HHS Public Access

Author manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2018 May 01.

Published in final edited form as:

Mol Cell Endocrinol. 2017 February 05; 441: 7–16. doi:10.1016/j.mce.2016.07.036.

A brief history of the search for the protein(s) involved in the acute regulation of steroidogenesis

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Abstract

The synthesis of steroid hormones occurs in specific cells and tissues in the body in response to trophic hormones and other signals. In order to synthesize steroids *de novo*, cholesterol, the precursor of all steroid hormones, must be mobilized from cellular stores to the inner mitochondrial membrane (IMM) to be converted into the first steroid formed, pregnenolone. This delivery of cholesterol to the IMM is the rate-limiting step in this process, and has long been known to require the rapid synthesis of a new protein(s) in response to stimulation. Although several possibilities for this protein have arisen over the past few decades, most of the recent attention to fill this role has centered on the candidacies of the proteins the Translocator Protein (TSPO) and the Steroidogenic Acute Regulatory Protein (StAR). In this review, the process of regulating steroidogenesis is briefly described, the characteristics of the candidate proteins and the data supporting their candidacies summarized, and some recent findings that propose a serious challenge for the role of TSPO in this process are discussed.

Keywords

Steroidogenesis; Mitochondria; Translocator Protein (TSPO); Steroidogenic Acute Regulatory Protein (StAR); Conditional knockout; Global knockout

1. Introduction

Steroidogenesis is the process by which important steroid hormones are synthesized by specific tissues and cells in the body. Examples of these important steroids are the glucocorticoids that are synthesized in the adrenal cortex, the mineralocorticoids that are synthesized in the adrenal glomerulosa, the ovarian and placental progestins and estrogens, the testicular androgens and several neurosteroids such as pregnenolone, progesterone, 5α-dihydroprogesterone, allopregnanolone and DHEA, that are synthesized in the brain. The adrenal glucocorticoids serve to regulate carbohydrate metabolism and manage stress and the mineralocorticoids are involved in salt balance and the maintenance of blood pressure.

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Ovarian progesterone and estrogen are involved in the maintenance of female secondary sex characteristics and reproductive function while testicular testosterone is involved in maintaining male secondary sex characteristics and is essential for male fertility. Neurosteroid functions include stimulation of GABAergic responses, modulation of the response of Purkinje cells to excitatory amino acids and the enhancement of memory function. Other tissues and cells have also been reported to have the capacity for *de novo* steroid synthesis, but the localization of where steroids are synthesized and their respective functions are not the main focus of this review. Rather, this review will concentrate on the manner in which the synthesis of steroid hormones are regulated and the history of the efforts that have been made to uncover the components and the mechanisms involved in this regulation. This history dates back approximately six decades when it was first observed that the synthesis of steroid hormones *in vitro* could be stimulated with trophic hormones and that this synthesis required the production of a new protein(s), as will be described later in this review. This singular observation formed the basis for what has been a long and most interesting search for the putative regulatory protein(s). We will briefly summarize the early studies that were performed in the search for this regulatory protein(s), the necessary characteristics of the candidates required to perform this function and some of the controversies that have arisen along the way, and indeed, remain to the present time. To be sure, this has been an interesting undertaking by a number of investigators in the field and it would seem safe to say that at this juncture in time, the entire story of what factors are involved in the acute regulation of steroid hormone biosynthesis and how they function is not yet completely understood.

2. Characteristics of the regulation of steroid hormone biosynthesis

The initial step in steroidogenesis is the conversion of cholesterol to the first steroid formed, pregnenolone, which occurs in all steroidogenic tissues (Miller, 1988, Pescador et al., 1997). This conversion is a result of the action of the cytochrome P450 side-chain cleavage enzyme (P450scc; CYP11A1), that is part of the cholesterol side chain cleavage system that resides on the matrix side of the inner mitochondrial membrane (Farkash et al., 1986). Pregnenolone then exits the mitochondria and is converted to progesterone and other steroids in the microsomal compartment and, in some cases, downstream steroids re-enter the mitochondrial compartment to be converted to the final product dependent upon the complement of steroidogenic enzymes present within specialized cells in those tissues (Miller, 1998). Understanding the enzymes involved in steroidgenesis and, most importantly, their intracellular location, is key to understanding how the synthesis of steroid hormones is regulated.

The biosynthesis of steroid hormones are mostly regulated by pituitary trophic hormones such as adrenocorticotropin (ACTH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) and this regulation occurs in two phases. The acute phase occurs very rapidly (within minutes) and is responsible for the production of steroids in response to immediate needs (Stocco and Clark, 1996). In addition, chronic regulation (on the order of hours) also occurs and consists of the longer-term expression of the mRNAs and proteins for the steroidogenic pathway enzymes to provide for continuing steroid synthesis following the acute phase (Miller, 1988, Simpson et al., 1992, Simpson and Waterman, 1988, Waterman

and Simpson, 1985). This review will focus only on elements involved in the acute phase of steroid hormone biosynthesis. Like most biosynthetic pathways, the steroidogenic pathway has a rate-limiting step and following a number of years of speculation, it was experimentally determined that the regulated and rate-limiting step in steroidogenesis was the delivery of the substrate cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) where the P450scc enzyme is located (Black et al., 1994, Farkash et al., 1986). This step was rate-limiting because the hydrophobic cholesterol could not cross the aqueous mitochondrial intermembrane space to the relatively cholesterol poor IMM within the time frame that was observed for the acute synthesis of steroids. There followed a significant period of investigation to determine the nature of the acutely regulated step. Early investigations were performed using adrenal perfusions *in vitro* and it was observed that ACTH could stimulate the biosynthesis of steroids (Stone and Hechter, 1954) and importantly, that acute steroid production had an absolute requirement for the synthesis of new proteins (Ferguson, 1962, 1963, Garren et al., 1965, Garren et al., 1966, Garren, 1968). Subsequent studies demonstrated that the putative regulator protein in all likelihood functioned at the level of the delivery of cholesterol to the P450scc enzyme in the IMM, the regulated step (Arthur and Boyd, 1974, Davis and Garren, 1968, Ohno et al., 1983, Privalle et al., 1983, Simpson and Boyd, 1966). The overall significance of all these early observations was that the search for the putative regulator now had a specific target on which to focus, namely, a newly synthesized protein.

The discovery and characterization of the putative protein regulator of steroidogenesis was the goal of several laboratories and over the ensuing decades candidate proteins for the acute regulator emerged. These candidates included the Sterol Carrier Protein 2 (SCP2) (Vahouny et al., 1987), the Steroidogenesis-Activator Polypeptide (SAP) (Pedersen and Brownie, 1983, 1987), the Peripheral Benzodiazepine Receptor/Translocator Protein (PBR/TSPO) and the Steroidogenic Acute Regulatory protein (StAR), and all were placed in contention for being the protein responsible for the acute regulation of cholesterol transfer to the IMM and thus, steroidogenesis.

Experimental evidence revealed that each of these proteins appeared to have characteristics that rendered them as viable candidates. However, after the initial studies demonstrating that SCP2 could enhance steroid synthesis in isolated mitochondria, further research on this candidate based on observations in knockout mice ruled out an involvement for SCP2 in steroidogenesis (Seedorf et al., 1998). Also, while first isolated as a small molecular weight peptide (Pedersen and Brownie, 1983, 1987), SAP was later identified as a fragment of the much larger glucose-regulated protein 78 (GRP78) (Li et al., 1989). GRP78 is a regulator of the unfolded protein response (UPR) (Wang et al., 2009), and it was demonstrated that GRP78 knockout mice died at an early embryonic stage (Luo et al., 2006). Later studies examining GRP78 conditional knockouts indicated a phenotype in oncogenic signaling (Wey et al., 2012), and examination of GRP78 structure showed that it contained a nucleotide-binding domain and a peptide substrate-binding domain (Wisniewska et al., 2010), but had no characteristics that indicated the potential for lipid transport. Therefore, a function for GRP78 in the acute regulation of cholesterol transport appeared unlikely. In contrast to SCP2 and SAP, the candidate proteins TSPO and StAR have continued to be studied in great detail.

3. The Steroidogenic Acute Regulatory Protein

A candidate protein for the acute regulator of steroidogenesis was first described by Orme-Johnson and her colleagues as an ACTH-induced 30 kDa phosphoprotein in hormone-treated rat and mouse adrenocortical cells, and as an LH-induced protein in rat corpus luteum cells and mouse Leydig cells (Alberta et al., 1989, Epstein and Orme-Johnson, 1991a,b, Krueger and Orme-Johnson, 1983, Pon et al., 1986a,b, Pon and Orme-Johnson, 1988). These studies demonstrated that a close relationship existed between the appearance of the 30 kDa proteins and steroid hormone biosynthesis and that their synthesis was sensitive to cycloheximide. Our laboratory was engaged in similar studies in hormone-stimulated MA-10 mouse Leydig tumor cells and described a family of proteins which were identical to those described by Orme-Johnson (Stocco and Kilgore, 1988, Stocco and Chaudhary, 1990, Stocco and Chen, 1991, Stocco and Sodeman, 1991, Stocco, 1992, Stocco and Ascoli, 1993, Stocco et al., 1995). These proteins were found localized to the mitochondria and consisted of several forms of a newly synthesized 30 kDa protein. The proteins, as identified by 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE), are shown in Fig. 1. Later studies determined that the 30 kDa mitochondrial proteins were processed from a 37 kDa precursor protein that contained a mitochondrial signaling sequence in its N-terminus (Epstein and Orme-Johnson, 1991a, b, Stocco and Sodeman, 1991). In general, all of the studies that were performed demonstrated tight correlations between the synthesis of steroids and the synthesis of the 30 kDa mitochondrial proteins and thus, they represented good candidates for the regulatory protein (Alberta et al., 1989, Epstein and Orme-Johnson, 1991a, b, Krueger and Orme-Johnson, 1983, Pon et al., 1986a, b, Pon and Orme-Johnson, 1988, Stocco and Kilgore, 1988, Stocco and Chaudhary, 1990, Stocco and Chen, 1991, Stocco and Sodeman, 1991, Stocco, 1992, Stocco and Ascoli, 1993, Stocco et al., 1995). The purification of the 30 kDa protein, the cloning of the cDNA for the 37 kDa protein precursor and its sequencing were successfully accomplished in 1994 (Clark et al., 1994). Both the nucleic acid sequence of the cDNA and amino acid sequence of the 37 kDa protein were found to be unique, indicating it represented a novel protein. Transient transfection experiments demonstrated that expression of the cDNA-derived protein in both MA-10 mouse Leydig tumor cells and COS-1 monkey kidney cells co-transfected with the P450scc enzyme system to render them steroidogenic resulted in a significant increase in steroid production in the absence of hormone stimulation (Fig. 2). As a result of these findings, this protein was named the Steroidogenic Acute Regulatory Protein or StAR (Clark et al., 1994). Also, transient transfection of COS-1 cells (that had been rendered steroidogenic by transfection with the cholesterol side chain system proteins), with the cDNA for the 37 kDa protein resulted in a several fold increase in the conversion of cholesterol to pregnenolone (Lin et al., 1995, Stocco and Clark, 1996, Sugawara et al., 1995). Taken together, these results indicated a direct role for the 37-30 kDa proteins in hormone-regulated steroid production.

Shortly following the cloning of the StAR cDNA, collaborative studies with Walter Miller and Jerry Strauss demonstrated that mutations in the StAR gene resulted in the disease congenital lipoid adrenal hyperplasia (lipoid CAH) (Lin et al., 1995). Lipoid CAH is a lethal condition characterized by an almost complete inability of the newborn to synthesize

steroids. These afflicted patients also have large adrenals containing high levels of cholesterol and cholesterol esters and an increased amount of lipid accumulation in testicular Leydig cells indicating an inability to transfer cholesterol to the P450scc enzyme for conversion to pregnenolone. A recent review provides an up-to-date summary of the mutations in StAR that have been uncovered as of the present time (Miller, 2016). These observations added compelling evidence for the essential role of this protein in the regulation of steroidogenesis since, in essence, with lipoid CAH nature provided a StAR knockout and the phenotype had all of the expected characteristics.

At approximately that same time we also performed collaborative studies with Keith Parker and his colleagues. In one of these studies we were able to demonstrate that during the course of embryonic development in the mouse, StAR mRNA expression was tightly correlated with the appearance of steroidogenic cells and the timing of steroidogenesis in the adrenal gland and the testis (Clark et al., 1995). These studies clearly demonstrated the presence of StAR transcripts in the adrenal cortex but not the adrenal medulla and in the testicular Leydig cells, but not the seminiferous tubules during embryonic development. As expected, there were no StAR transcripts in the ovary during the course of development, as estrogen is not produced in this organ until puberty. In another study in collaboration with Dr. Parker's laboratory, targeted disruption of the StAR gene in mice was used to successfully produce StAR knockout mice (Caron et al., 1997). All StAR knockout mice had female external genitalia, failed to grow normally and died within a short period of time, presumably as a result of adrenocortical insufficiency. Serum levels of corticosterone and aldosterone were depressed while levels of ACTH and CRH were elevated indicating impaired production of adrenal steroids with an accompanying loss of feedback regulation at the level of the hypothalamus or pituitary. The adrenal glands had a normal medulla but an abnormal cortex, that displayed a disrupted fascicular zone, and staining with oil red-O revealed elevated lipid deposits in the cortex of the StAR knockout mouse. Images depicting grossly elevated lipid deposits in both the adrenal cortex and in the Leydig cells in the interstitial compartment of the testis are shown in Fig. 3. Thus, the StAR knockout mouse demonstrated characteristics very similar to human lipoid CAH and further substantiated the necessity for StAR action in steroid biosynthesis.

With the availability of StAR reagents, results obtained with methodologies such a Western analysis, Northern analysis, *in situ* hybridization, immunocytochemistry, RNAse protection assays and RT PCR have all been employed to show that StAR expression is essentially confined to steroidogenic tissues. Importantly, these studies were conducted in many different laboratories and were able to successfully show that StAR was present in the steroidogenic cells of various steroidogenic tissues. Thus, StAR expression has been demonstrated in the adrenal cortical layers (both glomerulosa and fasciculata-reticularis cells) (Brand et al., 1998, Cherradi et al., 1998, Fleury et al., 1998, Lehoux et al., 1999, Liu et al., 1996, Nicol et al., 1998, Nishikawa et al., 1996, Peters et al., 1998, Pollack et al., 1997), ovarian theca cells (Brand et al., 1998, Cherradi et al., 1998, Fleury et al., 1998, Kiriakidou et al., 1996, Lehoux et al., 1999, Nicol et al., 1998, Peters et al., 1998, Pollack et al., 1997), ovarian granulosa cells (Balasubramanian et al., 1997, Bao et al., 1998, Kerban et al., 1999, Kiriakidou et al., 1996, LaVoie et al., 1999, Pescador et al., 1996, Ronen-Fuhrmann et al., 1998, Sekar et al., 2000, Thompson et al., 1999), ovarian corpora lutea cells

(Chen et al., 1999, Chung et al., 1998, Juengel et al., 2000, Kiriakidou et al., 1996, Mamluk et al., 1999, Pescador et al., 1996, 1999, Pollack et al., 1997, Townson et al., 1996), fetal mouse giant trophoblast cells (Arensburg et al., 1999), both fetal and adult testis, ovary and adrenal tissues (Pilon et al., 1997), adrenal tumors (Liu et al., 1996) and testicular Leydig cells (Bosmann et al., 1996, Kanzaki and Morris, 1999, Leers-Sucheta et al., 1999, Lejeune et al., 1998, Lin et al., 1998, Luo et al., 1998, Mauduit et al., 1998, Pollack et al., 1997). Since those early studies and using more sensitive technologies StAR protein has been detected in a wider variety of tissues where its roles are, so far, not completely known, but have been subjected to most interesting discussion that is beyond the scope of this review (Anuka et al., 2013).

Uncovering the role of StAR in the mechanism of cholesterol transport to the IMM has so far proved to be quite elusive even after more than two decades of effort. An early model depicted that import of the StAR protein into the mitochondrial matrix temporarily formed contact sites between the OMM and IMM and might thus allow the hydrophobic cholesterol to transfer between the membranes (Stocco and Clark, 1996). A later model, resulting from solving the crystal structure of a StAR homolog, MLN64, suggested that StAR mediated cholesterol transport by shuttling cholesterol molecules to the IMM (Tsujishita and Hurley, 2000). It was also proposed that StAR might alter the mitochondrial membranes to allow for the passage of cholesterol to the IMM, before it is transported to the mitochondrial matrix (Stocco, 2001). However, it was soon found that these models for StAR action would have to be modified when it was shown that deletion of 62 amino acids at the N-terminus of StAR prevented mitochondrial import, but did not affect cholesterol transfer and steroid production (Arakane et al., 1996, Wang et al., 1998). Further demonstrating that StAR need not enter the mitochondria to be active in cholesterol transfer, an OMM protein, TOM20, when fused to StAR resulted in a complex that could not enter the matrix but could still induce steroidogenesis (Bose et al., 2002). In an effort to explain what might be occurring at the level of the OMM Miller and colleagues performed a series of biophysical studies, which demonstrated that StAR undergoes a conformational alteration when it binds to cholesterol (Baker et al., 2005, Bose et al., 1999, Christensen et al., 2001). This alteration, which is caused by an acid-induced breaking of hydrogen bonds, appears to be required for StAR activity and is a result of StAR's transition to a molten globule form (Bose et al., 1999, Christensen et al., 2001). This observation was confirmed in another laboratory (Rajapaksha et al., 2013). Recent studies have revealed involvement of the voltage-dependent anion channel 2 (VDAC2) and TOM22 for StAR activity at the OMM membrane (Prasad et al., 2015) (Rajapaksha et al., 2016). In addition to the involvement of VDAC2 in this process, it has also been shown that the OMM proteins voltage-dependent anion channel 1 (VDAC1) and phosphate carrier protein (PCP) are also required for the production of steroids (Bose et al., 2008). The authors demonstrated that phosphorylation of StAR by protein kinase A requires PCP followed by the interaction of phospho-StAR with VDAC1 in order to promote cholesterol transfer and steroidogenesis. In spite of these efforts, there has yet to arise a model that adequately explains the role of StAR in the transfer of cholesterol into the IMM for conversion to pregnenolone.

By using the TOM20-StAR fusion protein, work performed by Papadopoulos and colleagues demonstrated that knockdown of PBR using antisense oligonucleotides resulted in an

inhibition of steroid synthesis (Hauet et al., 2005). These findings suggested that that there existed a cooperation between StAR and PBR that mediated mitochondrial cholesterol transport and a model in which StAR carried cholesterol from cellular stores to the OMM where PBR acted as a protein tunnel for the transfer of cholesterol to the IMM was proposed (Papadopoulos and Miller, 2012).

4. The Peripheral Benzodiazepine Receptor (PBR)/Translocator Protein (TSPO)

The Peripheral Benzodiazepine Receptor (PBR), whose name was recently changed to the Translocator Protein (TSPO; hereafter referred to as TSPO in this review) was first described in the late 1970s (Gavish et al., 1999). TSPO was demonstrated to have high binding affinity for benzodiazepines but was a distinctly different receptor from the central benzodiazepine receptor, the γ-aminobutyric acid type A receptor/$GABA_A$ receptor. Since that time there have been numerous studies involving TSPO that have attempted to characterize its pharmacological and physiological functions (Gavish et al., 1999, Rupprecht et al., 2010). Shortly after the discovery of TSPO, it was found that it was present in many different tissues, it appeared to be most highly concentrated in steroidogenic cells and that it was localized to the mitochondria in those cells (Mukhin et al., 1989, Papadopoulos et al., 1990). In those studies it was further demonstrated that treatment of adrenocortical and testicular Leydig cells with the TSPO ligands PK11195 and Ro5-4864 could stimulate steroid synthesis.

There followed over the next decade a series of studies in several different steroidogenic cell types that also indicated that TSPO ligands could increase steroid hormone production (Krueger and Papadopoulos, 1990, Papadopoulos et al., 1991a, b, c). These observations were further strengthened when it was reported that an intracellular TSPO binding protein, the diazepam binding inhibitor (DBI) increased steroid synthesis in steroidogenic cells (Garnier et al., 1993, Papadopoulos et al., 1991a, b, c, 1992). Knockdown technology to inhibit TSPO expression indicated that the presence of this protein was an absolute requirement for steroid synthesis (Boujrad et al., 1993, Papadopoulos et al., 1997a, b). The fact that knockdown of TSPO could, by itself, inhibit steroid biosynthesis indicated that its absence could prevent steroidogenesis while in the presence of the StAR protein. This was interpreted as indication that it acted downstream from StAR and functioned as a cholesterol channel, receiving intracellular cholesterol from StAR to be transported to the IMM (Papadopoulos et al., 1990, Papadopoulos and Miller, 2012). It was not possible to confirm the effects of knocking out TSPO *in vivo* as attempts to produce TSPO knockout animals was reported to result in early embryonic lethality in the mice (Papadopoulos et al., 1997a, b). Another study that seemed to strongly support the role of TSPO in steroidogenesis was that steroid production in the constitutively steroid synthesizing cell line, the R2C rat Leydig tumor cell line, occurred as a result of the presence of a higher affinity TSPO ligand-binding site in R2C cells (Garnier et al., 1994). In later experiments, knockdown of TSPO in these R2C cells was shown to almost completely inhibit their ability to produce steroid hormones (Papadopoulos et al., 1997a, b). It was also reported that TSPO contained a cholesterol-binding amino acid consensus (CRAC) motif that was described by the authors as providing

a mechanism for the binding and transport of cholesterol into the mitochondria (Li et al., 2001). The collective consideration of all of these characteristics of TSPO resulted in the authors concluding that TSPO played an indispensable role in cholesterol transfer to the IMM and steroidogenesis. This conclusion has persisted for over two decades with a number of models having been proposed as to how TSPO functioned in cholesterol transport into the mitochondria. Several recent reviews summarize the current state of the role of TSPO in steroidogenesis (Aghazadeh et al., 2015, Midzak et al., 2015, Papadopoulos et al., 2015).

A more recent model that has been proposed for the role of TSPO in cholesterol transport to the IMM involves its participation in an 800 kDa protein complex (Rone et al., 2012). This complex is composed of TSPO, the voltage dependent anion channel (VDAC), P450scc, the ATPase family AAA domain-containing protein 3A (ATAD3A) and optic atrophy type 1 proteins. Knockdown of ATAD3A or VDAC in this complex results in an inhibition of steroid synthesis, suggesting that an intact form of the complex is required for cholesterol transport. This model also indicated that the addition of StAR to the complex could increase steroid synthesis by mobilizing cholesterol that is bound to TSPO polymers present in the complex. Unfortunately, no knockdown of TSPO was included in these experiments so assessing its absolute requirement or role in this complex in cholesterol transfer and steroidogenesis could not be determined.

5. Controversies in the regulation of cholesterol transfer and steroidogenesis

A brief summary of the studies that have been performed in determining the roles of StAR and TSPO in regulating cholesterol transport to the IMM and thus stimulating steroid biosynthesis is given above. There have been a number of models proposed for the actions of each of these proteins. Some of the models have been challenged as a result of direct experimentation that demonstrated the mechanisms that they proposed to be unlikely, as were the cases with the candidate proteins SCP2 and SAP. However, in spite of the mechanisms that may be involved in their respective roles in transporting cholesterol to the IMM of steroidogenic cells, both protein candidates have been described as being absolutely indispensable for steroid biosynthesis to occur for a number of years. The basis of this belief for TSPO has been grounded in the studies that have shown that binding of various ligands to TSPO have resulted in an increase in steroid synthesis and that knockdown of TSPO using various methodologies have all resulted in very significant decreases in steroidogenesis (Boujrad et al., 1993, Garnier et al., 1993, 1994, Mukhin et al., 1989, Papadopoulos et al., 1990, 1991a, b, c, 1992, 1997a, b). Such results have been deemed to demonstrate the unequivocal requirement for TSPO in order for the acute stimulation of steroidogenesis to occur. For the StAR protein, the belief for its indispensable role in steroidogenesis comes mainly from the observations that mutations in the StAR gene in humans result in an almost complete inability of those individuals to synthesize steroids and from the fact that StAR null mice have essentially the same phenotype as see in the human condition (Caron et al., 1997, Lin et al., 1995). Also, strong evidence for the role of StAR in steroid biosynthesis came from transient transfection experiments that demonstrated expression of the StAR protein resulted in increased steroid production in the absence of hormone stimulation

(Clark et al., 1994, Lin et al., 1995). As a result, many attempts have been made to determine the mechanism of action of these two proteins and, if possible, to illustrate how they may work together in cholesterol transfer to the IMM. Despite several models that have been proposed, there remains a huge void in our knowledge of how these proteins might work, either separately or in concert with each other.

This was the state of affairs until two years ago. Since that time, experiments that demonstrated the requirement for TSPO in regulating cholesterol transport and steroidogenesis have been very seriously challenged. The first of these challenges arose as a result of studies performed by Vimal Selvaraj and colleagues at Cornell University which demonstrated that mice with a conditional knockout of TSPO in testicular Leydig cells were viable, fertile and had no differences in serum testosterone nor in testis weight or seminal vesicle weight from that of wild type animals (Morohaku et al., 2014) (Fig. 4). This observation dealt a serious blow to the earlier observations that TSPO was an absolute requirement for the production of steroids. Soon after the description of the conditional Leydig cell TSPO knockout appeared, a second manuscript from the Selvaraj laboratory was published describing a global knockout of TSPO in mice (Tu et al., 2014). These mice had essentially an identical phenotype as the wild type with regards to steroid synthesis and displayed none of the effects that would be expected if steroids were not being synthesized normally. They were viable, fertile and had normal circulating levels of adrenal (Fig. 5B) and gonadal steroids in spite of TSPO not being present in any of the tissues examined. Further, there were no histological differences found in the steroidogenic tissues of these animals, a sharp contrast to the results seen in the StAR knockout animals (Fig. 5A). In addition to the studies described above, another TSPO knockout mouse was generated separately in an entirely different setting and displayed a phenotype similar to the animals generated by the Selvaraj laboratory (Banati et al., 2014). Also, since many of the TSPO knockdown studies were performed in steroidogenic cell lines, we attempted to duplicate those studies to determine if *in vitro* studies differed from *in vivo* studies. Knockdown of TSPO in MA-10 mouse Leydig cells (Fig. 5C) and Y-1 mouse adrenocortical cells (Fig. 5D), resulted in approximately an 80% decrease in TSPO, but had no effect on steroid biosynthesis (Tu et al., 2014). Most interestingly, another cell line, the human adrenocortical H295R cell line, contained no detectable TSPO in wild type cells, yet synthesized steroids abundantly (Fig. 5E) (Tu et al., 2014). These results were completely opposite to those that had earlier been reported for the knockdown of TSPO in steroidogenic cell lines (Boujrad et al., 1993, Li et al., 2001, Papadopoulos et al., 1997a, b). These observations were further supported in studies in which TSPO was completely deleted using CRISPR/Cas9 technology in MA-10 Leydig cells and in the complete absence of TSPO, three separate clones of TSPO knockout cells synthesized steroids at both basal and stimulated levels identical to wild type cells (Tu et al., 2015). In an attempt to discern a possible linkage between TSPO ligands and steroid synthesis, we performed studies in which increasing concentrations of the TSPO ligand PK11195 was added to unstimulated wild type MA-10 cells as well as TSPO deleted cells (Tu et al., 2015). In these studies it was clearly shown that both wild type and TSPO deleted cells demonstrated similar increases in steroid production, indicating that TSPO was not required for these cells to increase steroid output and that the addition of the TSPO ligand in all probability caused off target effects.

In response to the findings reported from the Selvaraj laboratory, a report indicating that conditional knockout of TSPO in the adrenals and the gonads of mice resulted in a decrease in ACTH stimulated levels of corticosterone in these animals (Fan et al., 2015). Strangely, there appeared to be no correlation between the expression levels of TSPO and corticosterone synthesis in the stimulated adrenal glands from heterozygous and homozygous animals. Further, in the testes of these animals it was found that both basal and stimulated levels of testosterone were identical in the wild type, heterozygote and homozygote knockout animals, despite low levels of TSPO in the knockout. While it was shown that the loss of TSPO in the adrenal cells decreased stimulated steroid production it is extremely difficult to reconcile this loss with what is seen in the testis, in which steroidogenesis remains unimpaired. This would indicate that the regulation of steroidogenesis occurs differently in these two organs, a concept that has not been previously described to our knowledge.

Thus, some consider that the controversy as to whether or not TSPO is an absolute requirement for regulating cholesterol transport and steroidogenesis in steroidogenic cells remains. In the face of the data showing no effect on steroid production obtained from both the conditional and global TSPO knockout animals, the knockdown of TSPO in several different steroidogenic cell lines and the total deletion of TSPO in MA-10 cells, it is very difficult to understand how TSPO can be an absolute requirement for steroid synthesis. The specifics of this discussion can be found in much greater detail in several reviews that have recently been published on this topic (Selvaraj and Stocco, 2015, Selvaraj and Tu, 2016, Tu et al., 2015). Nevertheless, fundamental misinterpretations continue to sustain this debate [see commentary (Selvaraj et al., 2016)]. Despite decades of investigation (Fig. 6), it is clear that additional studies will need to be conducted to understand the precise function of TSPO and explain this gap in understanding, as without resolution, more time and resources may be spent in pursuing the wrong course of action.

Acknowledgments

Funding

This work was funded by the National Institutes of Health Grant # HD17481 (DMS), Grant B1-0028 from the Robert A. Welch Foundation (DMS) and startup funds from Cornell University (VS).

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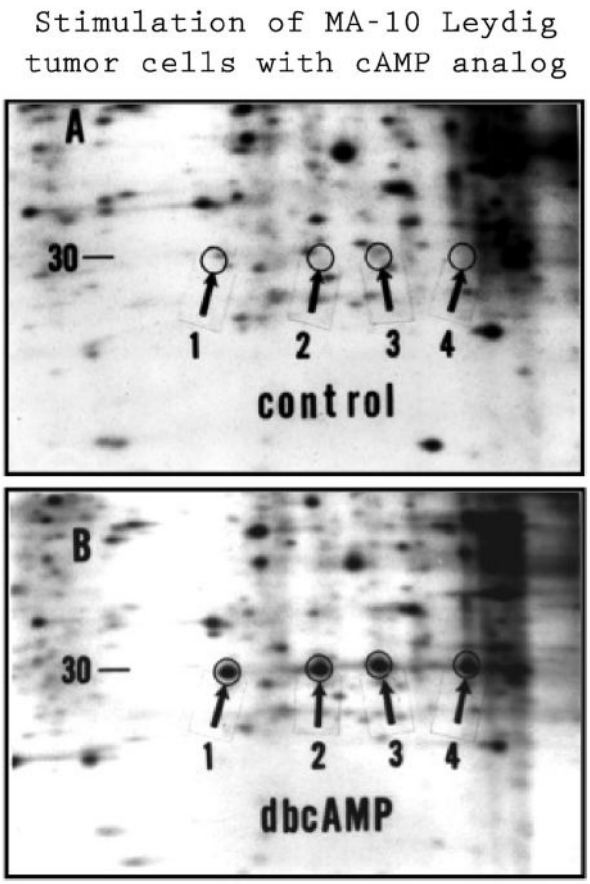

Fig. 1.

Illustration of Newly Synthesized StAR Proteins. Representative fluorograms of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of ^{35}S-methionine labeled mitochondrial proteins isolated from control and stimulated MA-10 mouse Leydig tumor cells. Cells were incubated in the presence of 1 mCi/ml ^{35}S-methionine and either in the presence or absence of 1 mM dibutyryl cAMP (dbcAMP) for 6 h. Mitochondria were isolated and the proteins were prepared for 2-D PAGE. Following electrophoresis the gels were dried and placed in cassettes with X-ray film. After suitable periods of time, the films were developed and the radioactive proteins could be seen. Shown are selected areas of fluorograms from control (A) and stimulated (B) cells. The arrows (1–4), illustrate the positions of the 30 kDa StAR protein forms. Proteins 3 and 4 represent the phosphorylated forms of proteins 1 and 2 respectively. Adapted from Stocco and Clark (1993).

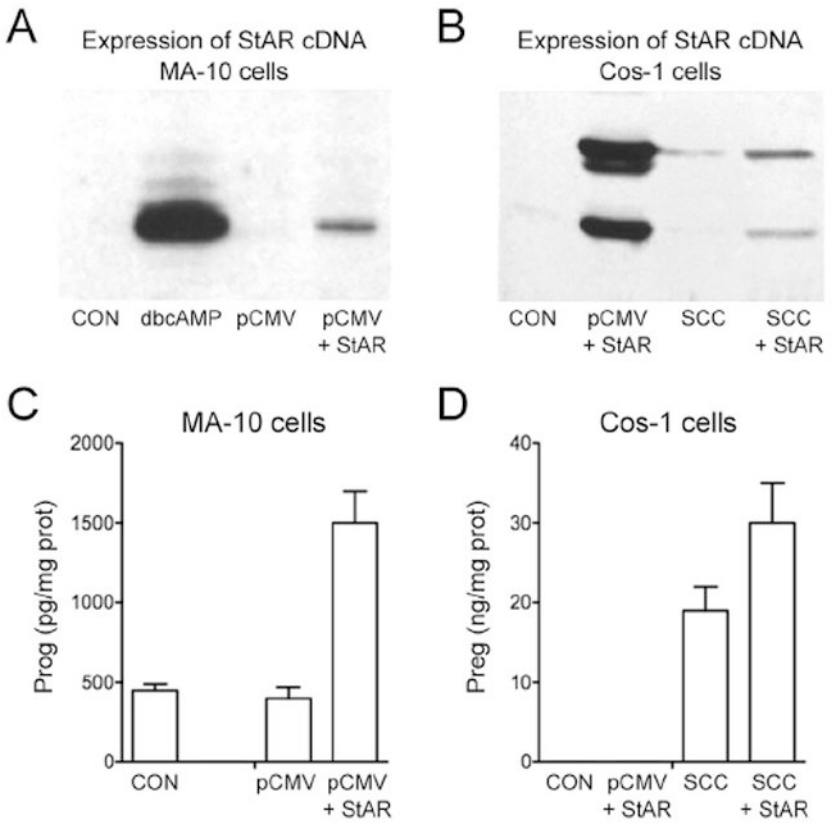

Fig. 2.

In vitro Expression of StAR Protein and Steroid Production in MA-10 and COS-1 Cells. A, MA-10 cells were stimulated with dbcAMP and subjected to Western blot analysis as were unstimulated MA-10 cells that had been transfected with pCMV vector alone or pCMV containing the 37 kDa cDNA for StAR. C, Progesterone production in control and StAR transfected MA-10 cells were measured by RIA 16 h post transfection. B, COS-1 cells rendered steroidogenic through transfection with the proteins for the cholesterol side chain cleavage enzyme system (SCC) were transfected with control pMCV and pCMV containing 37 kDa StAR cDNA and western blot analysis was performed. D, pregnenolone synthesis was measured in cells transfected with empty vector, StAR alone, SCC proteins alone and cells containing both StAR and the SCC system. It can clearly be seen that in both MA-10 and COS-1 cells, expression of the StAR protein is required for the cells to increase steroid synthesis.

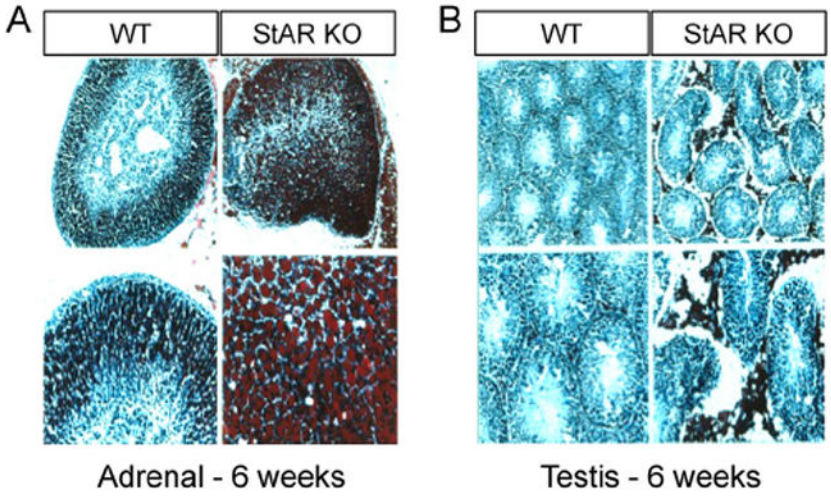

Fig. 3.

Adrenal Glands and Testes in StAR Knockout Mice. Adrenal glands and testes were isolated from wild type and StAR knockout mice at six weeks after birth. Frozen sections from each tissue were stained with oil red O for the detection of neutral lipids. A, the adrenal glands from StAR null mice contained significantly higher levels of lipid than did wild type animals. In the upper panels of A, the whole gland is shown, and a higher magnification of the gland is shown in the lower panels. B, similar procedures were performed in the testis and in the interstitial areas, the location of the steroidogenic Leydig cells, significantly higher deposits of lipid can be seen at both the lower and higher magnifications.

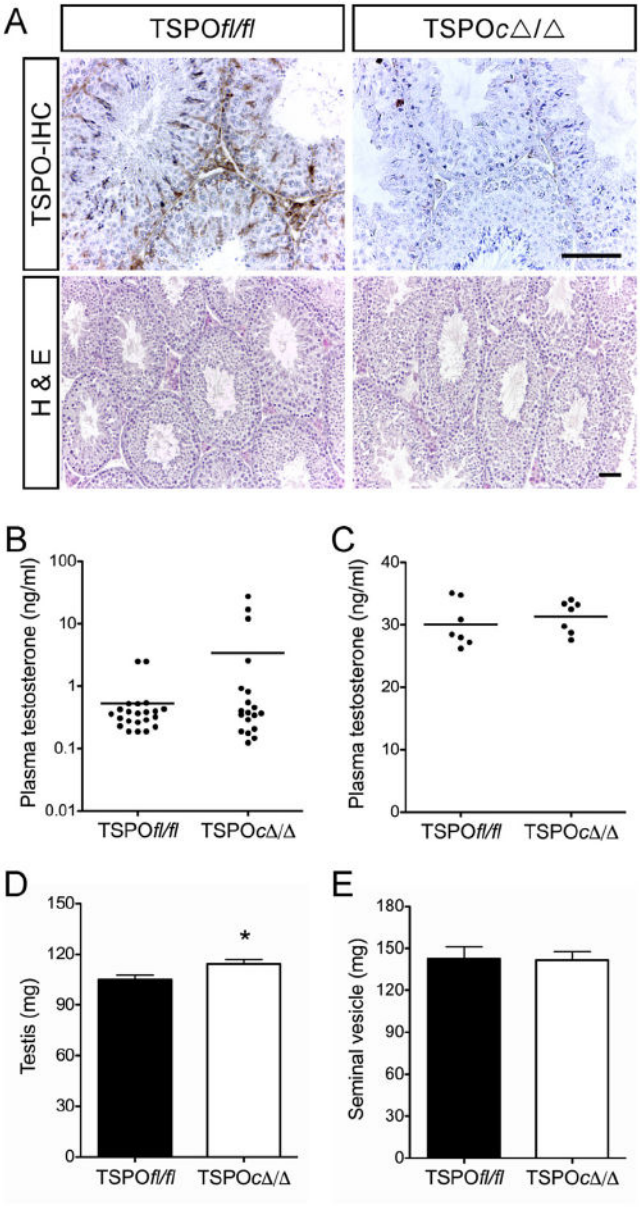

Fig. 4.

Effects of Conditional Knockout of TSPO in Mouse Testis. A, Immunohistochemical (IHC) localization showing complete absence of TSPO in Leydig and Sertoli cells of TSPO*c*–/– testes. Hematoxylin and eosin (H&E) staining showing unaltered seminiferous tubule morphology and spermatogenesis in TSPO*c*–/– testes (n = 5). Scale bars, 50 μm. B, Plasma testosterone levels were not significantly different between TSPO*fl*/*fl* and TSPO*c*–/– mice (n = 19-22/group), before, or C, when sampled 1 h after hCG stimulation (n 7/group). D, a modest but significant increase in testis weights was observed in TSPO*c*–/– mice compared with TSPO*fl*/*fl* mice ($P < 0.05$; n = 18/group). E, seminal vesicle weights were not significantly different between TSPO*fl*/*fl* and TSPOc–/– mice (mean SEM; n = 18/group). Panels from Morohaku et al., 2014, *Endocrinology*, 155 (1): 89–97.

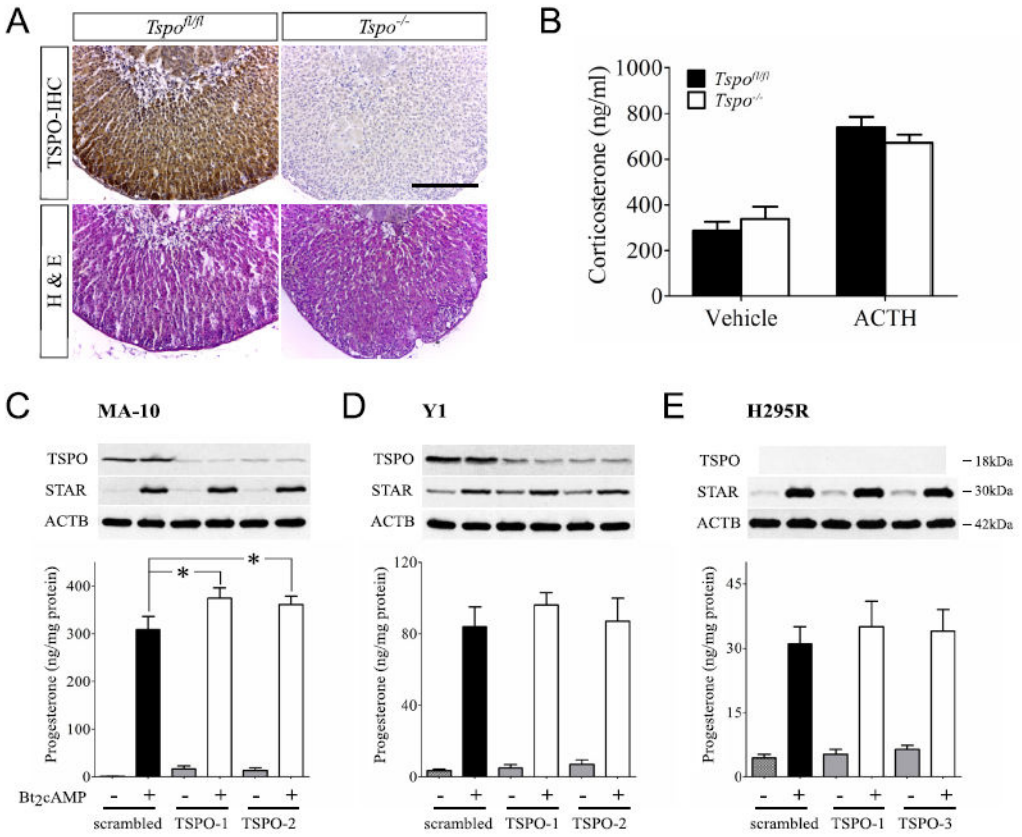

Fig. 5.

A-B: Effect of TSPO Global Knockout on Adrenal Gland Morphology and Steroid Synthesis. A, Adrenal sections from Tspo*fl*/*fl* and *Tspo*–/– mice showing TSPO localization in adrenocortical cells with a higher density in the zona glomerulosa; no staining was observed in *Tspo*–/– adrenal. No difference in adrenocortical morphology was apparent between the two genotypes. B, both basal and ACTH stimulated plasma corticosterone levels were similar in *Tspofl*/*fl* and *Tspo*–/– mice (n = 10–14/group). C-E: TSPO Knockdown in Steroidogenic Cells Does Not Affect Steroid Hormone Biosynthesis. C and D, TSPO knockdown resulted in substantial decreases in TSPO protein in MA-10 and Y1 cells compared to controls. E, no base-line TSPO was detected in H295R cells. Bt_2cAMP treatment resulted in similar increases in StAR protein expression and higher progesterone production in MA-10, Y1 and H295R cells. TSPO knockdown in MA-10 cells showed significantly higher progesterone production ($p < 0.05$) while progesterone production in TSPO knockdown Y1 cells was similar to the scrambled controls. H295R cells showed no TSPO expression but still produced progesterone upon Bt_2cAMP treatment. Panels from Tu et al., 2014, *Journal of Biological Chemistry*, 289 (40): 27444-54.

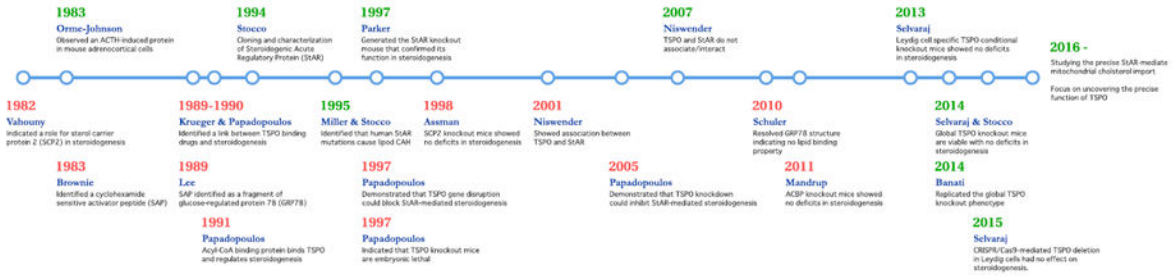

Fig 6.

Timeline representing key studies focused on the mechanism of mitochondrial cholesterol import for steroidogenesis and their outcomes. References in chronology: (Chanderbhan et al., 1982), (Krueger and Orme-Johnson, 1983), (Pedersen and Brownie, 1983), (Mukhin et al., 1989), (Krueger and Papadopoulos, 1990), (Li et al., 1989), (Papadopoulos et al., 1991a, b, c), (Clark et al., 1994), (Lin et al., 1995), (Caron et al., 1997), (Papadopoulos et al., 1997a, b), (Seedorf et al., 1998), (West et al., 2001), (Hauet et al., 2005), (Bogan et al., 2007), (Wisniewska et al., 2010), (Neess et al., 2011), (Morohaku et al., 2014), (Tu et al., 2014), (Banati et al., 2014), (Tu et al., 2015).