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DOES SIGNALING OF ESTROGEN-RELATED RECEPTORS AFFECT STRUCTURE AND FUNCTION OF BANK VOLE LEYDIG CELLS?

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To get a deeper insight into the function of estrogen-related receptors (ERRs) and dissect underlying mechanism in Leydig cells, ERRs (type α, β and γ) were blocked or activated in testes of adult bank voles (*Myodes glareolus*) which show seasonal changes in the intratesticular sex hormones level. Both actively reproducing animals (long day conditions; LD) and those with regression of the reproductive system (short day conditions; SD) received intraperitoneal injections of selective ERRα antagonist 3-[4-(2,4-*Bis*-trifluoromethylbenzyloxy)-3-methoxyphenyl]-2-cyano-*N*-(5-trifluoromethyl-1,3,4-thiadiazol-2-yl)acrylamide (XCT 790) or selective ERRβ/ERRγ agonist *N*-(4-(Diethylaminobenzylidenyl)-*N*'-(4-hydroxybenzoyl)-hydrazine (DY131) (50 μg/kg bw; six doses every other day). Markedly more, XCT 790 ($P < 0.05$) but also DY131 affected interstitial tissue histology whose volume increased in both LD and SD males while seminiferous epithelium structure was untouched. Ultrastructure analysis revealed alterations in mitochondria number as well as endoplasmic reticulum and Golgi complexes volume and structure especially after ERRα blockage. Diverse and complex ERRs regulation at mRNA level and protein expression ($P < 0.05$; $P < 0.01$ and $P < 0.001$) of steroidogenic (lutropin receptor (LHR), translocator protein (TSPO), steroidogenic acute regulatory protein (StAR)) and secretory (insulin-like protein 3 (INSL3) and relaxin (RLN)) molecules were revealed in relations to endogenous estrogen level in treated males. Notably, immunolocalization of ERRs and above proteins, exclusively in Leydig cells, indicated their involvement in Leydig cell function control based on interactions with endogenous estrogen level and/or estrogen signaling *via* ERRs. Treatment with XCT 790 or DY131 significantly decreased ($P < 0.05$; $P < 0.01$ and $P < 0.001$) intratesticular estrogens concentration, with exception in SD DY131 males. In addition, androgens level was decreased, but not in LD DY131 voles. Similarly, ERRβγ activation significantly reduced ($P < 0.05$; $P < 0.01$ and $P < 0.001$) cAMP and calcium ions (Ca^{2+}) concentrations particularly in DY131 voles. Overall, for the first time, we have shown that ERRs are involved in maintenance of Leydig cell architecture and supervision of its steroidogenic and secretory activity that is closely related to endogenous estrogen status in the testis. Further understanding of mechanism(s) by which individual types of ERRs can control Leydig cell function is relevant for predicting and preventing steroidogenic and spermatogenic disorders.

Key words: *estrogen-related receptors, Leydig cells, ultrastructure, rough endoplasmic reticulum, steroidogenic proteins, insulin-like family peptides*

INTRODUCTION

In 1934, for the first time, Zondek *et al*., (1) documented estrogen production in the male testis, through the discovery of the intratesticular conversion of androgens into estrogens in equine. For next decades estrogen role in the male physiology remained not elucidated and linked rather to unimportant or inhibitory function of these hormones. In 1988, pioneering study by Kula *et al*., (2) in immature rats, clearly demonstrated that estrogen signalization is crucial for initiation of spermatogenesis. Moreover, discovery in the middle 90ties of the estrogen receptors (ERs) in the male, and data showing functional disturbances of the reproductive system and infertility in transgenic mice lacking ERα or estrogen synthase (aromatase) together with clinical data from patients with mutations in ER and aromatase genes (3, 4) considerably extended the knowledge and strongly established stimulatory role of estrogens in males. Besides regulation of formation of bone stroma and its linear growth as well as metabolism of lipids, estrogens control cellular processes within the testis since a fetal period. In fact, in fetal rodent gonad, in steroidogenic cells (Leydig cells) ERs are expressed before androgen receptor (5). Leydig cells are the main source of estrogens in the adult testis (6). Moreover, these cells are under autocrine and paracrine as well as endocrine control of estrogen either during their differentiation, proliferation or pathological processes as

apoptosis and hyperplasia (7-9). Based on the current data, it should be added here that in Leydig cell estrogens signalize concomitantly by ERs and the membrane estrogen receptor (GPER) (9, 10). Control of estrogens biosynthesis, which are converted from androgens, is based on the lutropin action (LHR) in Leydig cell. This gonadotropin in multiprotein signaling pathway promotes the activation of steroid acute regulatory protein (StAR) and translocator protein (TSPO) (11).

Several recent lines of evidence indicate that members of nuclear receptor subfamily, estrogen-related receptors, (ERRs) control cell metabolism e.g. tricarboxylic acid cycle, oxidative metabolism, and mitochondrial biogenesis in tissues with high-energy demands (12). Three closely related members (ERR α, β and γ), each encoded by different gene, were identified. All ERRs share homology with ERs particularly in their DNA-binding and ligand-binding domains, DNA-response elements, target genes as well as co-regulatory proteins although it is not a general role (13-15). ERRs modulation of estrogen responsiveness, substituting for ER activities was well-confirmed in studies of breast and other cancers (13).

In target cells and tissues, these receptors are constitutively active without binding to natural estrogen but also show affinity to different ligands including these with estrogenic properties e.g. phytoestrogens, tamoxifen, factors contained in blood serum (14-17). Thus, as a result, both ligand-independent and -dependent ERR signaling may functionally crosstalk with ER signaling or modulate estrogen responsiveness in target cells *via* competition, including modulation of aromatase activity (18) and/or ERRs may act independently through novel signaling pathways (19). Steroid receptor coactivator (SRC)-1, -2, and -3, which regulate hepatic metabolism, fat storage, and energy balance, have been shown to interact with one or more ERR isoform stimulating their transcriptional activity (16). PPARγ coactivator (PGC)-1α and PGC-1β, which play essential roles in metabolic programs, interact directly with the ERRs, positively regulating their expression and activity (20). On the other hand, ERRs transcriptional activity is generally inhibited through physical interaction with receptor interacting protein 140 (RIP140), a corepressor that competes for interaction with PGC-1α for ERRs binding negatively regulating gene expression (19).

It should be mentioned here that, an early study in mice by Bonnelye *et al.*, (21) pointed the role of ERRs in numerous physiological or developmental functions in such tissues as muscle, central and peripheral nervous system and skin. The authors also showed correlation between ERRs expression with post-mitotic cells stage, suggesting role of these receptors in the differentiation process. Intensive studies of the next decades confirmed involvement of ERRs in control of cell proliferation and metabolic demands in many body organs during tumorigenesis (22). However, in organs of male reproductive system in physiological state, ERRs were not studied before. Their role has been highlighted only in prostate tumors (23).

Since our recent data in mouse Leydig cells (both primary and tumor) confirmed ERRs expression and its modulation after treatment with synthetic estrogenic compounds (24, 25), we were further prompted to examine mechanisms of ERR signaling and their interaction with endogenous estrogens. We hypothesized here that ERRs can regulate steroidogenic Leydig cell function and/or they can be implicated in estrogen signal transduction. With a reference to reasons presented above, the interplay between ERRs and estrogens, steroidogenic factors and secreting proteins (insulin-like 3; INSL3 and relaxin; RLN) reflecting functionality of Leydig cells will be investigated. For the study, Leydig cells of bank voles, which physiologically show pronounced changes in estrogen production dependently on seasonal testis activity (26) will be used. This unique and natural laboratory model allow for examination of testis disturbances, due to sex hormones insufficiency, that occur in mammals including human.

MATERIALS AND METHODS

Animals and experimental design

Bank vole males (*Myodes glareolus*; formerly *Clethrionomys glareolus*, Schreber) was originally derived about 30 years ago from wild bank voles colony living in the immense primeval forest in Bialowieza, Poland (27). Nowadays, there is a few vole colonies bred in research laboratories worldwide. In the Animal Facility of the Department of Endocrinology, bank voles (breeding pairs, separated pups, male and females in various age) are bred throughout the life in one of defined light regimes; long day (LD; 18 hours light : 6 hours darkness) and short day (6 hours light : 18 hours darkness; short day). Animals are bred in separate rooms without windows and well-isolated walls from external environment with automatic control of artificial light at 250 lux at cages level, stable temperature of 18°C and a relative humidity of 55 ± 5%. Voles are housed in polyethylene nontransparent cages (42 cm × 26 cm × 15.5 cm) furnished with sawdust and wood shavings for bedding according to the Directive of the European Union Parliament and the Council (2010/63/EU of 22 September 2010 regarding to the protection of animals used for scientific purposes). A standard pelleted diet (LSM diet, Agropol, Motycz, Poland) supplemented with seeds of wheat or oat, red beets, apples, and water is provided *ad libitum*.

For the previous study LD (n = 15) and SD (n = 15) animals isolated at the age of 3 to 4 weeks from mother and their sisters and bred in separate cages (five males per cage) were used. The use of the animals was approved by the National Commission of Bioethics at the Jagiellonian University in Cracow, Poland (No. 151/2015).

LD and SD animals (from summer generation) were allotted into groups (each group including 5 animals); control (Cont.) and experimental groups receiving selective ERRα antagonist 3-[4-(2,4-*Bis*-trifluoromethylbenzyloxy)-3-methoxyphenyl]-2-cyano-*N*-(5-trifluoromethyl-1,3,4-thiadiazol-2-yl)acrylamide (XCT 790) (Tocris Bioscience, Bristol, UK) and selective ERRβ/ERRγ agonist *N*-(4-(Diethylaminobenzylidenyl)-*N*'-(4-hydroxybenzoyl)-hydrazine (DY131) (Tocris Bioscience). Both XCT 790 and DY131 were dissolved in dimethyl sulfoxide (DMSO) and the stock solutions were kept at –20°C. LD and SD animals from the experimental groups were injected subcutaneously with freshly prepared solutions of XCT 790 (50 μg/kg bw) and DY131 (50 μg/kg bw) in phosphate buffered saline (six doses each dose injected every other day). Voles from control groups received vehicle only. Dose, frequency and time of XCT 790 and DY131 administration were based on literature data (28-33) and it was finally selected upon our preliminary study in bank voles *in vivo* (doses range 5, 50, 100, 150, 200 μg/kg bw) and in Leydig cells (mouse cell line MA-10) *in vitro* (dose range 1, 10, 100 μg/L). We performed microscopic analyses of hematoxylin-eosin stained testicular sections and ultrathin testicular slides. Next, we have analyzed mRNA level and protein expression of LHR, TSPO and StAR as well as INSL3 and RLN that are vital in this study and represents Leydig cells functionality alerts. The main purpose of this study was to determine specific effect of ERRs blockage or activation on Leydig cells function. As end points, concentration of signaling molecules, cAMP and Ca^{2+} as well as concentration of sex steroid hormones in vole testes (control versus experimental) were measured.

Tissue preparation

Bank voles were sacrificed by cervical dislocation and testes were surgically removed. Both testes of each individual of control and XCT 790 and DY131-treated bank voles were surgically removed and were cut into small fragments. For histology and immunohistochemistry, tissue samples were fixed in 10% formalin and embedded in paraplast. Concomitantly, small pieces of the testicular tissue were fixed in mixture of formaldehyde and glutaraldehyde for analysis in transmission electron microscope. Other tissue fragments were immediately frozen in a liquid nitrogen and stored at –80°C for RNA isolation, protein extraction, and determination of steroid hormone and signaling molecules levels in testes homogenates.

Morphology

For routine histology, hematoxylin-eosin staining was performed. The sections were examined under Nikon Eclipse Ni-U microscope (Nikon, Tokyo, Japan). A tubus setting of 1.25, a×10 ocular, and a×10 objective were used for the measurements. Detailed morphologic analysis was performed with the use of NIS-Elements software (Nikon, Tokyo, Japan), as previously described (34). The area of the interstitium occupied by Leydig cells was determined in 40 random fields of vision (which corresponds to 17.7 mm^2) for each animal from control and treated groups. A mean was determined for control LD and SD animals and those treated with XCT 790 and DY131.

Transmission electron microscopy (TEM) studies

The fixation procedure described below was based on the protocols proposed by Russell and Burguet (35). The modification developed in our labs had important advantages: it improved the quality of fixation and enhanced the contrast of plasma membrane and the organelles. Briefly, dissected testes of control and XCT 790 and DY131-treated voles were immersed in ice-cold pre-fixative containing 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. The tissues were then rinsed and post-fixed in a mixture of 2% osmium tetroxide and 0.8% potassium ferrocyanide in the same buffer for 30 min at 4°C. The material was embedded in Glycid Ether 100 resin (Serva, Heidelberg, Germany). Semi-thin sections (0.7 μm thick) were stained with 1% methylene blue and examined under a Leica DMR (Wetzlar, Germany) microscope. Prior to embedding small (3 – 5 mm) pieces of testicular tissue were carefully oriented in the mold to obtain accurate cross sections of the tubules. Ultrathin sections (80 nm thick) were contrasted with uranyl acetate and lead citrate and analyzed with a JEOL 2100 HT (Japan) TEM.

RNA isolation, reverse transcription

Total RNA was extracted from testes of control and XCT 790 and DY131-treated voles using TRIzol® reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. To remove contaminating DNA and DNase from RNA preparations, the RNA samples were incubated with reagents from the TURBO DNA-free™ Kit (Ambion, Austin, TX). The yield and quality of the RNA were assessed using a NanoDrop ND2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and by electrophoresis. Total cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

Real-time quantitative RT-PCR

Real-time RT-PCR was performed using the StepOne Real-Time PCR system (Applied Biosystems) and optimized standard conditions as described previously by Kotula-Balak *et al*., (36). Primer sets (Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw Poland) are listed in *Table 1*.

Table 1. Sequences of forward and reverse primers.

Genes	Primers (5'–3')	Product size (bp)	Annealing temperature (◦C)	Cycles
ERRα	5'- GCCTCTACCCAAACCTCTCT-3' 5'- AGCCAT CCCTCCTTCGCACA-3'	234	60	40
ERRβ	5'- GAGCCATCTTTACCGCTGGA-3' 5'- CAGCTTGTCAACAGGCAGTG -3'	239	60	40
ERRγ	5'- CTTGTAATGGGGTTGCCTC-3' 5'- TATCACCTTCTGCCGACCT-3'	222	62	35
LHR	5'- CTACGTCATCTCAGTTGTGTGG-3' 5'- GAGCAGAGCACTAAGCCAGT-3'	234	60	40
TSPO	5'- CCCGCTTGCTGTACCCTTACC-3' 5'- CACCGCATACATAGTTGACGACT -3'	195	60	40
StAR	5'- ATCTCCTTGACATTTGGGTTCCA-3' 5'- CGGTCTCTATGAAGAACTTGTGGAC-3'	389	62	35
INSL3	5'- CAT GCG CGC GCC GCT GCT AC-3' 5'- TCA GTG GGG ACA CAG ACC C-3'	596	62	30
RLN	5'- GTGAATATGCCCGTGAATTGATC-3' 5'- AGCGTCGTATCGAAAGG CTCT-3'	150	60	40
Tubα1a	5'-CGGAACCAGCTTGGACTTCTTTCCG-3' 5'-GGAACTGGCTCTGGCTTCACC-3'	321	60	40

Abbreviations: ERRα, estrogen-related receptor-alpha; ERRβ, estrogen-related receptor-beta; ERRγ, estrogen-related receptor-gamma; LHR, lutropin receptor; TSPO, translocator protein; StAR, steroidogenic acute regulatory protein; INSL3, insulin-like peptide 3; RLN, relaxin; Tubα1a, tubulin alpha1a.

Detection of amplification products for individual genes was performed with 10 ng cDNA, 0.5 μM primers, and SYBR Green master mix (Applied Biosystems) in a final volume of 20 μL. Amplifications were performed as follows: 55°C for 2 min, 94°C for 10 min, followed by 40 cycles of 30 s at 62°C and 45 s 72°C to determine the cycle threshold (Ct) for quantitative measurement. To confirm amplification specificity the PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis. The ERRs (α, β and γ) mRNA as well as LHR, TSPO, StAR, INSL3 and RLN mRNA expressions were normalized to the expression of Tubulin a1α mRNA with the use of the 2-ΔΔCt method (37). Control reactions either without the RNA template or without the reverse transcriptase enzyme were performed. Three independent experiments were performed, each in triplicate with tissues prepared from different animals. All PCR products stained with Midori Green Stain (Nippon Genetics Europe GmbH, Duren, Germany) were run on agarose gels. Images were captured using a Bio-Rad Gel Doc XR System (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

To optimize immunohistochemical staining testicular sections of control, XCT 790 and DY131-treated voles were immersed in 10 mM citrate buffer (pH 6.0) and heated in a microwave oven (2×5 min, 700 W). Thereafter, sections were immersed sequentially in H_2O_2 (3 %; v/v) for 10 min and normal goat serum (5%; v/v) for 30 min which were used as blocking solutions. After overnight incubation at 4°C with primary antibodies listed in *Table 2*. Next respective biotinylated antibodies (anti-rabbit, anti-goat and anti-mouse IgGs; 1:400; Vector, Burlingame CA, USA) and avidin-biotinylated horseradish peroxidase complex (ABC/HRP; 1:100; Dako, Glostrup, Denmark) were applied in succession. Bound antibody was visualized with 3,3'-diaminobenzidine (0.05 %; v/v; Sigma-Aldrich) as a chromogenic substrate. Control sections included omission of primary antibody and substitution by irrelevant IgG. The whole procedure was described in detail elsewhere (38, 39). Experiments were repeated three times. The sections were examined with a Leica DMR microscope (Wetzlar, Germany).

Western blot analysis

Lysates were obtained by sample homogenization and sonication with a cold Tris/EDTA buffer (50 mM Tris, 1 mM EDTA, pH 7.5), supplemented with a broad-spectrum protease inhibitors (Sigma-Aldrich). The protein concentration was estimated by the Bio-Rad DC Protein Assay Kit with BSA as a standard (Bio-Rad Labs, GmbH, Munchen, Germany). Equal amounts of protein were resolved by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany) and analyzed by Western blotting with antibodies listed in *Table 2*. The presence of the primary antibody was revealed with horseradish peroxidase-conjugated secondary antibodies diluted 1:3000 (Vector Lab., Burlingame, CA, USA) and visualized with an enhanced chemiluminescence detection system as previously described (40). All immunoblots were stripped with stripping buffer containing 62.5-mM Tris-HCL, 100-mM 2-mercaptoethanol, and 2% SDS (wt:v; pH 6.7) at 50°C for 30 min, and incubated in antibody against β-actin (*Table 2*), which served as a loading control. Three independent experiments were performed, each in triplicate with tissues prepared from different animals. To obtain quantitative results the bands (representing each data point) were densitometrically scanned using the public domain ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) (41). The data obtained for each protein were normalized against its corresponding actin and expressed as relative intensity. Results of 10 separate measurements were expressed as mean ± S.D.

Radioimmunological analysis

Radioimmunological technique described elsewhere (35, 38, 42, 43) was used to determine testosterone and estradiol level in homogenized testicular tissues from control and XCT790 and DY131-treated bank voles. Testosterone levels were assessed using [1,2,6,7-^{3}H]-testosterone (specific activity 110 Ci/mmol; American Radiolabeled Chemicals, Inc.) as a tracer and rabbit antibody against testosterone-3-0-CMO:BSA (a gift from Dr. B. Ricarova, Institute of Radiology, Czech Academy of Sciences, Prague, Czech Republic). The lower limit of sensitivity was 5 pg. Cross-reaction

Table 2. Primary antibodies used for immunochemistry and Western blotting.

Antibody	Host species	Vendor	Dilution
ERRα	Rabbit	Abcam cat.no. ab16363	1:100 (IHC) 1:1000 (WB)
ERRβ	Rabbit	Abcam cat.no. ab19331	1:200 (IHC) 1:1000 (WB)
ERRγ	Rabbit	Abcam cat.no. ab49129	1:200 (IHC) 1:000 (WB)
StAR	Mouse	Abcam cat. no. ab5813	1:200 (IHC) 1:1000 (WB)
INSL3	Rabbit	Santa Cruz Biot. cat. no. sc-134587	1:100 (IHC) 1:500 (WB)
RLN ½	Goat	Santa Cruz Biot. Cat.no.sc-20491	1:100 (IHC) 1:500 (WB)
β-actin	Mouse	Sigma-Aldrich cat. no. A2228	1:3000

Abbreviations: ERRα, estrogen-related receptor-alpha; ERRβ, estrogen-related receptor-beta; ERRγ, estrogen-related receptor-gamma; LHR, lutropin receptor; TSPO, translocator protein; StAR, steroidogenic acute regulatory protein; INSL3. insulin-like peptide 3; RLN, relaxin.

of this antibody was 18.3% with dihydrotestosterone, 0.1% with androstenedione and less than 0.1% with other major testis steroids. Coefficients of variation within and between assays were below 5.0% and 9.7%, respectively.

Estradiol concentrations were measured using [2,4,6,7-^{3}H]-estradiol (specific activity 81 Ci/mmol: American Radiolabeled Chemicals, Inc.) as a tracer and rabbit antibody against estradiol-17-O-carboxymethyloxime: BSA (a gift from Prof. R. Rembiesa, Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland). The lower limit of sensitivity of the assays was 5 pg. Cross-reaction was 1% with keto-oestradiol-17b, 0.8% with oestrone, 0.8% with oestriol, 0.01% with testosterone and less than 0.1% with major ovarian steroids. Coefficients of variation within and between assays were below 4% and 7.5%, respectively. Assays were validated by demonstrating parallelism between serial dilutions of culture media and standard curve. Coefficients of variation within and between each assay were 7.6% and 9.8% respectively. The recovery of unlabeled steroids were also assessed (never less than 90%). In addition, to monitoring intra-assays and inter-assays, assay quality control was assessed by control samples representing low, medium and high concentrations of measured hormones. Samples (each in triplicate with tissues prepared from different animals) were counted in a scintillation counter (LKB 1209 RACKBETA LKB; Turku, Finland). The concentrations of sex steroids were calculated as pg/10^5 cells.

Determination of cAMP production and Ca^{2+} levels

The amount of cAMP produced in testes of LD and SD animals both control and treated with XCT 790 or DY131 respectively, was determined by Direct cAMP Elisa kit assay (Enzo, Life Sciences, AG, Lausen, Switzerland), according to the manufacturer's instructions for the acetylated assay. The sensitivity of the assay was 0.037 pmol/mL. The cAMP levels were calculated as pmol/ml.

Control and XCT 790 or DY131-treated testes were lysed in cold RIPA buffer (Thermo Scientific, Inc., Rockford, IL, USA) and next sonicated for 60 s on ice and centrifuged at 10,000 *g* for 15 min. Ca^{2+} was estimated in the supernatants using Arsenazo III (Sigma-Aldrich, St Louis, MO, USA) according to the modified method by Michaylo and Illkova (44). The intensity of the purple complex formed with the reagent was read at 600 nm in a spectrophotometer (Labtech LT-4000MS; Labtech International Ltd., Uckfield, UK) with Manta PC analysis software. The proteins were estimated by modified Lowry's method (45). The Ca^{2+} levels were calculated as µg/ml.

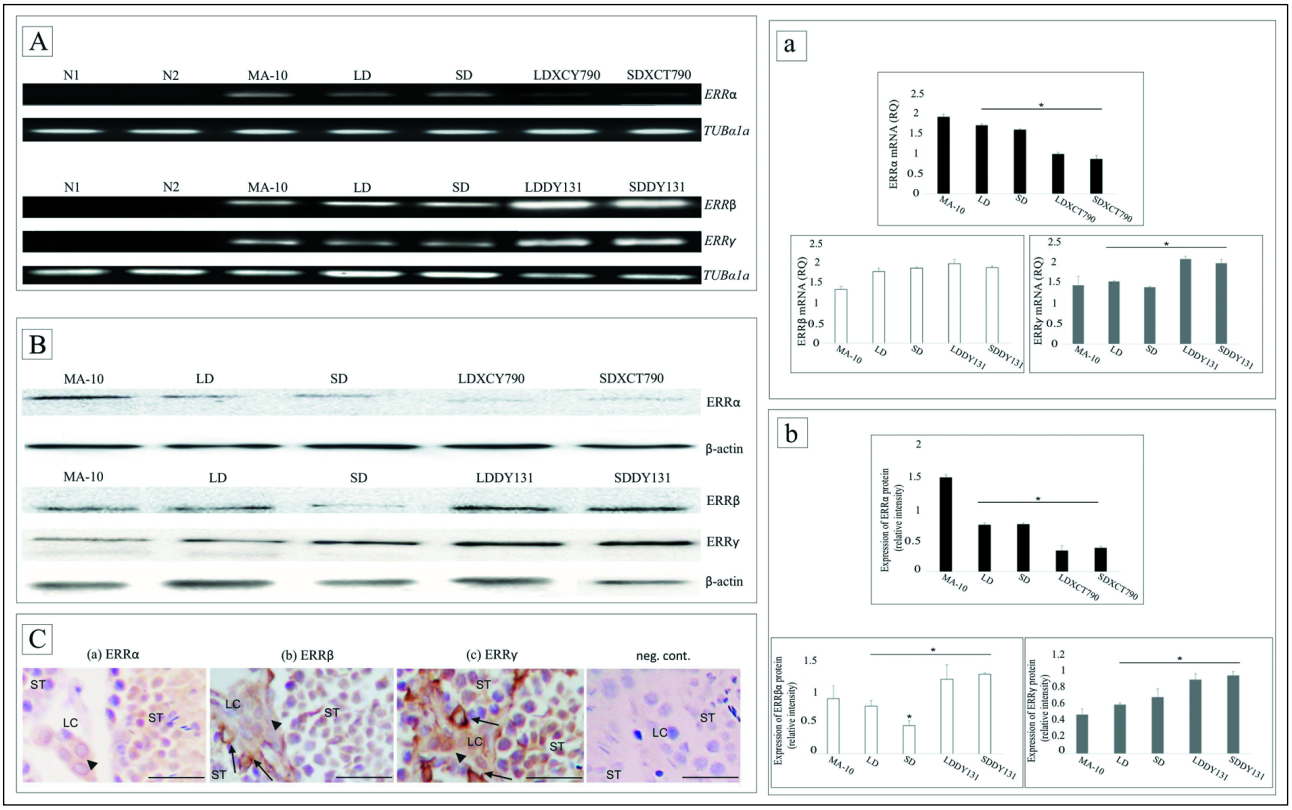

Fig. 1. Expression of ERRα, ERRβ and ERRγ at mRNA and protein level and cellular localization in bank vole testes (A-C). (A) Representative gel electrophoresis of qualitative expression, (line N1-negative control without complementary DNA template, line N2-negative control without nonreverse transcribed RNA, line MA-10; mouse Leydig cell line-positive control (a) and relative quantification; (RQ) of mRNA for ERRα, ERRβ and ERRγ. (B) Representative blots of qualitative expression (MA-10; mouse Leydig cell line-positive control and (b) and relative expression (relative quantification of protein density; ROD) of ERRα, ERRβ and ERRγ protein. RQ and ROD is expressed as means ± S.D. Asterisks show significant differences between LD or SD (control or XCT790 or DY131-treated) males. Values are denoted as $^*P < 0.05$. (C) Representative microphotographs of cellular localization of ERRα (a), ERRβ (b) and ERRγ in LD bank voles (c). Negative control - no immunostaining is visible when the primary antibodies are omitted. Immunostaining with DAB and counterstaining with hematoxylin. Scale bars represent 15 µm. Arrowheads show positive staining in cytoplasm of Leydig cells while arrows positive staining in cytoplasm of Sertoli cells. From each animal three mRNA/protein samples and 3 – 5 serial testicular slides were analyzed.

Each analysis was performed in triplicate with tissues prepared from different animals.

Statistical analysis

Each variable was tested by using the Shapiro-Wilk *W*-test for normality. Homogeneity of variance was assessed with Levene's test. Since the distribution of the variables was normal and the values were homogeneous in variance, all statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* comparison test to determine which values differed significantly from controls. The analysis was made using Statistica software (StatSoft, Tulsa, OK, USA). Data were presented as mean ± S.D. Data were considered statistically significant at $P< 0.05$.

RESULTS

Estrogen-related receptors mRNA level and protein expression in bank vole Leydig cells

Either dependently on animal group (LD or SD) or ERR type, differences in mRNA level were not found (*Fig. 1A, 1a*). At protein level, unlike ERRα and ERRγ, expression of ERRβ was significantly lower ($P < 0.05$) especially in SD animals (*Fig. 1B, 1b*). After XCT790 or DY 131 treatment changes ($P < 0.05$) at mRNA level and protein expression of ERRs were revealed (*Fig. 1A, 1a, 1B, 1b*). Localization of ERRα was restricted only to Leydig cells (*Fig. 1C*) while ERRβ and ERRγ were expressed besides Leydig cells also in Sertoli cells of the seminiferous tubules (*Fig. 1Ca-1Cc*) in testes of LD and SD (not shown) voles.

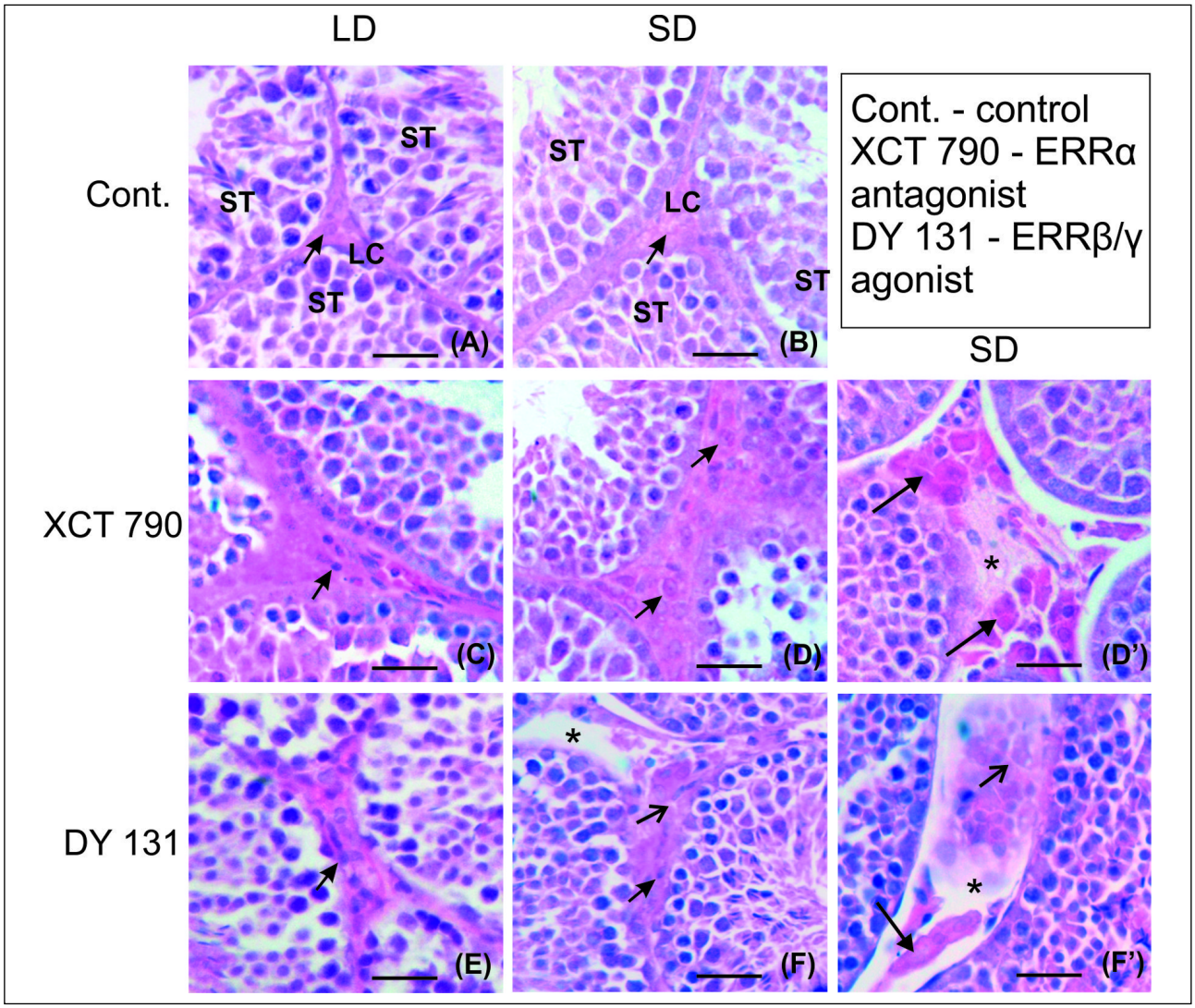

Fig. 2. Effect of ERRs blockage or activation on bank vole testis histology and Leydig cells morphology (A-F'). (A, B): control testes of LD (A) and SD (B) males. (C, D, D'): testes of XCT 790 treated males; LD (C) and SD (D, D'). (E, F, F'): testes of DY 131 treated males; LD (E) and SD (F, F'). Hematoxylin-eosin staining. From each animal 3 – 5 serial testicular slides were analyzed. Scale bars represent 15 µm. Full spermatogenesis in seminiferous tubules (ST) of both control and XCT 790 or DY131 testes (A-F') and enlarged interstitial tissue with Leydig cells (LC) after exposure is visible. Note, that in control SD males interstitial tissue is more abundant than in LD males (short arrows) (A, B). The most significant overgrowth of interstitial tissue is visible in DY131-exposed males when compare to XCT 790 treated ones (C-F'). In addition, in both groups of treated animals the enlargement of interstitial tissue is higher in SD testes than in LD ones (short arrows). In SD animals of both treated groups accumulated cytoplasmic debris between Leydig cells are visible (open arrows) (D, F, F') and occasionally lack of Leydig cells is seen (asterisks) (D', F, F'). Moreover in SD XCT 790 testes individual Leydig cells showed higher affinity to hematoxylin (long arrows) (D').

Effect of estrogen-related receptors blockage or activation on bank vole testis histology and Leydig cells morphology

Neither treatment with XCT 790 nor with DY131exerted effect on testes weight. Testes of animals exposed to XCT 790 were of similar relative weights in LD animals (15.03 ± 1.17 mg/g) and SD ones (11.05 ± 3.23 mg/g) when compared to their respective controls (16 ± 1.03 mg/g versus 10.97 ± 0.97 mg/g). Correspondingly, in DY131 treated animals comparable weights of testes of LD animals (16.32 ± 2/19 mg/g) and SD animals (9.89 ± 1.66 mg/g) similar to respective controls (see data above) were found.

No effect of XCT 790 and DY131was observed on testis histology (*Fig. 2A-2F*). In treated LD animals full spermatogenesis was observed whereas in SD animals slightly less active spermatogenesis (reduced number of elongated spermatozoa in seminiferous tubule lumens and some closed seminiferous tubule lumens - no spermatogenically active were found) in comparison to respective LD and SD controls (partly seen in *Fig. 2*).

In control LD animals, properly developed interstitial tissue was observed (short arrows) (*Fig. 2A*). In SD animals a slight increase in the interstitial tissue volume was revealed in contrast to LD ones (3.53 ± 0.33 versus 2.47 ± 0.28 mm^2) (short arrows) (*Fig. 2B*). Exposure to XCT 790 caused significant increase of interstitial tissue volume in both LD and SD voles (4.03 ± 0.15* versus 5.42 ± 0.12* mm^2) (short arrows) (*Fig. 2C* and *2D*). In addition, occasionally individual cells of SD voles showed enlarged volume and strong affinity to hematoxilin (long arrows) (*Fig. 2D'*). Also, empty spaces in the interstitial tissue reflected destruction and/or lack of Leydig cells (asterisks) (*Fig.* 2D'). In both LD and SD DY 131-treated males increased volume of interstitial tissue (3.17 ± 0.04 versus 3.98 ± 0.09 mm^2) was observed however not as abundant as in LD and SD XCT 790-treated animals (short arrows) (*Fig. 2E-2F'*). In SD voles treated with DY131, sporadically increased size of some Leydig cells was noticed together with places depriving of Leydig cells and visible debris (open arrows) (*Fig. 2F*). Remains of cytoplasm of destructed cells, in the places lacking Leydig cells, were observed too (open arrows) (*Fig. 2F'*).

Effect of estrogen-related receptors blockage or activation on bank vole Leydig cells ultrastructure

In Leydig cells of control voles both LD and SD (not shown) spherical nuclei (nu) were visible (*Fig. 3A* and *3B*). In the

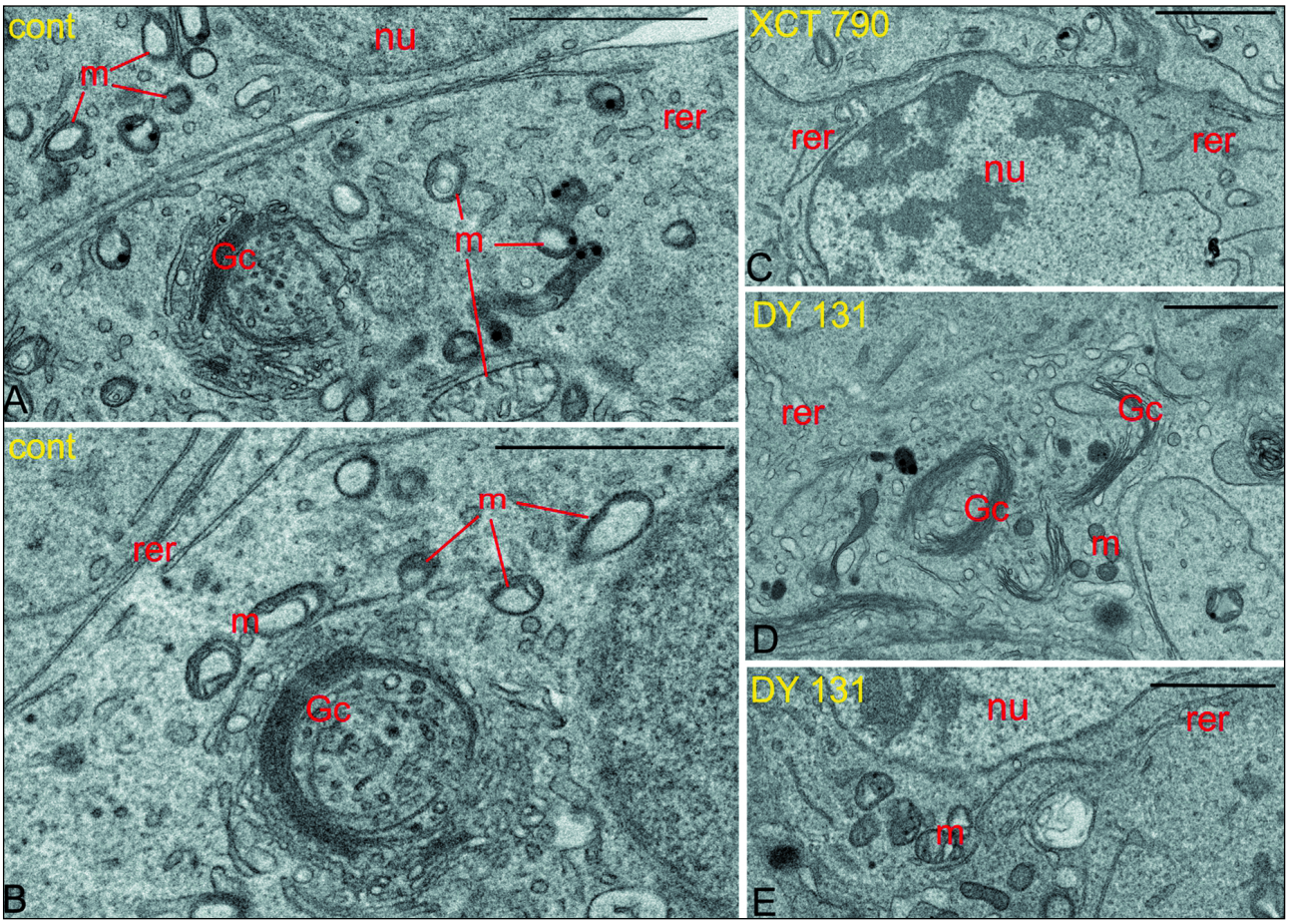

Fig. 3. Effect of ERRs blockage or activation on Leydig cells ultrastructure. (A-E) Representative microphotographs of LD Leydig cells of control and XCT 790 and DY131-treated voles. Each testicular sample in epoxy resin block was cut for at least three ultrathin sections that were analyzed. Bars represent 1 µm. (A, B) Control (C) XCT 790-treated (D, E) DY 131 testes. (A, B) In Leydig cells of control Leydig cells spherical nuclei (nu), numerous mitochondria (m) frequently formed relatively huge accumulations and well-developed and numerous Golgi complexes (Gc) are seen. (C-E) In treated with XCT 790 and DY131 Lydig cells folded nuclei, reduced number of mitochondria distributed more or less uniformly as well as smaller and composed of less numerous flattened cisternae Golgi complexes are observed. Note, abundant elements of rough endoplasmic reticulum (rer) with some of the cisternae arranged in characteristic circular complexes.

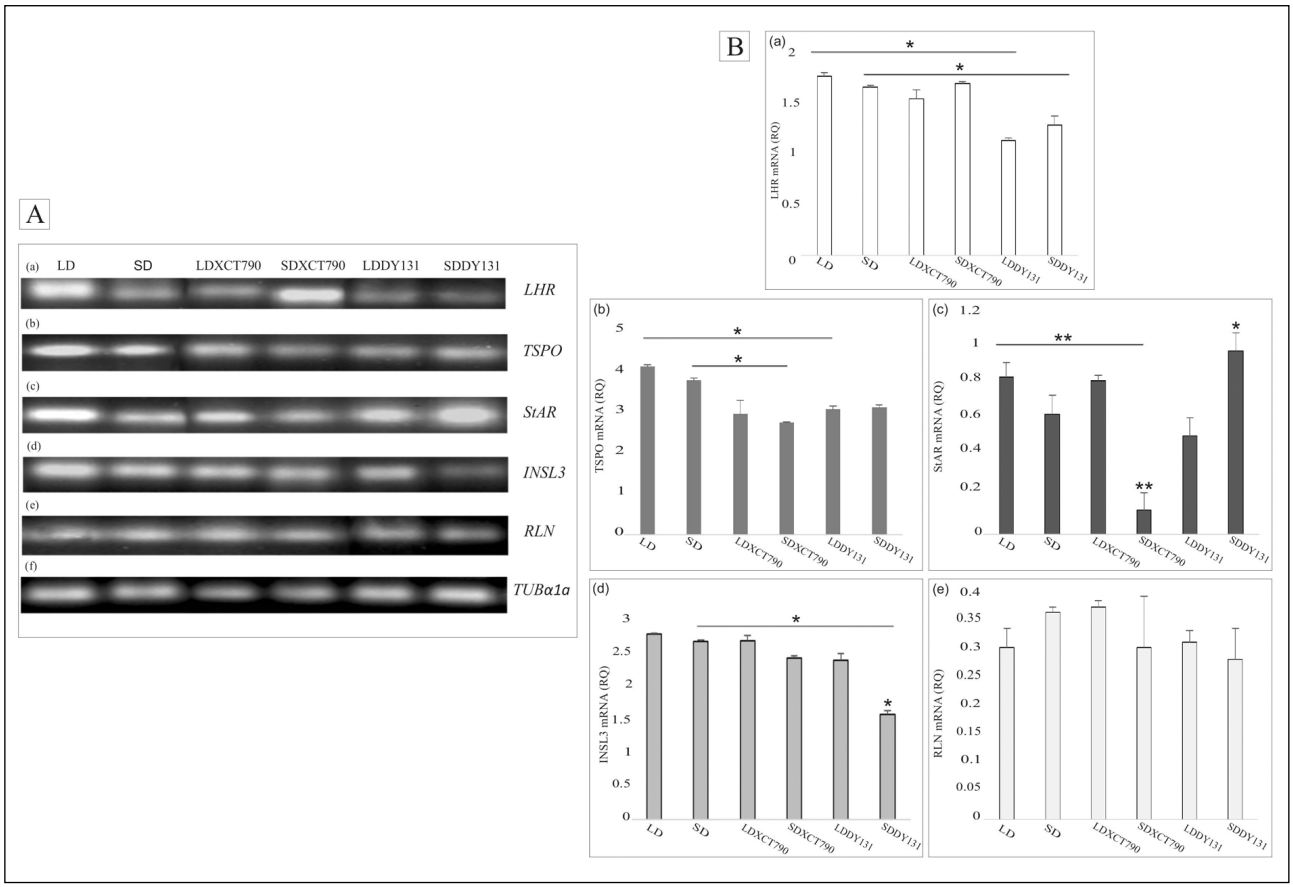

Fig. 4. Effect of ERRs blockage or activation on mRNA level of steroidogenic proteins and secretory proteins in bank vole Leydig cells (A, B). (Aa-Af) Representative gel electrophoresis of qualitative expression (LHR (a), TSPO (b), StAR (c), INSL3 (d) and RLN (e)). (Ba-Be) Relative level (relative quantification; RQ) of mRNA for (LHR (a), TSPO (b), StAR (c), INSL3 (d) and RLN (e)) determined using real-time RT-PCR analysis 2–ΔCt method. As an intrinsic control, tubulin α1a mRNA level was measured in the samples (Af-qualitative expression). RQ from three separate analyses is expressed as means ± S.D. Asterisks show significant differences between respective controls (LD or SD) males and those treated with XCT 790 or DY131 (LD and SD males), respectively. Values are denoted as *P < 0.05 and **P < 0.01. From each animal at least three samples were measured.

cytoplasm numerous mitochondria (m) frequently forming relatively huge accumulations were observed. Within the cytoplasm, well-developed and numerous Golgi complexes (Gc) were evenly distributed.

Independently on treatment (XCT 790 or DY131), disturbances of similar type and severity in Leydig cells ultrastructure in LD and SD (not shown) voles were observed (*Fig. 3C-3E*). However, these alterations were observed more frequently in XCT 790 exposed animals than in DY131 ones. In treated cells folded nuclei were noticed. The most serious alterations were revealed within the cytoplasm. Slightly reduced number of mitochondria were located more or less uniformly. Smaller and composed of less numerous flattened cisternae Gc were observed. In addition, number of elements of rough endoplasmic reticulum (rer) were found. Some of the cisternae of endoplasmic reticulum were arranged in characteristic circular complexes.

Effect of estrogen-related receptors blockage or activation on mRNA level of steroidogenic proteins and secretory proteins in bank vole Leydig cells

Electrophoresis revealed PCR-amplified products of the predicted sizes: 234 bp for *LHR*; 195 bp for *TSPO;* 389 bp for *StAR*; 596 bp for *INSL3*; 150 bp for *RLN* and 321 bp for *tubulin a1α* (*TUBa1α;* reference gene) in both LD and SD testes (*Fig. 4Aa-4Af* and *4Ba-4Be*). Real-time RT-PCR analysis in testes of LD and SD males both control and experimental (XCT790 and DY131-treated) revealed changes in the level of *LHR, TSPO, StAR, INSL3* and *RLN* in both LD and SD testes (*Fig. 4A* and *4B*). No significant differences in *TUBa1α* level were revealed in testes of LD and SD voles both control and experimental ones (*Fig. 4Af*).

In control LD bank vole testis, level of *LHR, TSPO, StAR* and *INSL3* was always higher than in SD males (*Fig. 4A* and *4B*). In contrast, level of *RLN* was higher in SD males compared to LD ones.

The mRNA for LHR was significantly decreased (P < 0.05) after DY131 treatment of LD and SD males that was not the case in XCT 790 treated males compared to respective controls (*Fig. 4Aa*). Also a decrease (P < 0.05) in XCT 790 and DY131- treated males was revealed for *TSPO* in both groups of voles (LD and SD) (*Fig. 4Ab*). Marked change, four-fold lower (P < 0.01) level of *StAR* was revealed in SD males after XCT 790 exposure. Level of StAR mRNA in LD XCT 790 voles was similar to that of control. While in DY131 males significant *StAR* increase (P < 0.05) was observed in SD voles. In LD ones slight decrease of *StAR* was found (*Fig. 4Ac*). Compared to respective controls, *INSL3* level was slightly decreased in all groups of treated males, including significant decrease (P < 0.05) in SD DY131-exposed ones (*Fig. 4Ad*). Level of RLN mRNA was increased

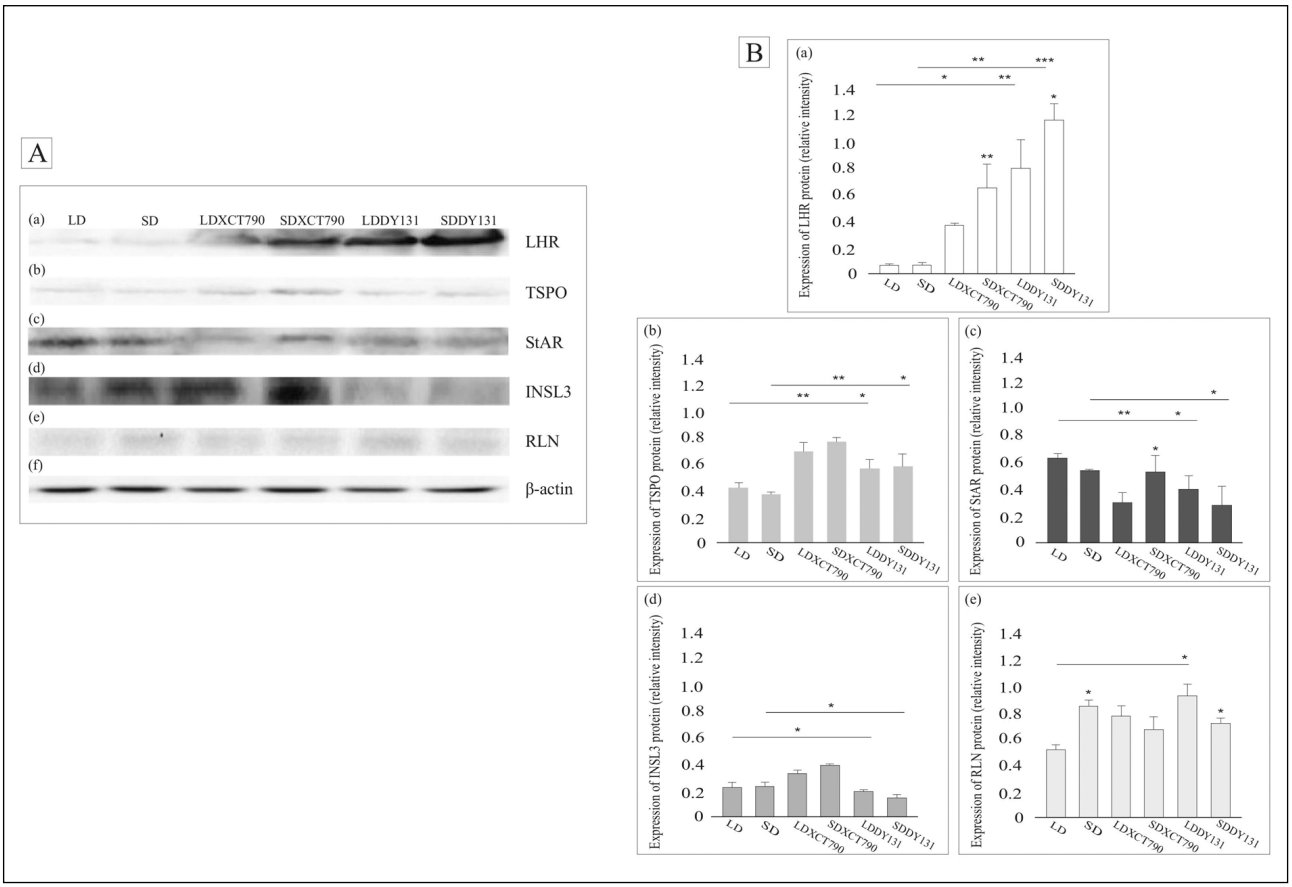

Fig. 5. Effect of ERRα blockage on expression of steroidogenic proteins and secretory proteins in bank vole Leydig cells (A, B). (Aa-Af) Representative blots of qualitative expression (LHR (a), TSPO (b), StAR (c), INSL3 (d) and RLN (e)). (Ba-Be) Relative expression (relative quantification of protein density (ROD); arbitrary units) of proteins (LHR (a), TSPO (b), StAR (c), INSL3 (d) and RLN (e)). The relative amount of respective proteins normalized to β-actin (Af; qualitative expression). ROD from three separate analyses is expressed as means ± S.D. Asterisks show significant differences between respective controls (LD or SD) males and those treated with XCT 790 or DY131 (LD and SD males), respectively. Values are denoted as *P < 0.05, **P < 0.01 and ***P < 0.001. From each animal at least three samples were measured.

after treatment with XCT 790 in LD males while in SD XCT 790 and DY131 it was decreased when compared to respective controls (*Fig. 4Ae*).

Effect of estrogen-related receptor-α blockage on expression of steroidogenic proteins and secretory proteins in bank vole Leydig cells

Immunodetectable LHR, TSPO, StAR, INSL3 and RLN proteins were observed as single bands near the 85 kDa, 18 kDa, 32 kDa, 15 kDa, and 6 kDa position of the SDS gel, respectively, in testicular homogenates of LD and SD voles both control and treated with XCT 790 and DY131 (*Fig. 5Aa-5Ae* and *5Ba-5Be*). Stripped immunoblots were also used for β-actin (*Fig. 5Af*). No differences were found in the expression level of LHR, TSPO, StAR and INSL3 between LD and SD voles what was not the case for RLN expression. The latter was higher (P < 0.05) in SD males (*Fig. 5A* and *5B*). LHR expression was increased in treated animals, however pronounced increase (P < 0.05, P < 0.01) was revealed after treatment either with XCT 790 or DY131 in SD voles (*Fig. 5Aa* and *5Ba*). No changes in expression of TSPO were revealed between LD and SD males of both treated groups. After exposure to XCT 790 or DY131 increased expression (P < 0.05, P < 0.01) of TSPO was found in both LD and SD males in comparison to respective controls (*Fig. 5Ab* and *5Bb*). The increase was always higher after XCT 790 exposure. Treatment with XCT 790 or DY 131 decreased (P < 0.05, P < 0.01) expression of StAR in all groups of males with exception of XCT 790 SD males where expression increased (P < 0.05) when compared to respective LD males (*Fig. 5Ac* and *5Bc*). Increased INSL3 expression (P < 0.05) was found in XCT 790 exposed animals of both LD and SD groups. In both LD and SD DY131 males expression of INSL3 was decreased (P < 0.05) when compared to respective controls (*Fig. 5Ad* and *5Bd*). No differences were found in expression of INSL3 between LD and SD males of treated groups. Exposure to XCT 790 and DY131 increased expression of RLN (P < 0.05) in LD males while in SD males expression of RLN was decreased (P < 0.05) in comparison to controls (*Fig. 5Ae* and *5Be*).

Effect of estrogen-related receptor-α blockage on localization of steroidogenic proteins and secretory proteins in bank vole Leydig cells

After treatment with XCT 790 immunostaining for LHR was stronger in membrane of LD than SD Leydig cells when compared to controls (*Fig. 6A*, *6G* and *Fig. 7A*, *7G*). Strong staining for TSPO was found in both LD and SD Leydig cells of control and experimental groups (*Fig. 6B*, *6H* and *Fig. 7B*, *7H*), however in treated males sometimes individual Leydig

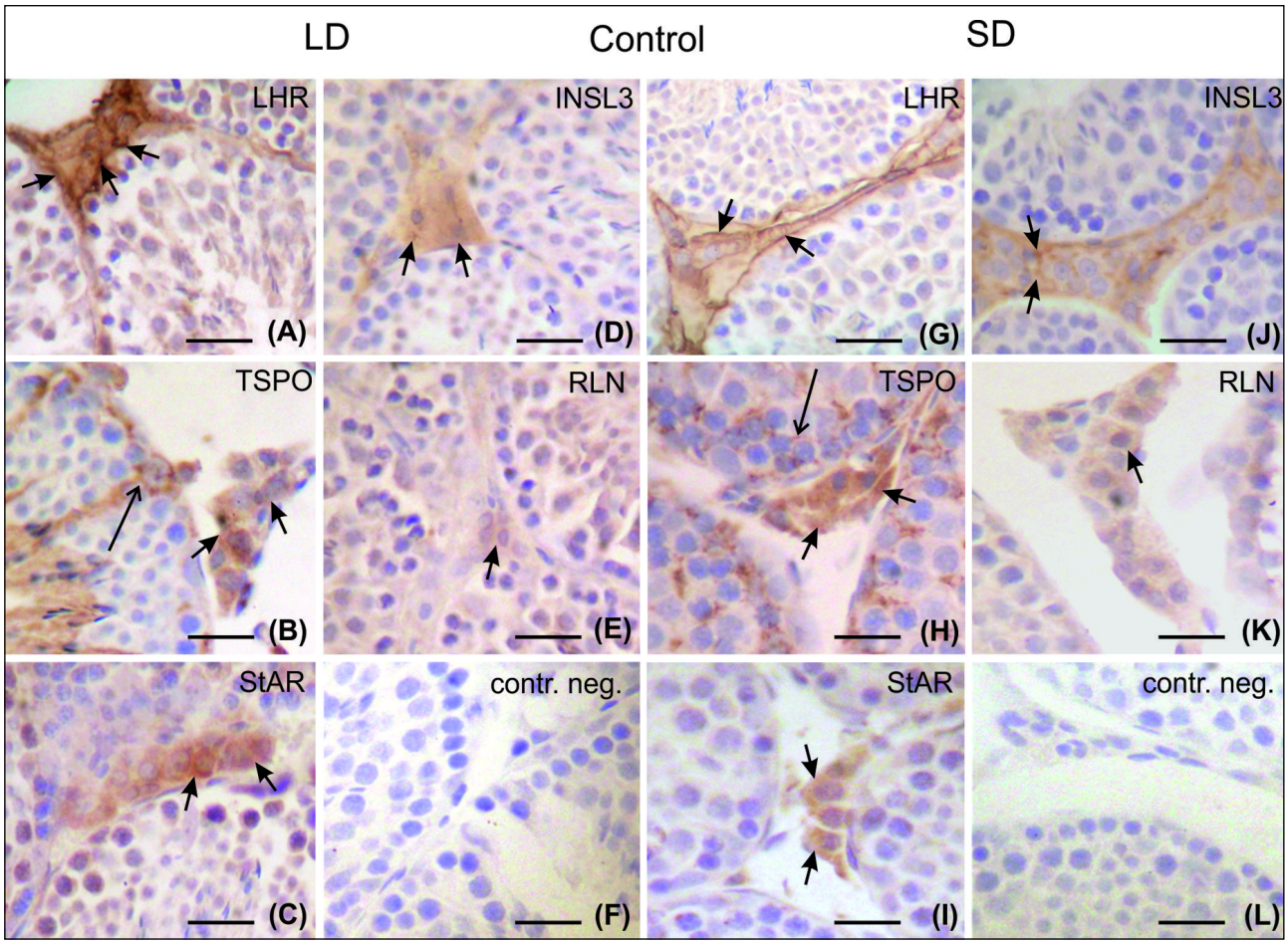

Fig. 6. Localization of steroidogenic proteins and secretory proteins in bank vole Leydig cells (A-L). (A-F): control testes of LD and (G-L) SD males. (A and G): localization of LHR; (B and H) localization of TSPO; (C and I): localization of StAR; (D and J) localization of INSL3; (E and K): localization of RLN in testes of LD and SD males, respectively. Immunostaining with DAB and counterstaining with hematoxylin. From each animal 3 – 5 serial testicular slides were analyzed. Scale bars represent 15 μm. (A) Strong immunostaining for LHR is visible in Leydig cell membranes of LD testes in comparison to moderate one in those of SD males (G) (arrows). (B and H) in both LD and SD Leydig cells moderate signal for TSPO is noted in cytoplasm of Leydig cells (arrows). In addition, positive, moderate signal for TSPO is detected in cytoplasm of Sertoli cells of seminiferous tubules (open arrows). (C and I) In both groups of males weak to moderate signal for StAR is visible in cytoplasm of Leydig cells (arrows). (D and J) INSL3 signal is exclusively located in Leydig cell cytoplasm and is of similar, moderate intensity, in both groups (arrows). (E) In LD males RLN exhibited very weak immunoexpression in Leydig cells cytoplasm while in SD males (K) the signal is slightly more intensive (arrows). (F, L) No positive staining is visible when primary antibody is omitted.

cells showed very weak immunoreaction for TSPO. Besides Leydig cells (arrows), TSPO expression was found in somatic cells of seminiferous tubules (open arrows) (partly visible in *Fig. 6B*, *6H* and *Fig. 7B*, *7H*). Immunoexpression of StAR was markedly reduced in SD males but not in LD males that was similar to respective controls (*Fig. 6C*, *6I* and *Fig. 7C*, *7I*). Positive signal for INSL3 and RLN was found exclusively in cytoplasm of Leydig cells (*Fig. 6D*, *6J*, *6E*, *6K* and *Fig. 7D*, *7J*, *7E*, *7K*). Although immunoexpression of INSL3 was of various intensity in single cells, expression of RLN was weak to very weak in all cells. No significant differences were found in RLN expression in control and treated males what was not the case in INSL3 expression that was always decreased in experimental animals when compared to controls (*Fig. 6D*, *6J*, *6E*, *6K* and *Fig. 7D*, *7J*, *7E*, *7K*).

In negative controls of control and experimental testicular sections no positive staining was observed (*Fig. 6F*, *6L* and *Fig. 7F*, *7L*).

Effect of estrogen-related receptor-βγ activation on expression of steroidogenic proteins and secretory proteins in bank vole Leydig cells

In membrane of Leydig cells of DY131 treated voles immunostaining for LHR was weak in both LD and SD animals in comparison to respective controls (*Fig. 8A*, *8G* and *Fig. 7A*, *7G*). Similarly to LD and SD controls, immunoexpression of TSPO in Leydig cells (arrows) was of strong-to moderate intensity in treated LD and SD voles (*Fig. 8B*, *8H* and *Fig. 7B*, *7H*). TSPO was also expressed in somatic cells of seminiferous tubules (open arrows) (partly visible in *Fig. 8B*, *8H* and *Fig. 7B*, *7H*). Increased expression of StAR was revealed especially in LD voles, in SD ones and controls it was comparable (*Fig. 7C*, *7I* and *Fig. 8C*, *8I*). No differences in immunostaining for INSL3, that was of moderate intensity, and RLN, that was weak, in cytoplasm of DY 131 Leydig cells were observed (*Fig. 7D*, *7J*, *7E*, *7K* and *Fig. 8D*, *8J*, *8E*, *8K*).

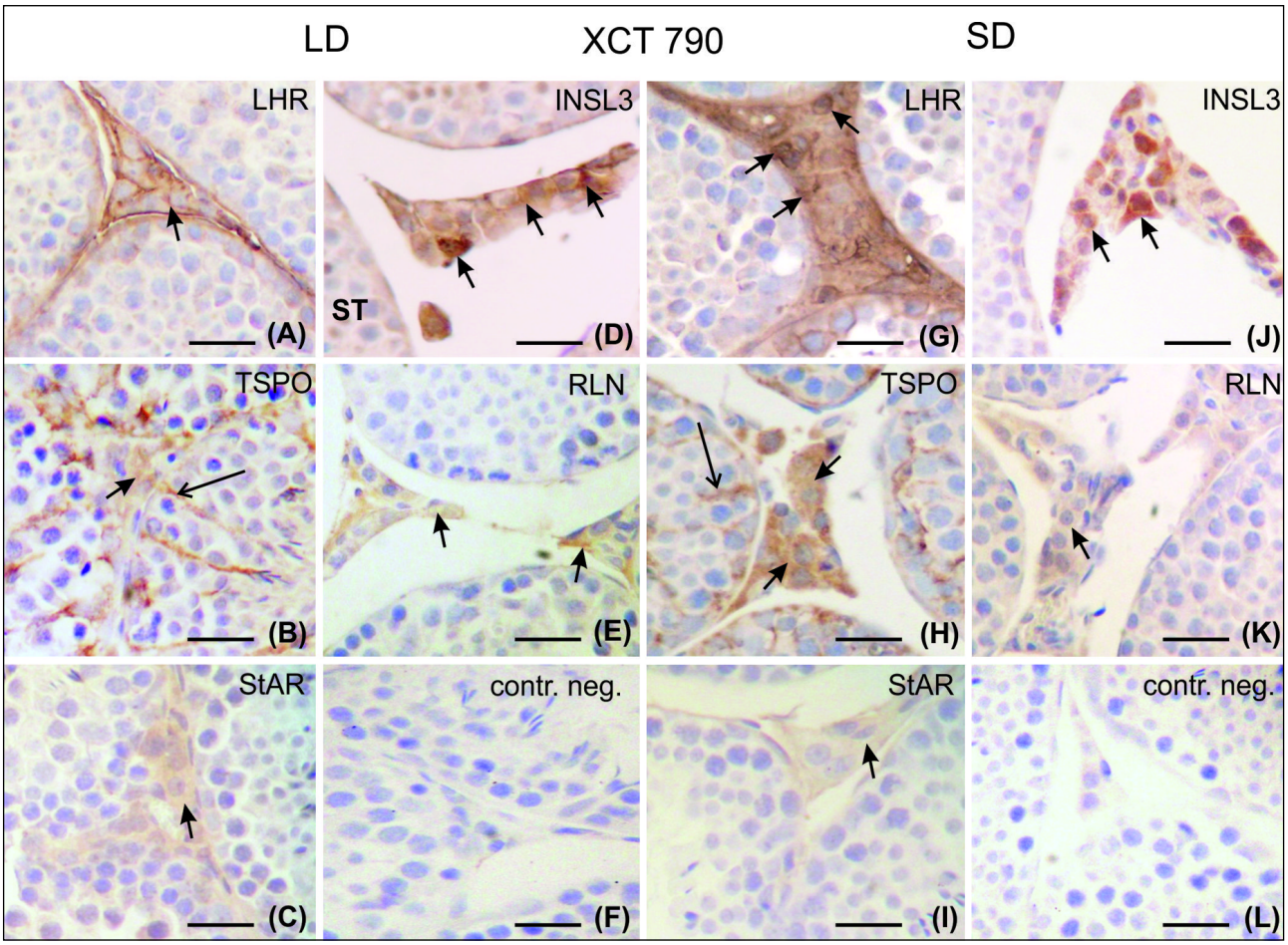

Fig. 7. Effect of ERRα activation on localization of steroidgenic proteins and secretory proteins in bank vole Leydig cells (A-L). (A-F): testes of LD and (G-L): SD XCT 790-treated males. (A and G): localization of LHR; (B and H): localization of TSPO; (C and I): localization of StAR; (D and J): localization of INSL3; (E and K): localization of RLN in testes of LD and SD males, respectively. Immunostaining with DAB and counterstaining with hematoxylin. From each animal 3 – 5 serial testicular slides were analysed. Scale bars represent 15 µm. (A and G) Strong expression of LHR is visible in membrane of Leydig cells of both LD and SD testes (arrows). (B and H) Moderate (in LD) and strong (in SD) signal for TSPO is visible in cytoplasm of Leydig cells (arrows) and cytoplasm of Sertoli cells (open arrows). (C and I) Note reduced expression of StAR in cytoplasm of SD Leydig cells when compared to LD ones (arrows). Expression of INSL3 is of various intensity in individual Leydig cells of LD and SD testes (D, J) while expression of RLN is weak to very weak in cytoplasm of Leydig cells of both groups (E, K), (arrows). (F, L) No positive staining is visible when primary antibody is omitted.

In negative controls of control and experimental testicular sections no positive staining was observed (*Fig. 7F*, *7L* and *Fig. 8F*, *8L*).

Effect of estrogen-related receptors blockage or activation on testosterone and estradiol concentration in testes of bank voles

After treatment with XCT 790 testosterone level within the testes decreased in both LD ($P < 0.05$) and SD males when compared to respective controls (*Fig. 9A*). Similar decrease ($P < 0.001$) in testosterone concentration was revealed in SD DY131 exposed males. In LD DY131 voles marked increase was noted ($P < 0.001$) when compared to control. Treatment with XCT 790 and DY131 decreased estradiol concentration in all treated groups with exception of SD DY131 voles where pronounced increase when compared to control ($P < 0.01$) and to LD DY131 males ($P < 0.001$) was found (*Fig. 9B*).

Effect of estrogen-related receptors blockage or activation on cAMP and Ca^{2+} level in testes of bank voles

In the testicular homogenates of LD and SD bank voles, control and treated with XCT 790 and DY131, significant differences in the levels of both cAMP and Ca^{2+} were found (*Fig. 10A* and *10B*). In SD voles, cAMP level was significantly lower ($P < 0.001$) than in LD ones. After treatment with XCT 790 or DY 131 the level of cAMP was always decreased ($P < 0.01$) in LD males while it was increased ($P < 0.01$) in SD ones (*Fig. 10A*). The same tendency like in control LD and SD animals was revealed in males treated with XCT 790 where the cAMP level was lower ($P < 0.01$) in SD group in comparison to LD XCT 790. In contrary, after treatment with DY131 in SD voles cAMP level was increased ($P < 0.01$) while in LD was decreased ($P < 0.01$).

In testes of LD voles, the level of Ca^{2+} was higher when compared to SD ones (*Fig. 10B*). Treatment with XCT 790 or DY131 did not change Ca^{2+} level in LD animals. In SD treated

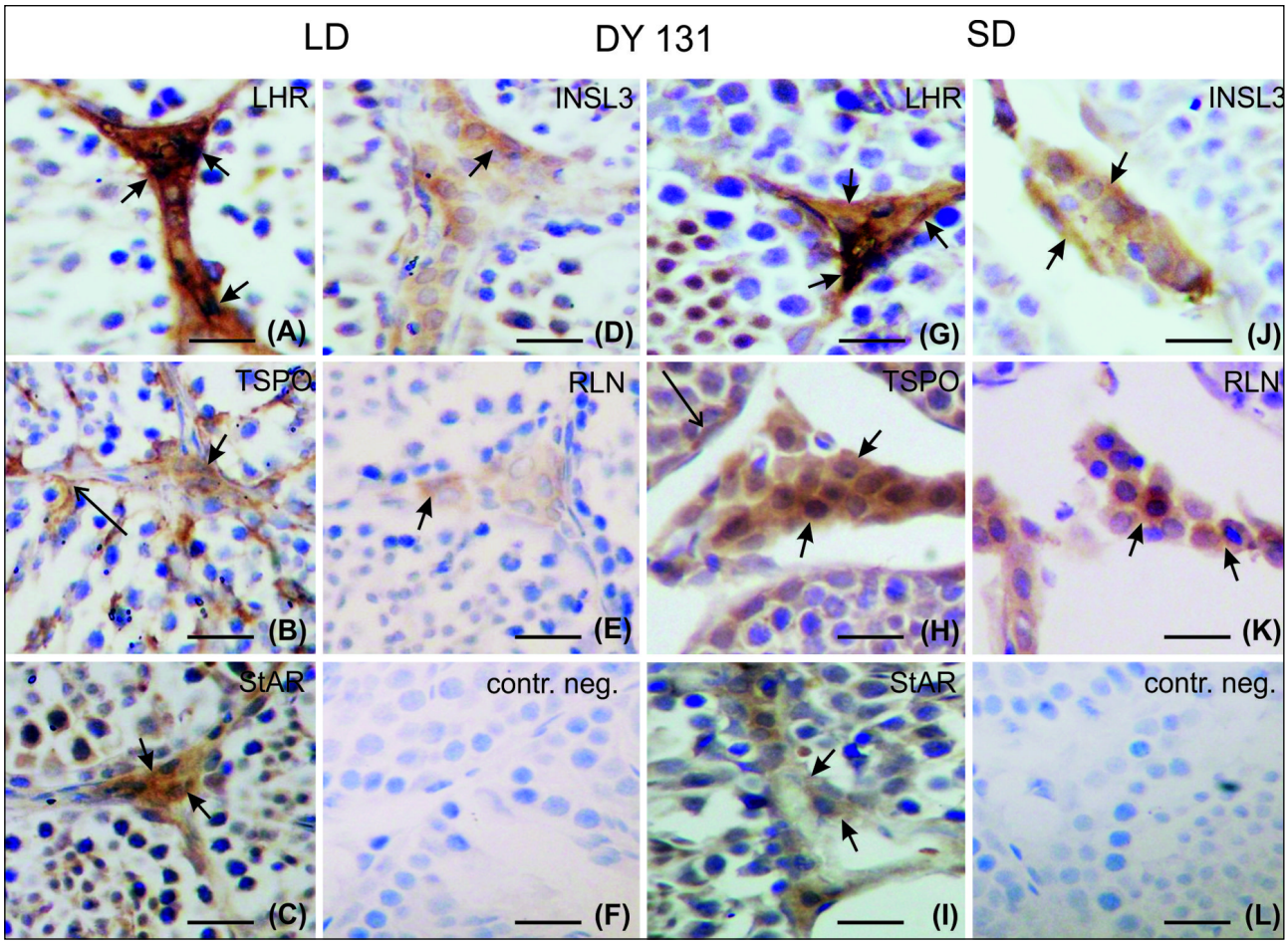

Fig. 8. Effect of ERRβγ activation on expression of steroidogenic proteins and secretory proteins in bank vole Leydig cells (A-L). (A-F): testes of LD and (G-L) SD DY131-treated males. (A and G): localization of LHR; (B and H): localization of TSPO; (C and I): localization of StAR; (D and J): localization of INSL3; (E and K): localization of RLN in testes of LD and SD males, respectively. Immunostaining with DAB and counterstaining with hematoxylin. Scale bars represent 15 µm. From each animal 3 – 5 serial testicular slides were analyzed. (A and G) Weak signal for LHR in membrane of Leydig cells of both LD and SD testes is seen (arrows). (B and H) Strong to moderate expression of TSPO in cytoplasm of Leydig cells of LD and SD testes is visible (arrows). TSPO exhibits signal also in cytoplasm of Sertoli cells of seminiferous tubules (open arrows). (C and I) Strong expression of StAR in cytoplasm of LD Leydig cells is noted while in SD ones the expression is moderate to weak. (D and J), (E and K) Note similar intensities of INSL3 (D and J) and RLN (E and K) immunostaining in cytoplasm of Leydig cells of both LD and SD groups (arrows). (F, L) No positive staining is visible when primary antibody is omitted.

males the Ca*2+* concentration was always significantly lower ($P < 0.05$, $P < 0.01$, respectively) than in LD exposed males. The most significant decrease ($P < 0.01$) was observed in SD XCT 790 males.

DISCUSSION

In testis physiology, functional studies of ERRs have been absent thus far. Recent evidences have revealed important roles for ERRα and ERRγ in regulating mitochondrial and lipid metabolism in hepatocytes, cardiomyocytes, adipocytes and other cells (12, 45-48). In mouse intestine ERRα controls lipid uptake (48). On the other hand, ERRγ regulates mainly cardiovascular system, and its loss results in a reduced ventricular mass and increased mitochondrial DNA (49). Little is known about the physiological role of the ERRβ due to the confounds of mid-gestational lethality and placental defect in knockouts (50). Concomitantly, that result is an initial evidence on ERRβ involvement in a control of the function of reproductive system. However, considering similarity of ERRs, ERRβ can play a yet unappreciated role in metabolic function. Of note, new dimensions to the functions of ERRs have been recently discovered in *Drosophila melanogaster* (51). Knockout males showed developmental disturbances of testes and serious anomalies in spermatozoa. By Northern blot and reverse transcription-PCR, ERRs transcripts were detected in various fetal and adult human tissues and organs, including prostate, with particularly high levels in brain, gastrointestinal tract, heart, kidney, skeletal muscle, and placenta (50, 52, 53). Similar ERRs expression patterns were identified in mouse and rat adult tissues (54). To our knowledge no reports demonstrating ERRs mRNA level and protein expression and the relation between ERRs function and endogenous estrogen concentration as well as its impact on steroidogenic and secretory proteins in testicular Leydig cells are available at present. Herein, α, β and γ types of ERRs at mRNA and protein level in the vole Leydig cells with respect to reproductive system activity is reported. Cytoplasmic localization of ERRs similarly to ERs can be linked to a dynamic equilibrium

between cytoplasm and nucleus thus ERs are not detected at all times in a single subcellular compartment, in particular when hormonal milieu is changing (55).

No spermatogenic defects but those relevant to interstitial tissue alterations were found in males administered with XCT 790 and DY131. This result clearly shows high importance of especially ERRα, but also ERRβγ, signaling in relation to endogenous estrogen level for architecture of interstitial tissue compartment. Moreover, histological changes in the interstitial tissue may have a close relationship with steroidogenic and secretory function of Leydig cells.

In this study we hypothesized that limited blockage of ERRα by XCT 790 antagonist and activation of ERRβγ by DY131agonist may have a direct effect on Leydig cell function and may precede a percussion on cell ultrastructure. Our detailed analysis of Leydig cells in transmission electron microscope showed differences in

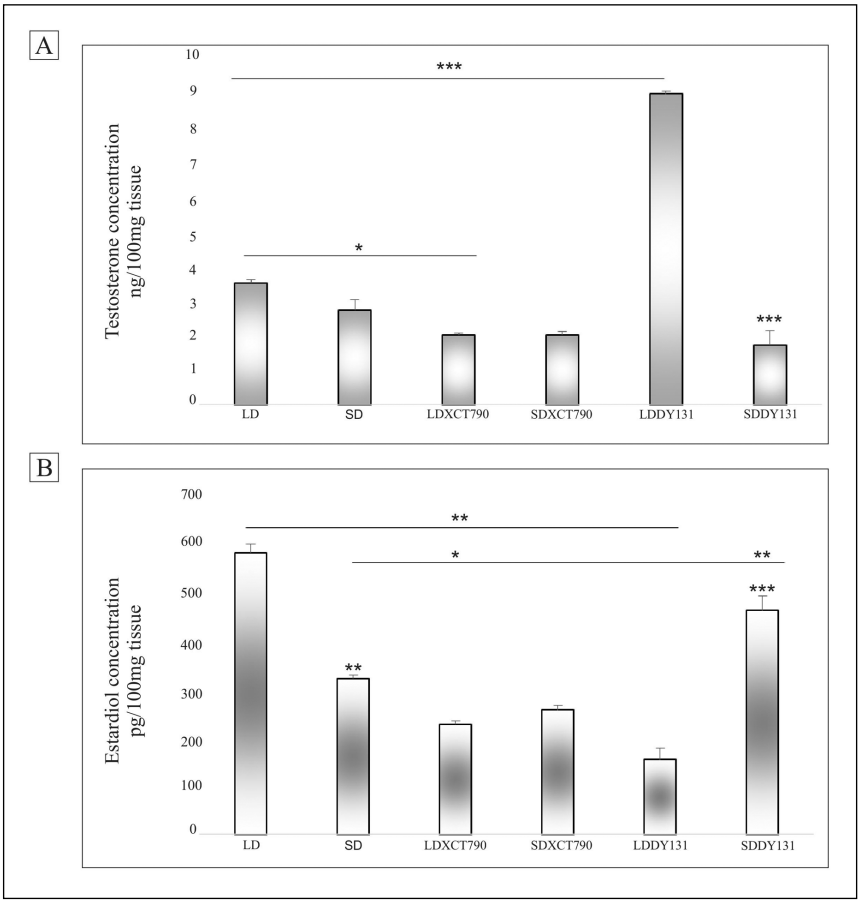

Fig. 9. Effect of ERRs blockage or activation on estradiol concentration in testes of bank voles. Data from three separate analyses is expressed as means ± S.D. From each animal at least three samples were measured. Asterisks show significant differences between testosterone and estradiol concentrations in control and XCT790 or DY 131 treated males. Values are denoted as *P < 0.05, **P < 0.01 and ***P < 0.001.

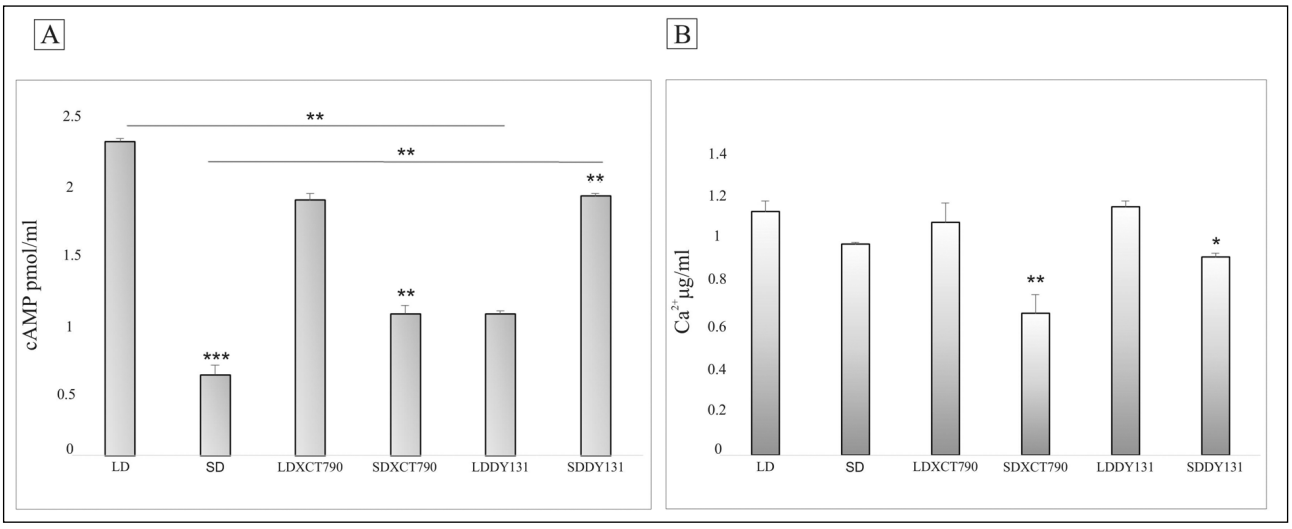

Fig. 10. Effect of ERRs blockage or activation on cAMP and Ca^{2+} level in testes of bank voles. Data from three separate analyses is expressed as means ± S.D. From each animal at least three samples were measured. Asterisks show significant differences between in cAMP and Ca^{2+} level in control and XCT790 or DY 131 treated males. Values are denoted as *P < 0.05, **P < 0.01 and ***P < 0.001.

number and structure of organelle after ERRs activity modulation (blockage or activation). Significant changes in ER volume and Golgi complexes (Gc) were observed during development of rat liver (56). Almost all of the proteins that are secreted to the cell exterior plus those destined for the lumen of the ER, Gc, or lysosomes are initially delivered to the ER lumen (57). The ER is also the principal organelle involved in Ca^{2+} homeostasis (58). Ultrastructural results of the present study indicate on interplay of ERRs signaling with regulation of the cell architecture and modulation of the function under various endogenous estrogen microenvironment. Estrogens have cell-specific effects on a variety of physiological endpoints including regulation of mitochondrial biogenesis and activity. The identification of estrogen receptors within mitochondria of various cells and tissues reflects estrogen control of the mitochondrial genes expression (59). In our recent work, we have reported modulation of arrangement and number of mitochondria in Leydig cells exposed to mixture of xenoestrogens (25). Differences in the number of these organelle can be based on mitophagy. Hence, possible estrogen signaling modulation may be reflected in changes in mitochondrial transcription control. Although deprivation/stimulation of ERRs (α, β, γ) signaling consistently effected steroidogenic Leydig cell function, it caused diverse repercussion in secretory activity of these cells. It seems possible that normal level of estrogens prevents against lack of ERRα signaling, as no dramatic change was revealed in function of steroidogenic genes. In contrary, changes in *RLN* expression showed its being beyond estrogen control and/or being related to compensation of ERRα absence. Additionally, paracrine or autocrine RLN action on ERRs should not be excluded. In *Danio rerio* RLN has input on estrogen production thus in this way controls follicle development and steroidogenic function (60). The important role of insulin-like peptides in control of ovary steroidogenesis has been described in insects too (61). Interestingly, deprivation of ERRα signaling influenced *TSPO* and *StAR* expression and this was referred diversely to various endogenous estrogen concentration. Thus, in voles *TSPO* and *StAR* seem to be differently regulated by ERRα and estrogens. In contrary, lack of ERRβγ signaling markedly effected *LHR* and *TSPO* expression independently on estrogen level while, in SD males, *StAR* and *INSL3* and slightly *RLN* exhibited modulation of their expression additionally related to endogenous sex hormones concentration. This result points out very distinct and complex estrogen regulation of *StAR* and insulin-like peptides together with estrogen interplay with different types of ERRs in the Leydig cells of bank voles. Unique sensitivity as well as diverse cell response and pattern of protein expression in testes of voles with various hormonal status was demonstrated in voles treated with xenoestrogen (4-*tert*-octylphenol) where variations in expression of steroidogenic enzymes (hydroxysteroid dehydrogenase and aromatase) and receptors (androgen and estrogen receptors) as well as junctional proteins were demonstrated (38, 62). Undoubtedly, level of sex steroids interplays with proper or disturbed microenvironment of internal organs. It is well-known that estrogens diversely influence synaptic plasticity, neurotransmission, neurodegeneration, and cognition in male and females neuronal cells (63). Also, bone remodeling, is under specific control of estrogens and androgens in both sexes being related to the age, too (64).

ERRs regulate cell transcription in a similar manner to ERs, *via* a tethered pathway, by interacting with the Sp1 and AP-1 transcription factors at their respective DNA binding sites (13). Therefore, it is likely that ERRs may control a broad spectrum of genes in their target cells. Likewise estrogen responsive element (ERE), ERRs can also bind to a number of estrogen receptor-related responsive element-related sequences (ERRE) that is also true for nonmammalian organisms like Zebrafish and *Drospohila melanogaster* (65). Currently, ERRγ own distinct target genes e.g. ERRα, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX-1), and small heterodimer partner SHP have been characterized (66, 67). Based on initial data from the present study regulation of genes for LHR, TSPO, StAR as well as INSL3 and RLN by ERRs need further detailed consideration. Subsequently, understanding of mechanisms of diverse and/or reverse protein expression during lack of ERRs will be a future issue to resolve. One the other hand, localization of exanimated here steroidogenic and secretory proteins was revealed regardless to vole reproductive status and without/or with performed ERRs arrest or stimulation. Only presence of TSPO was observed besides Leydig cells in basal region of Sertoli cell cytoplasm. In control as well as in treated males, expression at protein level of steroidogenic and secretory proteins differed sometimes from their mRNA level. It should also be added here, that cell protein production is based on the level of molecular efficiency and control of gene expression, protein production machinery as well as organelles function (especially Golgi complexes), being sometimes inconsistently reflected. Protein degradation and its changes in response to cell physiological condition can be another preponderant cause of observed differences at transcription and expression level. Interestingly, in human skin cells, the presence of ERRβ and ERRγ is linked to StAR activity regulation and cutaneous estrogen signaling in this tissue (68, 69). Expression of LHR in Leydig cell surface differed especially between LD and SD voles that is in line with variable vole Leydig cells steroidogenic efficiency previously reported by us (70). Such mechanism is also possible for StAR and TSPO. Results of this study are in line with those by Chen *et al*., (71) who demonstrated modulation of TSPO and StAR by estradiol through protein kinase A signaling in hypothalamic astrocytes. On the other hand, in the testis, TSPO-StAR complex is a well-described target for endocrine disrupters action also those with estrogenic properties (72, 73).

Well-known marker of Leydig cells functionality, INSL3, participates in a control of transabdominal testis descent (74) but the specific role of the RLN in the testis and Leydig cells is still not clarified (75). Regulation of RLN secretion and action by estrogens in Leydig cells has never been studied before. Inactivation of RLN gene results in the interstitial tissue overgrowth based on not fully discovered mechanism (76). INSL3 signalization *via* its specific receptor increases cAMP level and steroid secretion (77). Lague and Tremblay (78) showed that estrogens repress INSL3 promoter gene activity in MA-10 Leydig cells. Therefore, estrogenic regulation of INSL3 and RLN and its interplay with other proteins in Leydig cells is an interesting issue. Higher level of RLN in SD males than LD ones and reversed changes in its expression after ERRs activity modulation can be related to sort of compensatory mechanism, but also possible function exchanging mechanism or its overlapping existing between insulin family peptides in various cells and tissues (75). However, weak expression of *INSL3* and *RLN* in control and treated males reflects very low production of insulin family peptides in voles. In mice, microarray hybridization (GEO database) generally fails to indicate RLN mRNA signals in the testes, although specific RT-PCR did identify testicular RLN mRNA (79). In human, INSL3 shows high expression in fetus Leydig cells and mature ones, thus it is powerful sentinel biomarker for the effects of gestational exposure to hormonally active chemicals (80). Importantly, INSL3 is expressed constitutively and is acutely independent of the hypothalamic-pituitary-gonadal axis but it only depends on Leydig cell number which is rather small in rodents compared to human.

It is worth mentioning here that Robert *et al.*, (81) have identified the orphan nuclear receptor, nerve growth factor IB (NR4A1) as a novel regulator of INSL3 transcription in Leydig cells of mouse and human. It was co-expressed with INSL3 in

purified Leydig cells and in several Leydig cell lines. Finally, they found that NR4A1 is also implicated in cAMP-induced INSL3 transcription in Leydig cells. Last founding by Abdou *et al*., (82) has indicated on increased of NR4A1 expression in MA-10 Leydig cells by Ca^{2+} signaling. However, recent study in pigs tends to support the testes, as the major site of RLN production, as both RNA transcript and protein were detected in Leydig cells during porcine postnatal development (83). In rats, locally produced RLN in seminiferous tubules, acts as an autocrine or paracrine agent in the testis and vas deferens to affect spermatogenesis and seminal fluid composition (84). Furthermore, RLN is able to induce an increase in spermatozoa motility, intracellular Ca^{2+} and cAMP levels and acrosome reaction (85). Nonetheless, the potential roles of testicular RLN in male reproduction are still undiscovered, despite various knock-out studies conducted in rodents (75).

Adjustment of sex steroid biosynthesis in bank voles was detected after ERRs blockage or activation. Blockage of ERRα seems to inhibit steroid biosynthesis independently on endogenous level of steroids while activation of ERRβγ stimulates testosterone synthesis in LD voles and estrogen synthesis in SD ones. Inhibition of steroid biosynthesis in voles with modulated ERRs activity was clearly reflected by decreased expression of LHR, TSPO and StAR genes. It is possible that the leading role in steroid synthesis regulation plays LHR-StAR interaction with ERRβγ as expression of LHR and StAR was dramatically changed especially in SDDY131 males. Studies showed that disturbed action of StAR cannot be compensated by TSPO or other proteins causing always steroidogenesis failure (86). Thus, ERRβγ is possibly more important than ERRα for steroidogenesis regulation, being dependent on hormonal status of the voles. In Leydig cells, steroid hormone synthesis is finely regulated to ensure adequate amount but also to avoid conditions of hormone insufficiency. Estrogens overproduction as well as their lack lead to severe consequences in Leydig cells and other testicular cells function (87). Inadequate levels of sex steroid hormones are associated with various pathological conditions including autoimmune distinct Leydig cell populations: fetal and adult Leydig cells (88, 89). Hence, changes in secretion of steroid hormones indicate on involvement of ERR signaling in this process *via* modulation of function of steroidogenic factors like LHR, TSPO and StAR. Especially, the latter one showed significant differences between DY131 treated LD and SD males. In mouse Leydig cells, StAR level correlates with steroid hormone concentration (90). Chen *et al*., (91) reported that blocking of intracellular Ca^{2+} efflux by sodium orthovanadate, a Ca^{2+} pump inhibitor, blocks cAMP, progesterone secretion and StAR promoter activity in tumor Leydig cells-exposed to phytoestrogens. This is in accord with our results showing Ca^{2+} and cAMP levels decrease after DY131 treatment, suggesting alterations in estrogen signaling in treated voles. In fact, estrogens were found to increase cAMP level in breast cancer and uterine cells *in vitro* and in the intact uterus *in vivo* (92).

The increase in the intracellular cAMP levels, a transient increase in cytoplasmic Ca^{2+} concentration following LH stimulation is essential for proper steroidogenesis. It should be added here, that unlike in most of mammals in seasonal breeders such as bank voles spermatogenic testis function is also under LH control through sex steroid hormones but not foliculotropin (93). Herein, findings additionally support this thesis, as significant changes in LHR at both mRNA and protein level were found in control and treated voles. Murine Leydig cells contain two L-type Ca^{2+} channel receptors: ryanodine receptors (I, II and III) and the inositol triphosphate receptors I, II and III. Both types of receptors are required for LH-induced rise of cytoplasmic Ca^{2+} levels. Ca^{2+} signaling activates downstream of cAMP essential for maximal hormonal responsiveness (94). Then, cAMP in Leydig cells provides alternate signaling routes leading to proper gene expression in response to it. Estrogens are regulators of cellular Ca^{2+} level. Indeed, for many years, estrogenic therapy has improved Ca^{2+} balance in patients with postmenopausal osteoporosis (95). Decrease of Ca^{2+} level in XCT 790 treated animals indicates on direct regulation of Ca^{2+} level by ERR and estrogen signaling. As similar changes were observed also in cAMP level, it seems likely that in Leydig cells, ERRα regulates cAMP and Ca^{2+} level *via* different mechanism than ERRβ and γ. Action of ERRs, besides genomic route, directly *via* cAMP and Ca^{2+} signaling pathways cannot be now excluded.

Modulation of expression of ERRs at molecular and hormonal level can allow to maintain Leydig cell morfo-functional status and/or avoid and/or treat its disturbances opening new prospects for therapeutic intervention as it has recently been shown for other functional molecules in testicular and ovarian steroidogenic cells as well as pituitary cells (96-99).

In conclusion, these results illustrate that the ERRs signaling, especially ERRα, is essential to couple Leydig cell tissue architecture together with cellular metabolism (secretion of steroid and protein hormones) in order to maintain normal Leydig cell function in bank voles of various sex hormonal status. ERRs differentially and complexly regulate responsiveness of vole Leydig cells *via* modulation of transcription of ERRs-related genes and expression of their proteins; LHR, TSPO, StAR as well as INSL3 and RLN, that was mostly observed through ERRβγ activation. Similarly, mostly ERRβ/ERRγ act through or/and is involved in modulation of signaling molecules concentration and sex steroid secretion. It should be highlighted, that given the multiple of ERRs action and regulation further detailed analyses are desire to identify conditions, processes and targets of ERRs control of Leydig cell physiology and predicting fertility disorders.

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