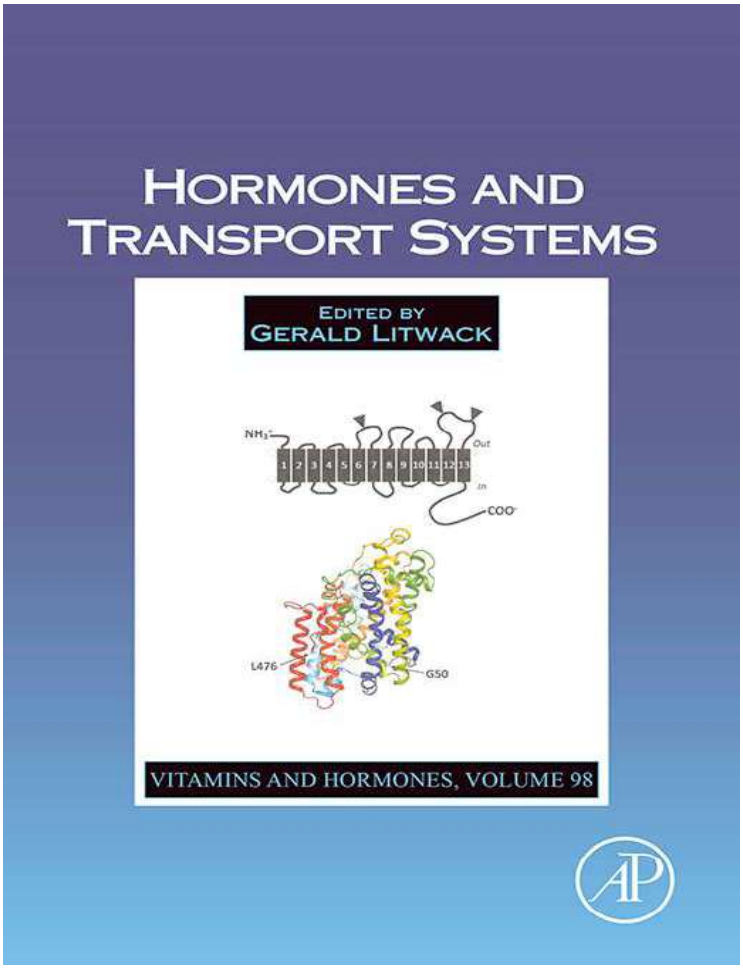

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CHAPTER SEVEN

Pharmacological Regulation of the Cholesterol Transport Machinery in Steroidogenic Cells of the Testis

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Contents

Abstract

Reduced serum testosterone (T), or hypogonadism, is estimated to affect about 5 million American men, including both aging and young men. Low serum T has been linked to mood changes, worsening cognition, fatigue, depression, decreased lean body mass and bone mineral density, increased visceral fat, metabolic syndrome, decreased libido, and sexual dysfunction. Administering exogenous T, known as T-replacement therapy (TRT), reverses many of the symptoms of low T levels. However, this treatment can result in luteinizing hormone suppression which, in turn, can lead to reduced sperm numbers

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and infertility, making TRT inappropriate for men who wish to father children. Additionally, TRT may result in supraphysiologic T levels, skin irritation, and T transfer to others upon contact; and there may be increased risk of prostate cancer and cardiovascular disease, particularly in aging men. Therefore, the development of alternate therapies for treating hypogonadism would be highly desirable. To do so requires greater understanding of the series of steps leading to T formation and how they are regulated, and the identification of key steps that are amenable to pharmacological modulation so as to induce T production. We review herein our current understanding of mechanisms underlying the pharmacological induction of T formation in hypogonadal testis.

ABBREVIATIONS

3βHSD/HSD3B 3β-hydroxysteroid dehydrogenase
17β-HSD/HSD3 17β-hydroxysteroid dehydrogenase
19-Atriol 3,17,19-androsten-5-triol
ACBD1/ABCD3 acyl-CoA binding domain 1/3
AKAP a kinase anchoring protein
ANT adenine nucleotide transporter
ATAD3 AAA+ ATPase domain 3
ATP adenosine three phosphate
Bcl-2 B-cell lymphoma 2
CAH congenital adrenal hyperplasia
cAMP cyclic adenosine monophosphate
CBP CREB-binding protein
CD spectroscopy circular dichroism spectroscopy
CDK5 cyclin-dependent kinase 5
CRAC cholesterol recognition/interaction consensus
CREB cAMP-response element-binding protein
CYP11A1 cytochrome P450 side-chain cleavage
CYP17A1 cytochrome P450 steroid 17-α-monooxygenase
ER endoplasmic reticulum
ERK1/2 extracellular signal-regulating kinase 1/2
FdR ferredoxin reductase
Fdx ferredoxin
FGIN-1–27 *N,N*-di-*n*-hexyl 2-(4-fluorophenyl)indole-3-acetamide
GABA gamma-aminobutyric acid
hCG human chorionic gonadotropin
Hsc70 heat-shock cognate protein of 70 kDa chaperone
HSP90 heat-shock protein 90
IMM inner mitochondrial membrane
IMS intermembrane space
LH luteinizing hormone
MEK1/2 mitogen-activated kinase kinase 1/2
MPTP mitochondrial permeability transition pore
MSF mitochondrial import-stimulating factor
NMR nuclear magnetic resonance

OMM outer mitochondrial membrane
P4 progesterone
P5 pregnenolone
PadX FdX precursor preadrenodoxin
PAP7 PKA-RIα-associated protein 7
PKA protein kinase A
PKA-C catalytic subunit
PKA-R regulatory subunit
PSA prostate-specific antigen
PTM posttranslational modification
RNAi interfering RNA
SAM Sorting and Assembly Machinery
SHBG sex hormone-binding globulin
STAR steroidogenic acute regulatory protein
START domain STAR-related lipid transfer domain
T testosterone
TIM translocase of the inner membrane
TOM translocase of the outer membrane
TRT testosterone replacement therapy
TSPO translocator protein
VDAC1 voltage-dependent anion channel 1

1. INTRODUCTION

Reduced serum testosterone (T), or hypogonadism, is estimated to affect about 5 million American men, including both aging and young. The condition is common in aging men, with 20–50% of men over age 60 reported to have serum T levels significantly below those of young men (age 20–30 years) (Lutz, Sanderson, & Scherbov, 1997; Oeppen & Vaupel, 2002). Age-related decline in serum T levels in these men typically is not in response to reduced luteinizing hormone (LH), but rather a consequence of Leydig cells becoming less responsive to LH (Huhtaniemi & Forti, 2011). This condition, referred to as primary hypogonadism, also occurs in many infertile men. Approximately 15% of couples seek infertility-related medical appointments, with male factor effects contributing to 40–50% of these cases (Carruthers, 2009; Huhtaniemi & Forti, 2011; Mitchell, Hollis, Rothwell, & Robertson, 1995; Perheentupa & Huhtaniemi, 2009; Rohrmann et al., 2011). About 30% of infertile men are diagnosed as idiopathic, among whom about 50% have primary hypogonadism (Ishunina et al., 2005; Valenti, 2005; Veldhuis, 2008). Whether in aging or young men, reduced serum T is linked to a number of metabolic

and quality-of-life changes, including decreased lean body mass and bone mineral density, decreased muscle mass and strength, obesity, cardiovascular disorders, decreased libido and sexual function, altered mood, and fatigue (Belchetz, Barth, & Kaufman, 2010; Cattabiani et al., 2012; Kaplan, Meehan, & Shah, 2006; Morris & Channer, 2012; Oeppen & Vaupel, 2002; Tuck & Francis, 2009; Valenti, 2005).

Administering exogenous T, known as T-replacement therapy (TRT), reverses many of the symptoms of low T levels. The primary objective of TRT is to raise serum T levels so as to reduce symptoms of hypogonadism. Ideally, serum T concentration within the physiological range of adult men is the desired outcome. The T preparations in use to achieve this are injections, scrotal and nonscrotal transdermal patches, and oral, buccal, and gel preparations (Lang, Samaras, & Samaras, 2012; Lutz et al., 1997; Morris & Channer, 2012; Muraleedharan & Jones, 2010; Oeppen & Vaupel, 2002; Somani, Khan, & Donat, 2010; Wu et al., 2010). With injections, serum T levels initially are supraphysiologic and then reduced (ASA Position Statement, 2006; de Ronde, 2009; Dobs et al., 1999). This commonly used mode of T delivery requires serum T levels to be measured and sometimes adjusted between injections. High T levels may pose a risk for aging males due to possible prostate (benign prostatic hyperplasia; prostate cancer) and cardiovascular consequences (Morris & Channer, 2012). T administered by gels and other transdermal methods produce more constant serum T concentrations, but with the potential for T transfer via skin contact and skin irritation (Busse & Maibach, 2011; Muram, Melby, & Alles, 2012; Stahlman et al., 2012). Moreover, the administration of exogenous T by any means can suppress LH and thus result in reduced Leydig cell T formation and suppression of spermatogenesis. Indeed, contraception in men can be achieved by administering LH-suppressive T. Thus, the exogenous administration of T to ameliorate hypogonadism is inappropriate for men wishing to father children (Carruthers, 2009; Huhtaniemi & Forti, 2011; Perheentupa & Huhtaniemi, 2009; Riggs et al., 1982). There are methods in use that increase serum T without T administration, including hCG treatment in the case of men with secondary hypogonadism. In individuals with primary hypogonadism, aromatase inhibitors can increase T-to-estradiol ratios, particularly in men with severe infertility, but often this approach is ineffective (Perheentupa & Huhtaniemi, 2009).

Increasing intratesticular and serum T by stimulating the Leydig cells themselves could have great advantages. Such an approach should not elicit significant fluctuations in T levels because T formation would be regulated at

least in part by negative feedback of T on LH. Nor would there be T transfer to others via contact. Fertility should be preserved, not suppressed, because the hypothalamic–pituitary axis should not be shut down as with exogenous T. Indeed, the local stimulation of Leydig cell T production by the use of drug ligands that target proteins involved in cholesterol import into mitochondria, the rate-determining step in T formation, might actually support or enhance spermatogenesis because intratesticular T levels should increase, not decrease. This advantage could be of great benefit to the many young men with primary hypogonadism who wish to father children. If successful, T replacement through the administration of drugs targeting proteins involved in T formation could constitute a paradigm shift in the treatment of hypogonadal men.

In summary, the design of new therapies that increase intratesticular bioactive androgen levels without affecting the hypothalamic–pituitary axis would be of great benefit to numerous patients. This approach requires an understanding of the series of steps leading to T formation and how they are regulated, and the identification of key steps amenable to pharmacological modulation to induce T.

2. ROLE OF T IN HEALTH AND WELL-BEING

Recent studies on human longevity indicated that women have a 10% longer life span than men (Lutz et al., 1997; Oeppen & Vaupel, 2002), suggesting that hormonal differences might play a role in general health and the rate and age of mortality (Cattabiani et al., 2012). T production by men is required for virilization, development of secondary male sex characteristics, fertility, and maintaining bone density and muscle mass. Thus, maintaining Leydig cell androgen formation is essential for well-being. However, reduced serum T occurs commonly in aging men (20–50% of men over age 60), subfertile/infertile young men (>50% of those diagnosed as "idiopathic infertile"), and many others (Huhtaniemi & Forti, 2011; Perheentupa & Huhtaniemi, 2009). A progressive decline in serum T levels can be caused by age-related reduction in the capacity of Leydig cells to produce T, a condition called primary hypogonadism, by interruptions in the hypothalamus–pituitary–testis axis, referred to as secondary hypogonadism (Belchetz et al., 2010; Mitchell et al., 1995; Veldhuis, 2008), or a mixed primary and secondary hypogonadism.

Approximately 1–2% of serum T is free and 30–50% is bound to albumin with low affinity, while the rest is bound to sex hormone-binding globulin

(SHBG) with high affinity. Therefore, free and albumin-bound T are considered bioactive. The assessment of total T levels of patients is initially used for diagnosis of hypogonadism. However, other factors could also affect the circulating levels of T, including (i) alteration in the levels of SHBG, leading to decreased free serum T levels while the overall T production is unaltered (Kupelian et al., 2006); (ii) increase in levels and activity of aromatase, an enzyme that converts T to estrogen (Ishunina et al., 2005; Valenti, 2005); and (iii) the levels of 5α-reductase which converts T to the active dihydrotestosterone (Valenti, 2005). Moreover, genetic and environmental factors including stress, smoking, and alcohol consumption, as well as health conditions such as obesity and metabolic syndrome (Kaplan et al., 2006; Muraleedharan & Jones, 2010; Somani et al., 2010), also can affect T production. Androgen deficiency has been found to be associated with conditions that include infertility, cardiovascular diseases, adrenal or testicular hyperplasia, neurodegeneration, altered mood, fatigue, decreased lean body mass, reduced bone mineral density, increased visceral fat, metabolic syndrome, and decreased libido and sexual function (Carruthers, 2009; Lang et al., 2012; Morris & Channer, 2012). These observations highlight the significance of reduced T in male health and well-being and provide the rationale for the development of therapeutic techniques to treat this condition.

3. T-REPLACEMENT THERAPY

The administration of T to hypogonadal men, referred to as TRT, has increased markedly in recent years. It is estimated that in 2011, there were 5.3 million prescriptions and a market of $1.6 billion for TRT, which was five times higher than that in 2010 (http://www.fda.gov/AdvisoryCommittees). TRT can reverse several symptoms of androgen deficiency. However, a large body of clinical studies suggests health-threatening side effects. Therefore, the Endocrine Society offered precautionary guidelines for the use of TRT (Bhasin et al., 2010; Valero, Marquez, Campos, Puigvert, & Prieto, 2013). TRT is not recommended for patients with breast or prostate cancer, a palpable prostate nodule, induration, or a prostate-specific antigen (PSA) estimate higher than 4 ng/ml for most men or 3 ng/ml in high-risk men (e.g., African-American men, or men with a family history of prostate cancer or cardiac conditions; Bhasin et al., 2010; Saad, 2009; Twiddy, Leon, & Wasan, 2011; Wang et al., 2009). On the other hand, TRT is recommended for many men showing symptoms of androgen deficiency, with the objectives to maintain secondary

sex characteristic, improve sexual function (Isidori, Giannetta, Gianfrilli, et al., 2005; Wang et al., 2000), increase a sense of well-being, bone mineral density and free-fat mass (Benito et al., 2005; Isidori, Giannetta, Greco, et al., 2005), improve lipid profiles and insulin resistance (Jones et al., 2011; Marin, 1995), increase muscle strength (Sih et al., 1997), and treat depression (Amore et al., 2012).

Although TRT can reverse symptoms associated with low T, there are disadvantages and side effects to its use. TRT can result in LH suppression and therefore contraception (Howell et al., 2001; Nakazawa et al., 2006; Winters & Atkinson, 1997), fluctuating T levels, skin irritation, and the potential for T transfer to others upon skin contact (de Ronde, 2009; Lawrentschuk & Fleshner, 2009). It is noteworthy that studies on a large sample population of men showed that moderate rather than high serum levels of bioactive T are required for the treatment of hypogonadal men (Ferrini & Barrett-Connor, 1998).

Clearly, the development of alternate therapies for treating hypogonadism would be highly desirable. One such approach could be to stimulate T production by the hypofunctional Leydig cells themselves rather than providing T exogenously. Such an approach requires an understanding of the series of steps leading to T formation by Leydig cells, and the identification of key steps that are amenable to pharmacological modulation so as to induce T production.

4. STEROID BIOSYNTHESIS

There are four steroidogenic tissues in humans: adrenal cortex, gonads, placenta, and brain. Leydig cells of the testis are the main site of androgen formation in men, regulated by LH from the pituitary. Cholesterol is an amphipathic lipid synthesized from acetyl-CoA. Most tissues are able to synthesize cholesterol through a chain of enzymatic reactions. More than half of the cholesterol storage of the body is from *de novo* synthesis, 10% of which is produced in liver and another 10% by intestine. The remainder is provided by the diet. Cholesterol is the precursor of steroids, principally stored in the plasma membrane and lipid droplets of steroidogenic cells either as free cholesterol or combined with long-chain fatty acids as cholesteryl esters (Ikonen, 2008; Mesmin & Maxfield, 2009). Steroidogenesis is a result of a series of enzymatic reactions that occur in steroidogenic tissues, with the steroid products dependent upon tissue-specific enzymes (Payne & Hales, 2004).

Steroidogenic cells are defined by their ability to convert the precursor cholesterol to pregnenolone in the mitochondrial matrix through the function of the cytochrome P450 side-chain cleavage enzyme (CYP11A1) (Jefcoate, 2002), which is a member of a large family of P450 enzymes. Seven members of this family are targeted to mitochondria and the remaining 50 members to the endoplasmic reticulum (ER). Six of the members of this family are involved in steroidogenesis. Mitochondrial CYP11A1 converts cholesterol to pregnenolone, with the latter then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD; HSD3B) present in both the mitochondria and ER. CYP17A1, present in the ER, possesses both 17α-hydroxylase and 17,20-lyase activities. In the gonads of men, CYP17A1 converts progesterone to dehydroepiandrosterone which is further metabolized to T by 17-βHSD (Fig. 1).

The rate-limiting step in steroidogenesis is the import of cholesterol into mitochondria to become accessible to CYP11A1. By limiting access of the hydrophobic cholesterol molecule to CYP11A1, steroidogenic cells are able to control the amount of steroids they produce (Rone, Fan, & Papadopoulos, 2009). Steroid hormones are continuously synthesized. However, in response to increased circulating peptide hormones (LH in the case of Leydig cells), the rate of steroid hormone synthesis is greatly increased. LH binds its cognate receptors on the surface of Leydig cells

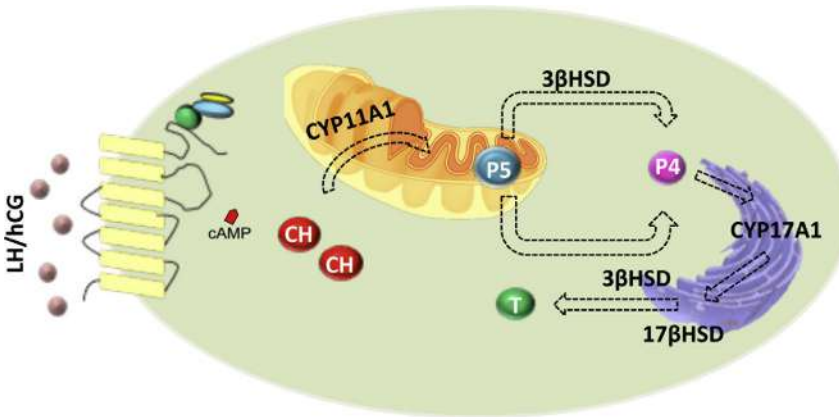

Figure 1 Summary of the steroidogenic pathway in the testis. The steroid hormone biosynthesis pathway in the Leydig cells of the testis is shown in this schematic representation. Cholesterol is the sole precursor of all steroids and it is through several enzymatic reactions in mitochondria, cytoplasm, and ER that different types of steroids are synthesized due to the presence and activity of specific enzymes. P5, pregnenolone; P4, progesterone; T, testosterone.

and stimulates intracellular signaling cascades, of which the cAMP pathway, through protein kinase A (PKA), is the most prominent. Acutely, these events stimulate the import of cholesterol to the OMM and then its transfer from OMM to IMM where it is converted to pregnenolone by CYP11A1 (Jefcoate, 2002; Papadopoulos, Liu, & Culty, 2007). In addition, hormonal and cAMP stimulation of steroidogenic cells are important for the chronic regulation of steroidogenesis, as continued stimulation is necessary to ensure proper expression levels of steroidogenic proteins and enzymes as well as steroidogenic metabolic flux (Simpson & Waterman, 1988).

The mitochondrion is a double-membrane organelle with an aqueous intermembrane space (IMS) between OMM and IMM, where CYP11A1 resides. Cholesterol molecules are hydrophobic. Thus, movement of cholesterol across the aqueous microenvironment of the mitochondria requires the involvement of intracellular machinery (Mesmin & Maxfield, 2009). Indeed, steroidogenic cells possess a multicomponent protein machine, the transduceosome, an ensemble of cytoplasmic and resident mitochondrial proteins that receives hormonal signals and is involved in the translocation of cholesterol across the IMS at contact sites between the OMM and IMM (Papadopoulos et al., 2007; Rone, Liu, et al., 2009) (Fig. 2). The transduceosome OMM proteins were identified as the 30-kDa voltage-dependent anion channel 1 (VDAC1) and the 18-kDa translocator protein TSPO, a high-affinity drug- and cholesterol-binding protein previously named the peripheral-type benzodiazepine receptor (McEnery, Snowman, Trifiletti, & Snyder, 1992; Papadopoulos et al., 2006). In addition to the mitochondrial proteins making up core structural and enzymatic components of the transduceosome, cytoplasmic proteins are instrumental in regulating its assembly and cholesterol transport activity. Hormonal stimulation was found to promote the clustering of TSPO, which was correlated with steroidogenesis and could be suppressed by the PKA inhibitor H-89 (Boujrad, Gaillard, Garnier, & Papadopoulos, 1994). This suggests an active role for PKA in the assembly of the transduceosome. Yeast two-hybrid screening for additional cellular partners of TSPO yielded the acyl-CoA-binding domain family protein ACBD3/PAP7 (Li, Degenhardt, et al., 2001), which serves to scaffold the cytosolic PKA-RI subunits to the transduceosome (Li, Degenhardt, et al., 2001; Liu, Rone, & Papadopoulos, 2006). An additional ACBD protein family member, ACBD1, participates in transduceosome function. Originally identified through its ability to displace benzodiazepine bound to GABA receptor sites in neurons (Costa & Guidotti, 1991), and hence originally named the diazepam-binding

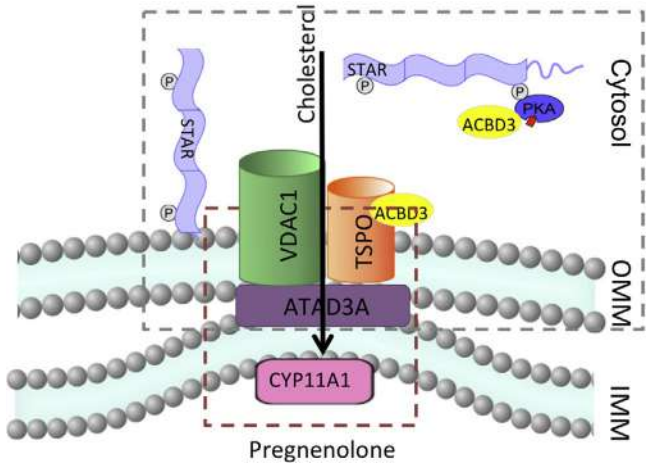

Figure 2 Transduceosome and metabolon complexes. The transduceosome consists of cytosolic and OMM proteins as indicated by gray margin. The metabolon, a protein complex that consists of OMM, IMS, and IMM proteins, is indicated in red (dark gray shade in the print version) margin. VDAC1 and TSPO are members of both the transduceosome and metabolon. CYP11A1 enzyme, located at the mitochondrial matrix side of IMM, and ATAD3, present at the contact sites of IMM and OMM, are members unique to the metabolon complex. The transduceosome mediates the import of cholesterol from cytosolic sources to mitochondria, while the metabolon serves to direct the cholesterol molecule to CYP11A1 where it can be converted to pregnenolone.

inhibitor, ACBD1 acts on TSPO and stimulates steroidogenesis (Boujrad et al., 1994; Papadopoulos, Berkovich, Krueger, Costa, & Guidotti, 1991).

While gene expression of the transduceosome components has been the focus of numerous studies, much less is known about mechanisms through which transduceosome components are targeted to their proper locations in the mitochondria, a factor critical to their function in steroidogenesis (Black, Harikrishna, Szklarz, & Miller, 1994).

4.1 Mitochondrial protein import and chaperones

Most proteins targeted to mitochondria are transcribed in the cytosol as preproteins containing mitochondrial signal sequences (Chacinska, Koehler, Milenkovic, Lithgow, & Pfanner, 2009). The import of such proteins occurs posttranslationally, shown by *in vitro* studies (Maccecchini, Rudin, Blobel, & Schatz, 1979) of cellular chaperones such as Hsc70 (heat-shock cognate protein of 70-kDa chaperone) and HSP90 (heat-shock protein 90). Upon interaction, Hsc70 and HSP90 stabilize the mitochondria-targeted, cytosolic preproteins and prevent protein malformation and/or

aggregation by protecting the hydrophobic sequences (Hsc70) or high-affinity interactions with non-native forms of these proteins (HSP90) (Scheufler et al., 2000). Therefore, the preproteins are kept in mitochondrial import-competent state (Fan, Bhangoo, & Young, 2006; Voos & Rottgers, 2002; Young, Hoogenraad, & Hartl, 2003) until docking with the appropriate receptors at the OMM. ATP hydrolysis leads to dissociation of chaperones from preproteins and the mitochondrial import machinery continues the import process (Young & Hartl, 2000; Young et al., 2003). Import machinery includes the Translocase of the Outer Membrane (TOM) complex at the OMM, which assists in the import of α-helices proteins, Translocase of the Inner Membrane (TIM) complex at the IMM, which is involved in the import of β-sheet structures, and the Sorting and Assembly Machinery (SAM) complex at the IMS (Wiedemann et al., 2003).

4.2 Transduceosome

The transduceosome is composed of the cytosolic members STAR, PKA, and ACBD3, and OMM proteins including TSPO and VDAC1, which function together to mediate cholesterol transport through the OMM upon hormonal stimulation (Liu et al., 2006) (Fig. 2). Hormonal stimulation triggers the assembly of the transduceosome at the OMM. This protein complex mediates the import of lipophilic cholesterol from the cytosol through the aqueous IMS and therefore regulates the rate-limiting step in steroidogenesis. The transduceosome consists of cytosolic and mitochondrial proteins which are enriched at the contact site of OMM and IMM.

4.2.1 Translocator protein

TSPO, previously known as the peripheral-type benzodiazepine receptor, is an 18-kDa protein ubiquitously expressed in all mammalian cells and abundant in steroidogenic cells. TSPO localizes at the OMM where it spans across OMM through its 5α-helical domains. Therefore, it is widely considered to function in the cholesterol trail from intracellular stores to the inner mitochondrial membrane (Jaremko, Jaremko, Becker, & Zweckstetter, 2014; Jaremko, Jaremko, Giller, Becker, & Zweckstetter, 2014). Unlike most mitochondrial proteins, TSPO does not possess mitochondrial targeting presequences. However, it contains information on the C-terminus which is essential for its mitochondrial import (Rone, Liu, et al., 2009). Mutational analysis indicates that C-terminal amino acids 103–109 of TSPO form Shellman motif through which Hsc70 and HSP90 target TSPO to mitochondria. The insertion of TSPO is further carried out by Tom70 in an

ATP-dependent manner (Otera et al., 2007; Rone, Liu, et al., 2009). A more intricate mechanism of OMM insertion was proposed for TSPO when RNAi knockdown studies indicated that the classic TOM complex import mechanism involving Tom20, Tom22, and Tom40 was not required for TSPO import. Rather, a role for Tim8 and Metaxin 1, homolog of Sam37, was suggested (Otera et al., 2007; Rone, Liu, et al., 2009). After proper integration at the OMM and in response to hormonal stimulation, TSPO forms clusters at the interface of OMM and IMM in complex with VDAC1 through which cholesterol can enter mitochondria (Chung et al., 2013; Liu et al., 2006). Nuclear magnetic resonance (NMR) studies elucidated the presence of the cholesterol- and ligand-binding domains on TSPO (Jamin et al., 2005; Murail et al., 2008).

The TSPO cytosolic C-terminus has a cholesterol recognition/interaction domain (CRAC) which binds cholesterol with low nanomolar affinity (Ostuni et al., 2009; Papadopoulos et al., 2007). NMR spectroscopy of the TSPO CRAC motif demonstrated that the side-chain amino acids form a groove which accommodates the cholesterol molecule. The tyrosine residue in this groove was shown to play a critical role in cholesterol binding. Moreover, molecular modeling identified a wide range of androstenetriol derivatives that potently subdue steroid biosynthesis by targeting the TSPO CRAC domain (Midzak, Rammouz, & Papadopoulos, 2012). In particular, 3,17,19-androsten-5-triol (19-Atriol) pharmacologically blocks cholesterol binding to the CRAC domain, thereby suppressing steroid biosynthesis. 19-Atriol is potent at low micromolar concentrations and its effect is dose dependent in both hormone-responsive MA-10 mouse Leydig tumor cells and constitutive steroid-producing R2C rat Leydig tumor cells (Midzak, Akula, Lecanu, & Papadopoulos, 2011).

TSPO also contains a drug ligand-binding domain and therefore is a target of numerous exogenous drug ligands which induce steroidogenesis. Numerous previous studies have shown that TSPO drug ligands are able to induce the translocation of cholesterol from the inner to the outer mitochondrial membrane, leading to increased steroid formation by isolated mitochondria and steroidogenic cells (Chung et al., 2013; Romeo et al., 1992) (Fig. 3). These effects of the TSPO drugs will be further discussed in detail below.

4.2.2 Voltage-dependent anion channel 1

VDAC1 is an OMM β-barrel protein involved in the regulation of ions and small molecules across the OMM that influence multiple cellular processes,

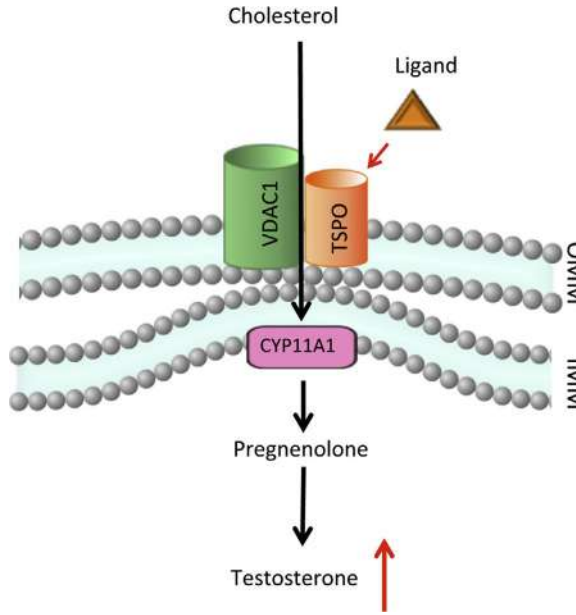

Figure 3 TSPO ligands induce endogenous T levels. TSPO is a high-affinity cholesterol- and drug-binding protein mediating cholesterol import into mitochondria. TSPO is closely interacting at the OMM with VDAC1. TSPO drug ligands ( ) stabilize the conformation of TSPO and induce the movement of cholesterol bound to TSPO into the mitochondria, leading to increased pregnenolone and then testosterone formation. Thus, these compounds can be used to induce endogenous T production.

including apoptosis and energy transduction (Vyssokikh & Brdiczka, 2003). The structure, but not amino acid sequence, of VDAC1 is similar to Tom40 as both proteins are β-barrel proteins that span the OMM 19 times (Zeth, 2010). shRNA knockdown of Tom40 in HeLa cells also reduced VDAC1 insertion into OMM (Kozjak-Pavlovic et al., 2007).

Inducing the loss of IMS proteins by using mitochondrial permeabilizing agents blocks VDAC1 import to this organelle (Krimmer et al., 2001), suggesting a role for Tim8–Tim13. As a B-barrel protein, VDAC1 is further transferred to SAM where Sam50 receives it and assists in its integration into mitochondria (Kutik et al., 2008). Knockdown of mammalian analogs of Sam37 (Metaxin 1) and Sam35 (Metaxin 2) in HeLa cells decreased VDAC1 assembly onto OMM by 50% and 90%, respectively (Armstrong, Komiya, Bergman, Mihara, & Bornstein, 1997; Armstrong, Saenz, & Bornstein, 1999; Kozjak-Pavlovic et al., 2007).

VDAC1 is a multifunctional protein that acts in cross talk between the cytosol and the mitochondria. VDAC1 interacts with TSPO (Liu et al., 2006; Papadopoulos & Miller, 2012), the Bcl-2 family of proteins (Brdiczka, Zorov, & Sheu, 2006), and adenine nucleotide transporter (ANT) (Liu et al., 2006). Upon hormonal stimulation, TSPO and VDAC1 form a cholesterol-binding complex through which the cytosolic cholesterol enters the mitochondria (Liu et al., 2006). VDAC1 also assists in anchoring the transduceosome to the OMM and in integration of STAR and OMM (Bose, Whittal, Ran, et al., 2008; Liu et al., 2006). The mechanisms and regulation of TSPO and VDAC1 interactions are not clear (Liu et al., 2006), but the presence of VDAC1 increases the ability of TSPO to bind to some of the TSPO drug ligands, such as benzodiazepines (Boujrad et al., 1994; Garnier et al., 1994). Through interactions with Bcl2, VDAC1 participates in apoptosis, and through its interactions with ANT, it forms the mitochondrial permeability transition pore (MPTP) through which small molecules and ions can enter mitochondria. In this way, it is involved in mitochondrial energy transduction (Sileikyte et al., 2011). Interestingly, TSPO ligands affect MPTP function (Sileikyte et al., 2011). It is noteworthy that VDAC1 knockout does not affect MPTP and mitochondrial function, opening a new area of investigation to identify the exact role of VDAC1 (Huang et al., 2013).

4.2.3 Steroidogenic acute regulatory protein

Of the cytoplasmic transduceosome components, the protein that has garnered the greatest research attention is the steroidogenic acute regulatory protein, STAR. STAR (STARD1) belongs to a family of proteins containing 15 members that are structurally related to STAR (Clark, 2012) as they contain the STAR-related lipid transfer (START) domain which binds a variety of lipids with low micromolar affinity. The members of this family STARD1–15, STARD1, STARD3 (MLN64), and STARD5 bind cholesterol. STARD5 also binds 25-hydroxycholesterol. STARD2 (phosphatidylcholine-transfer protein) binds phosphatidylcholine, and StarD10 binds phosphatidylcholine and phosphatidylethanolamine. Structural and knockdown studies suggested that among the cholesterol-binding members of this family, only STAR is involved in steroidogenesis (Akula, Midzak, Lecanu, & Papadopoulos, 2012; Midzak & Papadopoulos, 2014; Riegelhaupt, Waase, Garbarino, Cruz, & Breslow, 2010).

STAR was originally identified as a 30-kDa phosphoprotein located in the mitochondrial matrix and induced upon hormonal stimulation in adrenal and gonadal tissue (Pon, Hartigan, & Orme-Johnson, 1986; Pon &

Orme-Johnson, 1986). Later, it was shown to be synthesized as a 37-kDa cytoplasmic protein with a mitochondrial presequence tag that is imported into the mitochondrial matrix and processed to a 30-kDa form (Clark, Wells, King, & Stocco, 1994; King et al., 1995). STAR contains an N-terminal mitochondrial signal sequence that restricts its function to mitochondria (Clark et al., 1994), and a β-barrel START domain present at the C-terminus (Alpy & Tomasetto, 2005; Baker, Yaworsky, & Miller, 2005). Upon hormonal stimulation, STAR levels are induced in a manner parallel to steroidogenesis and STAR activation and action results to increased flow of cholesterol to mitochondria. STAR transcription in steroidogenic cells is rather rapid. In mouse Leydig cell lines, *Star* mRNA is induced 120 min following cAMP stimulation through the activity of cAMP-responsive element-binding protein 1 (CREB) and CREB-binding protein (CBP) (Clark, Combs, Hales, Hales, & Stocco, 1997; Lefrancois-Martinez et al., 2011). However, increase in STAR protein levels in these cells begins after 30 min of cAMP/hCG stimulation and reaches significantly higher levels after 60 min. Treatment of cells with actinomycin, a transcription inhibitor, does not block the induction in STAR protein levels, suggesting the presence of preexisting mRNA for STAR (Clark et al., 1997).

STAR activity is regulated by protein phosphorylation (Ascoli, Euffa, & Segaloff, 1987). Few protein kinases have been identified to date that regulate STAR function and activity. Extracellular signal-regulated kinase (ERK1/2) phosphorylates STAR on Ser232, which is a highly conserved ERK1/2-docking site. Such phosphorylation occurs at the mitochondria. However, the activity of ERK1/2 relies on cholesterol binding to STAR, whereas PKA phosphorylation occurs independently of cholesterol binding to this protein (Poderoso et al., 2008). Recent studies identified cyclin-dependent kinase 5 (CDK5) as interacting with and phosphorylating STAR. However, such phosphorylation is analogous to the role of PKA in regulating STAR phosphorylation (Lin, Chen, & Ku, 2009). Recently, the mitochondrial kinase MEK1/2 was also shown to phosphorylate STAR on S232, which contributes to STAR activation (Manna & Stocco, 2005).

PKA is the most prominent kinase that regulates STAR activity. Among the PKA phosphorylation sites, three residues are the most important, S554/56, S187, and S194. Mutational analysis suggests the presence of proteolytic cleavage sites at positions 39/40 and 55/56 of the bovine STAR protein, with similar predicted sites existing in other mammalian STAR homologs (Artemenko, Zhao, Hales, Hales, & Jefcoate, 2001; Yamazaki et al., 2006). In rodents, the STAR preprotein is targeted to mitochondria and proteolytically processed to yield a 32- and a final 30-kDa protein, the

latter located in the mitochondrial matrix (Epstein & Orme-Johnson, 1991; Stocco & Sodeman, 1991). This suggests that phosphorylation might be required for such cleavage. PKA phosphorylation of S194 induces STAR activity by twofold, required for maximum steroidogenesis (Arakane et al., 1997). Therefore, mutation of this residue is considered to be one of the major causes of lipoid CAH, a severe adrenal and gonadal disorder in which steroidogenesis is severely impaired and fatty deposits accumulate in steroidogenic cells (Lin et al., 1995; Miller, 2007), leading to lipoid hyperplasia (Bose, Sugawara, Strauss, & Miller, 1996; Fluck et al., 2011; Jo, King, Khan, & Stocco, 2005). Similar results were shown in mutations of S187 phosphorylation sites. This observation led to the suggestion that STAR's role is as a sole importer of cholesterol. In this hypothesis, STAR was believed to bind cholesterol through its START domain and import this molecule from the cytosol to the mitochondrial matrix. However, further studies indicated that STAR is only functional at the OMM, and upon reaching the matrix, the 30-kDa STAR is degraded, with a half-life of 4–5 h (Granot et al., 2003). An insufficient stoichiometry of 1:1 cholesterol binding was assessed for STAR (Tsujishita & Hurley, 2000), suggesting that STAR function in steroidogenesis is distinct from its cholesterol-binding capacity (Baker, Epand, Epand, & Miller, 2007). STAR knockout mouse models were developed and found to require corticosteroid supplementation for their survival (Hasegawa et al., 2000). These mice had high levels of lipid deposition in the adrenal cortex, and much lower levels in the testes (Caron et al., 1997). Although Leydig cell steroidogenesis seems to not be affected in the neonatal STAR knockout mice, T levels and spermatogenesis were reduced in the adult compared to wild-type mice (Hasegawa et al., 2000). However, it is not clear whether the reduced testicular function over time is due to the lack of STAR or to the detrimental effect of the long-term corticosteroid treatment on Leydig cell development and function (Bernier, Gibb, Collu, & Ducharme, 1984; Dong et al., 2004; Welsh, Bambino, & Hsueh, 1982; Xiao, Huang, Hardy, Li, & Ge, 2010). In female mice, the gonads were not affected by STAR knockout (Hasegawa et al., 2000). Therefore, it is now clear that STAR functions in a multiprotein dynamic along with other members of the transduceosome to import cholesterol to mitochondria (Liu et al., 2006; Papadopoulos et al., 2007; Papadopoulos & Miller, 2012). These studies also suggested the possibility of differential roles for STAR in adrenal versus testicular steroidogenesis.

The mechanism underlying STAR's contribution to steroidogenesis has been addressed in a number of additional in-depth studies. CD spectroscopy suggested a "molten globule model" for START domain interactions with

cholesterol. In this model, STAR was proposed to form Ω loops protecting the 259–282 residues from cholesterol docking. In the presence of OMM-resembling vesicles, STAR surface residues are protonated, leading to partial loosening of the tertiary structures. In this state, cholesterol can bind the START domain (Yaworsky et al., 2005). Furthermore, graphic modeling of the positioning and orientation of cholesterol in the START cavity led to high-throughput screening of steroid-structured compounds that can replace cholesterol. Two compounds that can dock onto the START domain with low energy decreased MA-10 steroidogenesis by 50% (Akula et al., 2012). As affixation of STAR at the cytosolic side of the OMM, but not on IMM or IMS, increased steroidogenesis (Bose, Lingappa, & Miller, 2002), it is now suggested that STAR binds cholesterol and is active at the OMM but does not shuttle cholesterol to CYP11A1 located at the IMM. Therefore, other candidate proteins such as TSPO and VDAC1 have been proposed to be integrally involved in cholesterol import.

Although observed at the IMS by crystallography studies, STAR import to mitochondria terminates its function, a mechanism proposed to be carried out by Lon protease with high turnover (Ondrovicova et al., 2005). However, the mitochondrial-binding partners of STAR are not yet identified. Interestingly, recent studies suggest the presence of an OMM acceptor for STAR. Knocking down TSPO or antagonizing its function in MA-10 cells suggested a role for TSPO in processing of the 37 kDa STAR preprotein to the mature 30 kDa form (Hauet et al., 2005; Midzak, Akula, et al., 2011), emphasizing the significance of protein–protein interactions in the transduceosome in driving steroidogenesis. This work was further complemented by a series of experiments in MA-10 cells in which mass spectrometry analysis identified STAR and VDAC1 in the same mitochondrial complexes, VDAC1 knockdown decreased STAR expression, and acute inhibition of VDAC1 led to accumulation of 32 kDa STAR preprotein in the cytosol (Bose, Whittal, Miller, & Bose, 2008). Despite the previous observations of STAR in complex with TSPO and VDAC1 (Bose, Whittal, Miller, et al., 2008; Liu et al., 2006), the lack of evidence of direct physical interaction between STAR with either VDAC1 or TSPO (Bose, Whittal, Gairola, & Bose, 2008; Bose, Whittal, Miller, et al., 2008; Liu et al., 2006) suggests the involvement of an adaptor or scaffold that mediates such physiological correlations.

4.2.4 Protein kinase A

Increase in cAMP levels leads to steroid production due to downstream activation of proteins such as PKA (Catt et al., 1980; Karlsson et al., 2010). PKA

holoenzyme is a heterotetramer of two regulatory (R) and two catalytic (C) subunits. Upon binding of two cAMP molecules to each regulatory subunit, the two catalytic subunits are released to phosphorylate Ser/Thr residues on target proteins, among which are proteins involved in cholesterol import to mitochondria. Therefore, PKA activation enhances and transduces the hormonal signal (Hansson, Skalhegg, & Tasken, 1999). The R subunits of PKA compartmentalize at the target membranes in order to recruit the holoenzyme at the sites of cAMP action, thus amplifying the cAMP signal at specific subcellular compartments. PKA regulates steroidogenesis through multiple steps that include inducing STAR phosphorylation, Star gene expression, mRNA stability, and posttranslational modifications (PTMs) enhancing the protein activity.

In steroidogenic cells, both PKA and PKC phosphorylate CREB in a coordinated, time-sensitive manner, required for *star* transcription (Chrivia et al., 1993; Gonzalez & Montminy, 1989). Phosphorylation by PKA occurs on CREB Ser133, which is indispensable for its interactions with CBP to recruit the transcription factors on the proximal STAR promoter (Clem, Hudson, & Clark, 2005; Hiroi et al., 2004). Moreover, similar coordination between PKA and PKC was shown in order to phosphorylate c-Jun on S63 and c-Fos at Thr325. Such phosphorylations lead to dimerization and DNA binding of the two proteins which assist the recruitment of CBP to the STAR promoter (Manna, Wang, & Stocco, 2003). The interactions between phospho-CREB and CBP and the recruitment of CBP by phosphorylated c-Jun and c-Fos ultimately induce *star*. DNA expression and therefore PKA are the regulator of such transcription (Clem et al., 2005; Duan & Jefcoate, 2007; Manna et al., 2006; Manna & Stocco, 2005). The best-characterized contribution of PKA in steroidogenesis is the PTMs of STAR protein. Briefly, PKA phosphorylates STAR on several sites, critical for STAR cleavage, enhancement of activity, and prolongation of the duration of STAR activity at OMM before import and termination of its function.

4.2.5 Acetyl CoA-binding domain 3

ACBD3 is a 52-kDa protein formerly known as peripheral-type benzodiazepine receptor and PKA-RIα-associated protein 7 (PAP7). ACBD3 is expressed in all human tissues and is highly expressed in steroidogenic cells of gonads and adrenals as well as in glial and hippocampal neuronal cells in the brain (Morin et al., 2012). The primary localization of ACBD3 is at the Golgi apparatus, but this protein translocates to mitochondria during

steroidogenesis (Li, Degenhardt, et al., 2001). ACBD3 contains a homology domain with AKAPs (A kinase anchoring proteins). These proteins are best known for their interactions with PKA enzymes and for enhancing the function of such kinases by increasing their proximity to their targets proteins and membranes (Esseltine & Scott, 2013). ACBD3 was shown to selectively interact with PKA-RIα *in vivo*, suggesting a role in transduction of PKA activity during steroidogenesis. ACBD3 has been shown to interact with TSPO at the OMM, suggesting that the translocation of this protein from the Golgi apparatus to mitochondria is through its interactions with TSPO (Fan, Liu, Culty, & Papadopoulos, 2010; Liu, Li, & Papadopoulos, 2003). Overexpression of ACBD3 induced hCG-stimulated steroidogenesis (Feliciello, Gottesman, & Avvedimento, 2001; Li, Degenhardt, et al., 2001), while overexpression of a sequence of ACBD3 that contained interaction sites with PKA-RIα and TSPO was shown to act as a dominant negative during steroidogenesis (Fan et al., 2010; Liu et al., 2003). Although the exact mechanism of ACBD3 action in steroidogenesis has not been clearly addressed as yet, it is likely that this protein tethers PKA-RIα to mitochondria in order to phosphorylate S194 on STAR and enhance the protein activity, and/or activates mitochondrial kinase MEK1/2 which phosphorylates STAR on S232 and contributes to STAR activation (Poderoso et al., 2008). Moreover, ACBD3 also anchors to mitochondria through interactions with TSPO, which serves to alter the topography of TSPO at the OMM so as to facilitate drug ligand binding during steroidogenesis (Fan et al., 2010; Liu et al., 2003).

4.3 Metabolon

The term "metabolon" refers to multiprotein complexes that perform a series of reactions on a particular substrate. The components of metabolons are tethered at membranes such as those of mitochondria. Their main function is to optimally increase substrate concentration and targeting to certain enzymes for further actions (Issop, Rone, & Papadopoulos, 2013). Recently, an 800-kDa metabolon complex was identified and characterized at the mitochondria consisting of OMM and IMM proteins (Moller, 2010). This protein complex links the transduceosome to CYP11A1 and therefore is an intermediate between cholesterol import into the mitochondria and its conversion to pregnenolone. The mitochondrial metabolon overlaps with the transduceosome at OMM as TSPO and VDAC1 are considered to be

members of both complexes. The IMM enzyme, CYP11A1, is a part of the steroidogenic metabolon. Interactions between the IMM and OMM proteins are mediated through the IMM–OMM protein, AAA+ ATPase, ATAD3 (Fig. 2) (Rone et al., 2012).

4.3.1 Cholesterol side-chain cleavage cytochrome P450 (CYP11A1)

CY11A1 is a nucleus-encoded mitochondrial matrix protein. Cytosolic protein translation yields a 521-amino acid polypeptide CYP11A1 preprotein containing a 39-amino acid mitochondrial targeting signal sequences with three positive amino acids that are essential for protein import (DuBois, Simpson, Tuckey, Lambeth, & Waterman, 1981; Payne & Hales, 2004). Inhibition of CYP11A1 presequences using the metal chelator *o*-phenanthroline did not alter the association of CYP11A1 with the matrix or its enzymatic activity (Nabi, Kominami, Takemori, & Omura, 1983) as the presequences are cleaved upon import. The mechanism of CYP11A1 import suggests the involvement of Tim22 and Tim23 (Minenko, Novikova, Luzikov, & Kovaleva, 2008). Studies on yeast showed that CYP11A1 is imported completely into the matrix and then reassembled, leading to decreased enzymatic activity (Minenko et al., 2008). Once imported, 66 N-terminal amino acids of CYP11A1 form an F-G loop anchor to the IMM; Tim22 was suggested to mainly facilitate the anchorage process rather than be involved in mitochondrial import (Minenko et al., 2008). The N-terminal of CYP11A1 is therefore protected from trypsin proteolysis of isolated mitochondria (Ou, Ito, Morohashi, Fujii-Kuriyama, & Omura, 1986). Topology studies of the isolated mitochondrial membranes (Ou et al., 1986), and three-dimensional modeling of CYP11A1 structure, indicated that the IMM-anchoring residues are located on the opposite surface from the substrate-binding site (Nelson & Strobel, 1988). When properly localized, CYP11A1 catalyzes the conversion of cholesterol to pregnenolone by cleaving the cholesterol aliphatic side chain. The rate of CYP11A1 activity relies on the rate of electron transfer from the matrix proteins Fdx and FdxR (Minenko et al., 2008). The Fdx precursor preadrenodoxin (Padx) is imported through the function of Hsc70 or MSF. Upon import, a 58-amino acid signal sequence of Padx is cleaved, creating an interaction site with CYP11A1 as shown in *Escherichia coli* (Nabi & Omura, 1980). A more intricate model for Padx import was suggested by studies indicating that the ribosomes synthesizing Padx bind to the OMM (Hachiya et al., 1993, 1995), suggesting that this protein can be translated in the cytosol or at the level of mitochondria (Nabi & Omura, 1980, 1983). FdR also contains a

mitochondrial signal sequence consisting of 32 amino acids that are cleaved after import (MacKenzie & Payne, 2004). Both proteins have matrix localizations and interact with CYP11A1 to provide electrons. The electrons are further utilized by CYP11A1 to activate an oxygen molecule during conversion of cholesterol to pregnenolone (Payne & Hales, 2004). The proteins are localized in the mitochondrial matrix where they interact with CYP11A1.

4.3.2 AAA+ ATPase, ATAD3

ATAD3 is an AAA+ ATPase protein that is present at the mitochondrial matrix and participates in maintenance of mitochondrial DNA integrity (He et al., 2007). This protein is enriched in OMM–IMM contact sites (Sagara, Takata, Miyata, Hara, & Horiuchi, 1987). The N-terminus of ATAD3 is anchored to OMM through which ATAD3 controls mitochondrial dynamics at the interface of the IMM and OMM (Gilquin et al., 2010). Recent studies reported that ATAD3 knockdown leads to reduced levels of Leydig cell steroidogenesis in response to hormonal stimulation (Rone et al., 2012). The mechanism through which ATAD3 contributes to steroidogenesis is under investigation.

5. CAN SERUM TESTOSTERONE LEVELS BE INCREASED BY STIMULATING THE LEYDIG CELLS THEMSELVES?

As indicated above, there are good reasons to increase serum T levels in hypogonadal men, but doing so by TRT is less than ideal for both young and aging men. There are methods to increase serum T without T administration, including administering LH (or hCG) for men with secondary hypogonadism. In men with primary hypogonadism, aromatase inhibitors can increase T-to-estradiol ratios, but often this approach is ineffective (Perheentupa & Huhtaniemi, 2009). Increasing intratesticular and serum T by stimulating the Leydig cells themselves could have great advantages in part because doing so would be expected to result in the physiological regulation of the T that is produced by its negative feedback on LH. This is in contrast to essentially flooding the system with T, as with exogenous T administration. Stimulating the Leydig cells should not elicit significant fluctuations in T levels, and nor there be T transfer to others via contact. Fertility should be preserved, not suppressed, because the hypothalamic–pituitary axis should not be shut down as with exogenous T. Indeed, the local stimulation of Leydig cell T production might actually enhance spermatogenesis because intratesticular

T levels should increase, not decrease. Moreover, Leydig cells produce more than just T, and so their stimulation might produce Leydig cell products in addition to T that might have effects on spermatogenesis. With an understanding of the series of steps leading to T formation derived from numerous studies conducted over the years, and the identification of key steps that have been shown to be amenable to pharmacological modulation so as to induce T production, it now might be possible to stimulate Leydig cells to produce additional T as a means by which to increase serum T levels.

5.1 TSPO drug ligands

These particular OMM proteins have attracted considerable interest in steroidogenesis research. Drugs binding to TSPO are able to stimulate steroid biosynthesis (Krueger & Papadopoulos, 1990; Papadopoulos, Berkovich, & Krueger, 1991), and the VDAC1–TSPO pair is the binding site of benzodiazepine and other drug ligands to the OMM (Garnier et al., 1994) (Fig. 2). TSPO itself is able to exclusively bind different classes of drugs with the capacity to stimulate steroidogenesis (Rupprecht et al., 2009). Moreover, blocking the interactions between TSPO and its ligands blocked steroidogenesis in primary cells (Chung et al., 2013; Lacapere & Papadopoulos, 2003; Midzak, Akula, et al., 2011).

Our recent *in vivo* studies showed that the pharmacologic activation of TSPO increased serum T in hypogonadal aged Brown Norway rats to the level of young rats (Chung et al., 2013). This exciting finding suggests that there is the potential to use of TSPO drug ligands to induce T formation in conditions of primary (or secondary) hypogonadism if their *in vivo* use proves to be specific for Leydig cells and safe. TSPO drug ligands have been shown to increase circulating corticosteroids in animals, though far less so in control than in hypophysectomized animals (Cavallaro, Korneyev, Guidotti, & Costa, 1992). These drugs also will induce neurosteroid formation in the brain *in vitro* and *in vivo* (Cavallaro et al., 1992; Costa & Guidotti, 1991; Romeo et al., 1992) and have been used to alleviate neurological and psychiatric disease symptoms (Romeo et al., 1992; Schule, Nothdurfter, & Rupprecht, 2014). These findings suggest that TSPO drug ligands may exert stimulatory effects on steroid formation by the adrenal and brain, with effects that are minor in normal *in vivo* settings but significant when TSPO levels are reduced due to conditions that include aging, hypophysectomy, depression, and anxiety. Compounds such as *N*,*N*-di-*n*-hexyl 2-(4-fluorophenyl) indole-3-acetamide (FGIN-1–27) are drug ligands of TSPO that can induce

neurosteroidogenesis in rat glioma cells (Romeo et al., 1992), or androgen formation in rat testes (Aghazadeh, Martinez-Arguelles, Fan, Culty, & Papadopoulos, 2014). Taken together, these studies clearly indicate a potential role for TSPO drug ligands for possible therapeutic use in increasing serum T levels in hypogonadal men.

Knockdown of TSPO was shown in previous studies to ablate steroid biosynthesis in steroidogenic cells (Hauet et al., 2005; Papadopoulos et al., 1997). However, in a recent study, testis-specific TSPO knockout mice in which TSPO monomers were not present were reported to produce T in amounts comparable to those of control mice (Morohaku et al., 2014). This suggests a potential compensatory mechanism for maintenance of mitochondrial integrity and function (Papadopoulos, 2014). However, the identification and characterization of several pharmacological agents of distinct chemical structure that target TSPO and stimulate steroid formation, as well as structural studies of TSPO, indicate that TSPO is a valuable drug target for the induction of steroid and neurosteroid biosynthesis (Jaremko, Jaremko, Becker, et al., 2014; Jaremko, Jaremko, Giller, et al., 2014; Lacapere et al., 2001; Murail et al., 2008).

5.2 14-3-3γ and ε proteins

14-3-3 proteins are a family of small, acidic proteins that, through functioning adaptors, scaffolds, and chaperones, regulate a variety of intracellular signaling pathways. All species express at least two isoforms of 14-3-3 proteins; mammals express seven isoforms, named β, γ, ζ, ε, η, σ, and θ (α and δ are phosphorylated forms of β and ζ) (Ichimura et al., 1997; Toker et al., 1992). Each 14-3-3 monomer is composed of nine α-helices. There is a high level of homology among the 14-3-3 family members, and they are expressed ubiquitously in mammalian tissues (except the endothelial cell-specific 14-3-3σ and T cell-specific 14-3-3θ) (Aitken, 2006). Nonetheless, proteomic analysis identified unique, tissue-specific networks (Choudhary et al., 2009), and global microarray data suggest tissue-specific isoform expression (www.biogps.org). Therefore, we now understand that 14-3-3 family members also have isoform and tissue-specific roles in addition to their ubiquitous functions. Such unique roles rely in part on hetero-/homodimerization, which offer functional diversity to 14-3-3 proteins. The N-terminal helices contain highly varied amino acid sequences, and therefore, each isoform exerts different dimerization affinity for others (Liu et al., 1995; Xiao et al., 1995). The dimerization of 14-3-3 isoforms

was previously believed to be essential for thermodynamic stability of the proteins of this family, but recent studies showed that dimer/monomer equilibrium for 14-3-3 proteins is influenced by PTMs, which also serve to stabilize 14-3-3 monomers. Moreover, a splicing variant was identified in 18 human tissues for 14-3-3ε, called sv-14-3-3ε, which lacks the 22 dimerization amino acids and is highly functional (Han et al., 2010). 14-3-3 dimers form a clamp-like shape where each monomer contains an amphipathic groove at the C-terminal through which it can bind to target proteins. Although the C-terminal of 14-3-3 proteins is highly homologous, the surrounding surface residues and tissue-specific PTMs dictate target specificity for each isoform. Thus, 14-3-3 proteins bind to more than 200 targets, and this list is expanding. The majority of target proteins contain 14-3-3-binding motif(s) with the following amino acid sequences: RSXpSXP (mode I) and RXY/FXpSXP (mode II) or -pS/pTX(1–2)–CO_2 H (mode III) (where X is not Pro) (Johnson et al., 2010). "Suboptimal" motifs are observed in majority of 14-3-3 targets, varying by 1–3 amino acids from the classical sequences. The presence of suboptimal motifs leads to transient association/dissociation of 14-3-3/target protein, required for adaptor characteristics of 14-3-3 family of proteins. The presence of more than one suboptimal motif induces 14-3-3-binding affinity by 30-fold (Yaffe et al., 1997). Upon interactions with targets, 14-3-3 proteins alter target protein modifications, activity, and cellular localization and therefore regulate five major cellular pathways: cell-cycle and apoptosis, signal transduction, metabolism, and intracellular protein trafficking.

14-3-3ε, also known as tyrosine 3/tryptophan 5-monooxygenase activation protein epsilon, has high affinity for heterodimerization with 14-3-3γ (Aitken et al., 2002). 14-3-3γ, also referred to as 3-monooxgenase/tryptophan 5-monooxygenase activation protein gamma, has high homodimerization affinity though its preferable heterodimerization partner is 14-3-3ε. A complex of the 14-3-3γ and ε is called the mitochondrial import-stimulating factor (MSF) (Alam et al., 1994; Hachiya et al., 1993, 1994). MSF is a cytosolic chaperone that delivers the mitochondrial-targeted β-barrel proteins and recognizes and binds to signal sequences on mitochondrial pre-proteins.

14-3-3ε is highly expressed in brain, lymphoblasts, adipocytes, and testes. The 14-3-3γ isoform is highly expressed in brain, skeletal muscle, heart, and embryonic stem cells (www.biogps.org). Recently, 14-3-3ε and γ were identified as negative regulators of androgen formation in the Leydig cells (Muslin, Tanner, Allen, & Shaw, 1996). *In silico* analysis predicted 14-3-3-binding motifs on transduceosome proteins; they were present on

structural domains critical for their function (Aghazadeh et al., 2014). STAR contains three predicted 14-3-3 motifs, on sites of cleavage and activation (Aghazadeh et al., 2012); TSPO contains a predicted 14-3-3 motif with one amino acid distance from the CRAC domain (Li, Yao, Degenhardt, Teper, & Papadopoulos, 2001). VDAC1 contains two 14-3-3 motifs at the dimerization site and at the lateral surface accessible to other OMM proteins for interactions (Geula, Naveed, Liang, & Shoshan-Barmatz, 2012; Sorgato & Moran, 1993). 14-3-3γ is hormonally induced, similar to STAR, whereas the 14-3-3ε expression is not hormonally regulated. Rather, 14-3-3ε localizes to mitochondria during steroidogenesis. Low-throughput analysis suggests that the principal target of 14-3-3γ is STAR, while 14-3-3ε mainly targets VDAC1 in the transduceosome. Moreover, VDAC1 and START domains are classified as β-barrel protein and protein domain, and MSF was shown to mediate the transport of mitochondrial proteins belonging to this class (Geula et al., 2012; Sorgato & Moran, 1993; Tsujishita & Hurley, 2000). Different mechanisms of action were proposed for 14-3-3γ and ε, with their roles consecutive and complementary to each other.

14-3-3γ was shown to bind to 194 phosphorylation site on STAR at the initiation of steroidogenesis. PKA phosphorylation of S194 was shown previously to induce STAR activation by twofold (Arakane et al., 1997). Therefore, a model was proposed in which 14-3-3γ protein physically blocks the accessibility of STAR S194 for PKA phosphorylation and therefore maintains STAR at basal activity. Such negative regulation, however, is terminated by the dissociation of 14-3-3γ from STAR, thus allowing significant induction of steroidogenesis (Aghazadeh et al., 2012) (Fig. 4A). In light of the observations that the transduceosome assembly, TSPO polymerization, and increased STAR expression occur shortly after cAMP/hormone stimulation, it was not clear why there is a delay in reaching maximum steroidogenesis induction. The time-sensitive mechanism of action of 14-3-3γ suggests that this protein contributes, at least in part, to the delay in steroidogenesis induction. Why this lag period might be necessary for steroidogenic cells is yet to be elucidated. The termination of 14-3-3γ function was shown to coincide with increased homodimerization of this protein (Aghazadeh et al., 2012), suggesting a protein regulation mechanism. Indeed, 14-3-3 phosphorylation and/or high expression were shown to contribute to protein self-homodimerization which alters the protein function (Shen et al., 2003).

Once 14-3-3γ releases STAR, a second regulatory mechanism for cholesterol import to mitochondria is activated that involves 14-3-3ε. The regulatory role of 14-3-3ε has a later onset but is maintained long term. 14-3-3ε anchors to mitochondria through interacting with TSPO, thereby

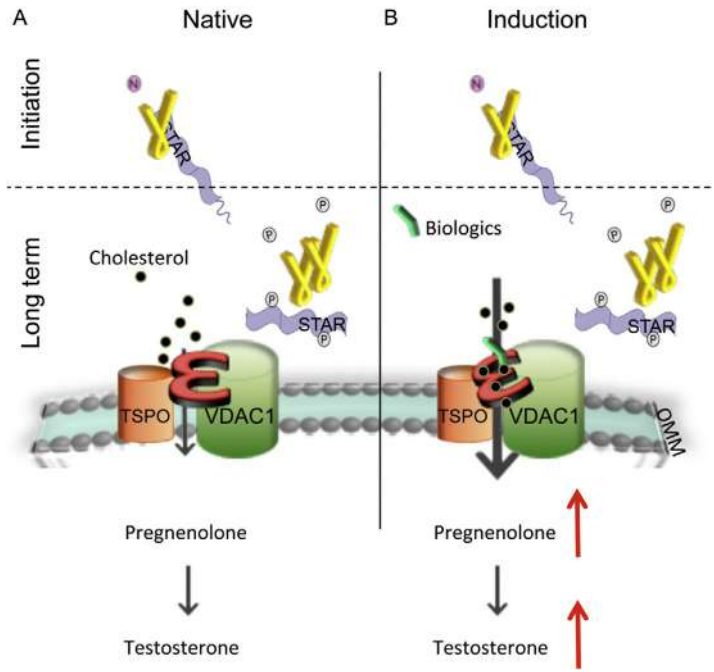

Figure 4 Induction of endogenous steroidogenesis via blockage of 14-3-3 protein negative regulation. 14-3-3γ and ε are negative regulators of steroidogenesis. Initially upon hormonal stimulation, acetylated 14-3-3γ monomers interact with STAR and block the PKA-dependent phosphorylation of this protein on S194, retaining STAR in its basal activity. Later on, 14-3-3γ phosphorylation and high levels of protein expression induce homodimerization of this protein and dissociation from STAR. (A) When steroidogenesis is highly increased, 14-3-3ε scaffold protein translocates to mitochondria where it regulates the TSPO microenvironment and, by intercalating between TSPO and VDAC1, negatively regulates the import of cholesterol into mitochondria, thus controlling the rates of T formation. (B) T production can be induced by using biologics that block the interactions between 14-3-3ε and VDAC1 on S167 ( ), which allows the formation of more efficient TSPO–VDAC1 interactions. This prompts cholesterol import into mitochondria and increases T formation in the absence of gonadotropin.

regulating cholesterol and ligand binding to TSPO throughout steroidogenesis. When steroidogenesis is highly induced, 14-3-3ε interactions with VDAC1 are increased. The primary interaction sites were identified to be VDAC1 S167. This Ser residue is located on the lateral side of the protein accessible for interaction with OMM partners such as TSPO. Therefore, 14-3-3ε intercalation between TSPO and VDAC1 blocks their efficient interactions, thus affecting the rate of cholesterol entry into mitochondria (Aghazadeh et al., 2014, 2012) (Fig. 4A).

Additional studies were conducted using a cell penetrating sequence conjugated to a short sequence of VDAC1 containing S167, and of STAR containing S194. Such fusion peptides successfully compete out 14-3-3ε and 14-3-3γ interactions in MA-10 cells. As a result, the negative regulatory role of 14-3-3γ and ε is ablated and therefore cells produce more steroids acutely at the initiation of steroidogenesis, or long term, respectively. Furthermore, peptides containing VDAC1 S167, administered directly to the testes of adult male Sprague–Dawley rats, induced testicular and plasma T levels in a dose-dependent manner (Fig. 4B), independently of LH (Aghazadeh et al., 2014). Modeling studies suggest that these peptides can mimic the VDAC1 docking to 14-3-3ε in rats as well as in humans due to high homology of 14-3-3ε and VDAC1 across species and the 100% conservation of the 14-3-3ε motif. These biological fusion peptides are therefore potential tools to induce T levels in men diagnosed with androgen deficiency (Aghazadeh et al., 2014).

6. CONCLUSION

Cholesterol is the main precursor of T. Its import into mitochondria is the rate-limiting step in steroidogenesis. After a decade of intensive research, it is now clear that cholesterol import into the mitochondria in response to hormonal stimulation is carried out through a multiprotein complex called transduceosome, and not by individual proteins (Midzak, Rone, Aghazadeh, Culty, & Papadopoulos, 2011; Papadopoulos & Miller, 2012; Rone et al., 2012).

In search of mechanisms inducing T production in the Leydig cell as an alternative to TRT, components of the transduceosome were identified to be critical for T formation and "druggable" candidates. Indeed, TSPO and 14-3-3ε–VDAC1 interactions were shown to be viable targets which, when activated, result in increased T formation both *in vitro* and *in vivo*, thus providing new means for the treatment of androgen deficiency in hypogonadal men.

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