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

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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 E90K) causes hypogonadotropic hypogonadism (failed puberty associated with low or apoplastic luteinizing hormone) in both humans and in the mouse model described. We find that pulsatile pharmacoperone therapy restores E\textsuperscript{90K} 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.

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.


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Results

Mutation GnRHR E90K Produces a Mouse with Hypogonadotropic Hypogonadism. A single base-pair substitution that produces GnRHR E90K 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 E90K phenotype. E90K/E90K males have slightly smaller testes compared with controls but are fertile. E90K/E90K 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 E90Kneo exhibits a more severe HH phenotype than E90K alone suggests that neo reduces transcription of Gnrhr. The combination of reduced transcription and E90K-induced protein misfolding produces the more severe HH phenotype in E90Kneo mice. E90Kneo/E90Kneo males and females exhibit a strong HH phenotype, but one that is less severe than the previously isolated L117P allele, which is equivalent to a null allele (9). These results indicate that the E90Kneo allele is a strong hypomorph.

Males were chosen for pharmacoperone infusion experiments; however, E90Kneo homozygous females exhibited HH as well. E90Kneo heterozygous females were fertile, but homozygous mutants were infertile. At the gross and microscopic levels, the ovaries of E90Kneo heterozygous females were indistinguishable from WT. The ovaries of homozygous mutants were small and lacked follicular development past the secondary follicle stage (Fig. S1).

A Catheter Enables Pharmacoperone Infusion to the Pituitary Gonadotropes. A catheter was inserted in the left carotid of 60-d-old male E90Kneo/E90Kneo 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 (25-[2-(2-azacycloc[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxo-ethyl]-2-(3,5-dimethylphenyl)-1H-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, n = 3. They were then infused with saline (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 E90Kneo/E90Kneo 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. 2A). The optimum drug infusion conditions produced, in 30 d, animals with a mean tests weight (0.11 g per 2 testes; n ≥ 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 ± 0.001 g per 2 testes, respectively.

We next compared tests weights for WT, E90Kneo heterozygotes, E90Kneo homozygotes, and E90Kneo homozygotes after IN3 infusion. Testis weights were similar for WT and E90Kneo heterozygotes. E90Kneo 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. 2B).

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.)
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 ≥ 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 ≥ 3 per group). (B) Comparison of testicular weights from WT, E90Kneo heterozygotes (Het), E90Kneo homozygotes (HOM), and E90Kneo homozygotes following a 30-d IN3 regimen (HOM+IN3). (C) Gnhr expression: animals containing the neo cassette. Real-time PCR was performed by using total RNA extracted from the testes. Data were analyzed by the ΔΔCT method and reported as copy number relative to that of Gapdh RNA. Females. Gnhr expression was reduced in E90Kneo heterozygous females compared with WT. Gnhr expression was further reduced in E90Kneo homozygotes, which exhibit the HH phenotype. For males, Gnhr expression was reduced in E90Kneo heterozygous males compared with WT. Gnhr expression was further reduced in E90Kneo homozygotes, which exhibit the HH phenotype. Pharmacoperone IN3 treatment did not affect Gnhr mRNA levels. For females, for WT, E90Kneo+ and E90KneoE90Kneo groups, n = 8, 3, and 6, respectively. For males, for WT, E90Kneo+, E90KneoE90Kneo, and IN3 groups, n = 5, 11, 8, and 4, respectively. (D) Gnhr qPCR: Comparison between males harboring only the IN3 mutation and those in which the neo cassette is retained. Real-time PCR was performed and data were analyzed as described in A. Gnhr expression was lower in E90Kneo heterozygotes than in WT or either E90K genotypes. E90Kneo homozygous males exhibited the lowest Gnhr mRNA expression of any genotype. For WT, E90Kneo+; E90KneoE90Kneo, and E90KneoE90Kneo 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.

Pharmacoperone IN3 Does Not Influence Transcription Levels of the Gnhr Gene. Gnhr mRNA transcript levels in both males and females were reduced in E90Kneo heterozygotes compared with WT and further reduced in homozygous mutants, consistent with the view that neo reduces transcription of the Gnhr gene. Pharmacoperone IN3 did not significantly influence transcript levels of Gnhr, consonant with a role of the pharmacoperone solely in protein folding (Fig. 2C).

To test whether the reduction in Gnhr mRNA expression was due to neo and not E90K, we compared Gnhr mRNA expression in pituitaries from male E90Kneo mice lacking neo versus E90Kneo by real-time PCR. The results of this assay showed no effect of E90K alone but a significant reduction in Gnhr mRNA levels in E90Kneo animals (Fig. 2D). These data are consistent with the idea that the reduction in Gnhr mRNA expression is due to the presence of neo and not due to the E90K mutation. Neo may negatively affect mRNA expression by suppressing transcription, causing aberrant splicing or destabilizing Gnhr transcripts.

Pharmacoperone Infusion Improves Testis Function. At the gross and microscopic levels, the testes of E90Kneo heterozygotes were indistinguishable from that of WT. The penetrance of the HH phenotype in E90Kneo 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. 3A 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. 3A).

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 E90Kneo heterozygotes was similar to that of WT animals. Mean seminiferous tubule diameter was reduced for E90Kneo homozygotes. IN3 treatment increased the mean seminiferous tubule diameter for E90Kneo homozygotes but did not restore it to the full WT diameter size (Fig. 3B).

Pharmacoperone Infusion Rescues Sperm Morphology. Because the mouse jacket interfered with mating studies, we isolated sperm from the epididymis of IN3-rescued E90Kneo/E90Kneo 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 E90Kneo homozygotes possessed defects, including reduced concentration, viability, and motility, and abnormal morphologies, including headlessness and looped tails. IN3 rescued 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 E90Kneo/E90Kneo males do not show castration cells (11), which form after gonadectomy in response to elevated stimulation by GnRH (Fig. 3C). The likely explanation for the absence of these cells is that E90Kneo/E90Kneo 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, E90Kneo homozygotes, and IN3-treated E90Kneo homozygotes by Western blotting. Compared with WT, STAR protein was markedly decreased in E90Kneo homozygotes. The loss was substantially reversed by IN3 treatment (Fig. 4A).
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 widespread in all mammalian tissues that specialize in the production of various steroid hormones. StAR protein levels may regulate the expression of CYP11A1, leading to increased pregnenolone production. Upon stimulation of a steroidogenic cell, the expression of CYP11A1 increases, allowing for the conversion of cholesterol to pregnenolone. This process is mediated by cholesterol from the outer to the inner mitochondrial membrane. The more modest reduction in pituitary morphology observed in IN3-treated males may reflect the fact that the pituitary is not the primary site of steroid hormone production in males. IN3 treatment increased the mean diameter of seminiferous tubules in IN3-treated 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, E90Kneo+, E90Kneo and IN3 groups, n = 6, 12, 10, and 12, respectively. (C) Pituitary morphology. Images of the anterior pituitary from WT, E90Kneo/E90Kneo, and IN3-rescued E90Kneo/E90Kneo males look similar. (Scale bars: A, Left; 2 mm; A Center and Right, 0.1 mm; C, 10 μm.)

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E90Kneo/E90Kneo</th>
<th>E90Kneo/E90Kneo + IN3</th>
<th>WT (IVF laboratory average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration, x 10^6/ml</td>
<td>1.1 ± 0.2</td>
<td>2.5 ± 0.2*</td>
<td>3.0</td>
</tr>
<tr>
<td>Viability, %</td>
<td>65 ± 0.0</td>
<td>66.7 ± 1.7*</td>
<td>75</td>
</tr>
<tr>
<td>Total motile, %</td>
<td>50 ± 0.0</td>
<td>51.7 ± 1.7*</td>
<td>60</td>
</tr>
<tr>
<td>Grade A motility, %</td>
<td>8 ± 1.2</td>
<td>30 ± 0.0*</td>
<td>30</td>
</tr>
<tr>
<td>Normal sperm, %</td>
<td>22.5 ± 4.8</td>
<td>56.3 ± 9.1*</td>
<td>75</td>
</tr>
<tr>
<td>Headless, % abnormal sperm</td>
<td>8.0 ± 2.1</td>
<td>13.7 ± 3.3</td>
<td>0</td>
</tr>
<tr>
<td>Thin head, % abnormal sperm</td>
<td>10.8 ± 4.7</td>
<td>9.0 ± 1.5</td>
<td>15</td>
</tr>
<tr>
<td>Looped tail, % abnormal sperm</td>
<td>59.0 ± 3.9</td>
<td>21.0 ± 6.0*</td>
<td>9</td>
</tr>
</tbody>
</table>

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.
Mice homozygous for E90Kneo, in which neo was not removed from the first intron, exhibit HH that is much more severe than E90K alone, but less than that of L117P. Both males and females are infertile. Testis size is smaller than E90K alone but larger than L117P. Histological sections show varied penetrance as to the degree of spermatogenesis, but testis histology is never as severely compromised as that observed for L117P. These phenotypic observations, combined with our Gnrhr gene expression data, support the conclusion that a portion of mutant E90K 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 E90K and a “store” of QCS-retain ed E90K in the ER that can be mobilized to the cell surface by IN3. Importantly, E90Kneo mice exhibit infertility and a degree of HH similar to that seen in human patients and, thus, are a good model for pharmacop erone therapy.

Gnrhr E90K Is Functional When Routed to the Cell Surface. Previous work in cell cultures demonstrated that both human and mouse Gnrhr E90K could be routed to the cell surface via pharmacop erone 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 E90K is retained in vivo, and E90Kneo mice exhibit infertility and a degree of HH similar to that seen in human patients and, thus, are a good model for pharmacop erone therapy.

Gnrhr E90Kneo Mice Are a Model for Pharmacop erone Therapy. In this study, we show the utility of pharmacop erone drugs to correct disease by rescuing PM expression rescue of a misfolded GPCR. Previous work using mouse models of lysosomal storage diseases demonstrated that pharmacop erone treatment can stabilize inactive misfolded soluble lysosomal enzymes and improve their function (18–20). These studies provided proof of principle that pharmacop erones 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 of the AVPR2 gene that cause misfolding (21). Similar to our previous work with Gnrhr, V2R-specific pharmacop erones 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 (GnrhrE90K) to directly test the usefulness of pharmacop erone 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 pharmacop erone to the target organ (anterior pituitary) increased routing of the misfolded GPCR (Gnrhr E90K) 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 pharmacop erones for the treatment of diseases caused by protein misfolding.

Pharmacop erone therapy is a unique approach for the treatment of disease when the underlying pathology is due to protein misrouting. The pharmacop erone used in this study (IN3) shows high specificity for the Gnrhr (5, 22–25) and acts by creating a surrogate bridge (D98-pharmacop erone-K121) 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).

Fig. 4. (A) Comparison of STAR and CYP11A1 protein expression in the testes of WT, E90Kneo/E90Kneo (HOM), and IN3-rescued E90Kneo/E90Kneo 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 E90Kneo heterozygotes, which are both phenotypically normal, exhibited similar levels of Hsd17b3 mRNA. E90Kneo/E90Kneo males, which exhibit hypogonadism, had reduced Hsd17b3 mRNA. Hsd17b3 expression was increased in E90Kneo/E90Kneo males following pharmacop erone treatment. For WT, E90Kneo+, E90Kneo/E90Kneo, and IN3-rescued E90Kneo/E90Kneo, n = 9, 14, 17, and 8, respectively. (C) IN3 restored testosterone levels in E90Kneo/E90Kneo males. The serum concentration of testosterone was measured by RIA. E90Kneo/E90Kneo males exhibited less serum testosterone than heterozygous and WT males. Testosterone levels were restored in E90Kneo/E90Kneo males after pharmacop erone 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 ≥ 3.
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.

Pharmacoperoine 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 pharmacoperoine 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. These procedures were approved by the Oregon Health Science University Institutional Animal Care and Use Committee.

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