

Chapter 9

Gonadotropin Receptor Expression in the Adrenal Gland of Healthy Ferrets and Ferrets with Hyperadrenocorticism

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Submitted

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Summary

Hyperadrenocorticism is a common disease in ferrets and is characterized by excessive production of sex steroids, which gives rise to vulvar swelling in neutered females, recurrence of sexual behavior in neutered males, and alopecia in both sexes. It is thought to be caused by increased gonadotropin secretion as a result of neutering. In this study the presence and function of gonadotropin receptors in the adrenal glands of healthy ferrets and ferrets with hyperadrenocorticism was investigated.

The adrenal cortices of healthy ferrets stained positively with an antibody against the LH receptor (LH-R). Fifty-five hyperplastic and/or tumorous adrenal glands from 46 hyperadrenocorticoïd ferrets also stained for the LH-R, with the exception of two metastasized carcinomas. RT-PCR analysis of 6 adrenal adenomas revealed the expression of the genes for LH-R and FSH-R.

Stimulation *in vitro* with ACTH and hCG of adrenal tissue from a healthy ferret led to significant increases in the concentrations of 17α -hydroxyprogesterone and cortisol in the culture medium, whereas the androstenedione concentration remained unchanged. FSH had no effect on steroid concentrations. In contrast, incubation of tumorous adrenocortical cells with hCG caused a sharp increase in androstenedione production in two of the three tumors investigated. FSH increased the production of 17α -hydroxyprogesterone and cortisol in the cells of one tumor, without having an effect on androstenedione release.

In vivo the plasma concentrations of 17α -hydroxyprogesterone, cortisol, and androstenedione of healthy ferrets did not change after stimulation with either FSH or hCG. In one of the two hyperadrenocorticoïd ferrets studied, hCG but not FSH, increased the plasma concentrations of 17α -hydroxyprogesterone, cortisol and androstenedione. Plasma concentrations of 17α -hydroxyprogesterone and androstenedione increased after GnRH administration in 10 of 12 ferrets with hyperadrenocorticism, with concomitant increases in the concentrations of LH in 11 ferrets and FSH in 8 ferrets.

We conclude that, in healthy neutered ferrets, adrenocortical LH-Rs are not, or hardly, functional *in vivo*. In contrast, these receptors are functional in most neutered ferrets with hyperadrenocorticism. Although FSH-R mRNA is expressed in adrenal adenomas, FSH appears to have a less prominent pathogenetic role than LH.

Introduction

Hyperadrenocorticism is a common disease in neutered pet ferrets (*Mustela putorius furo*). The syndrome differs from hyperadrenocorticism in other species, such as humans, dogs, cats, and horses, in that glucocorticoid excess is much less pronounced in ferrets.³⁰ Instead, in ferrets the disease is characterized by excessive production of sex steroids, giving rise to vulvar swelling in neutered female ferrets (jills), recurrence of sexual behavior in neutered male ferrets (hobs), and alopecia.^{19,29,30,31,34,46} In line with these physical and behavioral changes, plasma concentrations of androstenedione, 17 α -hydroxyprogesterone, dehydroepiandrosterone sulfate, and estradiol are often increased, whereas plasma cortisol concentrations only rarely exceed the upper limit of the reference range.³⁰ In approximately 85% of ferrets with hyperadrenocorticism there is unilateral adrenocortical enlargement without atrophy of the contralateral adrenal gland, while in the other 15% of cases bilateral enlargement is seen.^{31,46} The disease may recur after unilateral adrenalectomy due to enlargement of the contralateral adrenal gland.⁴⁶ The histological changes of the adrenals range from (nodular) hyperplasia to adenoma and adenocarcinoma.³¹

There are several reasons to suggest a relationship between gonadotropic hormones and hyperadrenocorticism in ferrets. First, some symptoms of hyperadrenocorticism, such as alopecia and swelling of the vulva, initially occur only during the breeding season, when plasma concentrations of gonadotropic hormones are high.¹³ Melatonin, which is released from the pineal gland during the dark period of the day, suppresses the release of gonadotropin-releasing hormone (GnRH). With increasing daylight melatonin release decreases accordingly, and when daylight exceeds 12 hours, melatonin exposure is insufficient to suppress the release of GnRH, leading to the start of the breeding season.¹ Second, as in some strains of mice^{8,21,38} in ferrets the age of neutering is related to the age at which hyperadrenocorticism develops.³⁴ Thirdly, the similar plasma adrenocorticotrophic hormone (ACTH) concentrations in ferrets with hyperadrenocorticism and in healthy ferrets makes it unlikely that the increased production of sex steroids is ACTH-dependent.³³ Fourthly, the depot GnRH agonist, leuprolide acetate, can be used successfully to treat ferrets with hyperadrenocorticism.⁴⁵ Finally, luteinizing hormone receptors (LH-R) have been detected in the adrenal glands of ferrets with hyperadrenocorticism. These receptors were considered to be functional because plasma concentrations of adrenal androgens increased after intravenous injection of a GnRH agonist.³⁵ These observations support the hypothesis that neutering leads to the persistent stimulation of the adrenal cortices, due to the loss of negative feedback, which may result in adrenocortical hyperplasia and tumor formation.³⁴ Daylight periods exceeding 12 h seem to promote this phenomenon.

The present study was designed to investigate the presence and functioning of LH and FSH receptors in healthy ferrets and in ferrets with hyperadrenocorticism by means of immunohistochemistry, RT-PCR, *in vitro* and *in vivo* stimulation tests.

Materials and Methods

1. Immunohistochemical staining for LH receptors

1.1 Tissues

Ovaries and testes from healthy, 6- to 9-month-old ferrets were used as positive controls. Adrenocortical control tissue was obtained from six intact (4 male, 2 female), 6- to 15-month-old ferrets that had died of diseases unrelated to hyperadrenocorticism.

Affected adrenal glands were obtained from 46 ferrets that had been presented with signs of hyperadrenocorticism. Most adrenal glands were obtained during adrenal surgery (n=31); the others were obtained post mortem (24 adrenal glands from 19 ferrets). From four ferrets, first an adrenal gland was obtained at surgery and later the contralateral adrenal gland was obtained post mortem. In total 55 affected adrenal glands were examined (39 left and 16 right).

For histological examination, all tissues were fixed for at least 24 hours in 4% phosphate-buffered formalin and then embedded in paraffin. Four-micrometer sections were cut: one section was routinely stained with hematoxylin and eosin (H&E) and the others were used for immunohistochemistry.

On the basis of the guidelines of Williams and co-workers,⁴⁸ the following criteria were used to categorize the adrenal cortices of diseased ferrets into hyperplasia, adenoma, or adenocarcinoma. Hyperplasia was defined as an increased tissue mass with maintenance of the normal cortical structure, with extra-capsular tissue or areas with ballooning cells (clear cells) within the cortex. Adenoma was defined as an increased tissue mass without the normal cortical structure; lesions usually contained a mixture of cell types ranging from clear cells to small cells with picnotic nuclei and spindle shaped cells. Adenocarcinoma was defined as infiltration of tumor cells into blood vessels and/or when metastases were found. Mitotic figures were not used as a selection criterion since they were absent in all slides examined.

Sixteen adrenal glands from 15 clinical cases were categorized as hyperplasia, 28 adrenal glands from 26 clinical cases were categorized as adenoma, and 11 adrenal glands from 10 clinical cases were categorized as adenocarcinoma. In three of the latter cases the tumor had metastasized.

1.2 Immunohistochemical Staining

Immunohistochemical staining for LH-Rs was performed as described previously.³⁵ The LH-R monoclonal antibody (PIB4), a gift from Dr. Wimalasena (Dept. Obstetrics and Gynecology, University of Tennessee, Knoxville, TN, USA), had been raised against purified rat LH-Rs, as described by Indrapichate *et al.*¹² The antibody binds specifically to LH-Rs in various tissues of different species.^{4,25,41,42}

2. *In vivo* stimulation tests

2.1 Stimulation tests in catheterized ferrets

In a first study, which was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University, four stimulation tests were performed with catheterized ferrets. The order of the first three tests (ACTH, hCG, and FSH) was

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determined by Latin square. The GnRH stimulation test was performed last as this test required twice the number of blood collections in comparison to the other tests. The tests were performed over two weeks with at least one day in between tests. The jugular catheter was placed 2 days before the first stimulation test. For the ACTH, hCG, and FSH stimulation tests, plasma concentrations of androstenedione, 17 α -hydroxyprogesterone and cortisol were measured. For the GnRH stimulation test, plasma concentrations of LH and FSH were measured; the corresponding concentrations of androstenedione, 17 α -hydroxyprogesterone, and cortisol are reported elsewhere.³⁵

2.1.1 *Animals*

Twelve ferrets (6 male and 6 female) were used in July and August 1999. Eleven 2-year-old ferrets had been gonadectomized at 6 weeks of age. The twelfth ferret, a 1-year-old male, had been gonadectomized at 9 months of age. The ferrets were either purchased at 6 weeks of age from a breeder or born and raised in our department. The ferrets were individually housed in outdoor suspended cages with a night box. Water and ferret pellets (FerRet, Hope Farms, Woerden, The Netherlands) were available *ad libitum*.

Unexpectedly, two ferrets had increased basal plasma androstenedione and 17 α -hydroxyprogesterone concentrations. This, together with ultrasonographically visualized unilateral adrenal gland enlargement, indicated the presence of hyperadrenocorticism, which was later confirmed by histological examination of the adrenal glands.

2.1.2. *Catheterization and sampling*

Jugular catheters were introduced, via vena section under isoflurane anesthesia, as described previously.³⁵ To keep the catheters patent they were filled with a mixture of polyvinyl pyrrolidone (PVP) and heparin (60 g PVP / 54 ml 0.9% sodium chloride + 6 ml heparin [5000 IU/ml]). Anesthesia was used only for catheter placement.

All blood samples were collected from the jugular catheter into pre-chilled EDTA-coated tubes and centrifuged. Plasma was stored in aliquots at -20 °C pending analysis. After collection of each blood sample the catheter was flushed with 0.3 ml heparin solution (50 IU/ml).

2.1.3 *Stimulation tests*

For the ACTH-stimulation test blood samples were collected at -5, 0, 30, 60, 120 and 180 min after an intravenous injection with 1 μ g of synthetic ACTH₁₋₂₄ (corticotropin, Synacten®, Novartis Pharma B.V., Arnhem, The Netherlands). For the FSH-stimulation test blood samples were collected at -5, 0, 60, 120, 240, 480 and 1440 min after a subcutaneous injection of 5 IU follitropin alfa (Gonal-F® 75, Serono Pharma S.P.A., Bari, Italy). For the hCG stimulation test blood samples were collected at -5, 0, 30, 60, 120 and 240 min after an intramuscular injection of 100 IU of the LH-R agonist hCG (Pregnyl®, Organon, Oss, The Netherlands). For the GnRH stimulation test blood samples were collected at -5, 0, 5, 10, 15, 30, 45, 60, 90 and 120 min after an intravenous injection of 10 μ g of the GnRH agonist gonadorelin (Fertagyl®, Intervet Nederland B.V., Boxmeer, The Netherlands).

2.2 *GnRH stimulation test with blood collection under anesthesia*

In a second study a GnRH stimulation test was performed in 10 privately owned neutered ferrets (5 male and 5 female; 3 – 7 years of age) with hyperadrenocorticism. The diagnosis of hyperadrenocorticism was based upon history, physical changes, and ultrasonography of the adrenal glands.

Blood samples were collected under isoflurane anesthesia by puncture of the cranial vena cava, immediately before and 30 min after an intravenous injection of 10 µg Fertagyl®, as described previously.³⁵ Plasma concentrations of androstenedione, 17 α -hydroxyprogesterone, LH, and FSH were measured.

2.3 *Hormone determinations*

Androstenedione concentrations were measured by radioimmunoassay (RIA) as described previously.⁴⁴ The lower limit of detection was 0.1 nmol/l and the interassay coefficients of variation were 10.5%, 9.3%, and 11.6% at 1.4, 4.8, and 11.8 nmol/l, respectively. 17 α -Hydroxyprogesterone concentrations were measured after toluene extraction by RIA as described previously.³⁵ The lower limit of detection was 0.2 nmol/l and interassay coefficients of variation were 9.0%, 7.4%, and 9.9% at 0.9, 5.1, and 26.0 nmol/l, respectively. Cortisol concentrations were measured by RIA (Coat-A-Count® Cortisol, Diagnostic Products Corporation, Los Angeles, USA). The lower limit of detection was 1 nmol/l and the interassay coefficient of variation was between 4.0% and 6.4%. FSH concentrations were measured by RIA as described previously.⁴⁷ This RIA has been validated for use in ferrets.⁶ Rat FSH-RP2 (NIAMD, Bethesda, MD) was used as a standard. The lower limit of detection was 0.8 µg/l and the intra-assay coefficient of variation was 7.2%. LH concentrations were measured in duplicate 100-µl samples of plasma in one run with a heterologous RIA method as described before⁵ with slight modifications. Anti-rat LH (1:11,200 CSU120; generously donated by Dr. G.D. Niswender, Colorado State University, Fort Collins, CO, USA), which recognizes ferret LH, was used. Radioiodinated rat LH (kindly donated by F.H. de Jong, Erasmus Medical Center, Rotterdam, The Netherlands) and canine LH LER 1685-1 (kindly donated by Dr. L.E. Reichert, Tucker Endocrine Research Institute LLC, Atlanta, GA, USA) were used as tracer and standard, respectively. The range was 0.39 to 50 µg/l at 94.6 to 6.5% relative bound with a maximum binding of 41%. Parallelism was tested by comparing the results of 50- and 100-µl samples of plasma from 5 ferrets (Fig 1). In four ferrets the difference between the 2 samples was 7.5%. In one ferret the results deviated by 43%. The intra- and interassay coefficients of variation were 5 and 10.5%, respectively. The limit of quantitation was 0.4 µg/l.

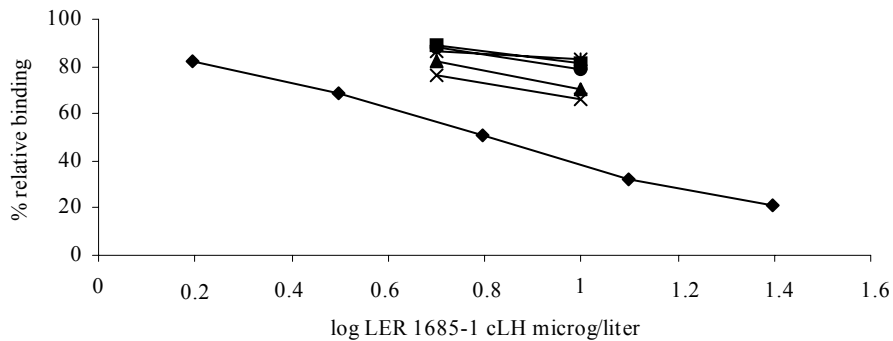


Figure 1. Ferret LH in plasma samples (n=5) of 50 and 100 μ l plotted vs. canine LH (LER 1685-1) standards in a radioimmunoassay with iodinated rat LH and anti-rat LH antibody (CSU120).

3. *In vitro* stimulation test

3.1 *Adrenal tissue*

Adrenal tissue was obtained at surgery or within 30 min of death from a healthy intact male ferret, which died of a disease unrelated to hyperadrenocorticism, and three neutered ferrets with signs of hyperadrenocorticism. The adrenal glands of the healthy ferret (a 5-year-old male) had no macroscopic alterations. To be able to obtain sufficient adrenal cells no histological examination was performed on the latter glands. Histological examination of parts of the adrenal glands of the hyperadrenocorticoid ferrets (1 male, 2 female; aged 4 to 6 years) revealed two adenomas and one carcinoma.

3.2 *Tissue preparation*

The adrenal glands were collected in Hanks' balanced salt solution (Life technologies, Breda, The Netherlands) and placed on ice. Within 30 min of collection the adrenals were cut into pieces and incubated with 3 mg/ml collagenase (type 1, Sigma-Aldrich, St. Louis, USA) in Hank's solution at 37°C for 60 min under gentle agitation. Cells were collected by centrifugation at 250 g for 3 min and resuspended in DMEM (Sigma-Aldrich, St. Louis, USA) with 0.2% BSA before plating into 24-well culture dishes at a density of 500,000 cells/ml/well. The cells were incubated in medium without or with ACTH₁₋₂₄ (Synacten®) in a final concentration ranging from 10⁻⁶ to 10⁻⁹ M, GnRH (gonadorelin, Fertagyl®) at 10⁻⁶ to 10⁻⁹ M, FSH (follitropin alfa, Gonal-F® 75) at 2 and 20 IU/ml, and hCG (Pregnyl®) at 5 and 100 IU/ml. Incubations were performed in triplicate during 2 h at 37°C. Supernatants were collected and stored at -20°C until measurement of cortisol, androstenedione, and 17 α -hydroxyprogesterone concentrations as described above.

4. RT-PCR

4.1 Primer design

The primers for the LH receptor and FSH receptor (Table 1) were based on sequences with a high degree of homology among other animals and came from Invitrogen (Breda, The Netherlands) and Isogen (Maarsen, The Netherlands), respectively.

Table 1. Sequences of primers used for PCR amplification of ferret LH-R and FSH-R cDNA

Name	Sequence	Expected product size
LH-Forward	5' ctaatgcctttgacaacctcctc 3'	208 bp
LH-Reversed	5' tgtaagttatcacaaatttccagaatg 3'	
FSH-Forward	5' ttggggacctggagaaaatagagatc 3'	447 bp
FSH-Reversed	5' aggctccgtggaaaacatcattagg 3'	

4.2 Tissues

Nine surgically removed adrenal glands of ferrets with hyperadrenocorticism were divided in two. One part was immediately frozen in liquid nitrogen and stored at -70°C , and the other part was fixed in 4% buffered formalin and submitted for histological examination, which revealed adrenocortical hyperplasia (n=2) and adenoma (n=7). All nine adrenal gland samples were examined for the presence of LH-R mRNA, while 6 adenomas were examined for the presence of FSH-receptor (FSH-R) mRNA. Ferret testes tissue, obtained during an elective castration and frozen in liquid nitrogen and stored at -70°C , served as a positive control.

4.3 RNA isolation and cDNA synthesis

Total RNA was extracted from tissue using the RNeasy mini kit (Qiagen, Valencia, CA), and 1 μg was reverse transcribed in 20 μl at 42°C for 60 min using the Reverse Transcription System (Promega, Leiden, The Netherlands). Each reaction contained 5 mM MgCl_2 , 1x Reverse Transcription Buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100), 1 mM each dNTP, 1 U RNasin, 15 U AMV Reverse Transcriptase, and 0.5 μg Oligo(dT)₁₅ primer.

4.4 PCR

PCR to detect LH-R and FSH-R mRNA was performed on 1 μl cDNA in a 50- μl reaction mixture containing 2 mM MgCl_2 , 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH9.0], 1% Triton X-100), 0.2 mM of each dNTP, and 1.25 U of Taq DNA polymerase.

Amplification was performed using the following thermo cycle parameters: 96°C for 4 min followed by 35 cycles of 30 sec at 96°C , 30 sec at 58°C and 1 min at 72°C . Final extension was performed at 72°C for 10 min. PCR products were analyzed on a 1% agarose gel. PCR products were also purified and sequenced for product verification.

Results

1. Immunohistochemical staining for LH receptors

Thecal cells in the ovary and Leydig cells in the testis of healthy ferrets (**Color section**; Fig 7) stained positive with the LH-R antibody. In the adrenal glands of healthy ferrets, the zona glomerulosa stained positively for LH-R, as did the zona fasciculata, but less intensely (**Color section**; Fig 8).

All adrenal glands from the clinical cases, except two adrenocortical tumors and their metastasis, stained positively for the LH-R. In another clinical case with a metastasizing adenocarcinoma, the primary tumor contained LH-R positive cells while the metastasis did not. Besides staining of the zona glomerulosa and zona fasciculata, clusters of clear cells in hyperplastic adrenal cortices stained for LH-R. Clusters of positively staining cells were found throughout the adenomas and adenocarcinomas (**Color section**; Fig 9).

2. *In vivo* stimulation tests

2.1 *Stimulation tests in catheterized ferrets*

Thirty minutes after intravenous administration of ACTH₁₋₂₄, plasma concentrations of cortisol and 17 α -hydroxyprogesterone had increased in all healthy ferrets. In one of the ferrets with hyperadrenocorticism the cortisol response was in the range. The other hyperadrenocorticoid ferret had a low basal cortisol concentration and only a moderate response to stimulation with ACTH. In the healthy ferrets plasma androstenedione concentrations remained unchanged after stimulation with ACTH, whereas they increased in both hyperadrenocorticoid ferrets (Fig 2).

In the healthy ferrets, plasma concentrations of cortisol, androstenedione, and 17 α -hydroxyprogesterone did not change after stimulation with recombinant FSH or hCG. In one hyperadrenocorticoid ferret, plasma concentrations of cortisol, androstenedione, and 17 α -hydroxyprogesterone increased 60 minutes after administration of FSH and returned to baseline 60 minutes later. In the other ferret with hyperadrenocorticism hormone levels did not change after stimulation with FSH. Plasma concentrations of androstenedione and 17 α -hydroxyprogesterone gradually increased after stimulation with hCG in the latter hyperadrenocorticoid ferret, but did not change in the former (Fig 3).

Within 10 minutes of intramuscular administration of GnRH, plasma LH concentrations increased in all ferrets and returned to baseline values after approximately 1 hour (Fig 4A), whereas plasma FSH concentrations did not change. In the two ferrets with hyperadrenocorticism plasma FSH concentrations increased slightly between 5 and 20 min after administration of GnRH (Fig 4B).

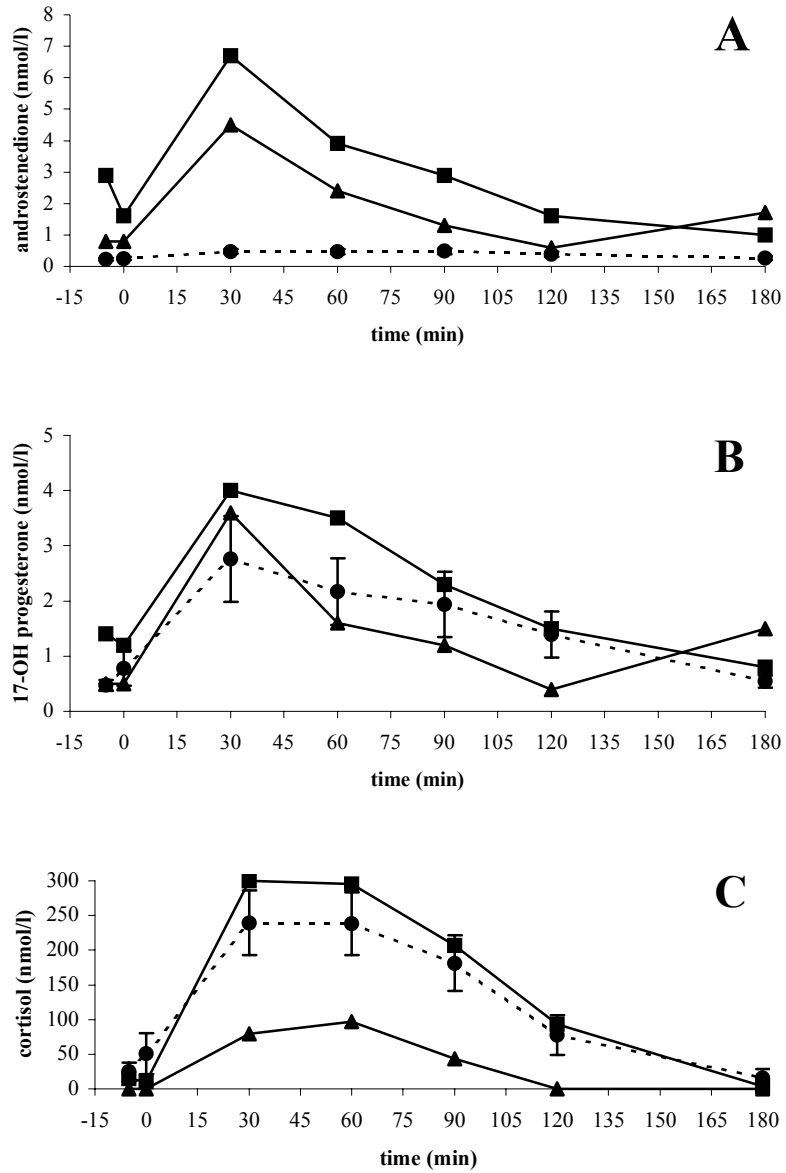


Figure 2. Mean (\pm SEM) plasma concentrations of androstenedione (A), 17α -hydroxyprogesterone (B), and cortisol (C) before and after the intravenous administration of ACTH₁₋₂₄ at t=0 min in 10 healthy neutered ferrets (●) and in 2 neutered ferrets (■, ▲) with hyperadrenocorticism.

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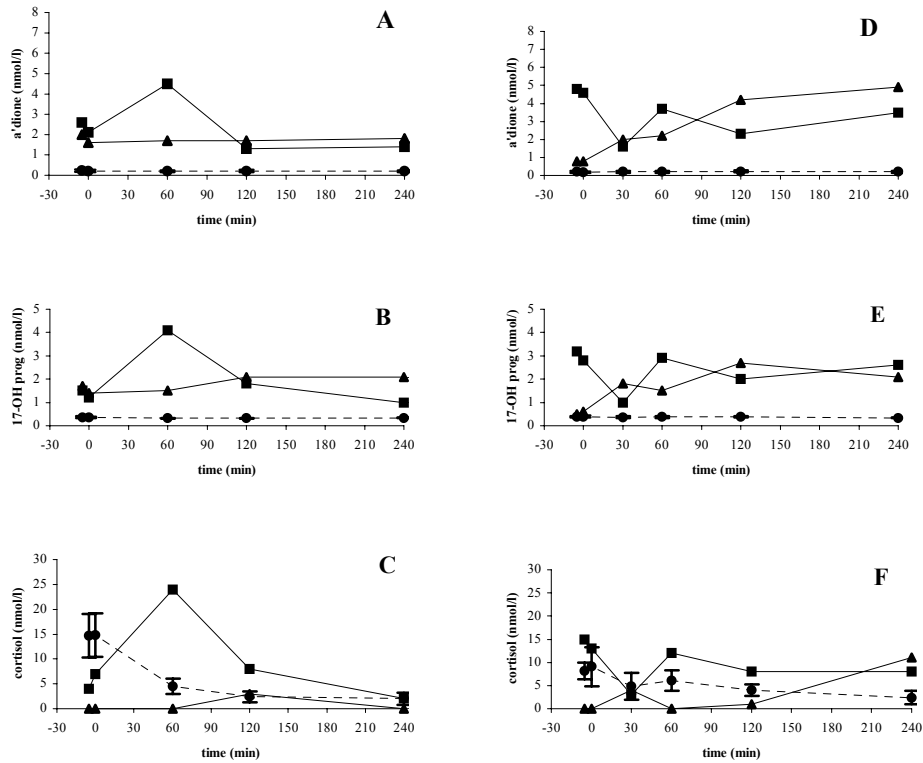


Figure 3. Mean (\pm SEM) plasma concentrations of androstenedione (A), 17α -hydroxyprogesterone (B), and cortisol (C) before and after the subcutaneous administration of recombinant FSH, and mean (\pm SEM) plasma concentrations of androstenedione (D), 17α -hydroxyprogesterone (E), and cortisol (F) before and after the intramuscular administration of hCG at $t=0$ min in 10 healthy neutered ferrets (●) and in 2 neutered ferrets (■, ▲) with hyperadrenocorticism. Note difference with figure 5 with regard to scale for cortisol.

2.2 GnRH stimulation test with blood collection under anesthesia

Intravenous injection of GnRH in 10 ferrets with hyperadrenocorticism increased the median plasma LH concentration from 1.6 $\mu\text{g/l}$ (range, 0.7 – 2.2 $\mu\text{g/l}$) to 3.2 $\mu\text{g/l}$ (range, 1.2 – 3.7 $\mu\text{g/l}$), and the median plasma FSH concentration from 23.8 $\mu\text{g/l}$ (range, < 0.8 – 67.2 $\mu\text{g/l}$) to 34.8 $\mu\text{g/l}$ (range, < 0.8 – 93.5 $\mu\text{g/l}$). In one ferrets the plasma LH concentration did not increase and in four ferrets the plasma FSH concentrations did not increase after GnRH administration. The median plasma androstenedione concentration increased from 1.1 nmol/l (range, 0.1 – 9.1 nmol/l) to 4.0 nmol/l (range, 0.1 – 22 nmol/l), and the median plasma 17α -hydroxyprogesterone concentration increased from 1.9 nmol/l (range, < 0.5 – 29 nmol/l) to 2.8 nmol/l (range, < 0.5 – 62 nmol/l). In 7 of the 10 hyperadrenocorticoïd ferrets, basal plasma concentrations of androstenedione exceeded the reference range (0.1 –

0.4 nmol/l)³⁵. In two of the ferrets with normal basal plasma concentrations of androstenedione, concentrations exceeded the reference range 30 min after stimulation with GnRH. In 6 of the 10 hyperadrenocorticotid ferrets basal plasma concentrations of 17 α -hydroxyprogesterone exceeded the reference range (0.3 – 0.7 nmol/l)³⁵. In three of the ferrets with normal basal concentrations, plasma 17 α -hydroxyprogesterone concentrations exceeded the reference range 30 min after stimulation with GnRH.

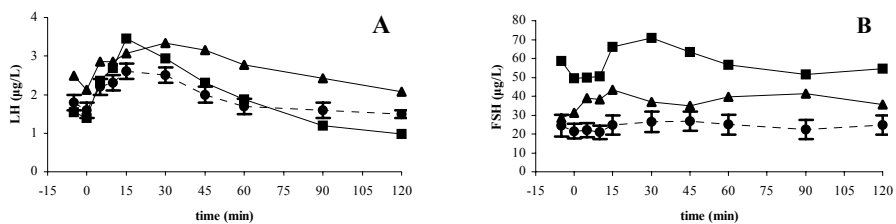


Figure 4. Mean (\pm SEM) plasma concentrations of LH (A), and FSH (B) before and after the intramuscular administration of a GnRH agonist at t=0 min in 8 healthy neutered ferrets (\bullet) and in 2 neutered ferrets with hyperadrenocorticism (\blacksquare , \blacktriangle).

3. *In vitro* stimulation tests

ACTH and hCG significantly stimulated the release of cortisol and 17 α -hydroxyprogesterone from normal adrenal cells in culture medium as compared to the control incubations. This was also true for the dispersed cells from the adrenal adenoma A and carcinoma B. In the medium of cells obtained from adrenal adenoma C cortisol and 17 α -hydroxyprogesterone concentrations only increased after incubation with hCG. The cells had been incubated with a far lower concentration of ACTH (10^{-11} M). Incubation with FSH only caused significant increases in cortisol and 17 α -hydroxyprogesterone concentrations in adrenal cells from carcinoma B (Fig 5).

None of the hormones used stimulated the release of androstenedione from normal adrenal cells in culture medium as compared to the control incubations, whereas ACTH (10^{-6} M) significantly increased androstenedione concentrations in culture medium of cells from adenoma A and carcinoma B. Incubation with hCG led to a significant increase in androstenedione concentrations in culture medium with carcinoma B and adenoma C cells (Fig 5). GnRH did not stimulate the release of any of the hormones measured.

4. RT-PCR

RT-PCR analysis showed that LH-R and FSH-R mRNA is expressed in the ferret adrenal gland (Fig 6). Sequence analysis of the partially amplified LH-R mRNA revealed high sequence homology to the corresponding regions of the mRNA encoding LH-R in the polar bear (98%), pig (96%), cow (95%), human (92%), and rat (92%). Part of the ferret FSH-R mRNA was homologous to the published sequences of the polar bear (85%), cow (84%), pig (83%), human (80%), and rat (78%).

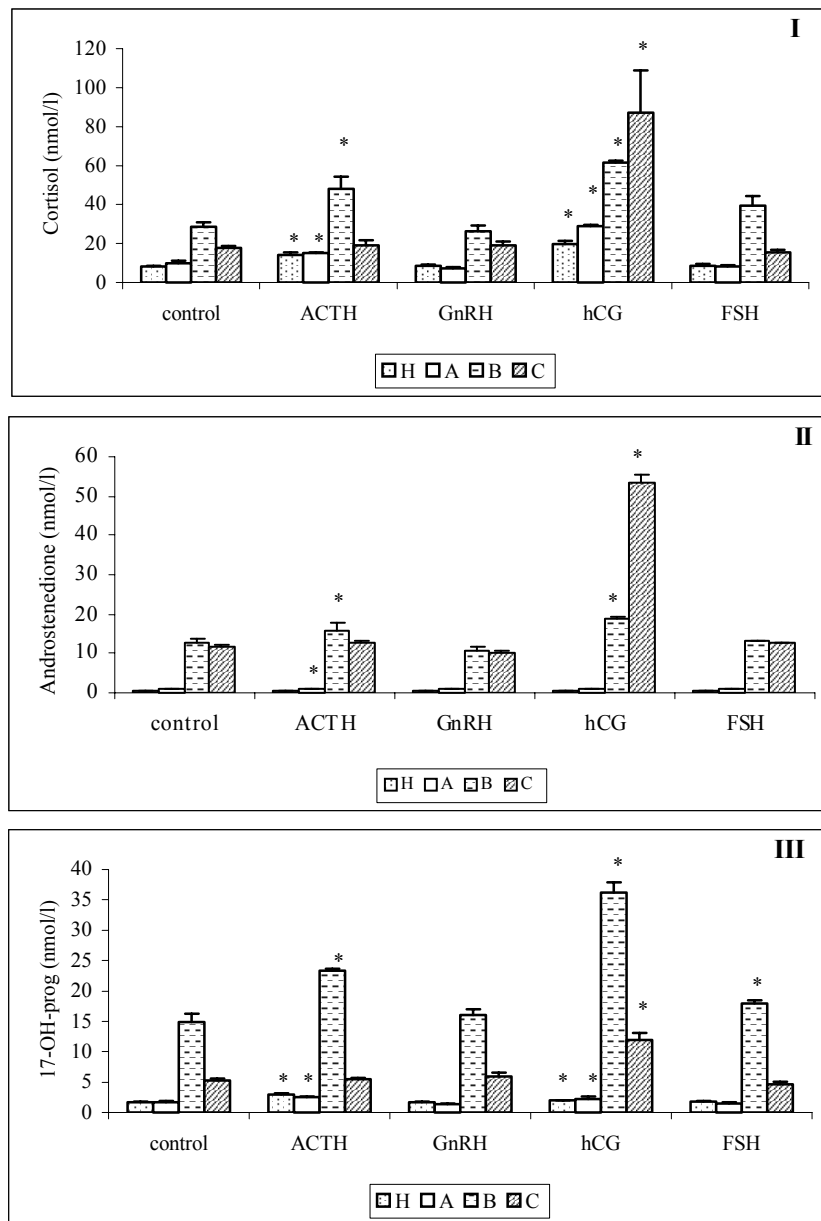


Figure 5. Concentrations of cortisol (I), androstenedione (II), and 17 α -hydroxyprogesterone (III) in culture medium after a 2-h incubation of dispersed cells from 1 normal adrenal gland (H), 2 adrenal adenomas (A, C) and 1 adrenal carcinoma (B) with ACTH (10^{-6} M), GnRH (10^{-6} M), hCG (100 IU/ml), and FSH (20 IU/ml). The dispersed cells of adenoma C were incubated with ACTH in a concentration of 10^{-11} M. An asterisk indicates a significantly ($P < 0.05$) higher concentration than the control

Discussion

The present data provide further support for the concept that gonadotropic hormones play an important role in the development of hyperadrenocorticism in ferrets. The results of immunocytochemical studies, *in vivo* and *in vitro* stimulation tests, and RT-PCR studies point to the importance of the expression of receptors for gonadotropic hormones, and particularly LH-Rs, in adrenocortical cells. The results are discussed below according to the experimental approach used.

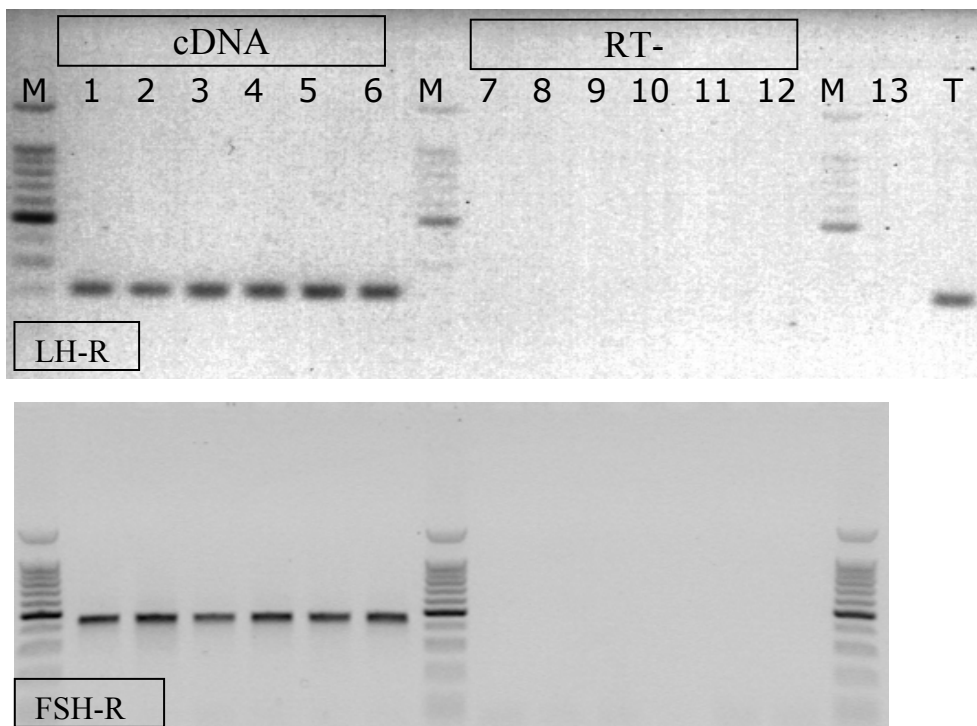


Figure 9. PCR-products obtained from 6 ferret adrenal adenomas (lanes 1-6) showing bands corresponding with the expected size for LH- and FSH-receptors. M = marker, T = testes as positive control. RT - = PCR reaction without the addition of reverse transcriptase.

Immunohistochemistry revealed the presence of LH-R protein in the adrenal cortices of intact 6- to 15-month-old ferrets. Thus, LH-R protein is already present in adrenal cortices of young, intact ferrets. The presence of LH-R protein in the zona glomerulosa and zona fasciculata is unexpected because LH-Rs are found in the zona reticularis and the distal zona fasciculata of the human adrenal gland.²³ These zones produce androgens, whereas the zona glomerulosa lacks the enzyme 17α -hydroxylase, which is essential to androgen synthesis.⁴⁰ The presence of LH-Rs in the zona glomerulosa, however, is not

unique to ferrets, and these receptors have been found throughout the adrenal cortex including the zona glomerulosa in mice with chronically elevated serum LH concentrations.¹⁵ Hämläinen *et al.*¹¹ detected positive β -galactosidase staining in the zona glomerulosa and zona fasciculata in transgenic mice carrying an LH-R promoter (7.4 kb) fused with β -galactosidase.

LH-R immunopositive cells were found in the adrenocortical lesions of 44 of the 46 ferrets with hyperadrenocorticism. Of the three ferrets with metastasizing adrenocortical adenocarcinomas only one had LH-Rs in the primary tumor but not in the metastases. These three ferrets had only minor signs of hyperadrenocorticism. In these animals LH-Rs may have been lost during dedifferentiation of the neoplastically transformed cells, as has been observed for steroid hormone receptors in malignant mammary tumors.³²

The detection of LH-R mRNA by RT-PCR further confirms the local expression of LH-Rs in the ferret adrenal gland. However, the mRNA transcript and the locally produced protein may not be functional because the transcript may comprise truncated (iso)forms of the receptor.^{11,12} In a previous study we demonstrated that plasma concentrations of androstenedione and 17 α -hydroxyprogesterone increased in neutered ferrets with hyperadrenocorticism in response to intravenous administration of a GnRH agonist. However, no such rise occurs in healthy ferrets,³⁵ suggesting that LH-Rs may become functional only after form of metabolic/hormonal derangement.

In all eight healthy neutered ferrets plasma LH concentrations, but not FSH concentrations, clearly increased after intravenous administration of GnRH. In 11 of the 12 neutered ferrets with hyperadrenocorticism plasma LH concentrations increased after intravenous administration of GnRH, whereas plasma FSH concentrations increased in only 8 of the 12 ferrets. In the two ferrets with hyperadrenocorticism in which hCG was administered intramuscularly, one responded with increased plasma concentrations of androstenedione and 17 α -hydroxyprogesterone. The other ferret appeared to respond only to subcutaneous administration of FSH. In the latter ferret, however, the response was seen only 60 min after administration, and then only after 60 min. Because ACTH can increase the plasma concentrations of these three hormones, this increase may well be due to the stress of handling before blood collection, rather than a direct effect of FSH administration. None of the healthy neutered ferrets responded to stimulation with either hCG or FSH, consistent with observations in healthy humans.^{24,26} In a young woman with an adrenocortical adenoma testosterone secretion could only be stimulated with hCG before resection of the adrenal tumor.² Thus in humans²³ and ferrets, LH-Rs may become functional only under pathophysiological conditions.

ACTH has been implicated as an important factor controlling the adrenal androgen secretion.^{9,24,27} In our study, however, only ferrets with hyperadrenocorticism responded to ACTH with an increased secretion of androstenedione; healthy ferrets showed no response. Adrenocortical cells can be categorized on the basis of their lipid content,⁴⁹ with lipid-rich cells producing more cortisol than androstenedione, and the reverse in lipid-poor cells. It is possible that the adrenal glands of ferrets with hyperadrenocorticism contain relatively more lipid-poor cells. However, in our ferrets the LH-R-positive cells seemed to be lipid-rich (clear cells). It is more likely that an alternative stimulatory pathway for the synthesis of androstenedione is activated in the hyperplastic or tumorous cells.

The *in vitro* stimulation tests gave the most cogent evidence for the existence of functional LH-Rs in the ferret adrenal glands. In the four adrenal glands, including a normal adrenal gland from an intact male ferret, cortisol secretion increased after incubation with hCG. In an adenoma and a carcinoma androstenedione concentrations increased after incubation with this hormone. Similar results have been reported for isolated guinea-pig adrenal cells, where hCG stimulated the production of cortisol and androstenedione.²² The increase in cortisol concentrations after incubation of ferret adrenal glands with hCG may explain why the urinary corticoid/creatinine ratio is increased in ferrets with hyperadrenocorticism.^{10, Schoemaker *et al.* submitted} However, *in vivo* this increase in cortisol secretion must be limited because plasma ACTH concentrations are not decreased in ferrets with hyperadrenocorticism.³³ An earlier study did not report an increase in cortisol after *in vivo* stimulation with hCG or GnRH in two ferrets with hyperadrenocorticism.³⁵

The LH-R belongs to the group of G protein-coupled receptors (GPCRs). While the precise mechanism for GPCR activation after agonist binding remains to be defined, it is generally postulated that GPCRs are in equilibrium between an activated state and an inactive state. These states presumably differ in the disposition of the transmembrane helices and, in turn, the cytoplasmic domains that determine G protein coupling. Since continued exposure to an agonist leads to desensitization,³⁹ the adrenocortical LH-Rs may have become desensitized in the healthy neutered ferrets, as a result of continuous exposure to high LH concentrations.

Some mutations in GPCRs can cause loss of function, while others lead to a gain of function. Activating missense mutations are thought to disrupt normal inhibitory constraints that maintain the receptor in its inactive conformation,¹⁴ shifting the equilibrium toward the activated state of the receptor. Some gain-of-function mutations are associated with increased sensitivity to the agonist.⁵⁰ As in mice, the tumorigenic consequences of the high LH levels^{20,28} may be associated with or mediated by activated LH-Rs, which may be due to a mutation in either the receptor protein or the G protein.^{3,43} In addition, receptor expression may be further increased after hormonal stimulation,³⁷ a supposition supported by studies in mice. In healthy intact mice LH-R mRNA is not expressed in the adrenal cortex. Increased LH concentrations following gonadectomy, however, resulted within 4 months in the expression of adrenal LH-R mRNA,¹⁵ indicating that prolonged elevation of LH levels may result in the expression of LH-Rs in adrenal cortex.

Both LH-dependent hypercortisolism and LH-dependent adrenal androgen secreting tumors have been described in humans.¹⁷ Patients with LH-dependent Cushing's syndrome usually have bilateral macronodular adrenal hyperplasia with high plasma concentrations of cortisol and suppressed concentrations of ACTH.^{7,16} Hyperadrenocorticism in ferrets is usually associated with unilateral adrenal pathology. However, the contralateral adrenal cortex is not atrophic and plasma ACTH concentrations are not significantly different from those of healthy ferrets.³³ Thus hyperadrenocorticism in ferrets is not identical to LH-dependent Cushing's syndrome in humans. Hyperadrenocorticism in ferrets may, however, be comparable with LH-dependent adrenal androgen-secreting tumors in women, in whom, as in ferrets, usually only one adrenal gland is involved without alterations in plasma ACTH concentrations.^{2,18}

Chapter 9

To our knowledge, the presence of FSH-Rs in adrenal tissue has thus far only been reported in adrenocortical carcinoma cells from a rat.³⁶ Here we detected FSH-R mRNA in ferret adrenal adenomas by RT-PCR. We also observed a significant increase in cortisol and 17 α -hydroxyprogesterone in response to FSH stimulation. However, since this response to FSH stimulation was demonstrated in only one of the three tumors examined, the response to an *in vivo* stimulation test was questionable, and plasma FSH concentrations did not increase during the GnRH stimulation test in 4 out of 12 ferrets, we speculate that the FSH-R plays a less prominent role than the LH-R in the development of hyperadrenocorticism in ferrets.

We conclude that the LH-Rs detected in the adrenal cortex of healthy neutered ferrets are hardly, or not, functional *in vivo*. In contrast, these receptors appear to be functional in neutered ferrets with hyperadrenocorticism. Although FSH-R mRNA is expressed in adrenal adenomas, these receptors appear to play a less prominent pathogenetic role than LH-Rs.

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