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► To cite this version:

Hélène Holota, Laura Thirouard, Mélusine Monroe, Manon Garcia, Angélique de Haze, et al.. FXR α modulates leydig cell endocrine function in mouse. *Molecular and Cellular Endocrinology*, 2020, 518, pp.110995 -. 10.1016/j.mce.2020.110995 . hal-03529553

HAL Id: hal-03529553

<https://uca.hal.science/hal-03529553v1>

Submitted on 5 Sep 2022

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FXR α modulates Leydig cell endocrine function in mouse.

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Running title: Leydig cell, FXR α and LH.

Key words: Leydig cell, testis, hCG signaling, nuclear receptors, steroidogenesis.

Disclosure. The authors declare to have no conflict of interests.

Grant supports. The study was funded by Inserm, CNRS, Universit e Clermont Auvergne. Volle’s team received support by the French government IDEX-ISITE initiative 16-IDEX-0001 (CAP 20-25).

Abstract.

The hypothalamic-pituitary axis plays a major control over endocrine and exocrine testicular functions. The hypothalamic-pituitary axis corresponds to a cascade with the Gonadotropin Releasing Hormone secreted by the hypothalamus, which stimulates the synthesis and the release of Luteinizing Hormone (LH) and Follicle Stimulating Hormone by the gonadotropic cells of the anterior pituitary. The LH signaling pathway controls the steroidogenic activity of the Leydig cell *via* the activation of the luteinizing hormone/choriogonadotropin receptor. In order to avoid a runaway system, sex steroids exert a negative feedback within hypothalamus and pituitary. Testicular steroidogenesis is locally controlled within Leydig cells. The present work reviews some local regulations of steroidogenesis within the Leydig cells focusing mainly on the roles of the Farnesol-X-Receptor-alpha and its interactions with several orphan members of the nuclear receptor superfamily. Further studies are required to reinforce our knowledge of the regulation of testicular endocrine function, which is necessary to ensure a better understanding of fertility disorders and then proposed an adequate treatment of the diseases.

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Introduction

1. The Hypothalamo-pituitary-gonad axis.

The hypothalamic-pituitary axis has major role on the control of testicular steroidogenesis. The hypothalamo-pituitary-testicular axis (HPT axis) is a neuro-endocrine regulatory circuit, with feedback loops (Kaprra and Huhtaniemi, 2018; Plant, 2015). Specific hypothalamic nuclei synthesize the Gonadotropin releasing hormone (GnRH). This later *via* the activation of a G-protein-coupled receptor stimulates the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by gonadotropic cells of the anterior pituitary gland.

Within the hypothalamus, the release of GnRH is pulsatile or by surge. It regulates the pulsatile secretion of the gonadotrophins; which are secreted in a pulsatile manner by the anterior pituitary (Kaprra and Huhtaniemi, 2018; Plant, 2015). In obese men, probably through excess of estrogen or the increased insulin resistance, LH pulse amplitude is diminished, explaining the central origin of hypogonadism (Gurung et al., 2020; Sen and Hoffmann, 2020; Vermeulen, 1996).

LH acts directly on Leydig cells to control their steroidogenic activity *via* the activation of the luteinizing hormone/choriogonadotropin receptor, LHCGR. In return, sex steroids exert central negative feedback on their own synthesis.

In adult, the Leydig cells constitute only 5% of somatic cells of testis (Kerr, 1991). These cells support part of the endocrine function of the testis through the production of androgens, mainly testosterone (Jin and Yang, 2014).. Testis also produces other hormones such as estrogen and progesterone. Maintenance of an adequate concentration of intra-testicular testosterone is essential for testicular function, especially for spermatogenesis (Woolveridge et al., 1998).

LH/hCG signaling pathway is the main regulator of Leydig cells functions. Through its receptor *Lhcgr*, the LH/CG activates the adenylate cyclase, which will stimulate cAMP pathway. This results in the phosphorylation of the C-AMP Response Element-binding protein (CREB). The phospho-CREB binds to CRE elements in the promoter regions of

steroidogenic genes and activation of cholesterol ester hydroxylase (Stocco et al., 2005). Numerous factors have also been identified to be involved in this response to LH such as Ca^{+2} dependent kinase system MAPK pathways, glucocorticoids, inflammatory cytokines, prolactin, transforming growth factors β ($\text{TGF}\beta$), Insulin growth factor-1 (IGF-1), Epidermal growth factor (EGF), factors, as well as sex steroids (Dufau and Catt, 1978; Manna et al., 2006; Tajima et al., 2005). LH also modulates the cholesterol homeostasis. Free cholesterol, the substrate for steroidogenesis, is transferred to the inner membrane of mitochondria. In addition, chronic LH stimulation controls expression of other enzymes involved in steroid synthesis (Payne and Youngblood, 1995; Wang et al., 2009).

Sex steroid hormones share a common biosynthesis pathway. The synthesis of steroids by the Leydig cells is a complex and multi-step process. The steroids are produced from cholesterol. Leydig cells are able to *de novo* produce cholesterol or can also use stored cholesterol ester to ensure steroidogenesis function. A recent study shows that the plasma membrane is one of the richest source of free cholesterol available for acute steroidogenesis (Venugopal et al., 2016). To ensure the production of steroids, Leydig cells also import cholesterol from circulating HDL (Hou et al., 1990). In that line, the homeostasis of Leydig cells involves factors of cholesterol homeostasis such as HMG-CoA reductase, HSL, and ACAT (Kabbaj et al., 2003, 2001).

The initiation of steroidogenesis, requires the transport of cholesterol from the outer mitochondrial membrane to the inner membrane. This limiting step is mainly ensured by the Steroidogenic Acute Regulatory protein (STAR) (D M Stocco, 2000). Mice lacking *Star* present gonadal insufficiencies due to steroidogenesis defects associated with lipid accumulation in the interstitial compartment (Caron et al., 1997). Recently, using a mitochondrial reconstitution assay system, it has been shown that SNARE proteins (Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor) are vital components contributing to cholesterol transport within the mitochondria (Lin et al., 2016). The mitochondrial transport of cholesterol could also be made by the peripheral-type benzodiazepine receptor (Pbr) (Papadopoulos et al., 2006). Then, the steroid biosynthesis

involves the oxidative cleavage of the side-chain of cholesterol by cholesterol side-chain cleavage enzyme (cytochrome P450_{scc}, CYP11A1), a mitochondrial cytochrome P450 oxidase, to give pregnenolone (Hu et al., 2004). Pregnenolone is converted to testosterone through two pathways called Delta 4 (Δ 4) and Delta 5 (Δ 5) (Conley and Bird, 1997). The Delta 4 (Δ 4) pathway involves conversion of pregnenolone to progesterone by the 3 β -Hydroxysteroid Dehydrogenase (3 β HSD). The cytochrome P450_{17 α -hydroxylase/17,20 lyase} (CYP17A1) converts the progesterone in androstenedione, which is the substrate of the 17 β -hydroxysteroid dehydrogenase enzyme (17 β HSD) giving rise to testosterone. The Delta 5 (Δ 5) pathway is initiated by the CYP17A1 enzyme, that allows the conversion of pregnenolone to dehydroepiandrosterone (DHEA). Then, 17- β HSD and 3 β HSD enzymes ensure its conversion into androstenediol or androstenedione, and testosterone. The 3 β HSD enzyme is responsible for the switch from Δ 5 to Δ 4 pathway. In primates including humans, the Δ 5 pathway predominates because CYP17A1 enzyme has a low activity 17, 20 lyase for the conversion of 17 α -hydroxyprogesterone to androstenedione (Hammar and Petersson, 1986; Rey et al., 1995). Then multiple enzymatic reactions allow the production of testosterone, which will then be transformed into dihydrotestosterone (DHT) by the 5 α -reductase, or into estradiol (E2) by the aromatase.

Sexual steroids are involved in the maintenance of fertility and the development of secondary sexual characters (for review: (Haider, 2004)) through the regulation of multiple functions within the testis. The critical role of the steroid synthesis pathway has been demonstrated using transgenic mouse models and through the identification of mutations in humans. For examples, mice lacking *Star* exhibit defective steroidogenesis, which is associated gonadal insufficiencies (Caron et al., 1997; Clark and Cochrum, 2007; D. M. Stocco, 2000). Such mutations are described in human and are associated lipoid congenital adrenal hyperplasia (Bose et al., 1997; Ishii et al., 2019).

The sexual steroids control many functions that all converge, within the testis, to the regulation spermatozoa production. Spermatogenesis is strictly controlled and depends on a

succession of signals provided by the local environment (Jégou, 1993; Mäkelä and Hobbs, 2019; O'Donnell, 2014; Skinner et al., 1991; Zhou et al., 2019). The review of the roles of sexual steroids within the testis and mainly on Leydig cells are not the aim of this review and for a complete overview, please read the following references (Edelsztein and Rey, 2019; Guercio et al., 2020; Gurung et al., 2020; Hess and Cooke, 2018; O'Hara and Smith, 2015). In addition, in the recent decades, numerous data have demonstrated the deleterious effects of endocrine disruptors with estrogenic or anti-androgenic activities on steroid homeostasis and thus on abnormal sperm leading to fertility disorders *via* the modulation of the steroid receptors (Ben Maamar et al., 2015; Casati et al., 2015; Sharma et al., 2017).

2. A Farnesol-X-Receptor alpha associated regulatory network in Leydig cells.

The nuclear receptor superfamily corresponds to transcriptional factors. In animal, nuclear receptors (NR) represent one of the most abundant classes of transcription factors. In response to ligand, NRs bind specific DNA sequences within the 5'-regulatory sequences of target genes and the recruit diverse transcriptional co-regulators. The activities of this protein complex modulate the structure of the chromatin and then lead to the transactivation or trans-repression of target genes. It is well defined that nuclear receptors, through the control of networks of target genes, control numerous physiological processes such as metabolism, inflammation and reproduction. In that line, abnormal functions of these NR signaling pathways can lead to the development of diverse diseases such as metabolic disorders, cancers or fertility disorders.

In the paragraphs below, we focus on the Farnesol-X-Receptor and its interactions with some other orphan members of the NR superfamily that have been defined over the last decades to control Leydig cell endocrine function. Here, the aim of this review is to highlight the roles of FXR α dependent network involved, within the Leydig cells, in a local regulation of steroid synthesis.

Farnesol-X-Receptor alpha (FXR α ; NR1H4) is expressed in many tissues and have been associated with the regulation of many physiological functions. Within the adult testis, FXR α is expressed in the interstitial space and mainly in Leydig cells (Volle et al., 2007a) suggesting its participation in the control of testicular steroids synthesis. It has been demonstrated that FXR α activation by a synthetic agonist (GW4064) results in a decrease of androgen production, independently of the hypothalamo-pituitary axis. This was associated with a decreased expression of steroidogenic genes encoding such as the steroidogenic acute regulatory protein (*Star*), the cytochrome *Cyp11a1* or the 3 β -Hydroxysteroid dehydrogenase/ Δ 5-4 isomerase (*3 β -Hsd*) (Volle et al., 2007a). The role FXR α in the inhibition of the steroidogenesis was also observed in boar taint (Gray and Squires, 2013).

At the molecular level, it has been demonstrated that FXR α acts in an indirect manner. Indeed, activation of FXR α in Leydig cells leads to the transcription of the gene encoding the orphan nuclear receptor Small Heterodimer Partner (SHP; NR0B2); which acts as a trans-repressor of transcription. SHP lacks the classical DNA-binding domain (DBD) (Seol et al., 1996). SHP is known to act as a negative regulator of transcription through the interactions to other nuclear receptors and the recruitment of co-repressors or histone methyltransferases (Båvner et al., 2005).

Within Leydig cells, SHP represses the expression of the steroidogenic genes such as *Star*, *Cyp11a1* and *3 β hsd* since puberty and in adult mice (Volle et al., 2007a). It has been clearly showed that SHP acts as a local regulator of testicular steroidogenesis as no modulation of LH concentrations are observed in SHP KO male mice. It has been demonstrated that in Leydig cells, SHP acts through the inhibition of the expression of two positive regulators of steroidogenesis, which are members of the nuclear receptor superfamily; the steroidogenic factor-1 (SF-1, NR5A1) and the Liver Receptor Homolog -1 (LRH-1; NR5A2) (Volle et al., 2007a). In addition, SHP was also demonstrated, at the molecular level, to inhibit the activity of LRH-1 on the promoter of steroidogenic genes such as *Star* (Volle et al., 2007a). The

impacts of SHP on the expressions/activities of these two nuclear receptors are important to understand its roles in the control of steroidogenesis..

In testis, *Sf-1* is highly expressed in many cells among which mature Leydig cells; where it directly controls the expression of steroidogenic genes in collaboration with many transcription factor such as phospho-CREB, NR4A1, GATA4, SP1, cJUN and C/EBP β , (Abdou et al., 2016; Martin and Tremblay, 2010). In addition, SF1 is involved in the control of steroidogenesis through the regulation of other cellular processed. In Leydig cells, SF-1 controls many aspects of cholesterol homeostasis. It regulates the expression of the scavenger receptor, class B, type I (SR-BI), which is critical for the uptake of plasma cholesterol (Cao et al., 1999). SF-1 seems to control *de novo* synthesis of cholesterol through the regulation of the HMGCoA synthase (Mascaró et al., 2000). Moreover, a recent study using genome-wide analysis identified Ferredoxin reductase (FDXR, also known as adrenodoxin reductase) as a SF-1 target gene (Imamichi et al., 2014). FDXR is a mitochondrial flavoprotein responsible for the transfers electrons from NADPH to mitochondrial cytochrome P450 enzymes, mediating the function of an iron-sulfur cluster protein, ferredoxin. This is critical for many processes including steroidogenesis. In addition, it has been hypothesized that stem cell transplantation could be method for the treatment of Leydig cell dysfunction-related diseases (Shima et al., 2018). At least *in vitro*, the expression of the aromatase gene and in turn of estrogen levels is controlled by FXR α (Catalano et al., 2010). The mechanism proposed is direct as it has been demonstrated that FXR α competes SF1 for the accessibility to DNA regulatory sequences.

Next to SF1, with the Leyddig cells, the FXR α target SHP controls the expression and the activity of LRH-1. LRH-1 shows a high degree of sequence conservation with SF1, and thus binds to the same hexameric response elements of the regulatory sequences of their direct target genes. Thus they regulate similar functions. In the adult testis, LRH-1 was found to be expressed in germ cells as well as in Leydig cells (Sirianni et al., 2002; Volle et al., 2007a). LRH-1 is thought to modulate steroidogenesis, as next to SF-1, most of

steroidogenic enzymes are up regulated by LRH-1 (Sirianni et al., 2002; Z. N. Wang et al., 2001). *In vitro* data demonstrate that LRH-1 controls the expression of the aromatase gene (Sierens et al., 2010). *In vivo*, the LRh-1^{+/-} males show altered testicular steroidogenesis (Volle et al., 2007a).

In addition, SHP has been previously described to interact and inhibit the Liver X Receptor alpha (LXR α ; NR1H3), a receptor of oxysterols (Macchiarulo et al., 2006). LXR play critical roles in testis (Jarvis et al., 2019; Maqdasy et al., 2016). LXR α was defined to regulate testosterone production within Leydig cells (Volle et al., 2007b). The *Lxr α ; β ^{-/-}* mice are infertile by 5 months of age. It has been demonstrated that LXR α -deficient mice had lower levels of testicular testosterone that is correlated with a higher apoptotic rate of the germ cells. If LXR β is in the Sertoli cells, it participates to regulate steroid synthesis, probably by the control of the expression of gene involved in the production of yet unidentified paracrine factors. Many enzymes implicated in steroidogenesis are targets for LXRs. Indeed, the absence or pharmacological stimulation of LXR in mice modulate the expression of *Star*, *3hsdB*, and *17 β hsd* and alter intra-testicular testosterone concentrations (Maqdasy et al., 2016). However, the published data do not support a potential interaction between LXR α and SHP in the Leydig cells (Volle et al., 2007b, 2007a).

Related to its roles in the control of steroid synthesis, FXR α signaling pathway must be of high importance in the context of endocrine disruption. Indeed, estrogen-signaling pathway regulates the expression of Fxr α with the testis, and it was recently highlighted that FXR α play a major role in the deleterious effects of endocrine disruptors with estrogenic activity such as BPA (Sedes et al., 2018). It is interesting to note that the expression of *Shp*, a known FXR α target gene, was also found to be control by estrogen (Lai et al., 2003). This regulation was also demonstrated to be essential within the Leydig cells as exposures of male mice to estradiol or endocrine disruptors with estrogenic activity lead to decrease of steroidogenesis in a SHP dependent manner (Volle et al., 2009). Consistently, *in vitro* analyses using the Ma-10 Leydig cell line showed that estradiol increased *Shp* mRNA

accumulation at 12 and 24 hours after treatment, and is then followed by a decreased mRNA accumulation of steroidogenic genes such as *Star* after 24 hours (Volle et al., 2009). These data highlighted the crosstalk between ER α and SHP. This was further validated through the use of the ER agonist (estradiol), or the antagonist ICI (Volle et al., 2009). Data suggest a significant contribution of SHP in the estrogenic effects of endocrine disruptors such as diethylstilbestrol. Indeed, estrogen exposure resulted in a decrease of the intra-testicular concentration of testosterone in *Shp*^{+/+} mice (Volle et al., 2009). The involvement of ER α was sustained by the fact that ICI exposure reversed the effect of DES on testosterone in *Shp*^{+/+} males, whereas no effect on testosterone level in *Shp*^{-/-} males (Volle et al., 2009). These effects of the ER α -SHP signaling pathways occur at the testicular level as no impact of DES on LH or FSH level was observed. At the molecular level, it was demonstrated that SHP was involved in the estrogenic signaling pathways as the mRNA accumulations ER target genes such as insulin-like-3 (*InsI3*) were impacted only on wild-type males. In addition, unlike wild-type males, *Shp*^{-/-} males are protected from the deleterious effects of diethylstilbestrol, as testosterone concentrations as well as *Star*, *Cyp11a1*, and *Cyp17a1* mRNA levels were not affected (Volle et al., 2009). This decrease in testosterone was also correlated with reduced mRNA abundance of the androgen-dependent genes *Pem* and *Osp* in *Shp*^{+/+} males (Volle et al., 2009). In this context of DES exposure, SHP decreased *Lrh-1* expression (Volle et al., 2009); suggesting that the regulation of *Lrh-1* and *Sf-1* by SHP must involve specific co-factors that must be gene specific within the Leydig cells.

From the above data, the regulation of SHP expression seems to be important to regulate testicular steroidogenesis following the modulation of FXR α expression and/or activity. But so far, in the Leydig cells the level of post-translational modifications of SHP has not been studied. This could be an important point to cover as recent findings have shown that through the ERK pathway SHP can be phosphorylated resulting in an increased stability of SHP protein (Miao et al., 2009). Indeed, this phosphorylation was associated with an inhibition of the ubiquitination-coupled degradation. This stabilization leads to an

accumulation of inhibitor complexes. In addition, studies have identified site of ubiquitination within SHP protein and it was suggested that these sites must be involved in transcriptional repression (Miao et al., 2009). It has also been shown that RanBP2 co-localizes with SHP at the nuclear envelope region and mediates SUMO2 modification SHP at Lysine K68 (Kim et al., 2016). This facilitates its nuclear transport and increases its interaction with repressive histone-modifying enzymes LSD1 and HDAC1, which results in the inhibition of BA synthetic genes (Talamillo et al., 2020). It could thus be hypothesized that such mechanisms exist in Leydig cells might control the process of steroidogenesis inhibition by SHP.

All the above data sustain the idea of the importance of the FXR&-SHP-SF1/LRH-1 network in the control of the Leydig cell endocrine function.

Next to its roles in adult Leydig cells, the potential role of FXR α in testis was studied during pubertal period, which is highly dependent of steroids. For that purpose, many experimental models of liver injury were used to modulate the concentrations of bile acids, FXR α natural ligands. In that line, bile-duct ligation in pre-pubertal males, which leads to an increase of bile acid concentrations, is associated with lower testosterone levels (Houten et al., 2007). Experimental models of liver injury show an altered puberty phenotypes (Van Thiel et al., 1985) with smaller testes and seminal vesicles. This suggests that cholestasis contributes, at least in part, in pubertal and maturational failure due to an alteration of the testicular endocrine function. Thus, the roles of bile acid signaling on puberty onset and male sexual maturation were defined (Baptissart et al., 2016). Pubertal mice (21-days old) exposures to bile acid supplemented diet lead to an increased percentage of sterile males at adulthood. This decrease of fertility was associated with a lower production of spermatozoa that is related to an alteration of germ cell survival rate. The increased apoptotic rate is a consequence of a decreased testosterone synthesis as sustained by the fact that supplementation with exogenous testosterone restores the germ cell survival. The role of FXR α was supported by the fact that no effect of its agonist (GW4064) was observed in *Fxr α*

deficient mice. Regarding what was published in adult mice on the regulation testosterone synthesis (Volle et al., 2007a), it was expected that a FXR α -SHP cascade might be involved in this alteration of sexual maturation by bile acids. However, the use of Shp^{-/-} male mice showed that the lack of SHP was not sufficient to counteracts the deleterious effects of BA on testosterone production (Baptissart et al., 2016). This suggested that other mechanisms might exist.

During puberty, the effects of the bile acids-FXR α pathways are, at least in part, mediated DAX1 (Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1), which has been described as a FXR α target gene (Baptissart et al., 2016). As SHP, DAX1 is an unusual orphan nuclear receptor that lacks the C-terminal portion of a ligand-binding domain (Zanaria et al., 1994). DAX1 interacts with other nuclear receptors to inhibit their activities and thus act as a negative regulator of transcription (McCabe, 2007). In that line, DAX1 interacts with SF1, LRH-1 and NGFIB three positive regulators of steroidogenesis (Martin and Tremblay, 2010).

The major role of Dax-1 in the testis is sustained by the fact that Dax-1-KO mice show hypogonadism (Yu et al., 1998). These mice show with reduced testis mass with altered spermatogenesis and Leydig cell hyperplasia (Meeks et al., 2003). Interestingly, the re-expression of *Dax-1* specifically in Leydig cell knock-out improved their fertility. *In vitro*, DAX-1 has been demonstrated to control the expression of most of the steroidogenic genes. However, *in vivo*, only the expression of *Cyp19* mRNA was modulated in mice with a specific deletion within Leydig cells (Z. J. Wang et al., 2001). Consistently, the selective estrogen receptor modulator, tamoxifen partly rescues fertility of Dax-1 KO males. Although testicular size was not restored, *Cyp19* expression was back to normal levels, and sperm production was increased (Meeks et al., 2003). However, more data will be necessary to define whether *in vivo* DAX-1 is the main mediator of the effect of bile acids on testosterone during pubertal period, or to define if SHP and DAX-1 can compensate each other. It also appears that DAX-1 must play a critical role in multiple pathophysiological contexts such as exposures to ED or

metabolic diseases. Interestingly, it has been demonstrated that androgens up-regulates *Dax-1* expression; which in turn represses the activity of SF1 on the aromatase proximal (Maris et al., 2015). In the context of the relation between DAX-1 and the androgen signaling pathways, it has been demonstrated that Triclosan, an ED that has anti-androgenic effects suppressed testicular steroidogenesis (Duan et al., 2020). The authors have demonstrated a new signaling cascade where Triclosan exposure induced the accumulation of the miR-142-5p, which in turn modulates a Sp1; DNMT1; DAX1 cascade leading to the inhibition of P450c17 and the decrease in testosterone levels (Duan et al., 2020).

In addition, DAX-1 was suggested to play a critical role in the repression of testosterone production observed in obese or diabetic patients (Ahn et al., 2013). Indeed, the authors have demonstrated that insulin induces the expression of *DAX-1* in Leydig both *in vivo* and *in vitro*. Using multiple molecular approaches, it was shown that insulin-induced *DAX-1*, which in turn inhibited LRH-1-mediated regulation of *Star*, *Cyp17a1* and *3 β -hsd* at the promoter level (Ahn et al., 2013). These data were supported by additional report on mouse showing studying the impact of exposure to 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47). BDE-47 is associated with increased diabetes prevalence; and co-exposure to high-fat diet can aggravates BDE47-inhibited testosterone production through hyperinsulinemia. This was associated with an increase of *Dax-1* expression and a down-regulation of steroidogenic proteins such as STAR and 3 β -HSD in Leydig cells (Zhang et al., 2017). These data must be of interest in the context of FXR α modulation of *Dax-1*, as FXR α is a known regulator of lipid metabolism.

It must also be highlighted that pro-inflammatory cytokine such as the tumor necrosis factor alpha (TNF- α) have an inhibitory impact in the steroidogenesis of Leydig cells (Syriou et al., 2018). This could be of interest in the case of pathologies, metabolic disorders or exposure to endocrine disruptors (Bini et al., 2015; Pivonello et al., 2019; Zheng et al., 2010). It has been demonstrated that, within the Leydig cells, TNF- α treatment leads to an increase of DAX-1 mRNA and protein accumulations via a JNK/ERK MAP kinase pathway; which then results in a repression of the steroidogenic enzyme genes in Leydig cells

(Sadasivam et al., 2015). In contrast to SHP, little is known about post-translation modifications of DAX-1 and the potential impacts on its activity. It was shown that the E3 Ubiquitin Ligase RNF31 and DAX-1 act as co-repressor complex on the promoter of steroidogenic genes such as *Star* and *Cyp19* (Ehrlund et al., 2009, p. 31). This leads to a mono-ubiquitination of DAX-1 that stabilizes the protein. This seems to be required for the repressive effect of DAX-1 on steroidogenic gene expression. These above data sustain the idea that DAX-1 must be key in the impact of FXR α signaling pathway in prepubertal testis.

As mentioned above, within the Leydig cells, the FXR α -SHP or FXR α -DAX1 pathways must play some roles in normal physiology or in multiple pathophysiological conditions. As highlighted in the above sections most of the effects of FXR α on Leydig cells are driven by two downstream targets, that are two other nuclear receptors, namely SF1 and LRH1. Their expression and/or the activity levels must be critical. Their expressions are modulated by a wide range of transcriptional regulatory processes, involving transcription factors, intron enhancers, and epigenetic elements (Meinsohn et al., 2019). These multiple regulations contribute to explain the specific, developmental, tissue and/or species expression of LRH-1 and SF-1 and their impacts on steroidogenesis. An exhaustive review of these processes is outside the purpose of this review as it has been extensively covered (Meinsohn et al., 2019). However, recently, important informations have been reported showing that post-translational modifications of SF1 and LRH-1 are key to control the endocrine function of the Leydig cells. Within the adult Leydig cells; data showed that transforming growth factor beta 1 (TGFB1) down-regulates the expression levels of SF1 and LRH1, through the canonical signaling pathway involving ALK5. This is associated with the down-regulations of estrogen production (Yang et al., 2017, p. 1). The protein accumulation (stability) and/or activities of SF-1 and LRH-1 are controlled by multiple post-translational modifications. For example, it has been demonstrated that SF1 can be acetylated by P300 following activation of the intracellular second messenger cAMP and protein kinase- A

pathway. This post-translational change was associated with an increase binding of SF1 to DNA and its interaction with regulatory cofactors. PKA has pathway promotes the dissociation SF1 and DAX1, leading to an increased transcriptional activity of SF1 (Chen et al., 2005, p. 1; Fan et al., 2004). In addition, both LRH-1 and SF-1 have phosphorylation sites modulated by activation of the protein kinase C and the MAPK/ERK pathways. Phosphorylations of these receptors are associated with an increase in the transcriptional activity of these receptors (Krylova et al., 2005; Lee et al., 2006)k. SF-1 and LRH-1 can be sumoylated reducing their binding to DNA sequences or their translocation from the chromatin to inactive nuclear bodies. Moreover, E3 ligase RNF31 and stabilize DAX1 and by the way induces SF-1 inactivation on promoter of target genes (Ehrlund et al., 2009). Interestingly, the permanent elimination in vivo of SF-1 SUMOylation in mice resulted in abnormal endocrine tissue development (Lee et al., 2011, p. 1). Interestingly, LRH-1 SUMOylation controls cholesterol metabolism (Stein et al., 2014) (Stein et al., 2017). This effect could be critical and must be studied in the context of steroidogenesis regulation. Another post-translational regulation process that affects SF1 and LRH-1 is ubiquitination (Lai and Hu, 2019; Lee et al., 2016). It leads to the inhibitory of their activity. SF-1 ubiquitination plays an important role in the reproductive and endocrine pathology, which then reduces steroidogenesis at least in adrenal tumor cells (Chen et al., 2007).

3. Crosstalk with the LH/CG signaling and FXR α signaling pathway within the Leydig cells.

If FXR α was shown to control testicular steroidogenesis the link with the hypothalamo-pituitary axis have been studied only very recently. A recent paper has demonstrated that the activation of FXR α by its agonist leads to the repression of the expression of the LH/CG receptor, namely *Lhcgr* (Baptissart et al., 2016). This FXR α signaling pathway alters LH/CG sensitivity of Leydig cells both *in vitro* and *in vivo* in pubertal males.

However, little is known about the regulation of the *Fxr α* gene. Within Leydig cells, a

recent report shows that hCG, at low dose, regulates the expression of the *Fxr α* gene via PKA signaling. It was demonstrated that this regulation is not mediated by subsequent steroid production induced by LH as sustained by the experiments using testosterone, R1881, flutamide or casodex (Holota et al., 2019). This suggests that *FXR α* could also participate in a local feedback of androgen synthesis

This potential role of *FXR α* in a negative local feedback of sex steroid synthesis was sustained by additional data showing that a metabolite of testosterone, androsterone, has been defined as *FXR α* agonist (Wang et al., 2006). In addition, recent data have demonstrated that Leydig cells must be able to produce bile acids that are endogenous *FXR α* ligands (Martinot et al., 2017). As bile acid and sex steroids have the same precursor, namely cholesterol, these data sustain the idea that the regulation of *FXR α* expression and/or activity could define a molecular pathway to regulate sex steroid synthesis. Next to the regulation of the expression of *Fxr α* , its activity level of *FXR α* is thus critical to control testicular steroidogenesis. Recently, *FXR α* activity has been demonstrated to be regulated by post-translational modifications such as sumoylation or phosphorylation (Hu et al., 2017). Further studies will be necessary to define whether such mechanisms could be involved in the regulation of steroidogenesis by *FXR α* .

So far, no clear data were published show that, within testis, DAX-1 and/or SHP regulates the expression of the *Lhcgr* and the sensitivity to LH/CG. It could be hypothesize that there are either not involved or that they could be both redundantly involved and in that case a double DAX-1/SHP knock-out mouse model must be necessary to prove such idea. However, it has been demonstrated that the two *FXR α* target genes *Shp* and *Dax1* cells are also modulated by the LH/CG signaling pathway. Regarding the association between Leydig cell activity of SHP and the hypothalamo-pituitary axis, it has been demonstrated that LH/CG represses the expressions of both *Shp* and *Dax-1*. Within the Leydig cells, the activation of the 5'-AMP activated protein kinase (AMPK) pathway, which might be activated following the decrease of ATP, led to a decrease of steroid synthesis which is correlated with an increased

expression of *Dax-1* (Abdou et al., 2014) and *Shp* (Vega et al., 2014). In addition, SHP seems to be involved in the repression of testosterone synthesis following anti-GnRH compound exposure (Vega et al., 2014). These data have highlighted a link between hypothalamo-pituitary axis and SHP in testicular androgen metabolism and thus testicular physiology.

Integration of data and Conclusions

These data suggest that local mediators are involved in a negative feedback of steroid synthesis. Combined all the above data improve our knowledge of the local control of the testicular steroidogenesis with the identification of the molecular links between the hypothalamo-pituitary axis and the FXR α /SHP and FXR α /DAX1 signaling pathways. These are important findings as the control of the Leydig cell functions is predominantly mediated by the hypothalamo-pituitary-gonadal axis *via* LH/CG. It highlights that FXR α might act as an important actor of the regulation of testicular physiology during puberty. This is of interest in order to identify the etiology of primary hypogonadism.

Infertility is defined as the failure of a couple to conceive after 12 months of unprotected sexual intercourse. It affects approximately 10–15% of all couples worldwide (Callister, 2010). Male factors contribute nearly 30–55% of all cases of infertility. The high rates of male infertility are governed by a variety of causative factors, including genetic defects, environmental pollutants, physiological and endocrine failure, and testis pathologies (Hauser and Sokol, 2008).

The present review highlights the links between nuclear receptors and the efficiency of the Leydig cell endocrine function (**Figure-1**). As reviewed here, several nuclear receptors, adopted or orphans, have been demonstrated to be key regulators of lipid homeostasis. Indeed, cholesterol plays an important role in maintenance of reproductive functions and can be responsible of fertility disorders. We thus propose here a model combining the reviewed data.

After the activation of the hypothalamo-pituitary axis, the LH will stimulate its receptor in the Leydig cells (**Figure-1**). There, in combination with the nuclear receptors such as NGFIB, SF-1 and LRH-1, Phospho-Creb induces the expression of steroidogenic genes. In the meantime, it has been demonstrated that the LH/CG, in part through AMPK signaling pathway, will repress the expression of SHP and Dax-1 two repressors of the steroid synthesis. These later act mainly through the inhibition LRH-1 and SF-1. Surprisingly, it has been demonstrated that the LH/CG signaling pathway induces the expression FXR α . This might prepare a kind of negative feedback. Indeed, it has been proposed FXR α first regulated the expression of SHP and Dax-1 for the repression of steroid synthesis. In addition, FXR α was also demonstrated to repress the expression of the *Lhcgr* gene that must also participates to reduce the response of Leydig cells to the hypothalamus-pituitary axis

However, it has not been clearly studied whether the nuclear receptors, highlighted in this review; could impact of the hypothalamus and LH and FSH production by the pituitary. Thus further data using transgenic mouse models (knock-out mice) will be needed to better define the complete crosstalk mechanisms at the hypothalamus/pituitary levels that could lead to an alteration of the hypothalamo-pituitary-gonadal axis and in turn the alteration of testicular physiology and particularly steroid synthesis.

New perspectives of research are now open. But several questions need to be answered. Studies must be conducted to analyze in human the alteration of the expression of all these nuclear receptors as well as to define if mutations (polymorphisms) of these NRs could be associated with male fertility disorders. In the future, these NRs may help to better understand, at the molecular level, the association between Metabolic Syndrome and infertility or the links between exposure to estrogenic endocrine disrupters and male fertility disorders associated with alteration of the hypothalamus-pituitary axis.

It will remain to define if some of these nuclear receptors could target by ligands to be used as therapeutical targets? Such ideas have been reported for the orphan nuclear receptors in different diseases. However, the difficulty remains to target specific nuclear

receptors in a particular developmental window in a specific cell type.

Regarding the complexity of the interaction throughout the hypothalamo-pituitary-gonadal axis, there still lot of effort to put on this research area to improve the treatment of human fertility disorders.

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Figure 1. Proposed model (see discussion section).

