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Lessons from the gonadotropin-regulated long chain acyl-CoA synthetase (GR-LACS) null mouse model: A role in steroidogenesis, but not result in X-ALD phenotype☆

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ABSTRACT

Gonadotropin-regulated long chain fatty acid Acyl-CoA synthetase (GR-LACS), is a member of the LACS family that is regulated by gonadotropin in the rat Leydig cell (LC). Its mouse/human homologs, lipidosin/bubblegum, have been suggested to participate in X-linked adrenoleukodystrophy (X-ALD), an adreno/neurodegenerative disorder with accumulation of very long chain fatty acids (VLCFA) in tissues and plasma. To further gain insights into its regulatory function, a GR-LACS/lipidosin null mouse was generated. No apparent phenotypic abnormalities were observed in the X-ALD target tissues (brain, testis, adrenal). Nuclear inclusions seen in mice >15 month-old, were present in LC of 9 month-old GR-LACS$^{-/-}$ mice. LC of the null mice showed refractoriness to the gonadotropin-induced desensitization of testosterone production that is observed in adult animals. LCFAs were moderately increased in the testis, ovary and brain, but not in the adrenal gland of GR-LACS$^{-/-}$ mice, with no major changes in VLCFA. No change in LACS activity was observed in these tissues, suggesting a compensatory mechanism exhibited by other LACS members. The GR-LACS$^{-/-}$ model did not support its association with X-ALD. These studies revealed a role of GR-LACS in reducing the aging process of the LC, and its participation in gonadotropin-induced testicular desensitization of testosterone production.

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1. Introduction

Fatty acyl CoA synthetases (FACS) are essential in the of CoA thioesterification of fatty acids, which are substrates for β-oxidation and phospholipid biosynthesis. These enzymes are also involved in protein/lipid transport, enzyme activation, protein acylation, cell signaling and transcription. Acyl CoA synthetases (ACS) are classified based on their specificities for the carbon chain length of the fatty acids substrates: ACSS, short chain (S) (C2-C4); ACSM, medium chain (M) (C6-C12); ACSL/LACS, long chain (L) (C12-C20) and ACSVL/VLACS, very long chain (VL) (C> = 22). Six ACSL genes, *ACSL1 and ACSL3-ACSL6* [1] and the *Acsl*/*GR-LACS*/*liposin*/*bubblegum* genes [2] have been identified. However, related family members show considerable overlap in chain length specificity [3]. ACSs contain two domains, the AMP-binding domain highly conserved in all species and a fatty acid-binding signature motif which participates in fatty acid binding. More recently, the long chain ACSs (ACSL/LACS) and very long chain ACSs (ACSVL/VLACS) relevance has been greatly enhanced because of their major importance in cell biology. Long chain fatty acids (LCFA) (C12-20) are activated by acyl-CoA synthetases present in mitochondria and perixosomes, where they undergo β-oxidation, and in microsomes for utilization in the synthesis of complex lipids. Long chain fatty acid esters in addition to serve as essential intermediates in lipid biosynthesis contribute to many cellular functions including regulation of ion fluxes, protein kinase C subtypes, of transcription and gene expression.

Members of the VLACS family, also known as fatty acid transport protein (FATPs), or solute carrier family 27 (SLC27), have acyl-CoA synthetase enzymatic activity and some of its members have dual functions in esterification of substrates and transport of fatty acids [4,5]. The enzymes are present in perixosomes, microsomes and plasma membrane. Accumulation of very long chain fatty acids (VLCFA) was found to be associated with X-linked adrenoleukodystrophy (X-ALD) [6]. X-ALD is a metabolic genetic disorder related to impaired degradation of VLCFA, with progressive central nervous system demyelination and adrenal insufficiency. Mutations of ABCD1 gene, which encodes a peroxisomal membrane ATP binding cassette (ABC) transporter result in various forms of phenotypic

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progression of neurological degeneration [6]. Although many tissues show accumulation of VLCFA, only the nervous system, adrenal glands, and testis are pathologically affected in these patients, with highly variable phenotypes.

Gonadotropin-regulated long chain fatty acid Acyl-CoA synthetase (GR-LACS), cloned in our laboratory from a rat cDNA Leydig cell library, is a distinct member of the ASCL family [7] capable of activating long chain fatty acids. It is transcriptionally regulated by gonadotropin in rat testis. GR-LACS is a 79 kDa protein that displays high similarity (>95%) with mouse lipidosin [8] and human bubblegum [9], which are products of the same gene subfamily in different species. GR-LACS contains the two characteristic domains of the ACS and luciferase super-families but shares only 23–28% similarity with other family members [7]. Rat GR-LACS and mouse lipidosin were found to have long chain ACS activity (ACSL, C12-20) [7,8]. The GR-LACS gene spans over 45 kb and is encoded by 14 exons. Exons 7 and 11 comprise the conserved ATP/AMP binding domain and the FACS signature motif, respectively [2]. GR-LACs expression is tissue, cell and species-specific [10]. It is expressed in rat and mouse gonads and brain, and only in the mouse adrenal gland and is found at cytoplasmic sites. In the ovary of both species it is associated with follicles undergoing atresia. The mRNA of this gene is expressed in the immature and prepubertal testis tubules, where it is poorly translated and low levels of GR-LACS protein are detected. In the adult testis, it is expressed in the Leydig cells and minimally in the tubules [10]. Since GR-LACS/lipidosin is constituitively expressed in Leydig cells and regulated by gonadotropin we hypothesize that this gene could affect biosynthesis of androgen in the male gonad.

In the brain [10], GR-LACS is predominantly concentrated in the hypocamppus including the dentate gyrus, thalamus and piriform cortex, and in the Purkinje cells of the cerebellum. A 64 kDa GR-LACS protein species lacking exons 6–8 that was more abundant than the 79 kDa long form was found in the rat brain but not in the mouse brain. Also, a minor 73 kDa species lacking exon 8 was present in rat brain and mouse ovary. Both alternatively spliced forms were devoid of fatty acyl CoA synthetase activity, suggesting that exon 8 is essential for the enzymatic function. These variants exhibit dominant negative effects on the FACS activity of the 79 kDa form, indicating that the short forms acting probably through oligomeric/dimer formation may regulate the long form's activity in the brain.

A mutant of the Drosophila melanogaster bubblegum gene, a member of the ACS family, was reported to display an increase in VLCFA and neurodegeneration [11]. In contrast to the rat GR-LACS [7] and mouse lipidosin [8], which exhibit ACSL/LACS activity, human bubblegum was shown to possess ACSVL/VLACS activity and was initially believed to predominantly activate VLCFA. This led to its given alias of "bubblegum" for the human form (although the fly form has only 50% similarity to human bubblegum and rodent GR-LCAS/lipidosin) and to the proposal of its role in X-linked ALD. However, much of the existing evidence predominantly derived from indirect studies, has questioned the involvement of ACSL in this disorder. Subsequent studies reported that this gene (human bubblegum) has a significant role in LCFA utilization and only a minor role in VLCFA metabolism in neuro2a cells [12]. Furthermore, human bubblegum was found in small vesicular structures that co-sedimented with mitochondria. Other studies indicated its VLACS function and localization mainly in microsomes and/or microsomal-like structures [13,14]. Mice lacking ABCD1 (X-ALD protein) exhibited elevated levels of VLCFAs in tissues with decreased VLCFA ß-oxidation in fibroblasts. However, inactivation of the murine ABCD1 gene did not lead to a clinical phenotype resembling the childhood cerebral X-ALD [15–17], and only caused mild neurological and behavioral abnormalities, similar to the adrenomyeloneuropathy (AMN) adult form of X-ALD [18]. Further observations showed that peroxisomal VLACS activity is normal in X-ALD mouse liver and the VLCFA ß-oxidation is normal in all tissues examined [19]. The assumption that human bubblegum is related to the X-ALD syndrome has been further challenged by the finding that no difference of GR-LACS/lipidosin/bubblegum mRNA level was present in the brain of wild type and ABCD1 knockout mice [13,14]. Further, of unquestionable importance indicating the non-participation of GR-LACS/lipidosin in X-ALD, was their lack of VLACS activity [7,8].

To conclusively determine the involvement of GR-LACS/lipidosin/human bubblegum in adrenoleukodistrophy, and to gain insights into its physiological function and its role in testicular steroidogenesis, we generated a GR-LACS/lipidosin/bubblegum knockout model in mouse. Our examination of the phenotype of this null model, demonstrated that GR-LACS/lipidosin/bubblegum is not involved in X-ALD, but has role a role in gonadotropin-induced desensitization of testosterone production in Leydig cells.

2. Materials and methods

2.1. Animal

Adult wild type ($^{+/+}$) and GR-LACS targeted ($^{+/-}$; $^{-/-}$) mice were housed in pathogen-free, in constant temperature (22 C) and light-controlled conditions. All animal studies were approved by the National Institute of Child Health and Human Development Animal and Care and Use Committee. Animals were killed by asphyxiation with CO_2 and decapitated. Organs were removed for gross and histological analysis.

2.2. Generation of GR-LACS null mice

The *GR-LACS* gene of 14 kb KpnI fragment containing the 5′ flanking DNA sequence, exon and intron 1 sequences (Accession # DQ009013) and 4.3 kb KpnI fragment containing exons 3–4 and the adjacent introns 2–4 sequences (Accession # DQ009015; DQ009016) was isolated using a rat GR-LACS cDNA probe from a mouse genomic BAC library derived from the ES 129/SVJ strain (Genome Systems, Inc, St. Louis, Mo). These two KpnI genomic fragments were used to generate 5′ and 3′ homologous arms in the targeting pPNT vector [20]. The 3.2 kb 5′ homologous arm was generated by PCR with primers located at −3326/−3314 tailed with Not1 and −104/−88 tailed with Kpn1/Xho I using 14 kb KpnI as the template. The 3′ homologus arm is a 2.6 kb BamH I/EcoR V fragment digested from 4.3 kb Kpn I fragment which contains exon 3 and adjacent intron sequences. The GR-LACS-pPNT (20 μg) was linearized at the NotI site and electroporated into 129/SVJ (R1) embryonic stem (ES) cells. Transformants were selected in G418 and Ganciclovir. The resistant colonies were isolated and screened by Southern analysis. Homologous recombinant ES clones were identified with KpnI digestion. Targeted ES clones were used to generate germ line GR-LACS$^{+/-}$ and GR-LACS$^{-/-}$ in C57BL/6 mice. Genotype screening of the offspring mice for the presence of the GR-LACS$^{-/-}$ allele were performed by PCR and verified by Southern analysis. PCR primers (P1 and 2) were derived from mouse GR-LACS genomic intron 2 DNA sequence (P1: TCCACAGACAGGTGACTAA; p2: CACTGAACATGGTTTGAGC). Primer 3 (P3: AGTACTGTGGTTTCCCAAA TGTGT CA) was derived from Neo gene in pPNT.

2.3. Northern and Western blot analyses

Poly (A) RNA samples (5 μg) extracted from adult (16 week-old) mice testis (GR-LACS$^{+/+}$, GR-LACS$^{+/-}$, GR-LACS$^{-/-}$) were resolved on 1% agarose gel and hybridized to full length GR-LACS cDNA probe. Protein samples (20 μg) extracted from adult mice tissues were

subjected to Western blot analysis using GR-LACS antibody [7] and β-actin for normalization (Sigma).

2.4. Gross and histological analyses

Body and organs weight of wild type (WT) and GR-LACS heterozygous and knockout (KO) mice from different ages were recorded. For histological analysis, testes, brain, adrenal gland and ovary and several other tissues from GR-LACS (+/+ and −/−) mice were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin-eosin (H&E). Testes, brain, adrenal gland and ovary for electron microscopic (EM) analysis were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, en bloc stained with 2% uranyl acetate, and subsequently dehydrated and embedded in Spurr's epoxy. Thin-sections were post-stained with lead citrate. For fertility testing, 8 weeks of wild

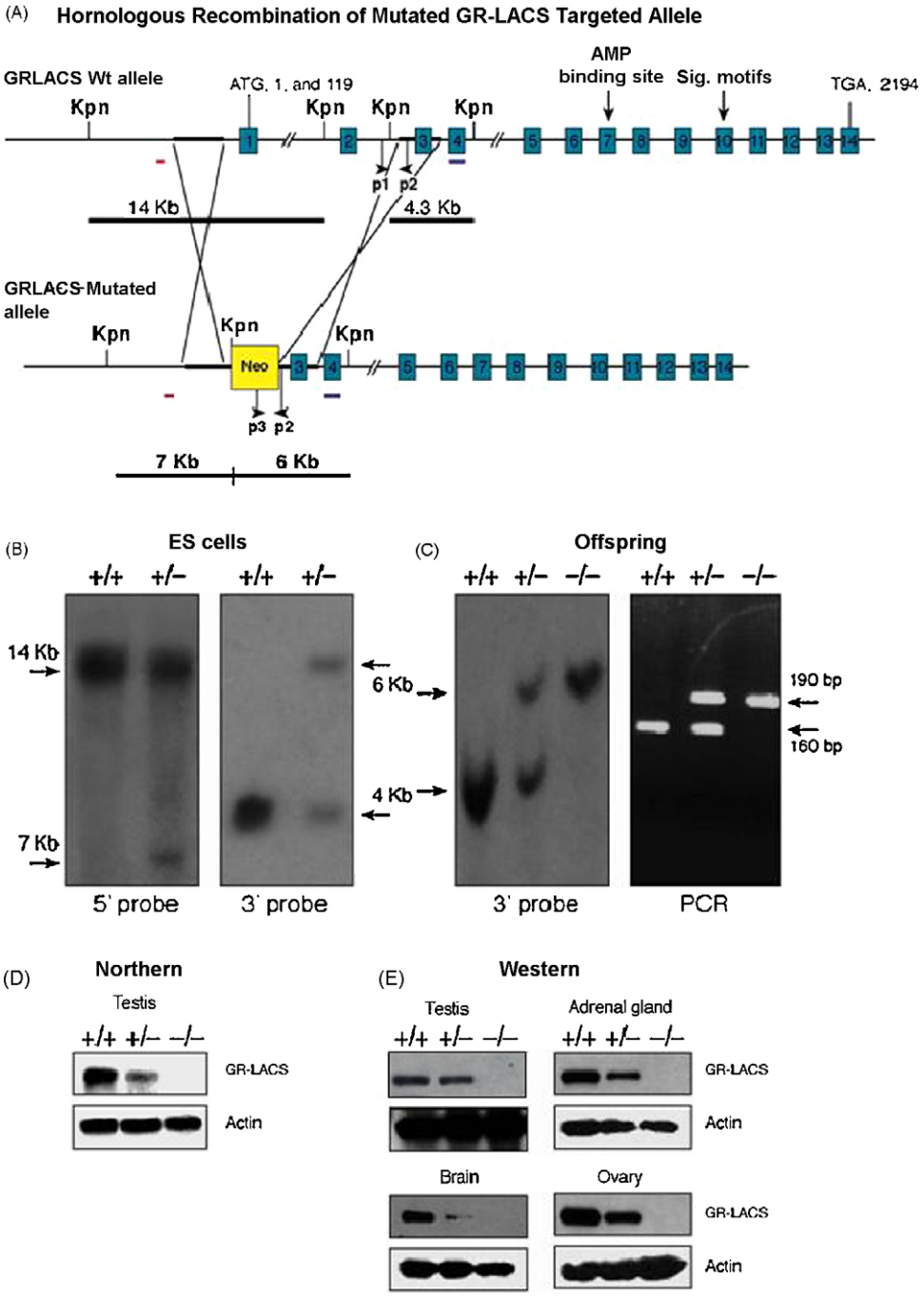

Fig. 1. Target disruption of GR-LACS gene (A) Top: wild type mouse GR-LACS gene allele. ATGs. Bold line indicates the homologus 5′ arm (3.2 kb PCR product: −3326/−88 bp upstream to ATG codon) and the 3′ arm (2.6 kb BAMHI/RV fragment with exon 3, 5′ and 3′ intron sequences) were subcloned at Not1/Xho and BAMHI/R1 site, respectively in the targeting vector pPNT with (Neo) and (TK) selection markers. Bottom: the GR-LACS mutated homologus recombinant allele. 5′ (red line, −4406/−3918 bp PCR product) and 3′ (blue line, exon 4) probes used for Southern analysis. PCR primers for genotype screening (p1-p3). (B) Screening ES clones by Southern analysis. Positive homologous recombinant clones were identified with KpnI digestion [predicted size: wild type with 5′ probe (14 kb) and 3′ probe (4.3 kb); recombinant allele with 5′ probe (7 kb), and 3′ probe (6 kb). (C) Genotyping of offspring mice by Southern analysis with 3′ probe (left panel). PCR (right panel), predicted size: GR-LACS$^{+/+}$, 160 bp (p1/p2), $^{+/-}$, 160 bp (p1/p2) and 190 bp (p2/p3), and $^{-/-}$ mice, 190 bp (p2/p3). Northern analysis of testis full length GR-LACS transcript (2.7 kb) (D) and Western (E) of tissues (testis, adrenal gland, brain and ovary) from adult GR-LACS$^{+/+}$, $^{+/-}$ and $^{-/-}$ mice (GR-LACS protein, 79 Kd).

type or GR-LACS null males were mated with C57BL/6 proven-breeder females, also GR-LACS null female were set up with C56BL/6 males. To determine total sperm counts, the cauda epididymis from 8 week-old wild type and GR-LACS null mice were cut into small pieces and placed into 2 ml 1X PBS at 37 C for 20 min. Sperm number was determined using the hemocytometer.

2.5. Animal treatment, Leydig cell (LC) preparation and hormone measurement

To cause gonadotropin induced down-regulation of testosterone production [21–26], adult male mice were treated by subcutaneous injection of hCG (Pregnyl, Organon, West Orange, NJ) 5 IU (equivalent to 0.5 μg of purified hCG) in 100 μl of phosphate-buffered saline, pH 7.4. Animals were sacrificed by asphyxiation with CO_2 and decapitated 24 h after hCG treatment unless otherwise indicated. Serum was collected for hormone measurements. Leydig cells were prepared by collagenase dispersion and purified by centrifugal elutriation, as previously described [27] and further incubated *in vitro* for 3 h in medium 199/0.1%BSA. Media were collected and analyzed for testosterone production. Samples (serum and media) were measured by ELISA kits from ALPCO (Windham, NH).

2.6. Analysis of fatty acyl-CoA synthetase (FACS) activity

FACS activities were determined in cell extracts as described previously [7] by using the following isotope-labeled fatty acids as substrates (Sigma): medium chain fatty acid - C^{14} decanoyl acid (C10:0) - also known as capric acid, long chain fatty acid - 9,10(n)-^{3}H palmitoyl acid (C16:0) and very long chain fatty acid - 1-^{14}C lignoceric acid(C24:0). The labeled acyl-CoAs were quantitated and normalized by the protein concentration and/or presented as fold-change from controls. All individual experiments were performed three times in triplicate.

2.7. Lipid analysis

Tissues (testis, ovary, adrenal and brain) were harvested from adult mice (wild type and KO) and used for fatty acid analyses. Total composition of lipids in these tissues ($n = 8$ mice) were analyzed by capillary gas chromatograph equipped with glass capillary column as previously described [28] and expressed either as per organ (adrenal, ovary) or per mg protein (testis, brain).

2.8. Statistical analysis

The statistical significance of differences of the biochemical parameters measured in normal and GR-LACS KO mice was determined by Bonferroni's multiple comparison test (one way ANOVA) using GraphPad Prism statistical software version 4.

3. Results

3.1. Targeted disruption of GR-LACS in ES cells and generation of GR-LACS$^{-/-}$ mice

GR-LACS gene was disrupted by deletion of exon 1 containing the 5′ the UTR sequence, ATG initiation codon, exon 2 and adjacent genomic intron sequences (Fig. 1 A) which was replaced by a neomycin resistance expression cassette. Positive ES cells which underwent homologous recombination were selected for the presence of Kpn1 digested genomic fragments. Southern analysis revealed the predicted restriction fragments of 14 kb and 7 kb, using 5′ probe, and of 4 kb and 6 kb with the 3′ probe for the wild type and mutated GR-LACS allele, respectively (Fig. 1 B). Chimeric mice were generated by injection of positive ES cells into the C57BL/6 blastocysts and further bred with C57BL/6 females. Positive GR-LACS$^{+/-}$ mice were interbred and the progeny was analyzed by southern or PCR analysis (Fig. 1C). The expression of GR-LACS mRNA in the testis was significantly reduced in the GR-LACS$^{+/-}$ mice, and no expression was detectable in GR-LACS$^{-/-}$ mice (Fig. 1D). Similarly, Western analysis of various tissues revealed decreased GR-LACS protein in the GR-LACS$^{+/-}$ mice and its absence in the GR-LACS$^{-/-}$ mice (Fig. 1E).

GR-LACS KO mice have normal growth rat during development

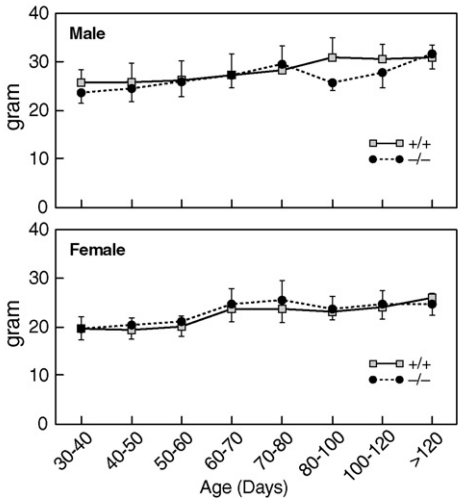

Fig. 2. GR-LACS KO mice have normal growth rate during development, body weight was compared between wild type and GR-LACS null mice from 1 to 5 month-old mice. Data represents the mean ± SE of at least 10 animals in each age group.

3.2. Gross phenotype of GR-LACS$^{-/-}$ mice

Genotype analysis demonstrated that interbreeding of heterozygous mice produced offspring with Mendelian segregation ratios. Newborn GR-LACS$^{-/-}$ mice of both genders were phenotypically normal. GR-LACS$^{-/-}$ and GR-LACS^{+}/$^{-}$ mice of both sexes were fertile, and did not exhibit obvious physical or behavioral abnormalities. No significant differences in body weight between GR-LACS null mice and wild type were observed during development (Fig. 2). There were no apparent anatomical or histopathologic abnormalities in all tissues examined, including testis, ovary, adrenal glands, brain, pituitary gland and sex accessory organ, of adult GR-LACS knockout mice (12 weeks, 9 and 15 months), compared with wild type mice of the same age (not shown). The weight of testis and accessory organs as well as the testis/body weight ratio, and sperm count, viability and motility in epididymis were similar in wild type and GR-LACS null male mice (data not shown).

3.3. Ultra-structure of GR-LACS expression tissues in GR-LACS$^{-/-}$ mice

No significant morphological differences were found in testis, ovary, adrenal gland and brain of adult wild type and GR-LACS$^{-/-}$ mice of same age by light (Fig. 3) and electron microscopic examination of male germinal cells, adrenal, ovary, and brain (not shown). However, in the testis, Leydig cells of 9 month-old GR-LACS null mice contained significant nuclear inclusions, which are commonly observed in 15 month-old wild type mice (Fig. 4B vs A and C, top and lower panel). These inclusions are surrounded by a single membrane contained degenerated cytoplasmic components (i.e. mitochondria, and endoplasmic reticulum). The number of Leydig cells was comparable in wild type and GR-LACS KO mice testis.

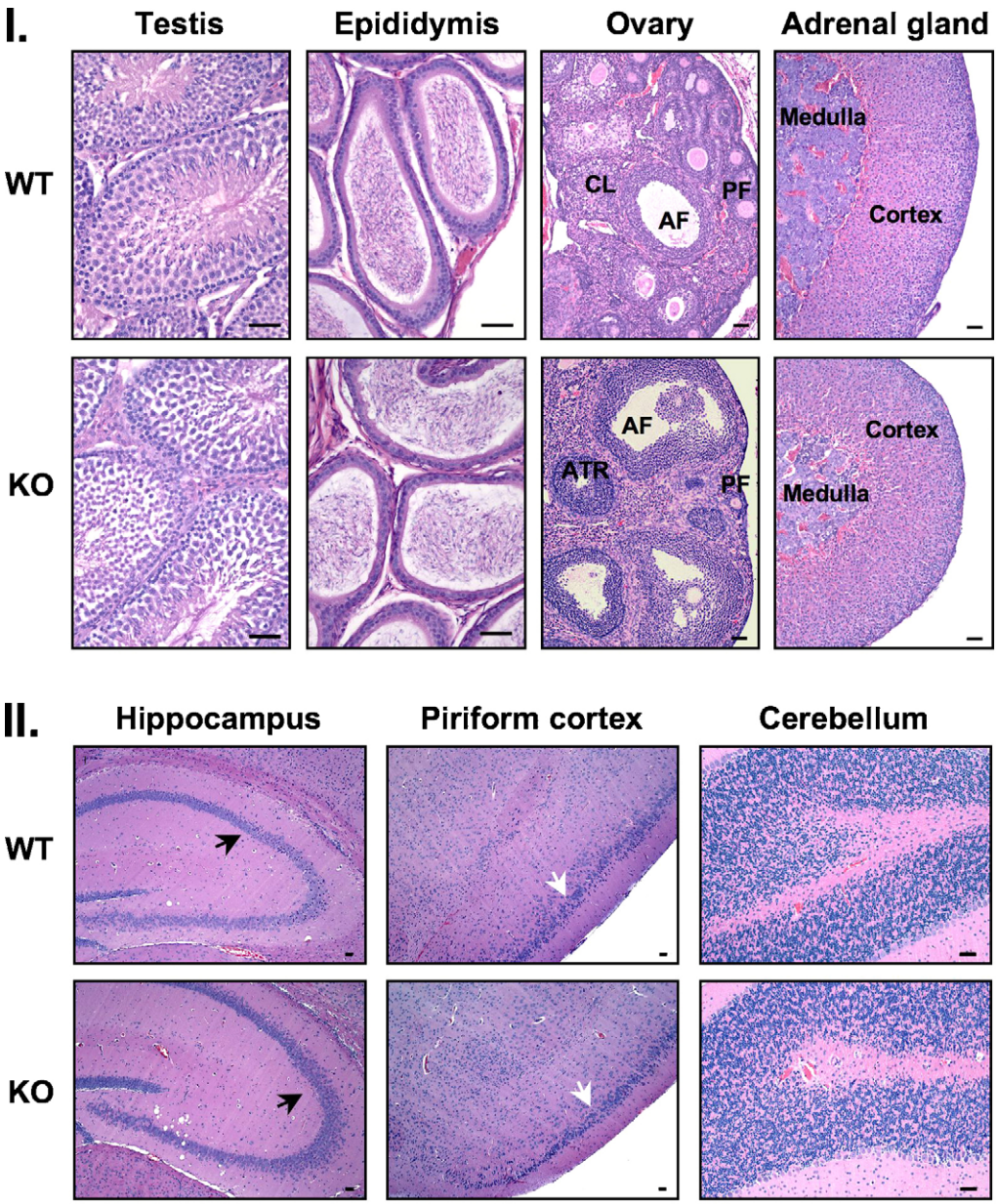

Fig. 3. Morphology of GR-LACS target tissues. Histology sections of adult mice tissues including testis, epidydimus, ovary, adrenal gland and brain were compared between wild type and GR-LACS null mice. Tissues were stained with H&E. Bar = 50 μm. CL: corpus luteum, AF: antral follicle, ATR: atretic follicle, arrow (black): dentate gyrus, arrow (white): piriform cortex.

3.4. Testosterone production in vivo and in vitro: basal and after hCG stimulation

Basal serum levels of testosterone were similar in both groups (WT and KO) (Fig. 5 above). However, serum testosterone levels in GR-LACS$^{-/-}$ were significantly increased over values in wild type mice after *in vivo* treatment with a single dose of hCG (0.5 μg) ($p < 0.05$). Testosterone production from cultured Leydig cells of control animals (WT, −hCG and KO, −hCG) showed no significant differences in basal levels. Treatment of these cells with hCG *in vitro* (100 ng) showed increases in testosterone production in both control groups. However, the increase in KO control (KO, −hCG) was significantly enhanced when compared to the WT control group (WT, −hCG) ($p < 0.001$). On the other hand LCs from *in vivo* hCG-treated WT animals incubated *in vitro* for 3 h in the absence of hormone (WT, +hCG) showed no significant basal differences from KO group (KO, +hCG). *In vitro* incubation of WT cells (WT, +hCG) with hCG for 3 h showed the expected desensitization [21–26] with marked reduction of testosterone response compared to LC from animals that did not received *in vivo* hCG treatment (by 87%) (WT, +hCG vs WT, −hCG). In contrast LCs from KO mice treated with hCG *in vivo* and subsequently incubated with hCG *in vitro* for 3 h showed marked increases in testosterone production compared to the wild type group (WT, +hCG) (6-fold vs 1.5-fold increase, $p < 0.001$). These values were comparable to WT animals that were not exposed to hCG *in vivo* (WT, −hCG), but significantly lower than those observed for the matching control KO group (KO, −hCG vs KO, +hCG: $p < 0.001$). The studies showed that lack on GR-LACS for the most part largely prevents gonadotropin induced desensentization. These findings also indicate an inhibitory role of GR-LACS in testicular steroidogenesis, which is revealed by the gonadotropin treatment.

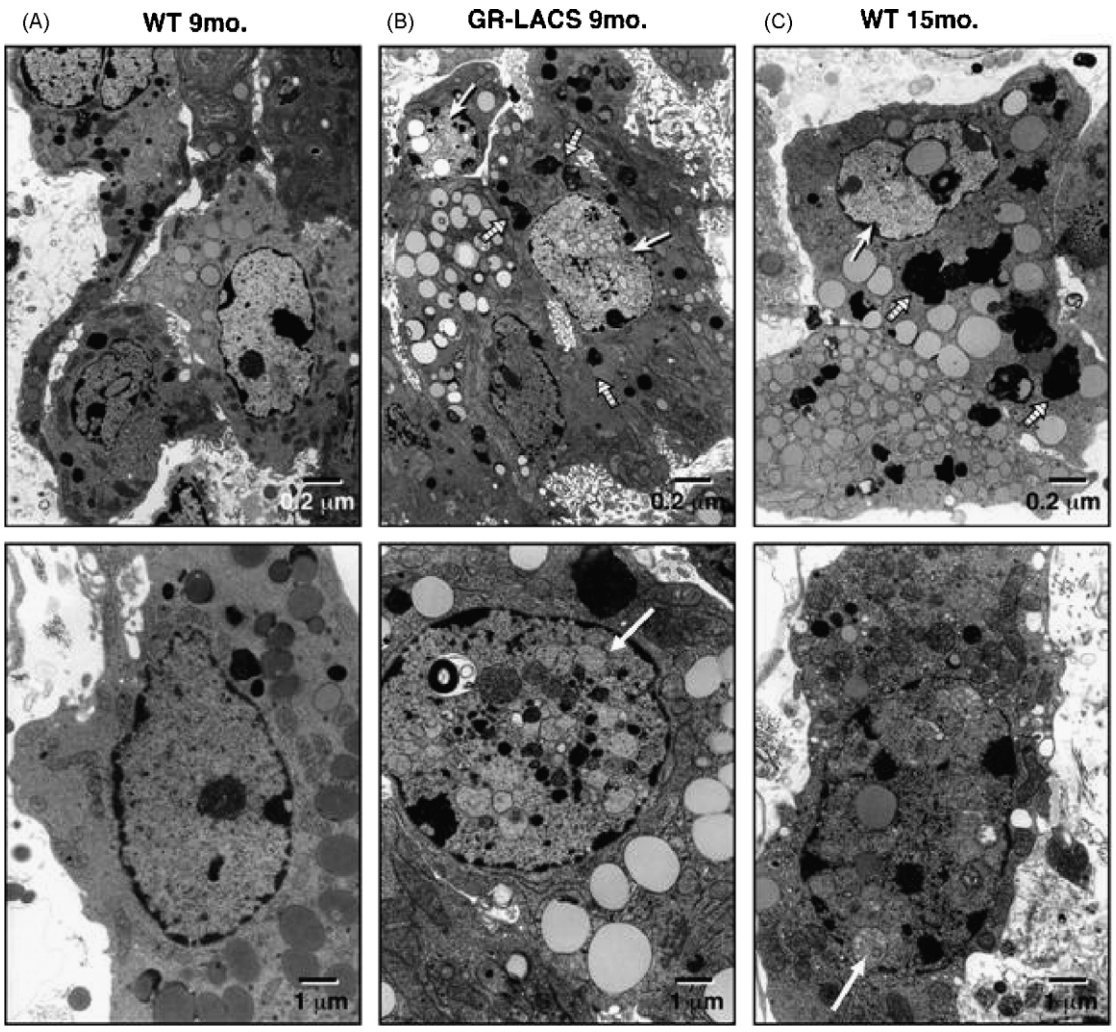

Fig. 4. Electron microscopic analysis of GR-LACS$^{+/+}$ and GR-LACS$^{-/-}$ mouse Leydig cells. Nuclear (N) structure (white arrow) in the Leydig cells of GR-LACS null adult mice. lysosome: stripped arrow. Top panel: bar = 0.2 μm. Lower panel: bar = 1 μm.

3.5. Fatty acid profile in GR-LACS$^{-/-}$ mouse tissues

Lipid analysis of the GR-LACS in expressing tissues are shown in Fig. 6. A minor but significant increase in the long chain fatty acids, C16:0 palmitic acid (ovary), C18:0 C18:1 steric acid (ovary and brain), oleic acid (testis, ovary, brain), C18:2 linoleic acid (testis, ovary) was observed in the GR-LACS knockout mice when compared with wild type. C22:6 docosahexanoic and C22:5 ($n-3$) very long chain fatty acids exhibited a significant increase only in the testis and brain respectively (Fig. 6 and Table 1) of null mice. No significant change was observed in the medium chain fatty acid including C8:0, C9:0, C10:0 capric acid (very low in these tissues) or most of very long fatty acid ($C \geq 22$) (Table 1). In contrast, some LCFAs, including palmitic (C16:0), oleic (C18:1), and linoleic acid (C18:2) was decreased in the adrenal gland of the null mice (Fig. 6).

3.6. FACS activity in GR-LACS null tissues

GR-LACS was proven to display LACS activity [7]. However, the FACS activity of testis, brain, adrenal gland and ovary from GR-LACS$^{-/-}$ mice did not show significant difference from wild type for medium chain FACS (decanoyl acid, C10:0), LACS (palmitic acid, C16:0), and VLCS (lignoceric acid, C24:0). A similar result was observed when using testis mitochondrial protein (not shown). These findings reflect a compensatory participation of other FACS.

4. Discussion

In this study, we report the generation and characterization of mice with a targeted disruption of the GR-LACS gene. There was no phenotypical abnormality in GR-LACS null mice. KO animals of both genders were fertile, and newborns were normal and displayed normal development. Leydig cells of GR-LACS KO mice had a higher number of the nuclear inclusions normally observed in older wild type animals, suggesting signs of premature aging. Significant increases of testosterone levels by *in vivo* and *in vitro* hCG treatment were observed in KO over those in wild type mice. Leydig cells of KO mice were refractory to the gonadotropin-induced desensitization that causes reduced testosterone responses to hCG/LH *in vitro* in wild type mice. This indicated a negative role of GR-LACS in androgen production. Minor accumulation of LCFA, but not of VLCFA known to be related to X-ALD, C24:0 (lignoceric acid) and C26:0 (hexacrosanoic acid) was observed in KO mice in most tissues where GR-LACS/lipidosin protein was predominantly expressed in wild type animals [10] (Fig. 6). This is consistent with our previous findings which demonstrated that LCFA but not VLCFA are the preferred substrates for GR-LACS [7]. This study demonstrated, that

Table 1
Comparison of lipid profile in the testis, ovary, adrenal gland and brain of wild type and GR-LACS mice.

	Mean		SE		*n*	*p* value
	W	K	W	K		
I. TESTIS						
MCFA (μg FA/mg protein)						
C8:0 - Octanoic	0.346	0.303	0.061	0.086	8	NS
C9:0 - Nonanoic	2.699	3.017	0.414	0.447	8	NS
C10:0 - Capric	0.162	0.221	0.030	0.032	8	NS
LCFA (μg FA/mg protein)						
C11:0 - Undecanoic	0.094	0.086	0.020	0.013	8	NS
C12:0- Laurie	0.927	1.566	0.154	0.339	8	NS
C13:0 - Tridecanoic	0.194	0.176	0.018	0.021	8	NS
C14:0 - Myristic	16.123	23.510	3.143	3.373	8	NS
C15:0 - Pentadecanoic	2.346	2.869	0.490	0.471	8	NS
C16:0 - Palmitic	479.071	505.863	29.260	26.401	8	NS
C17:0 - Heptadecanoic	2.778	3.082	0.325	0.357	8	NS
C18:0 - Stearic	147.782	146.244	5.982	5.630	8	NS
C20:0 - Arachidic	1.616	1.848	0.143	0.067	8	NS
C21:0 - Heneicosanoic	0.135	0.136	0.012	0.009	8	NS
c11:1 - Undecaenoic	0.186	0.161	0.030	0.024	8	NS
C12:1 - Dodecaenoic	0.027	0.026	0.004	0.002	8	NS
C13:1 - Tridecaenoic	0.003	0.005	0.003	0.003	8	NS
C14:1 - Myristoleic	0.824	0.886	0.357	0.289	8	NS
C15:1 - Pentadecaenoic	0.122	0.113	0.029		8	NS
C16:1(n-9)	10.326	15.353	1.561	1.983	3	NS
C16:1(n-7)-Palmitoleic	52.459	65.577	20.196	18.763	8	NS
C16:1(n-5)	0.015	0.006	0.004	0.004	8	NS
C17:1 - Heptadecaenoic	1.747	2.464	0.599	0.356	8	NS
C18:1(n-9)-Oleic	209.401	378.731	18.494	66.530	8	##
C18:1(n-7)-Vaccenic	52.833	67.556	5.879	6.238	8	NS
C18:1(n-5)	0.013	0.087	0.007	0.036	8	NS
C20:1(n-9)- Eicosenoic	5.725	8.415	0.956	0.990	8	NS
C14:2 - Myristolenic	0.051	0.034	0.006	0.008	8	NS
C16:2 - Palmitolenic	0.629	0.801	0.235	0.288	8	NS
C18:2(n-6)-Linoleic	108.380	299.125	20.496	75.203	8	##
C18:2(N-6)Conj - Rumenic	0.433	0.513	0.150	0.134	8	NS
C18:3(n-3) - Alpha Linolenic	9.032	11.742	3.290	2.992	8	NS
C18:3(n-6) - Gamma Linolenic	1.457	2.089	0.336	0.269	8	NS
C20:2(n-6) - Eicosadienoic	3.187	3.884	0.232	0.325	8	NS
C20:3(n-3)	0.126	0.174	0.028	0.035	8	NS
C20:3(n-6) - Dihomo-g-linolenic	24.411	35.687	1.148	2.080	8	NS
C20:3(n-7)	0.126	0.174	0.028	0.035	8	NS
C20:3(n-9)- Mead	2.909	6.843	0.133	0.329	8	##
C20:4(n-6) - Arachidonic	204.623	202.392	4.031	15.231	8	NS
C20:5(n-3) - Eicosapentaenoic	4.665	4.685	0.360	0.284	8	NS
VLCFA (μg FA/mg protein)						
C22:0 - Behenic	3.142	2.987	0.078	0.052	8	NS
C23:0 - Tricosanoic	0.548	0.472	0.016	0.013	8	NS
C24:0 - Lignoceric	1.962	1.985	0.043	0.044	8	NS
C25:0 - Pentacosanoic	0.047	0.047	0.002	0.003	8	NS
C26;0 - Hexacosanoic	0.050	0.052	0.003	0.006	8	NS
C28:0 - Octacosanoic	0.015	0.018	0.005	0.003	8	NS
C29:0 - Nonacosanoic	0.003	0.000	0.003	0.000	8	NS
C30:0	0.000	0.000	0.000	0.000	8	NS
C22:1(n-9)-Erucic	0.873	0.995	0.097	0.055	8	NS
C22:2(n-6) - Docosadienoic	0.146	0.142	0.015	0.013	8	NS
C22:3(n-3) - Docosatrienoic	0.009	0.020	0.005	0.004	S	NS
C22:4(n-6)-Adrenic	25.588	29.266	1.219	1.969	8	NS
C22:5(n-3)	9.611	10.318	0.884	0.705	8	NS
C22:5(n-6) - Docosapentaenoic	157.211	70.747	3.213	5.640	8	NS
C22:6(n-3) - Docosahexanoic (DHA)	107.820	167.347	3.186	4.032	8	##
C24:1(n-9)-Nervonic	6.083	6.519	0.261	0.317	8	NS
C24:2	0.472	0.360	0.023	0.027	8	NS
C25:1	0.003	0.008	0.003	0.004	8	NS
C26:1 - Hexacosaenoic	0.035	0.042	0.005	0.002	8	NS
C26:2 - Hexacosadienoic	0.000	0.000	0.000	0.000	8	NS
II. OVARY						
MCFA (μg FA/tissue)						
C8:0 - Octanoic	0.210	0.238	0.023	0.059	8	NS
C9:0 - Nonanoic	0.206	0.160	0.033	0.052	8	NS
C10:0-Capric	0.151	0.242	0.038	0.069	8	NS
LCFA (μg FA/tissue)						
C11:0 - Undecanoic	0.036	0.040	0.005	0.007	8	NS

Table 1 (*Continued*)

	Mean		SE		*n*	*p* value
	W	K	W	K		
C12:0- Laurie	0.951	2.148	0.326	0.824	8	NS
C13:0 - Tridecanoic	0.089	0.144	0.030	0.052	8	NS
C14:0 - Myristic	36.244	57.906	12.748	24.057	8	NS
C15:0 - Pentadecanoic	3.676	4.172	1.015	1.340	8	NS
C16:0 - Palmitic	996.740	1694.580	156.069	67.552	8	##
C17:0 - Heptadecanoic	8.545	12.156	1.766	3.391	8	NS
C18:0 - Stearic	592.990	941.810	44.150	8.661	8	##
C20:0 - Arachidic	7.658	10.618	1.205	2.009	8	NS
C21:0 - Heneicosanoic	0.373	0.780	0.057	0.235	8	NS
c11:1 - Undecaenoic	0.008	0.014	0.003	0.002	8	NS
C12:1 - Dodecaenoic	0.003	0.008	0.002	0.006	8	NS
C13:1 - Tridecaenoic	0.001	0.000	0.001	0.000	8	NS
C14:1 - Myristoleic	0.709	1.492	0.256	0.611	8	NS
C15:1 - Pentadecaenoic	0.020	0.004	0.013	0.002	8	NS
C16:1(n-9)	6.700	17.972	1.505	6.472	8	NS
C16:1(n-7)-Palmitoleic	54.200	80.140	20.385	31.465	8	NS
C16:1(n-5)	0.019	0.070	0.012	0.030	8	NS
C17:1 - Heptadecaenoic	2.528	3.548	0.748	1.290	8	NS
C18:1(n-9)-Oleic	801.311	1561.723	195.995	98.643	8	##
C18:1(n-7) - Vaccenic	39.914	68.304	6.804	18.846	8	NS
C18:1(n-5)	0.383	1.074	0.118	0.405	8	NS
C20:1(n-9) - Eicosenoic	27.755	46.926	5.093	12.414	8	NS
C14:2 - Myristolenic	0.020	0.024	0.005	0.013	8	NS
C16:2 - Palmitolenic	0.109	0.998	0.019	0.393	8	NS
C18:2(n-6)- Linoleic	989.068	1182.490	144.415	21.362	8	NS
C18:2(N-6)Conj - Rumenic	0.768	2.200	0.164	0.788	8	NS
C18:3(n-3) - Alpha Linolenic	21.043	28.674	8.007	11.585	8	NS
C18:3(n-6) - Gamma Linolenic	1.613	2.158	0.437	0.772	8	NS
C20:2(n-6) - Eicosadienoic	27.860	22.930	5.057	1.776	8	NS
C20:3(n-3)	1.205	1.068	0.190	0.205	8	NS
C20:3(n-6) - Dihomo-g-linolenic	23.733	29.388	4.092	6.263	8	NS
C20:3(n-7)	2.864	2.642	0.503	0.489	8	NS
C20:3(n-9)-Mead	1.796	2.794	0.1722	0.190	8	##
C20:4(n-6) - Arachidonic	338.208	398.924	36.563	18.236	8	NS
C20:5(n-3) - Eicosapentaenoic	10.203	13.250	1.568	3.758	8	NS
VLCFA (μg FA/tissue)						
C22:0 - Behenic	5.855	6.364	0.680	0.475	8	NS
C23:0 - Tricosanoic	1.378	2.498	0.164	0.581	8	NS
C24:0 - Lignoceric	4.688	6.182	0.509	0.888	8	NS
C25:0 - Pentacosanoic	0.155	0.370	0.018	0.110	8	NS
C26:0 - Hexacosanoic	0.145	0.342	0.021	0.107	8	NS
C28:0 - Octacosanoic	0.031	0.072	0.006	0.022	8	NS
C29:0 - Nonacosanoic	0.008	0.016	0.002	0.007	8	NS
C30:0	0.025	0.092	0.005	0.037	8	NS
C22:1(n-9)-Erucic	3.908	5.146	0.502	0.893	8	NS
C22:2(n-6) - Docosadienoic	2.560	2.436	0.438	0.246	8	NS
C22:3(n-3) - Docosatrienoic	0.170	0.230	0.030	0.063	8	NS
C22:4(n-6) - Adrenic	113.138	96.704	29.204	7.573	3	NS
C22:5(n-3)	84.605	82.456	14.717	11.384	8	NS
C22:5(n-6) - Docosapentaenoic	12.243	21.858	1.632	3.415	8	NS
C22:6(n-3) - Docosahexanoic (DHA)	146.704	147.102	15.004	22.195	8	NS
C24:1(n-9) - Nervonic	10.681	14.330	0.928	1.808	8	NS
C24:2	1.916	1.774	0.152	0.120	8	NS
C25:1	0.079	0.198	0.012	0.062	8	NS
C26:1 - Hexacosaenoic	0.190	0.406	0.023	0.108	8	NS
C26:2 - Hexacosadienoic	0.061	0.066	0.004	0.011	8	NS
III. ADRENAL						
MCFA (μg FA/tissue)						
C8:0 - Octanoic	0.098	0.220	0.042	0.109	8	NS
C9:0 - Nonanoic	1.343	1.308	0.337	0.329	8	NS
C10:0- Capric	0.200	0.208	0.111	0.068	8	NS
LCFA (μg FA/tissue)						
C11:0 - Undecanoic	0.055	0.080	0.012	0.018	8	NS
C12:0 - Lauric	1.088	1.340	0.724	0.554	8	NS
C13:0 - Tridecanoic	0.145	0.118	0.042	0.017	8	NS
C14:0 - Myristic	27.218	26.548	21.998	13.978	8	NS
C15:0 - Pentadecanoic	2.438	1.680	1.636	0.488	8	NS
C16:0 - Palmitic	333.433	301.098	54.281	28.099	8	NS
C17:0 - Heptadecanoic	3.188	3.040	1.746	0.556	8	NS
C18:0- Stearic	90.393	130.465	15.866	11.904	8	NS
C20:0-Arachidic	2.778	3.333	1.658	0.882	8	NS
C21:0 - Heneicosanoic	0.218	0.268	0.121	0.062	8	NS

Table 1 (*Continued*)

	Mean		SE		*n*	*p* value
	W	K	W	K		
c11:1 - Undecaenoic	0.078	0.070	0.017	0.017	8	NS
C12:1 - Dodecaenoic	0.008	0.010	0.003	0.000	8	NS
C13:1 - Tridecaenoic	0.000	0.000	0.000	0.000	8	NS
C14;1 - Myristoleic	1.425	1.118	1.196	0.627	8	NS
C15:1 - Pentadecaenoic	0.098	0.050	0.071	0.021	8	NS
C16:1(n-9)	7.958	4.400	6.152	1.842	8	NS
C16:1(n-7)-Palmitoleic	73.460	50.580	56.766	27.485	8	NS
C16:1(n-5)	0.020	0.013	0.014	0.006	8	NS
C17:1 - Heptadecaenoic	2.118	1.295	1.613	0.553	8	NS
C18:1(n-9)- Oleic	334.175	254.815	59.861	54.047	8	NS
C18:1(n-7) - Vaccenic	39.695	25.830	24.193	8.029	8	NS
C18:1(n-5)	0.113	0.068	0.099	0.042	8	NS
C20:1(n-9) - Eicosenoic	15.658	12.153	11.207	3.886	8	NS
C14:2 - Myristolenic	0.015	0.015	0.003	0.003	8	NS
C16:2 - Palmitolenic	0.725	0.483	0.593	0.206	8	NS
C18:2(n-6) - Linoleic	298.288	206.328	54.569	40.106	8	NS
C18:2(N-6)Conj - Rumenic	0.503	0.355	0.338	0.151	8	NS
C18:3(n-3) - Alpha Linolenic	14.053	8.938	10.843	3.935	8	NS
C18:3(n-6) - Gamma Linolenic	0.868	0.958	0.508	0.234	8	NS
C20:2(n-6) - Eicosadienoic	4.608	6.243	1.796	1.036	8	NS
C20:3(n-3)	0.348	0.420	0.207	0.078	8	NS
C20:3(n-6) - Dihomo-g-linolenic	10.033	12.428	3.067	2.821	8	NS
C20:3(n-7)	0.530	0.968	0.108	0.090	8	NS
C20:3(n-9)- Mead	0.800	0.730	0.235	0.158	8	NS
C20:4(n-6) - rachidonic	79.020	120.035	13.242	5.284	8	NS
C20:5(n-3) - Eicosapentaenoic	4.508	10.058	1.800	1.761	8	NS
VLCFA (μg FA/tissue)						
C22:0 - Behenic	2.825	3.900	0.541	0.372	8	NS
C23:Q - Tricosanoic	0.970	1.750	0.180	0.161	8	NS
C24:0 - Lignoceric	2.360	4.140	0.448	0.506	8	NS
C25:0 - Pentacosanoic	0.073	0.125	0.024	0.013	8	NS
C26:0 - Hexacosanoic	0.075	0.095	0.042	0.022	8	NS
C28:0 - Octacosanoic	0.020	0.020	0.010	0.007	8	NS
C29:0 - Nonacosanoic	0.003	0.000	0.003	0.000	8	NS
C30:0	0.008	0.013	0.008	0.006	8	NS
C22:1(n-9)-Erucic	1.753	1.670	1.068	0.429	8	NS
C22:2(n-6) - Docosadienoic	0.453	0.718	0.167	0.230	8	NS
C22:3(n-3) - Docosatrienoic	0.043	0.050	0.029	0.014	8	NS
C22:4(n-6)-Adrenic	19.010	20.330	3.750	4.756	8	NS
C22:5(n-3)	15.358	29.490	4.680	7.591	8	NS
C22:5(n-6) - Docosapentaenoic	2.453	2.533	0.844	0.622	8	NS
C22:6(n-3) - Docosahexanoic (DHA)	31.280	47.010	9.940	10.001	8	NS
C24:1(n-9)-Nervonic	4.485	6.890	0.847	0.521	8	NS
C24:2	0.470	0.853	0.093	0.089	8	NS
C25:1	0.030	0.043	0.014	0.009	8	NS
C26:1 - Hexacosaenoic	0.060	0.083	0.033	0.019	8	NS
C26:2 - Hexacosadienoic	0.008	0.015	0.005	0.003	8	NS
IV. BRAIN						
MCFA (μg FA/mg protein)						
C8:0 - Octanoic	0.12	0.159	0.070	0.047	8	NS
C9:0 - Nonanoic	1.78	1.341	0.319	0.215	8	NS
C10:0 - Capric	0.10	0.105	0.029	0.006	8	NS
LCFA (μg FA/mg protein)						
C11:0 - Undecanoic	0.08	0.051	0.016	0.010	8	NS
C12:0 - Laurie	0.42	0.359	0.049	0.007	8	NS
C13:0 - Tridecanoic	0.09	0.159	0.005	0.005	8	NS
C14:0 - Myristic	5.55	6.988	0.293	0.205	8	NS
C15:0 - Pentadecanoic	2.00	2.045	0.046	0.083	8	NS
C16:0 - Palmitic	640.25	654.482	37.268	15.229	8	NS
C17:0 - Heptadecanoic	6.75	7.669	0.186	0.472	8	NS
C18:0- Stearic	714.21	779.346	35.730	19.557	8	##
C20:0-Arachidic	20.08	26.172	2.529	0.904	8	NS
C21:0 - Heneicosanoic	2.43	3.379	0.125	0.112	8	NS
c11:1 - Undecaenoic	0.14	0.123	0.020	0.017	8	NS
C12:1 - Dodecaenoic	0.02	0.011	0.000	0.004	8	NS
C13:1 - Tridecaenoic	0.01	0.004	0.005	0.004	8	NS
C14:1 - Myristoleic	0.01	0.018	0.004	0.004	8	NS
C15:1 - Pentadecaenoic	0.04	0.025	0.008	0.004	8	NS
C16:1(n-9)	4.79	5.057	0.338	0.245	8	NS
C16:1(n-7)-Palmitoleic	18.35	21.547	1.225	0.656	8	NS
C16:1(n-5)	0.00	0.007	0.000	0.004	8	NS
C17:1 - Heptadecaenoic	1.58	1.708	0.068	0.079	8	NS

Table 1 (*Continued*)

	Mean		SE		*n*	*p* value
	W	K	W	K		
C18;1(n-9)-Oleic	599.91	661.674	13.303	14.943	8	##
C18:1(n-7)-Vaccenic	132.90	163.709	2.723	4.819	8	NS
C18:1(n-5)	0.00	0.000	0.000	0.000	8	NS
C20:1(n-9) - Eicosenoic	80.98	98.336	13.591	3.000	8	NS
C14:2 - Myristolenic	0.02	0.015	0.003	0.002	8	NS
C16:2 - Palmitolenic	0.01	0.007	0.004	0.004	8	NS
C18:2(n-6)-Linoleic	25.05	25.173	0.834	1.919	8	NS
C18:2(N-6)Conj - Rumenic	0.31	0.388	0.018	0.012	8	NS
C18:3(n-3) - Alpha Linolenic	0.06	0.268	0.005	0.027	8	NS
C18;3(n-6) - Gamma Linolenic	0.14	0.116	0.036	0.010	8	NS
C20:2(n-6) - Eicosadienoic	5.24	6.732	0.664	0.486	8	NS
C20:3(n-3)	0.11	0.312	0.008	0.016	8	NS
C20:3(n-6) - Dihomo-g-linolenic	22.77	28.049	0.841	1.092	8	NS
C20:3(n-7)	0.11	0.312	0.008	0.016	8	NS
C20:3(n-9)-Mead	4.59	4.999	0.181	0.338	8	NS
C20:4(n-6) - Arachidonic	318.94	301.374	29.072	8.653	8	NS
C20:5(n-3) - Eicosapentaenoic	4.92	2.918	0.534	0.113	8	NS
VLCFA (μg FA/mg protein)						
C22:0 - Behenic	27.93	37.681	4.342	1.510	8	NS
C23:0 - Tricosanoic	12.70	15.786	1.738	0.554	8	NS
C24:0 - Lignoceric	46.27	58.574	9.171	3.430	8	NS
C25:0 - Pentacosanoic	1.62	1.951	0.309	0.089	8	NS
C26:0 - Hexacosanoic	0.69	0.871	0.174	0.067	8	NS
C28:0 - Octacosanoic	0.03	0.029	0.008	0.000	8	NS
C29:0 - Nonacosanoic	0.00	0.000	0.000	0.000	8	NS
C30:0	0.00	0.000	0.000	0.000	8	NS
C22:1(n-9)-Erucic	9.15	12.487	1.171	0.361	8	NS
C22:2(n-6) - Docosadienoic	0.59	0.972	0.141	0.058	8	NS
C22:3(n-3) - Docosatrienoic	0.02	0.051	0.004	0.005	8	NS
C22:4(n-6)-Adrenic	81.55	91.609	2.544	2.741	8	NS
C22:5(n-3)	9.47	51.053	0.330	1.796	8	##
C22:5(n-6) - Docosapentaenoic	4.92	2.918	0.534	0.113	8	NS
C22:6(n-3) - Docosahexanoic (DHA)	411.95	417.290	33.405	13.621	8	NS
C24:1(n-9) - Nervonic	125.51	152.307	19.516	3.881	8	NS
C24:2	2.21	3.790	0.408	0.184	8	NS
C25:1	3.18	3.694	0.390	0.109	8	NS
C26:1 - Hexacosaenoic	1.25	1.523	0.270	0.063	8	NS
C26:2 - Hexacosadienoic	0.05	0.087	0.015	0.006	8	NS

MCFA: medium chain fatty acid, LCFA: long chain fatty acid, VLCFA: very long chain fatty acid, #: $p < 0.05$, NS: non-significant, SE: standard error, W: wild type, K: GR-LACS knockout.

GR-LACS is not essential for LCFA metabolism implying redundancy and/or compensation of its function/s by other members of the family. Therefore, GR-LACS/Lipidosin may not be involved and/or responsible in the X-ALD type disorder.

In rodent species, testicular GR-LACS mRNA expression reaches maximum levels at 14 days of age and is predominantly localized within the seminiferous tubules. After 22 days, the expression of GR-LACS shifts dramatically from the seminiferous tubules to the interstitial compartment. In the adult testis, GR-LACS protein is highly expressed in Leydig cells, and to much lesser extent in germ cells [7,10]. Thus, it is surprising that complete disruption of this "on and off" system of GR-LACS expression in specific testicular cells of KO mice had no effect in spermatogenesis. This could result from the functional compensation of some of GR-LACS metabolic functions by other member(s) of the family. GR-LACS/lipidosin is first detected in brain tissue at embryonic day 18 and increases steadily towards adulthood. However, no significant morphological or behavioral mating abnormalities are observed in GR-LACS null mice throughout development or adulthood. Because GR-LACS and isoforms are highly expressed in the brain, the late onset neurological and behavioral phenotype observed in old X-ALD-deficient animals, could not be excluded from our studies in adult mice.

Since Leydig cells are the primary site of GR-LACS expression in the mature rodent testis [7,10], we anticipated that its absence may affect energy metabolism and testicular steroidogenesis. It was not surprising to observe comparable basal levels of circulating testosterone in GR-LACS$^{-/-}$ and wild type mice, since null mice exhibited normal testicular morphology and spermatogenesis. However, the more significant increases in serum testosterone induced by hCG *in vivo* observed in KO mice indicated that GR-LACS could have a role in steroidogenesis. This was affirmed by the higher increases in testosterone production by LC in response to hCG *in vitro* observed in KO mice (50% over wild type). Furthermore, Leydig cells from KO mice treated with hCG *in vivo*, were largely refractory to desensitization by hCG *in vitro,* unlike findings in LC of wild type animals that displayed desensitization with profound decreases in testosterone production [21–26] (Fig. 5 II). Trophic hormone stimulation of steroidogenic cells is mediated by the activation of G proteins following binding to its cognate receptor, with subsequent activation of protein kinase A and steroidogenesis. Physiological endogenous LH levels cause positive regulation of membrane receptors and steroidogenesis. However, in adult rodents, elevations in circulating gonadotropin (i.e exogenous at comparable levels to LH increases at proestrus) can induce receptor down-regulation of homologous LH and a receptor-independent desensitization of steroid response with down-regulation of steroidogenic enzymes and marked reduction of testosterone production [21–26]. The unexpected refractoriness to desensitization observed in null mice

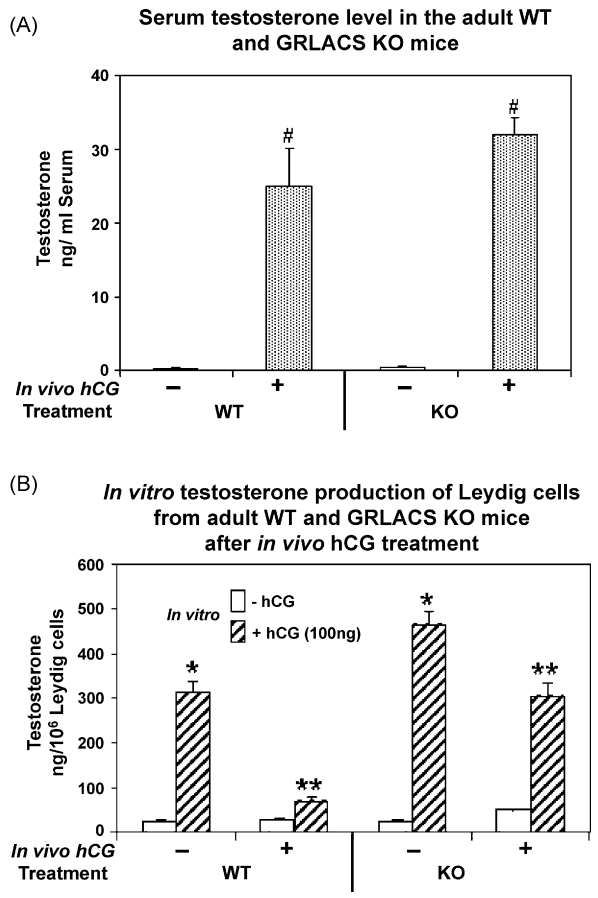

Fig. 5. *In vivo* hCG effect on serum testosterone level and Leydig cells response to 3 h *in vitro* hCG stimulation in wild type and GR-LACS null mice. Leydig cells were obtained and purified from the wild type (WT) and GR-LACS null mice (KO) 24 h after subcutaneous injection of a single dose of hCG (0.5 μg) or vehicle, phosphate buffered saline (control group). Testosterone was measured in the serum (A) and (B) LC cultures (1×10^6) incubated for 3 h in the presence or absence (control) of 100 ng hCG; #: $p < 0.05$, *, **: $p < 0.001$.

indicates that GR-LACS has a profound negative effect on steroidogenesis and contributes to gonadal function by regulating steroid production. However, a minor degree of desensitization was still observed in the KO (about 20%) when comparing KO mice desensitized *in vivo* (KO +) vs KO control (Fig. 5 II), indicating the participation of additional process(es) in desensitization. It is relevant to note that although hCG, through transcriptional events yet to be defined, causes marked reduction GR-LACS mRNA (by 80%), GR-LACS protein was moderately reduced by only 20–30% during the desensitization and recovery phases [7]. Thus, GR-LACS could effectively contribute to the desensitization process where the expression and activity of 17 α-hydroxylase [24] and 17 β-hydroxysteroid dehydrogenase [26] were nearly abolished, while 3β-hydroxysteroid dehydrogenase was affected to a lesser degree. [25]. The site(s) and nature of this negative regulation remain to be determined. GR-LACS could provide long chain acyl-CoA esters at specific cellular sites with regulatory effects on steroidogenic enzyme activity, membrane function, and gene expression. Alternatively, GR-LACS per se and/or an alternative spliced variant(s) of the enzyme [7,10] could abrogate the function of ASC4, an acyl-CoA synthetase with arachidonic acid substrate preference [29] and/or ASC3 which also prefers arachidonate [30], and reduce the availability of arachidonic acid metabolites which are known to induce STAR gene expression [31,32,33]. Furthermore, metabolites of the lipoxygenase and epoxygenase pathways have been demonstrated to stimulate 3β- and 17β-hydroxysteroid dehydrogenase activities and enhance the synthesis of testicular steroid hormones [33]. Thus, GR-LACS could limit/regulate steroidogenesis at one or more levels, including cholesterol formation/transfer and/or mitochondrial trafficking/steroid enzyme expression and regulation through different functional modalities.

The other distinct features observed in GR-LACS$^{-/-}$ null mice is the large number of nuclear inclusions in Leydig cells at 9 months of age that are normally observed in older wild type mice (>15 month-old). These nuclear inclusions are Leydig cell-specific and not found in other tissues of GR-LACS null mice. In normal mice, nuclear inclusions in Leydig cells are usually increased in old animals, indicating the change of Leydig cell functions at this stage [34]. Therefore, it is reasonable to assume that lack of GR-LACS in KO mice might reduce the lipid metabolism, and aging organelles would not be recycled properly. Specific changes in lipid profile with increase of LCFA could be linked to this aging process (Fig. 7). Thus, the absence of GR-LACS might trigger the aging clock prematurely. Although GR-LACS/lipidosin is known to exhibit LACS activity, it did not decrease in any tissues of GR-LACS null mice examined when capric acid (C10:0), palmitic acid (C16:0), and lignoceric acid (C24:0) were used as substrates in the present study (Fig. 7). This result suggests the contribution of compensatory genes on LACS enzymatic activity. Although LACS activity was found to be largely compensated, this was not complete as reflected by the moderate increase in LCFA in relevant tissues of KO mice (10–20% over wild type control), limited availability of compensatory gene(s) or some degree of exclusive participation of this enzyme in LACS metabolism.

The participation of GR-LACS/Lipidosin/bubblegum in X-ALD has long been debated. Although a number of indirect studies have questioned its involvement in this disease, the first direct evidence for its lack of a role in this neurodegenerative inherited disorder was provided by the analysis of the GR-LACS/lipidosin knockout mouse model generated in the present study. X-ALD is characterized by accumulation of VLCFAs due to reduced activity of the peroxisomal VLCS. However, the identified X-ALD gene, ABCD1, has no VLCS activity. It has been suggested that ABCD1 is involved in transport of VLCFA, VLACS, or a cofactor across the peroxisomal membrane and thus in the regulation of peroxisomal VLACS activity, VLCFA β-oxidation and VLCFA level [19]. Peroxisomal β-oxidation capacity is normal in tissues of ABCD1-deficient mice [35]. Furthermore, accumulation of VLCFAs in ABCD1 mice whole muscle homogenates does not affect mitochondrial function [36]. Aside from the Drosophila mutant bubblegum, which presented elevated levels of VLCFAs and adult neurodegeneration, similar to X-ALD [11], several other mouse models showed no association with X-ALD. Vlcs/Fatp2-deficient mice exhibit decreased peroxisomal VLCS activity and VLCFA β-oxidation without accumulation of VLCFA. Also, the abcd1/Vlcs double knockout did not display a more severe X-ALD phenotype [19]. Fatp4 targeted mice showed features of lethal restrictive dermopathy, combined with reduced esterification for C24:0 [5,37] and inactivation of Fatp1 prevents fat-induced insulin resistance in skeletal muscle [38].

In our GR-LACS/lipidosin deficient mice, LCFAs but not VLCFAs increased in X-ALD target tissues, including central nervous system, adrenal and testis, and no evidence was found supporting adrenal and/or neurological phenotype. We therefore conclude that GR-LACS/lipidosin is not involved in X-ALD. However, the question still remains whether deficiency in the human ortholog of Drosophila Bubblegum, which is 95% similar to GR-LACS/lipidosin and has been shown to possess VLACS, in addition to LACS activity has a role in X-ALD. The GR-LACS null mouse model presented in this study has revealed that GRLACS/lipidosin has a role in the regulation of steroidogenesis, exerts a negative effect on gonadotropin-induced stimulation of steroid production, and participates in gonadotropin-induced desensitization.

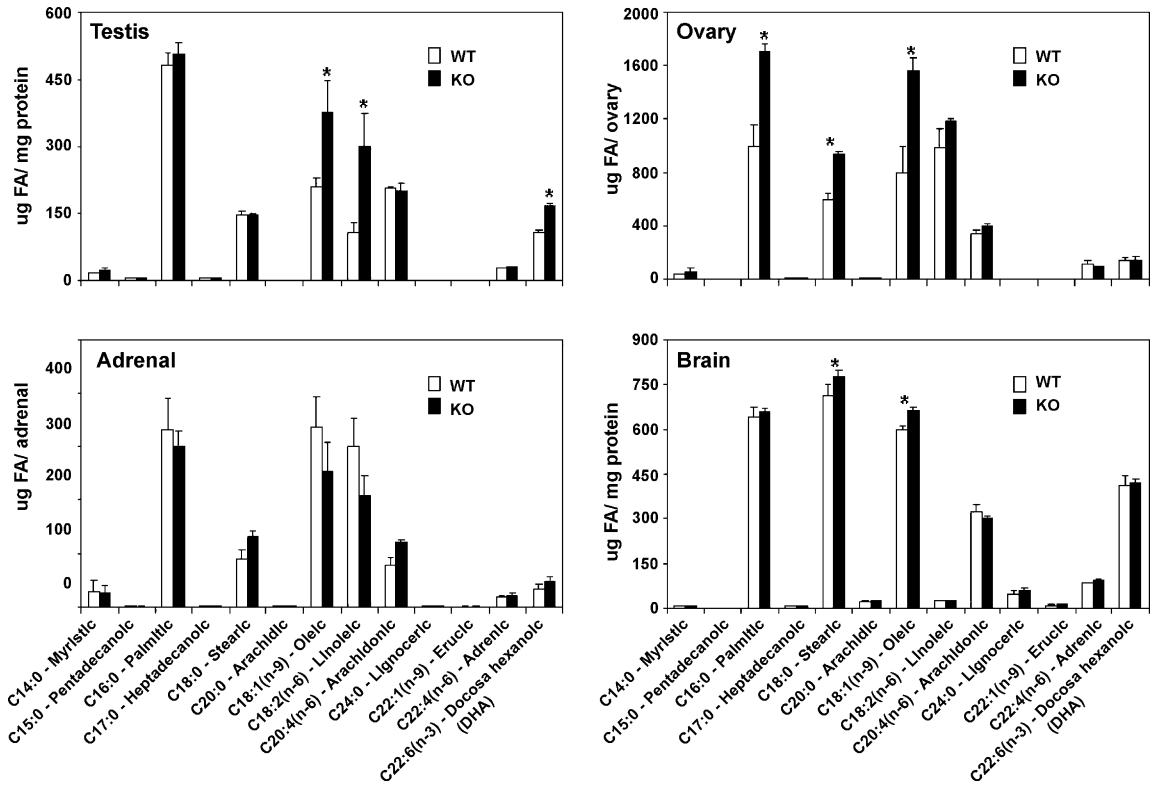

Fig. 6. Tissue lipid analysis in wild type and GR-LACS null mice. Endogenous lipid profile from tissues including testis, ovary, brain and adrenal gland were compared between wild type (WT) and GR-LACS$^{-/-}$ (KO) mice. Data represents the mean ± SE of at least triplicate from eight individual animals. MCFA: medium chain fatty acid. LCFA: long chain fatty acid. VLCFA: very long chain fatty acid. *: $p < 0.05$.

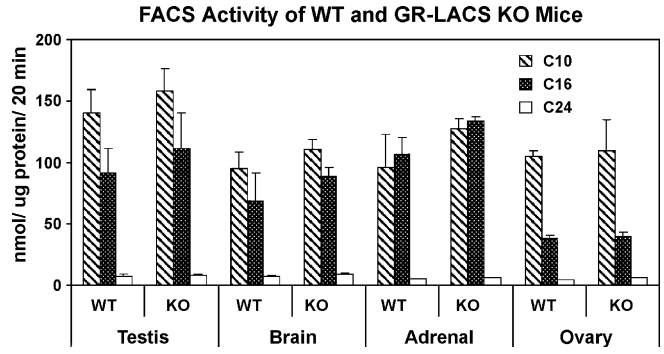

Fig. 7. Fatty acyl CoA synthetase (FACS) activity of wild type and GR-LACS null mice. FACS activity of extracts from wild type (WT) and GR-LACS knockout (KO) tissues assessed by using medium chain fatty acid C10:0, capric acid, long chain fatty acid C16:0 - palmitoyl acid, C18:0 - oleoly acid or and very long chain fatty acid C24:0 - lignoceric acid as substrates. $p > 0.05$. Mean ± SE. $n = 4$.

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