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Cholesterol transport in steroid biosynthesis: Role of protein-protein interactions and implications in disease states

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Abstract

The transfer of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in hormone-induced steroid formation. To ensure that this step is achieved efficiently, free cholesterol must accumulate in excess at the outer mitochondrial membrane and then be transferred to the inner membrane. This is accomplished through a series of steps that involve various intracellular organelles, including lysosomes and lipid droplets, and proteins such as the translocator protein (18 kDa, TSPO) and steroidogenic acute regulatory (StAR) proteins. TSPO, previously known as the peripheral-type benzodiazepine receptor, is a high-affinity drug- and cholesterol-binding mitochondrial protein. StAR is a hormone-induced mitochondria-targeted protein that has been shown to initiate cholesterol transfer into mitochondria. Through the assistance of proteins such as the cAMP-dependent protein kinase regulatory subunit I α (PKA-R1 α) and the PKA-R1 α TSPO-associated acyl-coenzyme A binding domain containing 3 (ACBD3) protein, PAP7, cholesterol is transferred to and docked at the outer mitochondrial membrane. The TSPO-dependent import of StAR into mitochondria, and the association of TSPO with the outer/inner mitochondrial membrane contact sites, drives the intramitochondrial cholesterol transfer and subsequent steroid formation. The focus of this review is on (i) the intracellular pathways and protein-protein interactions involved in cholesterol transport and steroid biosynthesis and (ii) the roles and interactions of these proteins in endocrine pathologies and neurological diseases where steroid synthesis plays a critical role.

Keywords

Steroid biosynthesis; translocator protein; steroidogenic acute regulatory protein; peripheral benzodiazepine receptor

Cholesterol and Steroid Synthesis

Cholesterol is the sole precursor of steroids. Steroid synthesis is initiated at the inner mitochondrial membrane (IMM), where the cytochrome P450 cholesterol side chain cleavage

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enzyme (CYP11A1) catalyzes the conversion of cholesterol to pregnenolone [1]. Pregnenolone then enters the endoplasmic reticulum (ER) where further enzymatic reactions occur to produce the final steroid products. It has been shown that the translocation of cholesterol from the outer mitochondrial membrane (OMM) to the IMM is the rate-limiting step in the production of all steroids [2,3]. Therefore, the ability of cholesterol to move into mitochondria to be available to CYP11A1 determines the efficiency of steroid production.

The production of steroids is regulated by trophic hormones, specifically, the adrenocorticotrophic hormone (ACTH) in adrenocortical cells and luteinizing hormone (LH) in testicular Leydig and ovarian cells [2,3]. The presence of these hormones activates the G protein-coupled receptors, which release the stimulatory subunit, resulting in the activation of adenylyl cyclase and a rise in intracellular cAMP [4]. This increase in cAMP leads to an increase in lipid synthesis, protein synthesis, and protein phosphorylation. All these processes have been shown to play a role in steroidogenesis and assist with cholesterol trafficking to the mitochondria.

Mitochondria are relatively cholesterol-poor organelles, with the majority of cholesterol located in the OMM. In the mitochondria of steroidogenic cells the pool of cholesterol available for steroidogenesis is segregated from the structural membrane cholesterol and is bound to the cholesterol-binding domain of the translocator protein (18-kDa, TSPO), formerly called the peripheral-type benzodiazepine receptor (PBR) [5]. It is from this site that cholesterol is released under hormonal stimulation to move to the matrix side of the IMM, where the cholesterol side chain cleavage enzyme CYP11A1, which will metabolize cholesterol to pregnenolone, is located. This pathway will be discussed in detail later.

As the initial translocation of cholesterol from TSPO is not sufficient to sustain the continuous production of elevated concentrations of steroids, additional free cholesterol must be moved from intracellular stores to the mitochondria. This intracellular cholesterol is known to come from three sources: i) de novo synthesis of cholesterol in the endoplasmic reticulum (ER) ii) mobilization of cholesterol in the plasma membrane with further uptake of circulating cholesterol esters from receptors found on the plasma membrane and iii) mobilization of cholesterol in lipid droplets (LD) (Fig. 1).

Cholesterol sources in the cell

With 65% to 80% of the total cellular cholesterol located in the plasma membrane, comprising 20% to 25% of the total lipids present, cholesterol plays a significant role in the structure and function of the plasma membrane. These interactions affect the organization of proteins and lipids in the membrane, alter the permeability of the membrane, and initiate the formation of lipid rafts [6]. The second highest concentration of cholesterol is found in the endosomal pathway, with the majority found in the endosomal to trans-Golgi compartment [6]. While the majority of total cholesterol found in the cell is located in the plasma membrane, the ER contains only 1% to 2% of the total cell cholesterol [7]. This gradient provides a mechanism for transport of cholesterol inside the cell from the ER to the plasma membrane and allows it to be recycled back [8].

ER cholesterol

The ER functions as the cholesterol sensing organelle of the cell, regulating endogenous cholesterol production primarily through the sterol regulatory element binding protein (SREBP) complex [9,10]. The SREBP proteins are translocated to the Golgi upon a decrease in cholesterol and upon arrival SREBP is cleaved by two proteases. The resulting N-terminus is an active transcription factor that translocates to the nucleus. This results in an increase in the activity of cholesterol transcription genes, including HMG-CoA reductase (HMGR) [11].

HMGR is the rate-limiting enzyme in cholesterol synthesis and is easily degraded under the high sterol conditions. HMGR is upregulated in the presence of hormones, resulting in an increase in cholesterol production under hormonal stimulation [9]. The increase of cholesterol via this pathway though has not been shown to play a primary role in steroid production [12].

As cholesterol synthesis is tightly regulated in the ER during hormonal stimulation, cholesterol transport out of the ER is tightly controlled as well. Cholesterol flux from the ER can occur through many pathways, including cytosolic lipid transfer proteins, through intracellular compartments or passive diffusion through contact sites. Contact sites are common between the ER and other intracellular organelles, facilitating cholesterol flux out of the ER. ER-mitochondrial contact sites have been identified in which mitochondria-associated membranes (MAM) cluster with stacks of ER [13]. The cholesterol generated in the ER could potentially use this pathway for receiving steroidogenic cholesterol (Fig. 1, Pathway 1). Recently an ER protein, acyl CoA:diacylglycerol acyltransferase 2 (DGAT2) was found associated with both LD and the mitochondria while still present in the ER. As DGAT2 functions in the final stage of triglycerol synthesis it is possible that this is a pathway for lipid transfer [14]. Currently it is unknown whether an interaction between the ER and mitochondria occurs in steroidogenic cells; therefore further studies will be necessary to investigate the presence and function of such an interaction in steroidogenesis.

Plasma Membrane

Cholesterol is primarily stored in the plasma membrane. Upon hormonal stimulation there is increased cholesterol absorption through the plasma membrane. When cholesterol is imported into the cell via the plasma membrane it greatly increases the cholesterol content stored elsewhere in the cell. This was observed when an increase of 50% in cellular cholesterol absorbed via the plasma membrane resulted in a 10-fold increase in ER cholesterol [15]. Currently, there are two known pathways for this cholesterol absorption and import into the cell: a non-selective endocytic pathway and a “selective” absorption pathway. In the non-selective pathway LDL molecules are specifically bound and internalized via the LDL receptors. Once the receptor has been internalized it fuses with the endosomal pathway for distribution of the lipoproteins (Fig. 1, Pathway 2). The “selective” pathway uses scavenger receptors class B type I (SR-BI) located at the plasma membrane to bind both LDL and HDL. Through local binding mechanisms the cholesterol present in the lipoproteins is transferred directly to the cell membrane without absorption of the lipoprotein particles [16] (Fig. 1, Pathway 3). Further analysis of these two pathways has shown that adrenal steroidogenesis is dependent primarily on HDL cholesterol absorbed from the plasma membrane, primarily via the SR-BI pathway [17].

The non-selective vesicular pathway is initiated primarily by LDL particles binding to the LDL receptor, followed by the endocytosis and budding of clathrin-coated pits into the cytoplasm [18] (Fig 1, Pathway 2). These vesicles fuse with early endosomes, releasing their clathrin coats and allowing the LDL receptors to cycle back to the membrane. This endosomal fusion and trafficking occur through interactions with microtubules which are controlled through Rabs [19]. Rabs are small GTPases that regulate membrane traffic through binding at their active site, currently there have been more than 60 proteins identified in mammalian cells [20,21]. The early endosomes bind to recycling endosomes coordinated by Rab5, and then to late endosomes via Rab 7, to further distribute cholesterol throughout the cell [20,22]. The endosomes also undergo a decrease in pH from 7.4 at the plasma membrane to 5.5 – 6 at the late endosome [23]. This decrease in pH helps further dissolve the absorbed lipoprotein and prepares the late endosomes to fuse with the lysosomes.

It has been shown that the LDL receptor is not necessary for acute adrenal steroidogenesis, suggesting that cholesterol absorbed via this pathway is not necessarily used for steroidogenesis [24]. However, in FSH- or FSH plus androstenedione-treated granulosa cells the rate of LDL receptor absorption increases while the time needed for the LDL to reach the lysosome decreases compared to non-hormone-treated cells suggesting this pathway is used for steroidogenesis [25]. As endosomes contain a large percentage of the cytosolic cholesterol present in the cell; they can function in the trafficking of intracellular cholesterol to the mitochondria without first absorbing cholesterol from the plasma membrane. This pathway could occur specifically through the cholesterol rich late endosomes, shown to fuse with the lysosomes and the Golgi apparatus and transiently interacting with the mitochondria, thus allowing for multiple sources of cholesterol to be available to the mitochondria. Endosomal trafficking in the cell has also been shown to be altered by cholesterol concentrations, specifically via Rab7, suggesting a mechanism by which trafficking to the mitochondria could be regulated [26].

The second pathway identified for cholesterol absorption and trafficking in steroidogenic cells occurs through the action of the SR-BI receptor [27] (Fig 1, Pathway 3). The SR-BI receptor is found in many tissues such as intestines, macrophages, and endothelial cells, though it is expressed in highest concentrations in steroidogenic tissues such as the adrenals, ovary, and testis [16]. Unlike the LDL receptor in which the apoprotein is absorbed, the SR-BI receptor forms a non-aqueous channel that allows a large influx of cholesterol directly into the plasma membrane [16]. This non-aqueous channel has also been shown to be regulated in the intestines through other proteins found in the plasma membrane, such as CD36 and other proteins yet to be identified [28]. Suggesting this pathway might require a complex, multiprotein interaction to regulate cholesterol absorption into the plasma membrane. Because SR-BI absorbs free cholesterol from HDL and stores it in the plasma membrane, this cholesterol can move spontaneously between bilayers and membranes in the cell without the assistance of any proteins. This process is slow and therefore not suggested as a pathway involved in the acute stimulation of steroidogenesis [29]. The esterified cholesterol absorbed from the SR-BI receptor must be converted to free cholesterol before it can be used for steroidogenesis via a cholesterol ester hydrolase [30]. In steroidogenic cells cholesterol ester hydrolysis is performed through hormone-sensitive lipase (HSL) [31]. HSL becomes activated when phosphorylated by cAMP; inhibition of HSL results in decreased steroidogenesis in the adrenals and inhibits sperm production in the testis [32–34]. The cholesterol absorbed via the plasma membrane has been shown to be hydrolyzed rapidly, presumably close to the plasma membrane, to form free cholesterol. Once the cholesterol esters have undergone hydrolysis, the HSL can interact with various cholesterol-binding proteins to direct the cholesterol to the OMM for steroidogenesis [35]. This pathway involving cholesterol-binding proteins will be discussed later in detail.

Lipid Droplets

Lipid droplets (LD) are bounded by a phospholipid membrane and function as a repository of cholesterol esters and triglycerides in the cell. It has been proposed that LD form from the ER when excess neutral lipids bud off, although there is no direct evidence to support this model [36]. The cholesterol esters found in the LD are the products of the ER enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT), which becomes active in the presence of high levels of cholesterol. This enzyme attaches an ester to the free cholesterol found in the ER, increasing the cholesterol ester content present in the cell [37]. Because lipids in the LD can be used for various biological activities their size fluctuates depending upon the cell's activity. In steroidogenic cells, LDs are small to increase the surface area for lipid retrieval [36].

The cholesterol esters present in the LD are also converted to free cholesterol in the same manner as cholesterol absorbed via the SR-BI receptor, i.e., through HSL. Transfer of

steroidogenic cholesterol from intracellular organelles to the mitochondria is thought to occur through cholesterol-binding proteins found in the cytosol (Fig 1, Pathway 4) [38]. Other mechanisms for lipid transport from the LD have been demonstrated. Rab5, which localizes to early endosomes, has been shown to interact with LD, suggesting a mechanism for cholesterol transfer from the LD to the endosome and vice versa, allowing for an increase in cholesterol in the endosomal pathway [39]. Rab18 also associates with LD, regulating the contact between the lipid droplet and the ER, which controls the flux of cholesterol during lipolysis [40]. Since LDs play an important role in regulating intracellular cholesterol through storage, trafficking, and esterification, further studies are needed to determine the endosome/LD interaction which would allow for transfer or fusion of the cholesterol from the LDs into the early endosomal pathway for steroidogenesis.

Targeting cholesterol to the mitochondria

The primary pathway for targeting cholesterol to the mitochondria has not been definitively identified. Two pathways have been proposed: (i) the non-vesicular pathway involving cholesterol-binding proteins transferring cholesterol through the cytosol to the mitochondria, and (ii) a vesicular pathway characterized by an increase in the fusion of vesicular membranes, such as endosomes and lysosomes, which results in an increase in cholesterol targeted to the mitochondria [41]. An overlap between these two pathways is highly likely as cholesterol binding proteins have been found on endosomes; this suggests that an increase in cholesterol targeting to endosomes could also have a direct effect on cholesterol-binding proteins which target cholesterol for transfer to the mitochondria.

Sterol carrier protein-2

Sterol carrier protein-2 (SCP-2) was one of the initial cholesterol binding proteins identified; SCP-2 was shown to play a role in the intracellular transfer of cholesterol, including from the lysosomal to mitochondrial membranes [42,43]. SCP-2 is found in tissues involved in cholesterol trafficking and oxidation, such as the liver, intestines, adrenal, testis, and ovary, suggesting it could play a role in steroidogenesis [44]. Further studies showed SCP-2 increases cholesterol uptake and transport throughout the cell while inhibiting the efflux of cholesterol from the cell through the HDL receptor [45,46]. Because no alteration in steroidogenesis was observed in SCP-2 knock-out mice, it has been assumed that SCP-2 does not play a primary role in steroidogenesis *in vivo*, though other interactions are still possible [47]. This focused attention on other identified cholesterol binding/transfer proteins.

START domain proteins

The steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain is an amino acid motif that has been proposed to play a role in cholesterol and lipid binding [48] (Fig. 2A). The motif first was identified in the StAR protein, discussed below, which has been shown to play a role in cholesterol transport to the mitochondria for steroidogenesis. The 210-amino-acid sequence forms a beta sheet core surrounded by two alpha helices, resulting in a hydrophobic channel that can hold a sterol molecule capped by the C-terminal alpha helix [38]. The structure becomes stable upon binding of the cholesterol molecule, which has been shown to bind at a 1:1 ratio [49].

MLN64, the second identified StAR domain protein, was shown to be upregulated in breast and ovarian cancers and found to have a C-terminal domain with 37% amino acid identity and 50% amino acid similarity to the C-terminal domain of StAR [50,51]. Further studies confirmed that this was a START domain that could bind cholesterol and transfer free cholesterol from sterol-rich vesicles to acceptor membranes. MLN64 was found to be localized primarily on late endosomes, integrating at the plasma membrane and functioning in the vesicular trafficking

of LDL cholesterol [52]. When the START domain was removed from MLN64, cholesterol accumulated in the lysosomes and altered late endosome trafficking [53]. In COS-F2 cells, this accumulation of cholesterol suppressed steroidogenesis, presumably by limiting the efflux of free cholesterol from the late endosomes and lysosomes to the mitochondria.

MLN64 was also found to be associated with Niemann-Pick type C disease protein 1 (NPC1) in the late endosomes [54]. Niemann-Pick type C disease (NPC) is a disorder characterized by the accumulation of LDL-derived unesterified cholesterol in the late endosomes and lysosomes caused by mutations in either NPC1 or NPC2 [55]. NPC1 gene expression has been shown to be responsive to cAMP and essential for the normal development of the adrenals [56]. NPC1 is found bound to late endosomal membranes while NPC2 is found inside the late endosomes, early lysosomes, and in the cytosol. The presence of NPC2 accelerates the transfer of free cholesterol from late endosomes to lysosomes and cholesterol efflux to the plasma membrane [57,58]. NPC1 is necessary for cholesterol efflux from the late lysosome for use in the cell [23]. When steroidogenic human granulosa-lutein cells deficient in NPC1 protein were used for studying cholesterol trafficking, steroidogenesis levels decreased to levels seen with LDL-deficient media [59]. This observation suggests that the NPC1/late endosome pathway is used primarily for LDL-derived steroidogenesis. As NPC1 and NPC2 function in the efflux of cholesterol from the endosome, this pathway could also interact with MLN-64 for steroidogenic purposes.

Steroidogenic acute regulatory (StAR) protein was first identified due to its rapid phosphorylation and protein expression soon after the addition of hormones and cAMP in steroidogenic cells [60–62]. Expression was confirmed in the adrenal cortex, testis, and ovary and later in the brain and placenta, suggesting a connection between StAR protein expression and steroid production [63,64]. Transfection of StAR expression vectors in both mouse Leydig MA-10 cells and COS F2 cells, which contain the components of CYP11A1, was found to increase steroidogenesis [65,66].

There have been many models proposed for the function of StAR in steroidogenesis [67]. It was suggested early that StAR assists with the transfer of cholesterol to the mitochondria, although no clear mechanism was identified. Because StAR contains an N-terminal mitochondrial-targeting sequence, which is cleaved from its active molecular weight of 37 kDa to its inactive weight of 30 kDa upon import into the mitochondrion, it was proposed that this targeting sequence could assist with the formation of a mitochondrial “contact site”. This would be accomplished by the interaction of StAR with mitochondrial protein import complexes found on both the OMM and IMM and allow the N-terminus to form a linker connecting the membranes. Cholesterol would then be able to flow from the OMM to the IMM and interact with CYP11A1. However, removal of the N-terminus targeting sequence in the construct N-62 StAR and transfection into steroidogenic cells had no effect on steroidogenesis, suggesting this is not the mechanism by which StAR facilitates cholesterol transfer [68]. It was later observed that N-62 StAR was able to insert cholesterol into cytosolic membranes other than the mitochondria, suggesting that the primary function of the N-terminal sequence is not to form mitochondrial contact sites but to limit StAR’s cholesterol targeting abilities solely to the OMM [69].

The effect of StAR on mitochondrial steroidogenesis was further confirmed by fusing mitochondrial translocases to the N-62 StAR construct. Fusion of N-62 StAR with Tim9, a mitochondrial inner membrane space protein, or Tim40, an IMM protein, resulted in no increase in steroidogenesis while fusion of N-62 StAR with Tom20, an OMM protein, resulted in maximal production of steroids [70]. This observation demonstrated that StAR’s site of action was confined to the OMM and that, once imported, StAR does not stimulate steroidogenesis. Because StAR interacts only transiently with the OMM before being imported,

the amount of time StAR spends at the OMM would be able to alter the rate of steroid production. This was shown when a StAR/StAR construct, which is imported at a slower rate than wild-type StAR, was shown to increase pregnenolone production over both wild-type and N-62 StAR construct levels in COS-F2 cells [70]. This finding shows that StAR functions primarily at the OMM, possibly activating a pathway of cholesterol transport for steroidogenesis.

Another proposed model built to explain StAR's activity suggested that it could function as an intermembrane cholesterol shuttle, moving cholesterol from the outer to the inner mitochondria membrane one molecule at a time during StAR's import [49]. Tsujishita and Hurley proposed that during StAR's import into the mitochondria several molecules of cholesterol could be transferred through the import of one StAR molecule through transient openings. Several issues were raised with the model, including the lack of understanding of how StAR could both bind cholesterol and then release it in the IMM space. It was also uncertain how StAR would reside in the IMM space as it does not have a mitochondria targeting sequence for the inner mitochondria membrane space.

One of the more current proposed models is the "molten globule" model identified through the studying of the StAR's C-terminus. Removal of the last 10 C-terminal amino acids resulted in decreased steroidogenesis, while removal of the 28 C-terminal amino acids resulted in a biologically inactive protein, suggesting that StAR's mode of action was occurring at the C-terminus. Further studies showed that the C-terminus forms a sterol-binding domain (SBD) with which a cholesterol molecule is proposed to interact. The SBD forms a pocket that prevents the release of the bound cholesterol molecule, which can occur only following a conformational change. This is proposed to occur through the "molten globule" configuration in which tertiary structures are removed, allowing the remaining secondary structures to undergo a conformational shift and allow the cholesterol molecule to enter the mitochondria. The molten globule model has been proposed to occur through the protonation of the C-terminus at the OMM. Since binding of cholesterol to the SBD in the StAR protein has not been definitively demonstrated, it is still not clear if this is the mechanism by which cholesterol is transferred to the OMM. It should be noted that the cholesterol-binding activity of StAR is independent of its activity at the OMM, because StAR mutant R182L can still function in the binding and transfer of cholesterol in isolated liposomes, although this results in an increase in cholesterol present in the cell [71]. This suggests that cholesterol binding is necessary but not sufficient for StAR's function on the OMM.

Further homology modeling and biophysical studies recently indicated the existence of a two-state model [72]. The first and open state of the model proposes that the C-terminal alpha-helix 4 of StAR, acting primarily as a gating mechanism to the cholesterol binding site, undergoes partial unfolding allowing cholesterol to bind. This cholesterol bound state, in theory, would lead to the stabilization and the refolding of alpha-helix 4; resulting in a well-defined tertiary structure [72]. This stable tertiary structure could be necessary for protein-protein interactions formed for cholesterol transfer at the surface of the mitochondria, suggesting that both StAR conformation and cholesterol binding are necessary for its proper steroidogenic activity.

These models of StAR's function have been able to provide many descriptions of cholesterol transport to the mitochondria which can relate to the function of START proteins. As six other START-domain families have been identified, such as StarD4, StarD5, and StarD6, the understanding of how the START domains function can be applied to these proteins as well [73]. This can have far reaching effects, as compared to StAR and MLN64; StarD4, StarD5, and StarD6 lack an organelle-targeting sequence and therefore are thought to be cytoplasmic. StarD4 and StarD5 are widely expressed, while StarD6 is found primarily in the testis [48]. This ability of the START domain to target and transport cholesterol from multiple sources,

including the plasma membrane, ER, and endosomes, could ensure a large source of cholesterol for steroidogenesis.

Importing Cholesterol into the Mitochondria

Cholesterol successfully imported via the plasma membrane or accessed in LDs and transported to the OMM remains segregated in the OMM until translocation to the IMM. This, the rate-limiting step in steroidogenesis, has been suggested to occur primarily through TSPO.

TSPO and cholesterol

TSPO was first identified by the presence of radiolabeled diazepam binding in the kidney [74] and it was later found to be present in most tissues of the body [75]. It was proposed that TSPO plays a role in steroidogenesis when ligand-binding studies revealed increased expression of TSPO in steroidogenic tissues and subcellular localization studies indicated that it was primarily localized to the OMM [75–78].

Because the benzodiazepine diazepam is a widely used drug ligand specific for the GABA_A receptor in the central nervous system the subsequent identification of TSPO-specific ligands allowed for the pharmacological differentiation between TSPO and the GABA_A receptor. This was accomplished by use of the isoquinoline carboxamide PK 11195, which binds with nanomolar affinity to TSPO [79] but has no affinity for the GABA_A receptor [80]. Many endogenous TSPO ligands exist in the cell, with porphyrins being able to bind TSPO with high nanomolar affinity [81]. The diazepam binding inhibitor (DBI) is another endogenous ligand. This 10-kDa protein, which also binds the GABA_A receptor with low affinity, is expressed in many tissues but is primarily expressed in steroidogenic tissues where it is localized in the cytosol in contact with the OMM [82]. Naturally processed peptides of DBI, octadecaneuropeptide (ODN, DBI_{33–50}) and triakontatetrapeptide (TNN, DBI_{17–50}), expressed in a hormone-dependent manner, were functional in binding TSPO in the brain, adrenal, and testis [74,83]. DBI and its peptides stimulated mitochondrial pregnenolone formation. When DBI expression was suppressed in the presence of antisense oligonucleotides MA-10 Leydig cells failed to respond to hormonal stimulation and steroid production was inhibited.

Early experiments in multiple steroidogenic cell systems showed that pregnenolone production was stimulated upon exposure of the cells to TSPO ligands [84,85]. When these experiments were repeated in isolated mitochondria incubated with TSPO ligands a similar increase in pregnenolone was observed [76,85]. This increase was not seen in mitoplasts, mitochondria devoid of their OMM and therefore deficient in TSPO. To determine the effect of TSPO ligands on the mitochondria, cholesterol content in the OMM and the IMM was measured both before and after TSPO ligand treatment [86]. This study revealed that ligand binding to TSPO induced the translocation of cholesterol from the OMM to the IMM and confirmed that TSPO participates in the binding and release of cholesterol at the OMM, an initial step in the production of steroids.

To further confirm TSPO's role in cholesterol binding and translocation a bacterial expression system was devised because bacteria contain no endogenous cholesterol. *E. coli* were transformed with an inducible mouse cDNA TSPO vector, resulting in fully expressed TSPO. Ligand-binding experiments were performed to verify that the bacterial TSPO possessed the same pharmacological binding properties as native TSPO; this was confirmed when bacterial TSPO bound both cholesterol and PK 11195 with nanomolar affinities [87]. When bacterial TSPO was incubated with radiolabeled steroids, time- and temperature-dependent uptake of cholesterol was seen in the protoplasts although no uptake of other steroids was seen. When the bacterial cholesterol-loaded membranes were treated with PK 11195, the cholesterol was

released [87]. These findings confirm that TSPO functions as a cholesterol translocator and suggest that TSPO might further function as a cholesterol sink, holding cholesterol until it is released by the binding of a ligand.

To identify the basis of the interaction of TSPO with cholesterol, molecular modeling and site-directed mutagenesis were used to identify potential binding sites. Previous studies had shown that TSPO spans the OMM in five alpha helices, composed of approximately 21 amino acids each. The 3-D models produced suggested the five alpha helices come together to form a channel with a hydrophilic but uncharged interior surface [87] (Fig. 2B). It was shown that the interior of the channel could bind a cholesterol molecule that had not been significantly modified, suggesting that TSPO could function as a transporter of cholesterol to the IMM. To identify the cholesterol-binding domain several deletion constructs were generated. A region on the C-terminus ($\Delta 153$ –169) was identified as necessary for cholesterol binding by virtue of the mutant's reduced ability to take up cholesterol, although PK 11195 binding was unaltered [88]. Further site-directed mutagenesis experiments identified the specific amino acids necessary for cholesterol binding, yielding a CRAC (cholesterol-recognition amino acid consensus) domain (Fig. 2C & D). The CRAC domain showed the high nanomolar affinity for cholesterol that had been observed in other proteins interacting with cholesterol [88]. These data suggest that the C-terminus of TSPO plays an important role in the uptake and translocation of cholesterol into the IMM.

To confirm the role of TSPO in ligand-binding and cholesterol translocation, a peptide antagonist was developed using a random seven-mer peptide library attached to an HIV-TAT domain [89]. The TAT domain allows receptor-independent entry of the protein or peptide attached to the sequence [90]. The random seven-mer peptides attached to the TAT domain were incubated with MA-10 Leydig cells and peptides eluted with ligand Ro5-4864. It was shown that the domain STXXXXP, specifically STPHSTP, competed with the highest efficiency for the ligand-binding domain [91]. This was further confirmed when hormone-induced steroidogenesis was shown to be inhibited by this peptide in a dose-dependent fashion. The CRAC domain was then also fused to the TAT domain, allowing entry into MA-10 cells in a dose-dependent manner [88]. This was shown to inhibit steroidogenesis through a dominant-negative effect by altering the translocation of cholesterol from the mitochondria to the TAT-CRAC domain. These data confirmed the importance of the C-terminus in the binding and translocation of cholesterol. In both cases the production of steroids from 22R-hydroxycholesterol was not altered, demonstrating that the peptide affected only TSPO and its ability to bind cholesterol and endogenous ligands [88,91].

From these experiments it was shown that TSPO is a high-affinity cholesterol- and drug ligand-binding protein. It functions in the translocation of cholesterol from the OMM to the IMM in the presence of its ligands. It should be mentioned that due to the important role of cholesterol in mammalian cells and the diverse localization of TSPO in many tissues, TSPO may play a more extensive role in the cell, participating in targeting cholesterol to mitochondria for membrane biogenesis and also cholesterol transport in the cell. This idea gained further support when treatment of both steroidogenic and non-steroidogenic cells with TSPO ligands resulted in a redistribution of cholesterol from the plasma membrane to LD [92], suggesting a possible role for TSPO in the intracellular regulation and trafficking of cholesterol, independent of cell type.

The next step was to determine if TSPO was solely responsible or if other proteins could be assisting with this translocation of cholesterol in the mitochondria. To do this the R2C rat Leydig cell line, derived from rat Leydig tumors and shown to constitutively produce steroids [93], was used. The TSPO gene was disrupted by homologous recombination, resulting in a dramatic decrease in steroid production to 10% of control values [94]. However, when 22R-

hydroxycholesterol, a hydrophilic CYP11A1 substrate that can pass directly into the IMM, was added the levels of steroid production returned to normal [94]. The role of TSPO in steroidogenesis was further verified when a TSPO knock-out mouse model proved to be embryonic lethal [95], demonstrating that TSPO is not only necessary for steroidogenesis but it also plays a critical role in early embryonic development.

Hormonal stimulation in steroidogenic cells initiates the transfer of cholesterol from the OMM to the IMM. As TSPO is suggested to play a role in this process, the effect of hormones on TSPO activity was examined. Upon the addition of the gonadotropin hCG in MA-10 Leydig cells, TSPO was shown to cluster in groups of four to six molecules [96,97]. This clustering results in increased ligand binding, cholesterol dispersal into the IMM, and steroid production and has been shown to increase the formation of contact sites. The clustering of TSPO caused by hCG can be inhibited by the addition of a cAMP-dependent protein kinase (PKA) inhibitor, suggesting that localization and clustering of TSPO is a cAMP-inducible event [98]. Because antibodies against TSPO recognize immunoreactive proteins of molecular weight greater than 18 kDa, it has been suggested these were polymers of TSPO formed through the hormonally induced clustering. The clusters of TSPO have been shown to be due to the formation of permanent dityrosine bonds [99]. Bond formation is achieved through the generation of reactive oxygen species (ROS) triggered by the presence of hormones in Leydig cells. This has been further confirmed to follow the pathway of induction of cAMP-induced and PKA-dependent ROS formation via the mitochondrial respiration complex I [100].

To better understand the role of drug ligands in cholesterol transport in the mitochondria NMR analyses were performed. The results showed that the alpha helical structure of TSPO was present in the monomer form, while the overall tertiary structure was somewhat less structured [101]. The presence of the drug ligand PK 11195 stabilized TSPO, providing a more stable environment for the translocation of cholesterol to the IMM.

Interactions of TSPO and mitochondrial proteins

Once cholesterol has been bound to TSPO it is committed to use in steroidogenesis. Because TSPO is located primarily at mitochondrial contact sites [102], it has been suggested that TSPO does not function alone in the OMM. Native TSPO in digitonin solubilized mitochondrial extracts elutes on gel filtration column chromatography in a digitonin containing buffer as a 200- to 240-kDa complex (unpublished results) while cross-linked TSPO solubilized with digitonin elutes at 170 to 210 kDa [103,104]. Studies identifying proteins in these complexes showed TSPO to be eluting at 18, 36, and 54 kDa, presumably representing the monomer and polymers of TSPO induced by hCG. Other proteins eluted were identified as voltage-dependent anion channel (VDAC), adenine nucleotide transporter (ANT), and unidentified proteins at 60 kDa (Fig 3A) [105,106].

VDAC is an OMM channel-forming protein that regulates the passage of ions and small molecules through the OMM. This function determines membrane potential, thus assisting with the regulation of apoptosis and cell metabolism [107]. VDAC's interaction with ANT forms the mitochondrial permeability transition pore (MPTP), which is located primarily at mitochondrial contact sites and regulates the cell's response to apoptotic events. ANT's role in the MPTP has been shown not to be essential though it is believed that it might function in a regulatory manner [108]. Its interactions with TSPO are currently unknown though it has recently been shown that ANT can also bind an identified TSPO ligand, protoporphyrin IX, and transport it into the mitochondrial matrix [109]. It has been suggested that interactions of TSPO, VDAC, and ANT might modulate the cell's response to apoptotic signals. Because TSPO has been shown to be involved in ROS production, it has also been suggested that TSPO might function in the apoptotic response [110]. It has also been suggested that MPTP can alter

steroidogenic rates as it is known that Leydig cell mitochondria need to be fully functional for steroidogenesis and that the opening and closing of the MPTP alters the cells' ability to produce steroids (unpublished data). As the number of contact sites can be modulated (increased) by hormone treatment; which in theory, could increase cholesterol transport between the OMM and the IMM, the permeability of the mitochondria could also affect steroidogenesis, regulated in part by TSPO [111].

Modeling has shown that VDAC binds cholesterol and it has been independently demonstrated that VDAC influences cholesterol distribution in the mitochondria [106,112,113]. It is also known that PK 11195 affects TSPO tertiary structure by stabilizing the protein in the cell [101]. TSPO's loss of flexibility as a result of binding to VDAC could translate into a more rigid VDAC, altering the respiratory state and possibly cholesterol distribution in the mitochondria as well.

TSPO and cytosolic cholesterol import

TSPO and StAR

Steroidogenic cholesterol is targeted to the mitochondria through proteins containing the StART domain - StAR and MLN64 - as mentioned earlier. For this cholesterol to be used effectively for steroidogenesis it must interact with TSPO. Because TSPO and StAR have been shown to interact by FRET [114], but not BRET [115] analysis, this might be a pathway through which cholesterol can be transferred.

To determine how TSPO and StAR interact at the OMM, antisense oligodeoxynucleotides (ODNs) were used to reduce expression of the two proteins. When StAR expression was reduced, hCG-stimulated MA-10 Leydig cells stopped producing progesterone after 20 minutes, while in TSPO-depleted cells steroidogenesis was inhibited after ten minutes [116]. Together these results show that both TSPO and StAR function in steroidogenesis; the difference in time of arrest of steroidogenesis was attributed to the presence of cholesterol on the OMM available to be used for steroidogenesis. Since we were unable to demonstrate a direct physical StAR/TSPO interaction we searched for a functional one. Thus, StAR expression was examined in TSPO-depleted cells. It was shown that StAR was not processed from the 37-kDa cytosolic protein to the mature intramitochondrial 30-kDa protein that is normally seen under hormonal stimulation [116]. This was further confirmed when a peptide antagonist shown to bind to the cholesterol-binding domain of TSPO also inhibited the intramitochondrial formation of the 30-kDa StAR [91,116]. Based on these results it was suggested that TSPO plays a direct role in the import of StAR into the IMM and that StAR is dependent upon TSPO for its activity.

To test this hypothesis, TSPO-depleted mitochondria were transfected with a Tom/StAR construct that would be targeted to and imported into the OMM. Previously it had been shown that this construct increased progesterone production two-fold in MA-10 cells; however, no effect was seen on steroid production in TSPO-depleted mitochondria [70,116]. TSPO was then reincorporated into the isolated mitochondria, restoring the ability of the mitochondria to produce pregnenolone. This effect was seen with both Tom/StAR and StAR constructs. Based on separate analysis and reintroduction of these two proteins into the cell system, it was proposed that StAR's primary function is in the hormone-induced transport of cholesterol to the OMM while TSPO regulates the translocation of cholesterol into the IMM.

Acute stimulation of steroidogenesis *in vivo* results in measurable hormone production within minutes. However, *in vitro* this effect is not observed until 10–20 minutes, which is interesting because StAR protein synthesis shows a lag of 20 – 30 min after hormone stimulation [117]. As previously mentioned, steroidogenic cholesterol can be found bound to TSPO in the OMM,

suggesting that this time frame would deplete TSPO of cholesterol and allow StAR to then replenish cholesterol concentrations. Limiting StAR protein expression and, therefore, its activity in the cell, would allow for cholesterol to be specifically targeted to the mitochondria for steroidogenesis and inhibit excess cholesterol transport. This delayed protein expression also confirms that StAR does not act alone on the OMM in steroidogenesis.

StAR has been shown to cycle sufficiently rapidly to transfer 400 molecules of cholesterol per minute into adrenal cells [118]. However, experimental observations have demonstrated that the stoichiometry of cholesterol transfer is 1.82 molecules of cholesterol per minute in isolated mitochondria [119], suggesting that further modification in the cell is needed for maximal StAR activity. One proposed mechanism for this modification is cholesterol binding to the SBD, which has been shown to be necessary for StAR's function [120]. StAR binds cholesterol with an affinity of 32 nM [121], although binding at an affinity of 95 μ M has recently been reported [122]. Because StAR rapidly shuttles cholesterol through the OMM, high-affinity binding of cholesterol to StAR would not favor this transfer. It is also important to note that this affinity is substantially lower than the affinity of 5 nM of TSPO for cholesterol [123], suggesting a possible mechanism by which TSPO removes cholesterol from StAR when it is in close proximity to the OMM.

TSPO and PAP7

To determine if other proteins are necessary for importing cholesterol into the mitochondria a yeast-two hybrid screen was performed with TSPO as the bait. This approach demonstrated the association of several PBR-associated proteins (PAPs) with TSPO, with PAP7 demonstrating the most compelling interaction [124]. PAP7 was found to have an expression pattern similar to TSPO and was localized to the Golgi and mitochondria. Interestingly, in another yeast-two hybrid screen with the regulatory subunit RI α of PKA as the bait, PAP7 was also identified [124]. These data provided useful insight into the function of PAP7 because PKA phosphorylates proteins in a hormone-specific manner through the activation of cAMP. When cAMP levels rise the proteins bind to the two regulatory subunits of PKA, RI and RII and their isoforms (I α and I β , II α and II β), which release the two catalytic subunits; these are then able to phosphorylate specific serine and threonine residues, activating select proteins (Fig. 3 B). It was then confirmed that PAP7 binds both TSPO and PKA-RI α *in vitro* in MA-10 mouse Leydig cells [124]. Overexpression of PAP7 was shown to stimulate progesterone production in MA-10 cells and transfection of the TSPO- and PKA-RI α -binding domain of PAP7 (a.a. 228 – 445) significantly inhibits steroidogenesis [124,125]. These results further confirm PAP7's role in steroidogenesis via interaction with TSPO and PKA-RI α . In these studies we identified an acyl-coA binding motif in PAP7, similar to the one identified in DBI [125]. More recently, DBI and PAP7 were renamed acyl-coenzyme A binding domain containing 1 (ACBD1) and 3 (ACBD3) proteins, respectively (Fan, Liu, Culty and Papadopoulos, manuscript submitted).

Since PAP7 is known to bind PKA-RI α , it was suggested that PAP7 functions as an A Kinase Anchoring Protein (AKAP). AKAPs are a family of proteins known to recruit the PKA holoenzyme into proximity to its substrate, confining its activity [126]. In this process, PAP7 is presumed to bring PKA-RI α into closer proximity to the proteins mediating cholesterol transport and thus steroidogenesis, playing a role in regulating their activity via phosphorylation. This mechanism would allow the signaling mechanism for steroidogenesis to be localized to certain areas on the mitochondria, limiting the concentration of protein needed for maximal stimulation. Thus, as the concentration of trophic hormone needed to stimulate maximal cAMP production is about 15 times higher than that needed to maximally stimulate testosterone secretion [127] such a mechanism would allow maximal steroid formation in the presence of submaximal cAMP accumulation. Targeting of PKA-RI α close to mitochondrial

TSPO would result in the localization and amplification of its ability to phosphorylate proteins involved in steroidogenesis.

It is known that cloned rat, bovine, and murine TSPO are phosphorylated in the C-terminal domain, although a phosphorylation site has not been identified in human TSPO [128]. StAR becomes rapidly phosphorylated upon the addition of trophic hormones in all species investigated to date [129]. Based on these observations it was proposed that PAP7 anchors PKA-R1 α , facilitating the phosphorylation of StAR and possibly TSPO, in a cAMP-dependent manner (Fig 3B). This mechanism would allow the activity of StAR to be regulated and activated by proteins localized to the OMM, controlled by proteins known to anchor to TSPO.

To determine if the proteins under investigation interact at the OMM, COS-F2-130 and MA-10 cells were transfected with TSPO, StAR, PAP7, and PKA-R1 α . The transfection of the four proteins together in the non-steroidogenic cells induced an increase in steroid production greater than that induced by each individual protein alone, suggesting that these proteins form a complex that performs a necessary role in steroidogenesis. Hormonal stimulation of non-transfected Leydig cells induced a greater increase in steroid synthesis, suggesting that the complex of proteins is still dependent upon stimulation through cAMP and not on the amount of proteins present. The interactions between TSPO, StAR, and PKA-R1 α were subsequently analyzed by microscopy. These studies indicated that, upon hormonal stimulation, PAP7 translocated from the Golgi to the mitochondria [130] and StAR translocates to the mitochondria, where PKA-R1 α and PAP7 colocalize with TSPO [130].

To further confirm that these proteins interact at the OMM, photoactivatable amino acids, specifically leucine and methionine, were used. These amino acids are functionally incorporated in the proteins and then can be cross-linked by exposure to UV light, allowing identification of protein-protein interactions [131]. Since hormonal stimulation of Leydig cells results in an acute response, the immediate cross-linking of proteins permits the identification of transient protein-protein interactions, which normally could not be observed using classical procedures such as immunoprecipitation. Upon transfection of TSPO, StAR, PAP7, and PKA-R1 α in the presence of photoactivatable amino acids in COS-F2-130 cells, cells were exposed to UV light. Immunoblot analysis identified a 210-kDa complex containing immunoreactive TSPO and StAR proteins [130]. MA-10 Leydig cells treated with hCG under the same conditions as described above were cross-linked under UV light. Immunoblot analysis identified a 240-kDa protein complex in which TSPO, PAP7, PKA-R1 α , and VDAC were observed to be associated in a time-dependent manner following hCG exposure. StAR levels peaked after a 30-min treatment with hCG and decreased after two hours of stimulation, while VDAC levels increased slightly and then also decreased at two hours [130]. Because StAR is dependent upon VDAC for import [132], it is interesting to note that VDAC also undergoes depletion during the hCG response [130]. This finding suggests that VDAC could play a role in the acute response to hormonal stimulation, assisting in the binding of StAR to the OMM. StAR would then be phosphorylated through the identified protein complex; TSPO, PAP7, and PKA-R1 α , would increase import. These experiments demonstrated that the inducible hormone complex of steroidogenic proteins, the “transduceosome,” interact to facilitate the transfer of cholesterol from the OMM to the IMM. This complex occurs at the OMM, not at the IMM, as confirmed by presence of VDAC and the absence of ANT in the cross-linked complex [130].

TSPO, StAR and cholesterol transport in disease

Improper storage and targeting of cholesterol can be toxic to cells, as seen in cells affected with NPC disease, mentioned previously. There are many opportunities for inappropriate storage and targeting to occur in the multiple steps required to target cholesterol to the mitochondria. Moreover, limiting or accelerating the transfer and access of cholesterol to CYP11A1 would result in changes in the levels of steroids formed that could affect the target

cell function as well as tissue and body homeostasis. We will discuss examples of diseases that arise when cholesterol targeting is altered below.

Endocrine pathologies

Lipoid congenital adrenal hyperplasia (Lipoid CAH) was initially proposed to be caused by deficiency of a steroidogenic enzyme but further study identified point mutations or stop codon insertion into the C-terminus of the StAR protein [63,133]. Patients with lipoid CAH suffer from a decrease in both adrenal and gonadal steroidogenesis, resulting in an increase in cholesterol accumulating in steroidogenic cells [67]. Patients affected by Lipoid CAH are still able to produce low levels of steroids, approximately 14% of normal, which was suggested to occur through cholesterol present on the mitochondria [67]. Since the steroidogenic cells are unable to correctly process the cholesterol esters, inhibition of the non-StAR-mediated steroidogenesis occurs. This results in a further increase in cell damage and sometimes death. This process has been confirmed in the StAR-knockout mouse model, which displays the same phenotype as human Lipoid CAH [134]. The mice could be kept alive if they were given corticosteroid replacement, although they still suffered from an increase in lipids with aging; this was not observed in females until after puberty [135]. Interestingly, the *Tspo* gene was found to be intact in Lipoid CAH [67]. This is not surprising, considering that knock-out of *Tspo* results in an embryonic lethal phenotype, as mentioned above.

Primary pigmented nodular adrenocortical disease (PPNAD) is a component of the Carney complex (CNC), in which the patient carries a PKA-RI α -inactivating mutation [136]. This mutation causes an adrenal disorder characterized by an ACTH-independent hypercortisolism, which is stimulated by exogenous steroids [137]. Analysis of the adrenal cortical nodular cells showed either very weak or no staining at all for PKA-RI α . Previously it had been proposed that PKA-RI α is a tumor suppressor [136], confirming the profile commonly seen in tumor cells, in which one allele contains an inactivating mutation and the other allele is missing. On further observation it was shown that the area around the nodular cells expressed PKA-RI α at levels higher than those seen in control cells, suggesting this area of the cortex could be compensating for the lack of expression of PKA-RI α [136].

Because it is known that PAP7 interacts with PKA-RI α in steroidogenesis, PAP7 expression was investigated in patients affected with PPNAD. In these patients PAP7 protein expression levels mirrored PKA-RI α expression levels, with very low expression in the adrenal cortical nodular cells and higher than wild-type expression of PAP7 in the area surrounding the nodular cells [138]; PKA-RII α expression was not affected. It was already known that PAP7 protein expression is not altered in adrenal hyperplasia or in brain, breast, or colon tumor mRNA levels, so this provided further evidence that PKA-RI α expression correlated strongly with PAP7 protein expression levels [138].

Previous data have shown that CNC tumors with PKA-RI α mutations respond to cAMP stimulation with a larger increase in PKA activity compared to cells lacking the mutations. The observation that PKA-RI α and PAP7 expression is higher in the adrenal cortical cells surrounding the nodules provided an explanation for the increase in hormones produced following even a low level of cAMP stimulation, compared to wild type. These findings further support the hypothesis that PKA-RI α and PAP7 interact in a cAMP-dependent manner to control steroidogenesis.

Neurological disorders

Steroids are able to diffuse across the blood-brain barrier, although concentrations present in the brain have been shown to be different from those found in the peripheral nervous system, suggesting that the brain is capable of locally synthesizing steroids [139,140]. In addition, the

brain is a major site of cholesterol synthesis, with cholesterol itself unable to pass through the blood-brain barrier. The term “neurosteroids” was first introduced to represent the steroid intermediates present in the brain; however, today it is accepted that the brain is a steroidogenic organ and the definition has evolved to represent steroids produced and metabolized in the brain [141]. Neurosteroids required to induce changes in neuronal activity are present and act at extremely low (nanomolar) concentrations, in contrast to the high concentrations of gonadal and adrenal steroids produced for distribution through the circulation and action at distant sites throughout the body. This may explain why no activator of neurosteroid biosynthesis has been identified to date.

TSPO is present in the brain and is primarily expressed in the steroid-producing glial cells, although it is also present at low levels in the neurons [142,143]. Since StAR and MLN-64 have also been identified in glial cells, it has been suggested that the steroidogenic pathway in the brain is similar to that in the periphery [144,145]. This was confirmed when activation of glial TSPO and isolated glial cell mitochondria with drug ligands of TSPO was shown to result in the production of pregnenolone and neurosteroids [146,147]. Neurosteroids have been shown to have an immediate, specific, and local effect on neural development; therefore, neural development would be significantly impacted if the regulation of neurosteroids were altered.

Examination of TSPO expression showed upregulation in many neuropathologies, neurodegenerative disorders, and during brain injury and inflammation. Upregulation of TSPO was confirmed in gliomas [148], multiple sclerosis [149], Parkinson’s and Huntington’s disease [150,151], and epilepsy [152]. TSPO expression was also shown to be increased after nerve degeneration and during regeneration, although once regeneration was complete the levels of TSPO decreased [153]. Microglia are more active in many of these neuropathologies and neurodegenerative diseases and, as TSPO is primarily localized in glial cells, it has been possible to use TSPO as a marker to diagnose and determine the rate of progression of many disease and injuries.

TSPO is also up regulated in Alzheimer’s disease (AD) with a resulting increase in pregnenolone levels in the hippocampal region of the brain [150,154]. Interestingly, it has been shown that a steroid intermediate in the conversion of cholesterol to pregnenolone, 22R-hydroxycholesterol, was found at lower levels in AD brain compared to control [155]. It was also later shown that 22R-hydroxycholesterol exerts neuroprotective effects against the neurotoxicant β -amyloid ($A\beta$) peptide, functioning through binding and inactivation of the peptide. This neuroprotectant is decreased in AD patients, allowing $A\beta$ to exert its toxic effect on the cells. Because $A\beta$ is an intermediate in the steroidogenic pathway, overexpressed TSPO would presumably compensate for the decrease in 22R-hydroxycholesterol. Because this does not occur, it has been suggested that TSPO does not function normally in Alzheimer’s patients. It will be necessary to further explore the role of TSPO in neurosteroid production and neuroprotective effects in AD.

TSPO has been shown to be altered in anxiety and mood disorders as well. The concentration of TSPO determined through assays of radiolabeled PK 11195 binding to platelets showed a decrease in anxiety, panic, and post-traumatic stress disorders. These data suggest that TSPO may exert an anti-anxiety effect through the production of neurosteroids, if the same reduction in TSPO is seen in the CNS. In agreement with these findings it was shown that many drugs used in the treatment of psychiatric disorders act on specific enzymes of the neurosteroidogenic pathway, resulting in normal levels of the neurosteroid allopregnanolone [156]. This was further confirmed when it was shown that DBI also has an effect on patients with schizophrenia [157] and suggests that TSPO ligands could play a role in the prevention and treatment of psychiatric disorders.

Conclusion

In the past few years many advances have been made in identifying the protein-protein interactions necessary for steroidogenesis. From the initial stages of cholesterol import into the plasma membrane, through lipoprotein binding to receptors, to the actual translocation of cholesterol into the IMM for pregnenolone production each step has been shown to be tightly controlled and regulated through these interactions. Newly identified proteins, such as PAP7 (ACBD3), contribute to a more complete picture of how steroidogenesis is regulated in each cell model and of the protein interactions involved in steroidogenesis. These discoveries have direct effects on the understanding of the bases of several neurological and endocrine diseases.

Despite recent progress in understanding the cellular and molecular mechanisms underlying the hormonal regulation of cholesterol transport into mitochondria and, thus, of steroidogenesis, there are several issues that remain to be addressed. These include acquiring a better understanding of the spatiotemporal and structural dependence of the mechanisms of both membrane and cholesterol movement in the cell under hormonal stimulation.

Mitochondria in general are cholesterol-poor organelles; however the mitochondria of steroidogenic cells contain a relatively high amount of cholesterol. Since TSPO also occurs at higher levels in steroidogenic cells, this cholesterol could be bound to the excess TSPO present in the OMM. Due to the location of the CRAC domain at the interface of the OMM and the cytosol, it is unclear where this excess cholesterol in the mitochondria is binding; it could be present in the interior of the pore formed by the alpha helices of TSPO or in the membrane. In the absence of the crystal structure of TSPO, this question remains to be answered. Moreover, the clustering of TSPO upon hormonal stimulation would increase the occurrence of cholesterol aggregation and possible cholesterol concentration in the OMM. This effect would cause alterations in both membrane permeability and ROS signaling, as is seen following hormonal stimulation. This would also allow easier access of cholesterol into the OMM since free cholesterol diffuses more readily into and out of areas of high cholesterol concentration. The clustering of TSPO could be conceived of as a reservoir of cholesterol molecules, which would be available for transfer to the IMM and use in steroidogenesis. The creation of a molten globule structure by StAR on the OMM is proposed to alter the OMM. This StAR-OMM interaction could cooperate with the clustering of TSPO, facilitating the formation of the "active" TSPO structures in which cholesterol could be transferred to the IMM and allowing StAR to directly transfer cholesterol to a cholesterol-rich area. However, this hypothesis remains to be tested. Ultimately, it is unclear how cholesterol locates and binds to CYP11A1 after it moves into the IMM.

In addition to these questions, it must be taken into consideration that steroidogenic cells have been shown to rapidly undergo morphological changes in response to hormone treatment. This well-established phenomenon further suggests that additional protein-protein interactions could be necessary for trafficking of cholesterol and for the reorganization of the mitochondria in preparation for optimal steroidogenesis.

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● Cholesterol

Figure 1. Trafficking of cholesterol to the mitochondria for steroidogenesis

Pathway 1: Cholesterol synthesized in the ER is trafficked to the Golgi apparatus where it can be targeted to the mitochondria via the PAP7 protein for steroidogenesis. Passive diffusion from the ER to the mitochondria, shown here with the corresponding cholesterol molecules present on both organelles, is another possible pathway for steroidogenic cholesterol to be transferred to the mitochondria. **Pathway 2:** Low density lipoprotein (LDL), containing cholesterol, binds to the LDL receptor and is trafficked through the endosomal pathway. MLN-64 assists with the transfer of cholesterol to the mitochondria from the late endosomes and lysosomes for use in steroidogenesis. NPC1 and NPC2 associate with MLN-64 for cholesterol transfer out of the lysosomes, although it is not known if this cholesterol is used for steroidogenesis. **Pathway 3:** Cholesterol is transferred from high density lipoprotein (HDL) to the plasma membrane by the SR-B1 receptor. Hormone-sensitive lipase (HSL) converts esterified cholesterol from the plasma membrane to free cholesterol, which can be used for steroidogenesis. **Pathway 4:** HSL also interacts with esterified cholesterol present in the lipid droplets (LD), which converts esterified cholesterol to free cholesterol for use in steroidogenesis as well. Free cholesterol from the LD can interact with lipid-binding proteins present in the cytosol for delivery to the mitochondria.

Figure 2. Cholesterol-binding domains of StAR and TSPO

A) Human StAR protein modeled with cholesterol (brown) present in the sterol-binding domain (PDB ID code: 2I93). Homology models are deduced via the Swiss-model services under project (optimize) mode using the crystal structures of human MLN64 (PDB ID code: 1EM2) as template (<http://swissmodel.expasy.org/SWISS-MODEL.html>). B) Molecular model of TSPO's five alpha helices in the presence of cholesterol, demonstrating a pore accommodating a cholesterol molecule. Homology modeling of mouse TSPO was performed using apolipoprotein-III from *Manduca sexta* as template (PDB ID code: 1EQ1) [158]. C) Ribbon diagram of mTSPO, showing the five helices as well as the CRAC domain, consisting of Leu/Tyr/Arg residues, and cholesterol (top view). D) Docking model of cholesterol to the TSPO CRAC domain. The accessible surface of the peptide and cholesterol molecules are represented in red and blue, respectively (Reprinted with permission from [159]).

Figure 3. Protein-protein interactions at the OMM

A) Proposed basal-state protein-protein interactions of TSPO, VDAC, and ANT. B) Transduceosome complex formed after acute hormonal stimulation. The TSPO, PAP7 (ACBD3), and VDAC complex recruits PKA-RI α , which is shown binding to PAP7 (ACBD3). The accumulation of cAMP activates PKA-RI α , releasing the catalytic subunits and phosphorylating StAR present at the OMM. StAR interacts with both TSPO and VDAC and is imported into the IMM. Cholesterol is imported into the IMM with the assistance of the transduceosome complex and the presence of DBI; there the cholesterol interacts with the CYP11A1 side chain cleavage enzyme to be converted to pregnenolone. ANT is represented in the IMM as identified from previous isolated complexes with TSPO.