*. WT and $E^{90}Kneo$ heterozygotes, which are both phenotypically normal, exhibited similar levels of *Hsd17b3* mRNA. $E^{90}Kneo/E^{90}Kneo$ males, which exhibit hypogonadism, had reduced *Hsd17b3* mRNA. *Hsd17b3* expression was increased in $E^{90}Kneo/E^{90}Kneo$ males following pharmacoperone treatment. For WT, $E^{90}Kneo/+$, $E^{90}Kneo/E^{90}Kneo$, and IN3-rescued $E^{90}Kneo/E^{90}Kneo$, $n = 9$, 14, 17, and 8, respectively. (*C*) IN3 restored testosterone levels in $E^{90}Kneo/E^{90}Kneo$ males. The serum concentration of testosterone was measured by RIA. $E^{90}Kneo/E^{90}Kneo$ males exhibited less serum testosterone than heterozygous and WT males. Testosterone levels were restored in $E^{90}Kneo/E^{90}Kneo$ males after pharmacoperone treatment. Significant differences ($P < 0.05$) are denoted by the lowercase letters above each bar. Equivalent means have the same letter; different letters indicate statistically significant differences (a,b,c). Error bars show SEM for the mean of $n \geq 3$.

a dominant negative, the idea was that this allele would suppress expression of the $E^{90}K$ mutant, prevent infertility, and allow us to maintain the line. There is support for this strategy in the literature (15, 16). *Neo* could be removed from the locus with cre recombinase to induce HH before experimentation. However, mice heterozygous for $E^{90}K$ alone or $E^{90}Kneo$ are phenotypically normal and fertile (17).

Interestingly, $E^{90}K$ homozygotes exhibit mild HH. Males have reduced testis size but are fertile. Females are infertile and fail to ovulate (17). These data suggested that mutant $E^{90}K$ is only partially retained by the QCS in mice and that PM-expressed $E^{90}K$ can respond to ligand (GnRH) with gonadotropin release. Albeit, the amount of gonadotropin released is not sufficient to stimulate ovulation.

These phenotypes are much milder than our previously characterized $L^{117}P$ allele. $Gnrhr^{L117P}$ homozygous males have very small testes with a complete absence of spermatogenesis. $L^{117}P$ homozygous females have small, immature ovaries with no follicular growth past the primary follicle stage (9), whereas heterozygotes are fertile and phenotypically normal. Unfortunately, mutant $L^{117}P$ is not rescuable by pharmacoperone, presumably due to the covalent conformational change caused by the leucine to proline substitution.

Mice homozygous for $E^{90}Kneo$, in which *neo* was not removed from the first intron, exhibit HH that is much more severe than $E^{90}K$ alone, but less than that of $L^{117}P$. Both males and females are infertile. Testis size is smaller than $E^{90}K$ alone but larger than $L^{117}P$. Histological sections show varied penetrance as to the degree of spermatogenesis, but testis histology is never as severely compromised as that observed for $L^{117}P$. These phenotypic observations, combined with our *Gnrhr* gene expression data, support the conclusion that a portion of mutant $E^{90}K$ is retained by the QCS and the level of *Gnrhr* transcription is reduced by *neo*. This scenario presumably creates a mouse with a low level of PM-localized $E^{90}K$ and a "store" of QCS-retained $E^{90}K$ in the ER that can be mobilized to the cell surface by IN3. Importantly, $E^{90}Kneo$ mice exhibit infertility and a degree of HH similar to that seen in human patients and, thus, are a good model for pharmacoperone therapy.

**GnRHR $E^{90}K$ Is Functional When Routed to the Cell Surface.** Previous work in cell cultures demonstrated that both human and mouse GnRHR $E^{90}K$ could be routed to the cell surface via pharmacoperone treatment. Once correctly routed to the PM, the mutant receptor responded to ligand by initiating signal transduction (9). This study extends these findings in two ways. First, collective evidence from comparing the phenotypes of our three *Gnrhr* mutants suggests that only a portion of $E^{90}K$ is retained in vivo, so any gonadotropin release is the effect of $E^{90}K$ localized to the gonadotrope PM. Second, in hypogonadotropic hypogonadal $E^{90}Kneo$ homozygotes, IN3 infusion increases LH release and improves testis function. These data support the conclusion that $E^{90}K$ recognizes its ligand (GnRH) and functions normally when correctly routed to the PM in vivo. This observation bodes well for the use of pharmacoperone therapy to correct diseases caused by GPCR misfolding.

***$Gnrhr^{E90K}$* Mice Are a Model for Pharmacoperone Therapy.** In this study, we show the utility of pharmacoperone drugs to correct disease by rescuing PM expression rescue of a misfolded GPCR. Previous work using mouse models of lysosomal storage diseases demonstrated that pharmacoperone treatment can stabilize inactive misfolded soluble lysosomal enzymes and improve their function (18–20). These studies provided proof of principle that pharmacoperones can stabilize a misfolded protein in vivo. Furthermore, a nonpeptide V1a antagonist was able to attenuate symptoms of diabetes insipidus in patients with missense mutations to the *AVPR2* gene that cause misfolding (21). Similar to our previous work with GnRHR, V2R-specific pharmacoperones were able to restore cell surface expression and signaling of misfolded V2R mutants in COS-1 cells (21). In this study, we expanded on this line of research by generating and characterizing a genetic mouse model ($Gnrhr^{E90K}$) to directly test the usefulness of pharmacoperone therapy to restore PM localization and function of a GPCR in vivo. Benefiting from our mouse model, we were able to show mechanistically that delivery of a pharmacoperone to the target organ (anterior pituitary) increased routing of the misfolded GPCR (GnRHR $E^{90}K$) to the gonadotrope cell surface, increased receptor activity (assayed by LH release), improved testis function (spermatogenesis and testosterone production), and restored fertility. These data support the utility of pharmacoperones for the treatment of diseases caused by protein misfolding.

Pharmacoperone therapy is a unique approach for the treatment of disease when the underlying pathology is due to protein misrouting. The pharmacoperone used in this study (IN3) shows high specificity for the GnRHR (5, 22–25) and acts by creating a surrogate bridge ($D^{98}$-pharmacoperone-$K^{121}$) between transmembrane segments 1 and 2 of this GPCR, enabling the mutant receptor to pass the QCS and traffic to the PM, restoring its activity (22, 25).

Four different chemical classes of pharmacoperones also rescue most of the HH-associated GnRHR mutants, even when the sites of mutations are distant to one another. These data suggest that these drugs stabilize the core component of the protein

required for correct trafficking (4). Further, the ability of individual pharmacoperones to rescue many different mutants of the GnRHR (4) is therapeutically advantageous because this observation suggests that each mutant of a particular protein will not require a separate drug. The observation that pharmacoperones rescue newly synthesized misfolded mutants as well as those that have previously accumulated in the ER (26) suggests that it is not necessary to have this drug present at the time of protein synthesis, an observation that will facilitate the timing of therapeutic administration.

Pharmacoperone rescue of misfolded molecules potentially has applicability to a broad range of proteins that cause diseases responsible for cystic fibrosis, nephrogenic diabetes insipidus, disorders of vision, digestion, and neurodegeneration, and hypogonadotropic hypogonadism (1). A short-term study in patients suffering from nephrogenic diabetes insipidus (caused by ER retention of a mutant of the V2 receptor) was conducted with pharmacoperone drugs (21). Although the drug showed efficacy, the use of oral administration (and the limited number of dose regimes in a small number of patients) did not enable optimization of the pattern of pulsatile administration. Pharmacoperones for the V2, GnRHR, and other targets have been identified from antagonist screens. The present work shows that considerable efficacy can be obtained for protein rescue if the pattern of administration is carefully selected. In the present study, the treatment corrected deficits of serum LH and androgen levels and enabled the elaboration of morphologically correct and functional sperm. New high-throughput screens (27, 28) for pharmacoperones will likely lead to additional interest in this approach.

## Materials and Methods

**Development, Breeding, and Genotyping of the Mouse Mutant.** These methods are described in *SI Materials and Methods*. Animal procedures were approved by the Institutional Animal Care and Use Committees of either the University of Texas M.D. Anderson Cancer Center or Oregon Health Science University, depending where the specific work was done.

**Endocrine Responses, Tissue Collection, Sperm Assessment, IVF, Endocrine Histology, Immunoblotting and Morphology, and Statistics.** These methods are described in *SI Materials and Methods*.

**Surgery and Infusion.** Insertion of the catheter and infusion is described in described in *SI Materials and Methods*. These procedures were approved by the Oregon Health Science University Institutional Animal Care and Use Committee.

**ACKNOWLEDGMENTS.** We thank Drs. Barry Zirkin, Marvin L. Meistrich, Richard Stouffer, Patricia Hinkle, Randy L. Johnson, Ilpo Huhtaniemi, and Hector Chemes for comments. This work was supported by National Institutes of Health Grants OD012220, DK85040, and OD011092 (to P.M.C.), NS061800 (Sue Aicher, Oregon Health and Science University Neuroscience Imaging Center), HD030284 (to R.R.B.), CA16672 (veterinary resources), HD063276, HD057121, and HD059946 (to S.M.), and HD17481 (to D.M.S.); the Ben F. Love Endowment (to R.R.B.); American Heart Association Grant 12BGIA11860006 (to M.D.S.); the Texas Heart Institute (M.D.S.); and Robert A. Welch Foundation Grant B1-0028 (to D.M.S.).

1. Castro-Fernández C, Maya-Núñez G, Conn PM (2005) Beyond the signal sequence: Protein routing in health and disease. *Endocr Rev* 26(4):479–503.
2. Morello JP, Petäjä-Repo UE, Bichet DG, Bouvier M (2000) Pharmacological chaperones: A new twist on receptor folding. *Trends Pharmacol Sci* 21(12):466–469.
3. Conn PM, Ulloa-Aguirre A, Ito J, Janovick JA (2007) G protein-coupled receptor trafficking in health and disease: Lessons learned to prepare for therapeutic mutant rescue in vivo. *Pharmacol Rev* 59(3):225–250.
4. Janovick JA, et al. (2003) Structure-activity relations of successful pharmacologic chaperones for rescue of naturally occurring and manufactured mutants of the gonadotropin-releasing hormone receptor. *J Pharmacol Exp Ther* 305(2):608–614.
5. Janovick JA, Maya-Nunez G, Conn PM (2002) Rescue of hypogonadotropic hypogonadism-causing and manufactured GnRH receptor mutants by a specific protein-folding template: Misrouted proteins as a novel disease etiology and therapeutic target. *J Clin Endocrinol Metab* 87(7):3255–3262.
6. Seminara SB, Hayes FJ, Crowley WF, Jr. (1998) Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): Pathophysiological and genetic considerations. *Endocr Rev* 19(5):521–539.
7. Janovick JA, et al. (2006) Regulation of G protein-coupled receptor trafficking by inefficient plasma membrane expression: Molecular basis of an evolved strategy. *J Biol Chem* 281(13):8417–8425.
8. Sharpe RM, McKinnell C, Kivlin C, Fisher JS (2003) Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* 125(6):769–784.
9. Pask AJ, et al. (2005) A novel mouse model of hypogonadotrophic hypogonadism: N-ethyl-N-nitrosourea-induced gonadotropin-releasing hormone receptor gene mutation. *Mol Endocrinol* 19(4):972–981.
10. Janovick JA, Conn PM (2010) Salt bridge integrates GPCR activation with protein trafficking. *Proc Natl Acad Sci USA* 107(9):4454–4458.
11. Arimura A, Shino M, de la Cruz KG, Rennels EG, Schally AV (1976) Effect of active and passive immunization with luteinizing hormone-releasing hormone on serum luteinizing hormone and follicle-stimulating hormone levels and the ultrastructure of the pituitary gonadotrophs in castrated male rats. *Endocrinology* 99(1):291–303.
12. Saloniemi T, Jokela H, Strauss L, Pakarinen P, Poutanen M (2012) The diversity of sex steroid action: Novel functions of hydroxysteroid (17β) dehydrogenases as revealed by genetically modified mouse models. *J Endocrinol* 212(1):27–40.
13. Brothers SP, Cornea A, Janovick JA, Conn PM (2004) Human loss-of-function gonadotropin-releasing hormone receptor mutants retain wild-type receptors in the endoplasmic reticulum: Molecular basis of the dominant-negative effect. *Mol Endocrinol* 18(7):1787–1797.
14. Leaños-Miranda A, Ulloa-Aguirre A, Ji TH, Janovick JA, Conn PM (2003) Dominant-negative action of disease-causing gonadotropin-releasing hormone receptor (GnRHR) mutants: A trait that potentially coevolved with decreased plasma membrane expression of GnRHR in humans. *J Clin Endocrinol Metab* 88(7):3360–3367.
15. Arango NA, Lovell-Badge R, Behringer RR (1999) Targeted mutagenesis of the endogenous mouse Mis gene promoter: In vivo definition of genetic pathways of vertebrate sexual development. *Cell* 99(4):409–419.
16. Ventura A, et al. (2007) Restoration of p53 function leads to tumour regression in vivo. *Nature* 445(7128):661–665.
17. Stewart MD, et al. (2012) Mice harboring Gnrhr E90K, a mutation that causes protein misfolding and hypogonadotropic hypogonadism in humans, exhibit testis size reduction and ovulation failure. *Mol Endocrinol* 26(11):1847–1856.
18. Ishii S, Yoshioka H, Mannen K, Kulkarni AB, Fan JQ (2004) Transgenic mouse expressing human mutant alpha-galactosidase A in an endogenous enzyme deficient background: A biochemical animal model for studying active-site specific chaperone therapy for Fabry disease. *Biochim Biophys Acta* 1690(3):250–257.
19. Matsuda J, et al. (2003) Chemical chaperone therapy for brain pathology in G(M1)-gangliosidosis. *Proc Natl Acad Sci USA* 100(26):15912–15917.
20. Shiozuka C, et al. (2011) Increased globotriaosylceramide levels in a transgenic mouse expressing human alpha1,4-galactosyltransferase and a mouse model for treating Fabry disease. *J Biochem* 149(2):161–170.
21. Bernier V, et al. (2006) Pharmacologic chaperones as a potential treatment for X-linked nephrogenic diabetes insipidus. *J Am Soc Nephrol* 17(1):232–243.
22. Conn PM, Janovick JA (2009) Drug development and the cellular quality control system. *Trends Pharmacol Sci* 30(5):228–233.
23. Conn PM, Janovick JA (2009) Trafficking and quality control of the gonadotropin releasing hormone receptor in health and disease. *Mol Cell Endocrinol* 299(2): 137–145.
24. Janovick JA, Park BS, Conn PM (2011) Therapeutic rescue of misfolded mutants: Validation of primary high throughput screens for identification of pharmacoperone drugs. *PLoS ONE* 6(7):e22784.
25. Janovick JA, et al. (2009) Molecular mechanism of action of pharmacoperone rescue of misrouted GPCR mutants: The GnRH receptor. *Mol Endocrinol* 23(2):157–168.
26. Janovick JA, et al. (2007) Refolding of misfolded mutant GPCR: Post-translational pharmacoperone action in vitro. *Mol Cell Endocrinol* 272(1-2):77–85.
27. Smithson DC, Janovick JA, Conn PM (2013) Therapeutic rescue of misfolded/mistrafficked mutants: Automation-friendly high-throughput assays for identification of pharmacoperone drugs of GPCRs. *Methods Enzymol* 521:3–16.
28. Conn PM, Smith E, Hodder P, Janovick JA, Smithson DC (2013) High-throughput screen for pharmacoperones of the vasopressin type 2 receptor. *J Biomol Screen* 18(8): 930–937.