Current status and future perspectives: TSPO in steroid neuroendocrinology

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Abstract

The mitochondrial translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), has received significant attention both as a diagnostic biomarker and as a therapeutic target for different neuronal disease pathologies. Recently, its functional basis believed to be mediating mitochondrial cholesterol import for steroid hormone production has been refuted by studies examining both *in vivo* and *in vitro* genetic *Tspo*-deficient models. As a result, there now exists a fundamental gap in the understanding of TSPO function in the nervous system, and its putative pharmacology in neurosteroid production. In this review, we discuss several recent findings in steroidogenic cells that are in direct contradiction to previous studies, and necessitate a re-examination of the purported role for TSPO in *de novo* neurosteroid biosynthesis. We critically examine the pharmacological effects of different TSPO-binding drugs with particular focus on studies that measure neurosteroid levels. We highlight the basis of key misconceptions regarding TSPO that continue to pervade the literature, and the need for interpretation with caution to avoid negative impacts. We also summarize the emerging perspectives that point to new directions that need to be investigated for understanding the molecular function of TSPO, only after which the true potential of this therapeutic target in medicine may be realized.

Key Words
- neurosteroid
- steroid
- brain
- spinal cord
- nervous system
- mitochondria
- cholesterol
- hormone
- inflammation

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Introduction

For more than 75 years, it has been recognized that steroid hormones can be potent modulators of nervous system function. Specific steroid-mediated mechanisms are capable of exerting a variety of physiological effects that can be either acute or persistent on different neurological processes. These include: specific gene expression by binding to nuclear receptors, modulation of neurotransmission via action on specific membrane receptors, development and establishment of specific neural circuitry, ameliorating neuroinflammation, and improving aspects of neuroregeneration. Due to their lipophilic nature that allows crossing of the blood–brain barrier, steroids from the adrenals or gonads can directly act on nervous system targets, or be converted to 'neuroactive' metabolites that elicit specific actions. Synthesis of steroids *de novo* in the nervous system was first demonstrated in 1981 (Corpechot *et al.* 1981); it was discovered that elimination of peripheral steroid sources by gonadectomy, adrenalectomy or removal of trophic stimuli through hypophysectomy only had modest effects on steroid levels in the central nervous system (CNS) (Corpechot *et al.* 1981, 1983, Robel *et al.* 1987, Jo *et al.* 1989). The term 'neurosteroid' was introduced to describe steroids locally synthesized by cells of the CNS and

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peripheral nervous system (PNS). Local concentrations of neurosteroids in the nervous system were found to exceed levels present in the blood stream, and differed based on brain regions examined (Cheney *et al.* 1995). Therefore, neurosteroid functions with a regional or localized concentrations, not achievable by peripheral steroids have been of particular mechanistic interest in studying nervous system homeostasis and pathologies.

Synthesis of steroids in the CNS and PNS is known to begin early during development and remain into adulthood (Compagnone *et al.* 1995, Pezzi *et al.* 2003, King *et al.* 2004). In these tissues, steroidogenesis does not occur in dedicated cells, but has been reported to be associated with several of the excitable and supporting cell types including neurons (different types), astrocytes, oligodendrocytes, Schwann cells and their progenitors (Le Goascogne *et al.* 1987, Hu *et al.* 1987*b*, Jung-Testas *et al.* 1989, Koenig *et al.* 1995, King *et al.* 2002, Sierra *et al.* 2003, Saalmann *et al.* 2007). In addition, specialized sensory elements, such as ocular neuro-retinal cells (Provost *et al.* 2003) and taste buds present in circumvallate papillae (Toyoshima *et al.* 2007), have also been reported to be capable of steroid production. Biosynthesis of neurosteroids in nervous tissue appears to be evolutionarily conserved across several vertebrate species examined (Tsutsui *et al.* 1999). However, interpretation of steroid mechanisms in the CNS and PNS presents a high degree of complexity not only because of the different cell types and potential actions, but also because of the nature and activity of the different neurosteroids that are being produced. Moreover, localization of neurosteroid production to specific regions within the nervous system and/or pathologies directly affects functional outcomes.

As a marker for localizing neurosteroid production, the mitochondrial translocator protein (TSPO) expression has been used in numerous studies. However, its purported steroidogenic function has been refuted in recent studies, challenging the basis of this interpretation in the nervous system. In this review, we carefully consider the past and assess the current state of understanding TSPO function.

Biosynthesis of neurosteroids

Neurosteroids act as paracrine or autocrine modulators of a variety of neuronal functions and activities. The primary neurosteroids whose functions have been studied can be broadly described as follows: 3β-hydroxysteroids (pregnenolone (PREG), pregnenolone sulfate (PS), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S)); pregnane steroids, which are progesterone (PROG) and progesterone metabolites (dihydroprogesterone (DHP), allopregnanolone (ALLO) and pregnanolone (THP), deoxycorticosterone (DOC), dihydrodeoxycorticosterone (DHDOC) and tetrahydrodeoxycorticosterone (THDOC)); and androstanes (androstanol and androsterone) (Fig. 1). Several recent reviews have addressed the topic of neurosteroids and their importance in nervous system physiology and pathology that include cognition, mood,

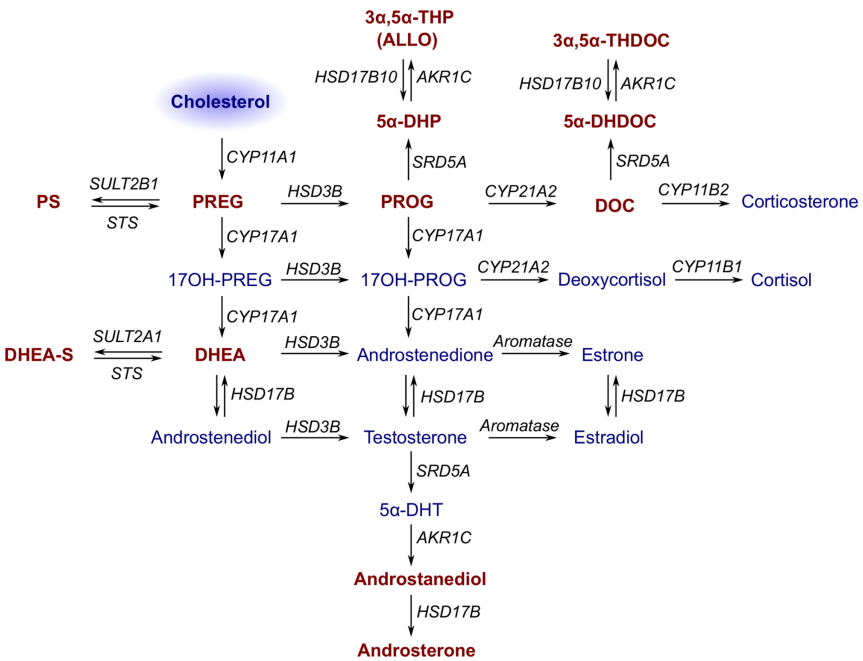

Figure 1
Pathways for neurosteroid synthesis. Steroids: pregnenolone (PREG), pregnenolone sulfate (PS), 17-hydroxypregnenolone (17OH-PREG), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S); progesterone (PROG), 17-hydroxyprogesterone (17OH-PROG), dihydroprogesterone (DHP), tetrahydroprogesterone (THP), allopregnanolone (ALLO), deoxycorticosterone (DOC), dihydrodeoxycorticosterone (DHDOC), tetrahydrodeoxycorticosterone (THDOC). Broadly studied neurosteroids are in bold-brown. Enzymes: cytochrome P450 side-chain cleavage (CYP11A1), cytochrome P450 11-hydroxylase (CYP11B), hydroxysteroid dehydrogenase (HSD), cytochrome P450 17α-hydroxylase/c17,20-lyase (CYP17A1), steroid sulfatase (STS), sulfotransferase (SULT), steroid 5α-reductase (SRD5A), aldo-keto reductase (AKR).

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personality traits, neuronal development, traumatic brain injury and neuroinflammation (Compagnone & Mellon 2000, Belelli & Lambert 2005, Charalampopoulos *et al.* 2008, Lambert *et al.* 2009).

Several reviews have also described the different enzymatic processes modifying cholesterol and intermediates in the steroidogenic pathway, and the conserved mechanisms in neurosteroid production (Compagnone & Mellon 2000, Do Rego *et al.* 2009). Among the different bioconversions, we will focus on the *de novo* production of neurosteroids, with an emphasis on the very first step leading to this enzymatic cascade, something that has often been used as an indicator of neurosteroid production by cells of the nervous system (Hu *et al.* 1987*a*). Enzymatic conversion of cholesterol by a mitochondrial cytochrome P450 side-chain cleavage (P450scc or CYP11A1) to generate PREG is the first and essential step for all steroid synthesis. The P450scc functions as the terminal oxidase in an electron-transfer chain where NADPH donated electrons to adrenodoxin reductase are transferred to adrenodoxin and then to P450scc (Kimura & Suzuki 1965, Omura *et al.* 1966, Shikita & Hall 1973). Overall abundance of P450scc transcripts in the rat brain was estimated to be only ~0.01% of that measured in the adrenal gland (Mellon & Deschepper 1993), perhaps indicating that only a small subpopulation of cells are capable of *de novo* steroidogenesis. Within the different brain regions, P450scc expression was observed in the cortex, basal ganglia, hippocampus, olfactory bulb, hypothalamus, thalamus and cerebellum (Mellon & Deschepper 1993, Compagnone *et al.* 1995).

Studies on steroids and their biosynthetic enzymes, mainly based on the adrenals and gonads, have established most of the currently accepted pathways involved in the steroid production (Fig. 1). For the bioconversion of PREG to other neurosteroids, most steroidogenic enzymes that are present in the adrenals and gonads have been identified in the nervous system, as enzymatic activity, presence of transcripts or detection of proteins in different neuronal cells (reviewed in Warner & Gustafsson 1995, Compagnone & Mellon 2000, Do Rego *et al.* 2009). Additional enzymes unique to the nervous system, responsible for bioconversion of peripheral steroids to neurosteroid forms, have also been described (Compagnone & Mellon 2000). Physiological distribution of this steroid biosynthetic framework in different forms within the nervous system has been demonstrated to be cell type specific with developmental regulation observed in different regions (Compagnone & Mellon 2000). However, the *in vivo* complexity in interpreting functional effects based on neurosteroid biosynthetic capability is further confounded by the fact that the enzymes involved can mediate bioconversion of multiple steroid substrates in the pathway. For example, P450c17 mediates both 17-α hydroxylase and 17,20-lyase activities (Kominami *et al.* 1982, Nakajin *et al.* 1983), on several neuroactive steroid intermediates. Although P450scc knockout/mutations have been phenotyped for steroid deficiency related to the adrenals and gonads in rabbits (Pang *et al.* 1992) and mice (Hu *et al.* 2002), *de novo* neurosteroid production and phenotypic deficits resulting from lack of P450scc in the nervous system remain unknown. To this time, there have been no attempts to study the effect of negating *de novo* neurosteroid production using *in vivo* genetic models. Therefore, the contribution of *de novo* steroid synthesis in the nervous system vs effects mediated by peripheral steroid-derived neurosteroids remains to be evaluated.

For *de novo* steroid production, upstream mechanisms that deliver cholesterol to P450scc have been demonstrated to form the rate-limiting step in PREG synthesis. The proteins and processes involved in this cholesterol delivery is a topic that has been subject to intense investigation in adrenal and gonadal steroidogenic cells for the past 50 years (reviewed in Stocco & Clark 1996, Stocco 2000, Miller & Bose 2011, Selvaraj *et al.* 2015). All functional elements for an identical system for cholesterol transport and *de novo* steroid production have been demonstrated in specific neuronal tissues (Furukawa *et al.* 1998).

Mitochondrial cholesterol import

The P450scc resides on the matrix side of the inner mitochondrial membrane (IMM) in steroid-producing cells (Churchill & Kimura 1979). Compared with the outer mitochondrial membrane (OMM), the IMM in steroidogenic mitochondria is cholesterol poor (Cheng *et al.* 1985). To produce steroids, cholesterol needs to be transported from the OMM to the IMM. As cholesterol molecules are highly hydrophobic, they cannot traverse the aqueous intermembrane space (IMS) at a rapid rate (Rennert *et al.* 1993). Rapid *de novo* synthesis of a protein was identified to be key for orchestrating cholesterol movement across the IMS (Ferguson 1963). Identification of this putative 'transporter' protein became the focus of research for decades, and the protein, now known as the steroidogenic acute regulatory protein (STAR), was observed in cultured adrenal, Leydig and corpus luteum cells during steroid production (Krueger & Orme-Johnson 1983, Pon *et al.* 1986), and was subsequently cloned and sequenced

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(Clark *et al*. 1994). It was determined that the expression of STAR in steroidogenic cells in the absence of hormone stimulation resulted in an increase in steroid biosynthesis (Clark *et al*. 1994), highlighting the rate-limiting nature of this step. It was further demonstrated that the expression of StAR in a nonsteroidogenic cell line rendered steroidogenic through transfection of the cholesterol side-chain cleavage system and also resulted in significant increases in steroid production (Lin *et al*. 1995). Supporting the indispensable role of STAR in steroidogenesis was the demonstration that mutations in the STAR gene were responsible for the potentially fatal lipoid congenital adrenal hyperplasia (lipoid CAH), a disease in which severely afflicted individuals are unable to synthesize steroids (Lin *et al*. 1995). In corroboration, STAR gene-deleted mice showed a similar inability to synthesize steroids, precisely replicating the human lipoid CAH phenotype (Caron *et al*. 1997). Crystal structure of the StAR-related lipid transfer (START) domain that was subsequently resolved confirmed the potential of StAR to function as a cholesterol binding and transport protein (Tsujishita & Hurley 2000).

Before the discovery of STAR, it was observed that chemicals capable of binding to the peripheral benzodiazepine receptor (PBR, the previous name for TSPO), which is present in the OMM (Anholt *et al*. 1986), could stimulate modest amounts of steroid synthesis in adrenal tumor cells (Mukhin *et al*. 1989) and Leydig tumor cells (Papadopoulos *et al*. 1990). Although TSPO was not a product of rapid *de novo* synthesis during steroid production, it was reported that TSPO knockdown could decrease steroid synthesis (Hauet *et al*. 2005). As supporting evidence, it was presented that a mono-allelic deletion of *Tspo* in the constitutively steroidogenic rat R2C Leydig cell line dramatically decreased their ability to produce steroids for a period of time, albeit followed by spontaneous recovery (Papadopoulos *et al*. 1997*b*). It was also declared in a review article that the *Tspo* gene-deleted mice were early embryonic lethal (Papadopoulos *et al*. 1997*a*). Examination of TSPO sequence led to discovery of a cholesterol-binding amino acid consensus (CRAC) motif, suggesting that it can bind to cholesterol in the OMM (Li *et al*. 2001). All these points appeared to make a strong case for TSPO as it was present at the site of mitochondrial import of cholesterol, and its expression was apparent in steroidogenic cells (Papadopoulos 1998). Extrapolating the descriptions, TSPO structure was then modeled as the cholesterol 'channel' (Bernassau *et al*. 1993, Papadopoulos *et al*. 1997*a*, Rupprecht *et al*. 2010), with the proposal that STAR delivered cholesterol to the OMM, and TSPO carried out the mitochondrial cholesterol import process. Collectively, these arguments led to the often stated conclusion that TSPO played an indispensable role in steroidogenesis (reviewed in Papadopoulos *et al*. 1997*a*, Papadopoulos & Miller 2012).

Effects observed using TSPO-binding chemicals/drugs have remained mysterious in that they did not conform to a specific functional pattern (reviewed in Gavish *et al*. 1999). These included a broad range of observations: cellular respiration, cell proliferation, stress response, apoptosis, reactive oxygen species production, protoporphyrin IX (PPIX) synthesis and steroidogenesis. However, with extant understanding at that period in time, the observations were most often explained as secondary effects resulting from steroid production in cells (Papadopoulos 1998, Papadopoulos & Lecanu 2009, Rupprecht *et al*. 2010). Over the ensuing three decades, the concept of steroid production has been used as the basis of interpretation of TSPO function in more than 700 manuscripts across different tissue types.

Dogma refuted: TSPO is not involved in steroidogenesis

Recent works on TSPO using more definitive genetic *in vitro* and *in vivo* models have demonstrated the irreproducibility of earlier results. The use of *Tspo*-floxed ($Tspo^{fl/fl}$) mice, to generate Leydig cell-specific TSPO conditional knockout ($Tspo^{c\Delta/\Delta}$) mice demonstrated that TSPO was not essential for testosterone production *in vivo* (Morohaku *et al*. 2014). Subsequently, global TSPO deletion ($Tspo^{-/-}$) in $Tspo^{fl/fl}$ mice did not affect viability, fertility and the ability to generate steroid hormones (Tu *et al*. 2014). Another group (Banati *et al*. 2014), independently reproduced this phenotype observed in global $Tspo^{-/-}$ mice, confirming that the nature of the floxed allele or the mouse background were not the criteria affecting this conclusion on viability and steroidogenesis.

These results were also supported by *in vitro* models, which were previously reported to indicate otherwise. Knockdown of TSPO expression to >80% did not affect steroid hormone biosynthesis in Leydig cells (MA-10 and MLTC cells), adrenocortical cells (Y1 cells) (Tu *et al*. 2014). Complete CRISPR/Cas9-based disruption of TSPO in the MA-10 mouse Leydig cell line ($MA\text{-}10^{Tspo\Delta/\Delta}$) did not have any effect on steroidogenesis (Tu *et al*. 2015).

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In support, direct examination of mitochondrial cholesterol import in isolated *Tspo*$^{-/-}$ steroidogenic mitochondria did not show any deficits, demonstrating that TSPO is not involved in this process (Banati *et al.* 2014). Most surprisingly, when the human adrenal H295R cell line was examined, it was determined that it did not express TSPO, but was still competent in making steroids (Tu *et al.* 2014). All these results were in direct contrast to previous reports in identical model systems (Papadopoulos *et al.* 1997*a*,*b*, Hauet *et al.* 2005).

Structural features of murine TSPO, as revealed by high-resolution NMR (Jaremko *et al.* 2014), did not show any indications of a channel-like structure with a hydrophobic core as previously modeled (Bernassau *et al.* 1993, Papadopoulos *et al.* 1997*a*, Rupprecht *et al.* 2010). The residues considered important for TSPO binding to cholesterol pointed away from the interior of the protein toward the hydrophobic environment of the membrane bilayer (Jaremko *et al.* 2015*b*), and did not present any structural basis for cholesterol transport.

Based on these investigations that systematically examined TSPO function *in vivo*, *in vitro* and in isolated mitochondria, it could be concluded that TSPO is not involved in mitochondrial cholesterol import for steroidogenesis. As these confirmations are in contrast to what was believed for decades, it also brought the realization that previous statements that appeared in the TSPO literature will require serious reinterpretation. Investigating how the field of steroid endocrinology got to this point, we have carefully reappraised previous studies from 25 years of TSPO research, and examined their limitations that led to the inaccurate association of TSPO and steroidogenesis in a recent review (Selvaraj *et al.* 2015). Nevertheless, as this proposition of TSPO and mitochondrial cholesterol import had pervaded the scientific literature across multiple fields connected in different organ systems, some have had difficulty in reconciling these differences (Midzak *et al.* 2015), and others not directly working on mechanisms of steroid production are likewise unable to do so (Campanella 2015, Gatliff & Campanella 2016). This is mainly because removal of TSPO from the steroid production equation has left the field without an explanation for its high expression in steroidogenic tissues and the different effects mediated by TSPO-binding chemicals/drugs.

Note, a recent manuscript from the research group involved in most of the early studies linking TSPO and steroidogenesis asserted that TSPO is crucial for viability and steroid biosynthesis in an attempt to revive the old model (Fan *et al.* 2015). Unfortunately, interpretations in this manuscript were seriously flawed (see commentary Selvaraj *et al.* 2016).

The TSPO paradigm shift: case closed or controversy?

The burden of proof for overturning popular paragidm is substantial, and to completely understand the new results, we need to examine the historical evidence in perspective, and consider the validity of claims that led to the misconception that TSPO is indispensable for steroidogenesis. For this, we need to dissect literature on adrenal and gonadal steroidogenesis, as these were extrapolated to form the foundation for neurosteroidogensis. It is not our intention to criticize work by other scientists, but we are indeed under obligation to point out some highly cited studies in this field that are not reproducible or have been too loosely interpreted. To make this section palatable for a diverse audience while remaining succinct, we present this information in chronology/relevance with answers, and make every effort to explain the interpretations for each point in our discussion. For a broader discussion on early events, please see our recent review (Selvaraj *et al.* 2015).

1. Regulation by trophic hormones from the pituitary: In 1985, the first circumstantial evidence presented for TSPO in steroid production was presented: hypophysectomy in rats that resulted in ACTH deficiency could decrease TSPO expression in the adrenal glands (Anholt *et al.* 1985*a*). After hypophysectomy, adrenal involution was at a severe stage weighing only 28% that of control adrenals (Anholt *et al.* 1985*a*), suggesting that all proteins associated with the adrenal cortex could decrease. Note that loss of adrenal weight after hypophysectomy is due to shrinkage of the adrenal cortex (Deane & Greep 1946). However, at that period in time, this possibility was not considered. Subsequent research by this same group documented abundant expression of TSPO in steroidogenic cells of the adrenal and testis (De Souza *et al.* 1985), and localization to the OMM (Anholt *et al.* 1986). These observations primed TSPO as a candidate for regulating steroid production.

Answer: A reinvestigation of the basis for the relationship between hypophysectomy and TSPO indicated that after cortical involution, ACTH-induced steroidogenesis was not temporally related to the expression of TSPO (Cavallaro *et al.* 1993), suggesting that TSPO was not driving the return of corticosterone production.

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However, this early indication that TSPO may not be associated steroid production was overlooked in subsequent studies.

2. TSPO pharmacology: In 1989, testing the effect of TSPO-binding chemicals (including the isoquinolone carboxamide PK11195 and chlorodiazepam Ro5-4864) on the Y1 adrenocortical cell line and the MA-10 Leydig cell line resulted in the induction of progesterone production (Mukhin *et al*. 1989, Papadopoulos *et al*. 1990). However, these effects were extremely modest and transient compared with physiological induction (80-fold lower response, and hormone levels plateauing within 40 min with no progressive accumulation as observed with physiological stimulation of these same cells). Although the effect on cellular physiology was unclear, it was postulated that TSPO played a role in mitochondrial cholesterol import (Krueger & Papadopoulos 1990).

Answer: Effects of TSPO-binding chemicals were not entirely consistent in other tissues including the nervous system (discussed later in the text) (reviewed in Selvaraj *et al*. 2015). In 2015, use of the same MA-10 Leydig cells made deficient in TSPO by CRISPR/Cas9-mediated gene targeting indicated that PK11195 could stimulate steroidogenesis even in the absence of TSPO (Tu *et al*. 2015). This work performed using three independent TSPO-deficient MA-10 cell lines indicated that the transient steroidogenic response mediated by TSPO-binding drugs might be off-target effects. A continued discussion on TSPO pharmacology with respect to neurosteroids is presented in an upcoming section in this review.

3. Role in cholesterol 'translocation': The proposed model for TSPO function (Krueger & Papadopoulos 1990) did not agree with existing knowledge of the cholesterol import system at that time. As steroidogenesis was sensitive to cycloheximide treatment, it was considered that rapid *de novo* synthesis of a protein was key for orchestrating this mitochondrial cholesterol import process (Ferguson 1963). However, expression of TSPO did not change with stimulation and hormone production, and transient steroid production by TSPO-binding chemicals was not cycloheximide sensitive (Krueger & Papadopoulos 1990). Nevertheless, this model was maintained, as no other candidates were identified at that point.

Answer: The STAR was identified as the mediator of mitochondrial cholesterol import that underwent rapid *de novo* synthesis in steroidogenic cells (Clark *et al*. 1994). (Please see previous section on mitochondrial cholesterol import). Although TSPO is present in these STAR-deficient mice and humans, it has no compensatory role in the cholesterol transport process. Recent research examining isolated *Tspo*-deficient steroidogenic mitochondria demonstrated that TSPO does not play a role in mitochondrial cholesterol import (Banati *et al*. 2014).

4. ACBP/DBI as endogenous ligand: In 1991, TSPO was reported to interact with a protein called the diazepam-binding inhibitor (also known as the Acyl-CoA binding protein/ACBP), which could stimulate steroid production in MA-10 Leydig cells and Y1 adrenocortical cells (Papadopoulos *et al*. 1991*a*,*b*), albeit only at very modest levels similar to TSPO-binding chemicals as described above. Antisense knockdown of ACBP in MA-10 Leydig cells appeared to block steroid biosynthesis demonstrating a vital role for ACBP in steroidogenesis (Boujrad *et al*. 1993). These reports appeared to provide some credibility in that a putative endogenous 'ligand' for TSPO existed, which could regulate its function in steroidogenic cells.

Answer: ACBP was demonstrated to play a role in maintaining the intracellular Acyl-CoA ester pool size (Mandrup *et al*. 1993), and synthesis of very long-chain fatty acids and sphingolipids (Gaigg *et al*. 2001). A spontaneous mutant mouse *nm1054*, cataloged at the Jackson Laboratory (Ohgami *et al*. 2005*a*,*b*), was subsequently identified to also contain a mutation in the ACBP gene locus (Lee *et al*. 2007). Loss of ACBP in these *nm1054* mice was linked to fatty acid metabolism abnormalities in skin and hair (Lee *et al*. 2007). The subsequent generation of viable *Acbp*-knockout mice ($Acbp^{-/-}$) displayed delayed metabolic adaptation to weaning (Neess *et al*. 2011), and a phenotype of fatty acid metabolic abnormalities in skin and hair similar to *nm1054* mutation (Bloksgaard *et al*. 2012, Neess *et al*. 2013). There was no phenotypic evidence that indicated defects in steroid hormone production in these $Acbp^{-/-}$ mice.

5. Simulated TSPO protein model: In 1993, a 3D model was presented for TSPO depicting its structure as a cholesterol carrier, accommodating a cholesterol molecule within the five alpha helices (Bernassau *et al*. 1993). This predicted model was used as explanation of the putative function for TSPO in intramitochondrial cholesterol transport.

Answer: Generation of this model was largely shaped by its perceived function in steroidogenesis. The only true prediction in this model was that TSPO potentially contained five transmembrane alpha helices; however, it was imprecise, in that it was calculated to traverse only one

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leaflet of the membrane bilayer (Bernassau *et al*. 1993), and side-chain orientations were highly speculative. In this structural model, features linking TSPO and cholesterol were assigned based on molecular dynamics simulations congruent with presumed functional requirements. Therefore, permutations and combinations sought for in developing this model were to just satisfy the basis for interpreting TSPO's steroidogenic function.

6. Cholesterol-binding property: In 1998, a cholesterol-binding amino acid consensus motif was characterized at the TSPO C-terminal region (Li & Papadopoulos 1998). The presence of this motif was initially postulated to indicate the ability of TSPO to bind and translocate cholesterol. Subsequently, it was demonstrated that expression of a HIV TAT-CRAC peptide could potentially compete for cholesterol and inhibit steroidogenesis in the MA-10 Leydig cell line (Li *et al*. 2001). Deletion of this CRAC motif reduced cholesterol binding when expressed in *Escherichia coli* (Li & Papadopoulos 1998). Although these studies offered no experimental evidence that TSPO could transport cholesterol, it was postulated as the mechanism supporting the putative pharmacological steroidogenic response mediated by TSPO.

Answer: The CRAC motif itself is a loose definition; it has previously been pointed out that almost all existing CRAC motifs in proteins (2.7/protein in *Streptococcus agalactiae*) have no association with cholesterol (Palmer 2004). Most functional CRAC motifs have been described in proteins that associate with cholesterol within the membrane, for example: myelin P0 (Luo *et al*. 2007) and caveolin-1 (Yang *et al*. 2014). There exists only biochemical evidence (in nonmembrane environments) that the C-terminal CRAC motif present in TSPO could bind cholesterol. In cells, association of cholesterol to TSPO has been shown to occur in two other distinct locations (Hulce *et al*. 2013), without any binding at the CRAC motif first described in the C-terminal region (Li & Papadopoulos 1998). Therefore, despite the strong claims (Li *et al*. 2001), it remains unclear if the C-terminal CRAC motif can bind cholesterol in cells. Further studies on the newly discovered cholesterol-membrane affinity sites on TSPO could improve understanding of OMM physiology and function.

7. TSPO structure: In 2008, the first experimental evidence for the murine TSPO as a protein with five transmembrane alpha helices was described (Murail *et al*. 2008). This was followed by a low-resolution structure of *Rhodobacter sphaeroides* TSPO, constructed using electron cryo-microscopy (Korkhov *et al*. 2010). These two structures formed the basis of early homology models that were used to speculate that TSPO could form a hydrophobic channel-like interior core lined by the CRAC motif for presumed cholesterol binding and translocation (Papadopoulos *et al*. 1997*a*, Rupprecht *et al*. 2010).

Answer: The key limitation indicated in these early studies is that it is not possible to assign amino acid sequences to low-resolution TSPO structures (Korkhov *et al*. 2010). A more recent high-resolution NMR structure of TSPO showed that it does not form a 'channel-like' structure and that the side chains of the C-terminal CRAC motif deemed essential for cholesterol binding are located on the outside of the TSPO molecule pointing toward the membrane environment (Jaremko *et al*. 2014), suggesting that previous models were not accurate. *Note*: At the time this new structure was published, TSPO was still considered important for steroidogenesis and the authors speculated alternative models for the putative cholesterol translocation; however, no credible structural evidence could be identified as discussed previously (Selvaraj *et al*. 2015).

8. TSPO knockdown in cells: A study performed in 1998 that examined a stable knockdown of gene expression to decrease TSPO protein levels in clones of MA-10 Leydig cells (Kelly-Hershkovitz *et al*. 1998) has often been cited to indicate that TSPO was involved in the steroidogenic machinery. Advances in research of STAR mechanism of action resulted in the development of OMM TOM20-STAR fusion constructs that showed maximal hormone production (Bose *et al*. 2002). This TOM20-STAR construct was subsequently used in conjunction with TSPO knockdown in MA-10 Leydig cells to demonstrate that TSPO was necessary for STAR function in steroidogenesis (Hauet *et al*. 2005).

Answer: The aforementioned study on TSPO knockdown in MA-10 Leydig cells did not observe differences in acute production of PROG (Kelly-Hershkovitz *et al*. 1998). They observed that in TSPO antisense 'knockout' cells, PROG levels decreased by about 20% and only at a late time point (24 h), leading to an interpretation that TSPO may play a role in PROG metabolism rather than synthesis (Kelly-Hershkovitz *et al*. 1998). They concluded that 'further studies are needed to confirm the involvement of the 18-kDa PBR subunit in MA-10 Leydig cell steroid biosynthesis' (Kelly-Hershkovitz *et al*. 1998). This study was perhaps the very first indication that the assumptions underlying TSPO function may be incorrect, but there

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was a key limitation, in that knockdown estimated by PK11195 or Ro5-4864 binding was only at 50% (Kelly-Hershkovitz *et al*. 1998). Nevertheless, at that time, this observation was overshadowed by claims to the contrary. Recent examination of TSPO knockdown (>80%) in MA-10 Leydig cells, MLTC Leydig cells and Y1 adrenocortical cells showed no effects on steroid hormone production (Tu *et al*. 2014). It was also discovered that the human H295R adrenocortical cell line does not express TSPO, but is still capable of producing steroids (Tu *et al*. 2014), demonstrating that TSPO is not necessary for STAR function and steroid production.

9. TSPO interaction with STAR: In 2001, visualization to evaluate a TSPO–STAR interaction using fluorescence resonance energy transfer (FRET) suggested that TSPO could interact with StAR in the OMM when coexpressed in nonsteroidogenic Cos-7 cells (West *et al*. 2001). Together with the above works on TSPO knockdown, the FRET study has been extensively cited to indicate that TSPO is part of the steroidogenic machinery.

Answer: Although this experiment appeared to provide direct evidence that TSPO could interact with STAR, 6 years later, this same research group followed up on their FRET analysis using bioluminescence resonance energy transfer (BRET) and concluded that there was no evidence for TSPO and STAR interaction (Bogan *et al*. 2007). The BRET analysis utilized cell populations rather than individual cells and three different cell types (CHO cells, MA-10 cells and Cos-7 cells). Also in this report, bacterial and mammalian two-hybrid assays failed to demonstrate a StAR–TSPO interaction (Bogan *et al*. 2007). The authors concluded that because few cells were selected for FRET analysis in their first report, 'it may have resulted in artifactual data' (Bogan *et al*. 2007).

10. TSPO deletion *in vitro*: As genetic evidence for TSPO being an 'indispensable element of the steroidogenic machinery', it was demonstrated that disruption of *Tspo* in the R2C rat Leydig cell line caused significant adverse morphological changes, lowered proliferation rate and obliterated steroidogenesis (Papadopoulos *et al*. 1997*b*). This highly cited manuscript has been used to indicate definitive genetic evidence for the role of TSPO in steroidogenesis, and justify the putative mechanism of action for TSPO ligands.

Answer: R2C Leydig cell line constitutively makes steroids without the need for trophic stimulation, due to the constitutive expression of STAR (Stocco & Chen 1991, Jo & Stocco 2004). The aforementioned study on TSPO deletion was based on a solitary distorted clone of R2C Leydig cells selected after homologous recombination. We say distorted because: (1) Disruption of one *Tspo* allele in the single clone used for this study resulted in a complete disappearance of TSPO protein even though the R2C cells are tetraploid (Papadopoulos *et al*. 1997*b*). Subsequent studies have shown that monoallelic deletion of *Tspo* does not affect TSPO transcript/protein expression (Tu *et al*. 2014), and complete deletion of *Tspo* studied in three independent clones of the MA-10 mouse Leydig cell line did not affect morphology, proliferation and/or steroidogenic function (Tu *et al*. 2015). (2) It was mentioned that proliferation rates of the single R2C clone used in this study spontaneously rebounded after 3 months in culture and hormone production resumed after 2 years in culture without any intervention (Papadopoulos *et al*. 1997*b*). Therefore, there are serious limitations to this interpretation, as experimental methods used in this study do not account for R2C cell line clonal effects. There were no attempts to select additional *Tspo*-disrupted R2C clones to examine if these results were consistent and specific for TSPO. The previous assumption that R2C cells may have a higher affinity TSPO ligand binding site (Garnier *et al*. 1994) was also not accurate. It was subsequently demonstrated that MA-10 cells may have more TSPO molecules and that their affinities are identical to R2C cells (Rao *et al*. 2002). R2C cells were demonstrated to contain higher levels of the scavenger receptor (SR-B1), hormone-sensitive lipase (HSL) and STAR, which may be associated with their constitutive nature of steroid synthesis (Rao *et al*. 2003). As this effect reported in the *Tspo*-disrupted R2C Leydig cell clone is not substantiated, and has not been reproducible in the MA-10 Leydig cell line (Tu *et al*. 2015), it is conceivable that genetic aberrations unrelated to *Tspo* in this selected R2C clone could have led to this misconception.

11. TSPO deletion *in vivo*: Another piece of genetic evidence was the report that global *Tspo*$^{-/-}$ mice were early embryonic lethal (Papadopoulos *et al*. 1997*a*). This conclusion was presented in a review article without experimental details or phenotypic characterization, but has been highly cited in the literature to indicate an important role for TSPO in development and other basic cellular functions that included steroidogenesis (Papadopoulos *et al*. 1997*a*).

Answer: Two independent reports published in 2014 provide solid data on generating and phenotyping global *Tspo*$^{-/-}$ mice, and demonstrating that TSPO is

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not involved in viability, fertility and steroidogenesis (Banati *et al*. 2014, Tu *et al*. 2014). Therefore, we can only speculate that experimental problems hindered the first *Tspo* knockout attempt.

12. Human TSPO polymorphism: Mutations/polymorphisms for TSPO were previously sought and excluded in lipoid CAH patients (Lin *et al*. 1993). However, a common human polymorphism in TSPO (*rs6971*, leading to amino acid change Ala147Thr) has been demonstrated to cause differences in affinity of TSPO-binding chemicals used for diagnostic imaging (Mizrahi *et al*. 2012, Owen *et al*. 2012). Ala147 is considered part of the TSPO PK11195-binding pocket (Jaremko *et al*. 2014), suggesting that a change to Thr147 could affect binding properties of PK11195 and other chemicals that bind to this region. Functionally, this *rs6971* polymorphism was linked to adult separation anxiety in patients with depression (Costa *et al*. 2009*b*). This same polymorphism was subsequently associated with decreased PREG production by immune cells in both Thr147 homozygous and heterozygous individuals (Costa *et al*. 2009*a*).

Answer: There is no evidence that this TSPO polymorphism suggests a link between TSPO and steroid biosynthesis. PREG production by activated lymphocytes, specifically T helper 2 cells, has been linked to functional immunosuppression (Mahata *et al*. 2014). TSPO upregulation has long been associated with immune activation and cellular responses (Liu *et al*. 2014). Therefore, this polymorphism may indicate an immune function for TSPO associated with its overexpression in inflammatory pathologies, and cannot be considered as evidence for steroidogenesis as suggested previously (Papadopoulos 2014). In contrast to previous views, recent studies have shown that PK11195 affinities are similar between the TSPO and the variant *rs6971* polymorphism (Jaremko *et al*. 2015*a*, 2016). Moreover, binding affinity was found to have no correlation to putative steroid synthesis mediated by TSPO-binding chemicals (Wolf *et al*. 2015).

13. TSPO functional redundancy: There has been no mention of functional redundancy in the previous TSPO literature. TSPO knockdown in MA-10 Leydig cells (Hauet *et al*. 2005) and TSPO monoallelic knockout in R2C Leydig cells (Papadopoulos *et al*. 1997*b*) were reported to result in dramatic decreases in steroid hormone production. Nevertheless, after the appearance of results refuting TSPO function, functional redundancy was offered as an explanation in several reviews on this topic (Midzak & Papadopoulos 2014, Papadopoulos *et al*. 2015).

Answer: Both the proclaimed studies using TSPO knockdown and 'knockout' Leydig cells, (Papadopoulos *et al*. 1997*b*) and (Hauet *et al*. 2005), have been highly cited as the genetic foundation for justifying research on TSPO pharmacology. Findings that in the same Leydig cells a complete TSPO deletion using CRISPR/Cas9-mediated targeting had no effect on steroid hormone production (Tu *et al*. 2015) suggest that this is not a case of functional redundancy, but a core problem with reproducibility, and needs to be considered as such. The TSPO homolog, TSPO2, is expressed almost exclusively in hematopoietic tissues, and does not present a case for functional redundancy in these studies (Fan *et al*. 2009) (discussed later in this review). If there is indeed a redundant protein or mechanism, it is not clear why these mechanisms did not become apparent in earlier studies that asserted an 'indispensable' role for TSPO in the steroidogenic machinery.

For readers not directly involved in steroidogenesis research, all these points might seem overwhelming, and perhaps hard to decode given the sheer volume of literature based on the fundamental premise that TSPO is linked to steroidogenesis. However, as progress is made with factual data, we believe that clarity for this paradigm shift will start to solidify with advancement of new directions for TSPO research, and oppositions, particularly presented by research groups involved in the early studies on TSPO and steroidogenesis, are addressed by identifying its true physiological function.

TSPO plight not just based on findings in mice

As indicated in the points above, evidence against TSPO link to steroidogenesis is not only based on studies in *Tspo*$^{-/-}$ mice. We reiterate this point because several recent reviews have greatly stressed this point and perpetuate the sentiment that evidence in mice is not conclusive, and that there could be compensatory mechanisms (Midzak *et al*. 2015). In contrast to previous propositions, recent research demonstrates that isolated steroidogenic mitochondria from *Tspo*-deficient cells do not exhibit any deficits in steroid production (Banati *et al*. 2014). Also, TSPO knockdown in Y1 adrenocortical cells and MA-10 Leydig cells does not affect steroid hormone production

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(Tu *et al*. 2014). Convincingly, complete TSPO deletion in MA-10 Leydig cells using the CRISPR/Cas9 system does not affect steroid hormone production (Tu *et al*. 2015). These new reports, unconnected to work performed on *Tspo*$^{-/-}$ mice, indicate that previous work on TSPO that used these same cell lines is not reproducible, and therefore challenge the very foundations of the premise that TSPO is involved in steroidogenesis.

As TSPO is considered an important therapeutic target and diagnostic marker for a broad range of inflammatory diseases/disorders (with 24 clinical trials that are either ongoing or recently completed; 17 in the US (source: www.clinicaltrials.gov) and 7 in the EU (source: www.clinicaltrialsregister.eu)), there has been pushback in several forms as these new considerations void its popular mechanism of action. According to this mechanism, TSPO is an essential cholesterol transport protein within the 'mitochondrial transport complex' (Midzak *et al*. 2011). The problem with this model is that TSPO is the key player, because it is the *only* cholesterol-binding member that cannot be compensated by STAR function (Hauet *et al*. 2005). This should mean that deletion of this critical conduit from this complex would offer no opportunity for cholesterol binding and essentially block all steroidogenesis. However, all the recent reports using TSPO-deficient models do not indicate a loss of steroid synthesis.

Neurosteroid production by TSPO-binding drugs

Based on several studies highlighting the therapeutic value of TSPO in preclinical studies, TSPO is widely popular as a drug target for a variety of diseases/disorders (Papadopoulos & Lecanu 2009, Rupprecht *et al*. 2010, Chua *et al*. 2014, Werry *et al*. 2015). The primary mechanism of TSPO-binding drugs, at least in the nervous system, is based on their putative ability to stimulate neurosteroid production. While TSPO is not involved in mitochondrial cholesterol import for steroidogenesis, it remains unexplained how different reports have linked the effect of TSPO-binding drugs to neurosteroid production. In steroidogenic cells of the adrenals and testes, TSPO is highly abundant. In contrast, TSPO expression in cells of the nervous system is extremely weak (Daugherty *et al*. 2013), and it is uncertain if upregulation seen during inflammation/injury approaches the levels seen in steroidogenic cells. Although complete deletion of TSPO from MA-10 Leydig cells did not affect steroid production, it has been demonstrated that supra-physiological overexpression of TSPO in the same MA-10 Leydig cells could increase steroid production (Liu *et al*. 2006). If we were to use this information for an alternate explanation, it is conceivable that the presence of TSPO in an organelle that is relatively cholesterol poor could increase cholesterol content by potentially enriching cholesterol molecules either through the CRAC motif or through other regions of affinity. As recently speculated (Midzak & Papadopoulos 2014), it may be possible that drugs that bind TSPO could induce a conformational change resulting in a decreased cholesterol affinity, making more molecules available for steroid synthesis. This mechanism could explain the transient steroidogenic effect observed with different TSPO-binding drugs (Mukhin *et al*. 1989, Papadopoulos *et al*. 1990). However, it does not explain how PK11195 could induce steroidogenesis in cells that are deficient in TSPO (Tu *et al*. 2015), and it is unclear if such a transient production would have physiological and/or therapeutic effect. In this section, we compare existing literature on TSPO-binding drugs and induction of neurosteroids in an attempt to reevaluate the possible mechanisms underlying this connection.

Although the basis of TSPO action through neurosteroid production is indicated as an explanation in numerous studies, only a handful of studies have directly measured neurosteroid synthesis induced by TSPO-binding pharmacological agents (Table 1). Critical comparisons of these studies taking into account the model systems, drug concentrations and the different neurosteroids measured revealed a degree of unexplained inconsistency. In some cases, effects observed were inconsistent even between studies that used the same dose and model system.

The PK11195 action on neurosteroid biosynthesis can be considered equivocal in that the effects range from stimulation to inhibition. Interestingly, PK11195 could block the neurosteroid-inducing effects of other TSPO-binding drugs like FGIN(1–27) (Korneyev *et al*. 1993), indoleacetamides (Kozikowski *et al*. 1993), YL-IPA08 (Zhang *et al*. 2014), CB-34 (Serra *et al*. 1999) and TTN (Do-Rego *et al*. 1998). On the other hand, the effect of Ro5-4864 on induction of neurosteroids appears reproducible, albeit with varying potency. Based on thermodynamic studies, even before a link was made between TSPO-binding drugs and steroidogenesis, it was predicted that PK11195 could be a TSPO antagonist, and Ro5-4864 a TSPO agonist (Le Fur *et al*. 1983). In the same time frame, a study examining TSPO drug effect on audiogenic seizures identified that Ro5-4864 could not only facilitate but also elicit seizures in the

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Table 1 Studies evaluating neurosteroid production by TSPO-binding drugs.

Reference	Model system(s)	Dose	Neurosteroids measured and drug effects	Inference
PK11195				
Korneyev *et al.* (1993)	Adrenalectomized and castrated rats	*In vivo:* 100 μmol/kg (i.p.)	No effect on PREG. Blocked effect of FGIN(1–27) when combined	The effect of PK11195 on neurosteroid production was highly inconsistent, ranging from inhibitory, no effect, to stimulatory effects. PK11195 could also antagonize neurosteroid-inducing effects of other TSPO binding drugs. Studies have identified different targets for PK11195 that are distinct from TSPO.
Kozikowski *et al.* (1993)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*: 1 μM	No effect on PREG. Blocked effect of indoleacetamides when combined	
Kozikowski *et al.* (1993)	Rat brain	*In vitro*: 1 μM	No effect on PREG. Blocked effect of indoleacetamides when combined	
Romeo *et al.* (1993)	Isolated mitochondria from rat brain	*In vitro*: 10 nM to 10 μM	*Mitochondria*: No effect on PREG. Slightly ↓ PREG at 10 μM	
Romeo *et al.* (1993)	Adrenalectomized and castrated rats	*In vivo*: 100 μmol/kg (i.p.)	*Rat*: No effect on PREG in the forebrain. Blocked effect of FGIN(1–27) when combined	
McCauley *et al.* (1995)	Isolated mitochondria from rat brain	*In vitro*: 1 μM	↑ PREG 7 folds. Most potent among ligands tested.	
Do-Rego *et al.* (1998)	Frog hypothalamic explants	*In vitro*: 100 μM	↓ 17OH-PREG, PROG, 17OH-PROG, 5α-DHT. Blocked effect of TTN when combined	
Lacor *et al.* (1999)	Rats	*In vivo*: 3 mg/kg (IP)	No effect on PREG. Blocked effect of Ro5-4864 when combined	
Serra *et al.* (1999)	Rats	*In vivo*:21, 40 mg/kg (i.p.)	↑ PREG, PROG, ALLO, THDOC in plasma and cerebral cortex	
Serra *et al.* (1999)	Rats	*In vivo*: 21, 40 mg/kg (i.p.)	Pretreatment at dose 40 mg/kg blocked effect of CB-34	
Primofiore *et al.* (2004)	Rat C6 glioma cells	*In vitro*: 40 μM	↑ PREG by 48%	
Selleri *et al.* (2005)	Rat C6 glioma cells	*In vitro*: 40 μM	↑ PREG by 48%	
Da Settimo *et al.* (2008)	Rat C6 glioma cells	*In vitro*: 40 μM	↑ PREG by 48%	
Scarf *et al.* (2012)	Rat C6 glioma cells	*In vitro*: 40 μM	↑ PREG by 37%	
Zhang *et al.* (2014)	Rats after the time-dependent sensitization procedure in post-traumatic stress disorder (PTSD) model	*In vivo*: 3 mg/kg (i.p.)	No effect on PREG. Blocked effect of YL-IPA08 when combined	
do Rego *et al.* (2015)	Frog hypothalamic explants	*In vitro*: 30 μM	↓ 17OH-PREG, PROG, DHEA, THP. Blocked effect of Etifoxine when combined	
Ma *et al.* (2016)	Mice injected with lipopolysaccharide (LPS)	*In vivo*: 3 mg/kg (i.p.)	Prevented LPS-induced reduction in PROG; ↑ ALLO in hippocampus.	
Santoro *et al.* (2016)	Rat C6 glioma cells	*In vitro*: 3 μM	↑ PREG by 14%	

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Table 1 (*Continued*).

Reference	Model system(s)	Dose	Neurosteroids measured and drug effects	Inference
Ro5-4864 (4′-chlorodiazepam)				
Guarneri *et al.* (1992)	Rat C6-2B glioma cells	*In vitro*: 1 nM to 100 nM	↑ PREG production from mevalonate within 1 min, max at 10 nM (2–3 folds)	Effect of Ro5-4864 appears consistent and increases neurosteroid production, although the potency varies between studies. Ro5-4864 also induced numerous secondary effects. Studies have identified Ro5-4864 effect as an anxiogenic is through effects on $GABA_A$ receptor.
Romeo *et al.* (1992)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*: 1 nM to 1 μM	↑ PREG 2 folds at nM concentrations	
Papadopoulos *et al.* (1992)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*: 0.1 nM to 0.1 μM	↑ PREG, max at 100 nM	
Korneyev *et al.* (1993)	Adrenalectomized and castrated rats	*In vivo*: 6, 12, or 18 μmol/kg (i.v.)	At 18 μmol/kg: ↑ PREG, no effect in DHEA in forebrain and cerebellum	
Romeo *et al.* (1993)	Isolated mitochondria from rat brain	*In vitro*: 10 nM to 10 μM	↑ PREG accumulation, max at 10 μM (6–7 folds). Most potent among ligands tested	
McCauley *et al.* (1995)	Isolated mitochondria from rat brain	*In vitro*: 1 μM	↑ PREG (3 folds)	
Do-Rego *et al.* (1998)	Frog hypothalamic explants	*In vitro*: 1 μM	↑ conversion of PREG into 17OH-PREG, PROG, 17OH-PROG, 5α-DHT, steroid X	
Lacor *et al.* (1999)	Rats	*In vivo*: 3 mg/kg (i.p.)	↑ PREG in plasma and sciatic nerve	
Primofiore *et al.* (2004)	Rat C6 glioma cells	*In vitro*: 40 μM	↑ PREG 41%	
Selleri *et al.* (2005)	Rat C6 glioma cells	*In vitro*: 40 μM	↑ PREG 41%	
Da Settimo *et al.* (2008)	Rat C6 glioma cells	*In vitro*: 40 μM	↑ PREG 41%	
Diazepam				
McCauley *et al.* (1995)	Isolated mitochondria from rat brain	*In vitro*: 1 μM	↑ PREG 1.7 fold	Effect of Diazepam is weak, but primary effect is through $GABA_A$ receptors.
Wolf *et al.* (2015)	Mouse BV2 microglia cells	*In vitro*:0.1–10 μM	*BV2 cells*:no effect	
Wolf *et al.* (2015)	Rat C6 glioma cells	*In vitro*: 0.1–10 μM	*C6 cells*: very mildly ↑ PREG at 1, 10 μM	
DBI and its fragments TTN: DBI (17–50), ODN: DBI (33–50)				
Romeo *et al.* (1992)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*: 1 nM to 1 μM DBI	↑ PREG 2 folds at nM concentrations	Effects are not consistent: TTN and ODN are reported to either increase or have no effect on neurosteroid production. In frog hypothalamic explants, the same group arrived at conflicting conclusions about TTN effect on DHEA and PROG.
Papadopoulos *et al.* (1992)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*: 0.1 nM to 0.1 μM DBI or TTN	DBI, TTN: ↑ PREG, max at 5–10 nM (2–3 folds)	
Papadopoulos *et al.* (1992)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*:0.1 nM to 0.1 μM ODN	ODN: no effect on PREG	
McCauley *et al.* (1995)	Isolated mitochondria from rat brain	*In vitro*: 1 μM ODN or DBI(42–50)	ODN, DBI(42–50): ↑ PREG (<2 folds)	

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Table 1 (*Continued*).

Reference	Model system(s)	Dose	Neurosteroids measured and drug effects	Inference
McCauley *et al.* (1995)	Isolated mitochondria from rat brain	*In vitro*: 1 μM TTN or TTN+ODN	TTN or TTN+ODN: no effect on PREG	
Do-Rego *et al.* (1998)	Frog hypothalamic explants	*In vitro*: 1 nM to 1 μM TTN	↑ 17OH-PREG, 17OH-PROG, 5α-DHT, steroid X in a dose-dependent manner; no effect on DHEA and PROG	
do Rego *et al.* (2015)	Frog hypothalamic explants (where membrane receptor signaling is disrupted)	*In vitro*: 30 nM TTN	*Explant*: ↑ 17OH-PREG, DHEA, PROG, THP. Had synergistic effect with Etifoxine to ↑ hormones	
do Rego *et al.* (2015)	Frog hypothalamic homogenates (where membrane receptor signaling is disrupted)	*In vitro*: 30 nM TTN	*Homogenates*: No effect on 17OH-PREG, DHEA, PROG, DHP, THP	
Etifoxine (Stresam)				
Verleye *et al.* (2005)	Rats	*In vivo*: 50 mg/kg (i.p.)	*Rats*: ↑ PREG, PROG, 5a-DHP, ALLO but not corticosterone in brain and plasma	Effects reported for Etifoxine are not consistent: an increase in neurosteroid production was observed in some studies, without effects in others. Neurosteroid induction by Etifoxine was suggested to be the most potent in one study, although its binding affinity for TSPO is lowest among the compounds tested. Etifoxine could stimulate neurosteroid production even in the absence of TSPO and other membrane receptors.
Verleye *et al.* (2005)	Adrenalectomized and castrated (ADX-CX) rats	*In vivo*: 50 mg/kg (i.p.)	*ADX-CX rats*: ↑ PREG, PROG, ALLO in brain to a lesser extent	
do Rego *et al.* (2015)	Frog hypothalamic explants (where membrane receptor signaling is disrupted)	*In vitro*: 0.3–30 μM	*Explant*: ↑ 17OH-PREG, DHEA, PROG, DHP, THP. ↓ DHP at >1 μM. Effect was not blocked by PK11195 or Flumazenil	
do Rego *et al.* (2015)	Frog hypothalamic homogenates (where membrane receptor signaling is disrupted)	*In vitro*: 0.3–30 μM	*Homogenates (no membrane receptors)*: Still ↑ 17OH-PREG, DHEA, PROG, THP but ↓ DHP	
Wolf *et al.* (2015)	Mouse BV2 microglia cells	*In vitro*: 0.1–10 μM	*BV2 cells*: ↑ PREG in a dose-dependent manner, but ↓ PREG at 10 μM	
Wolf *et al.* (2015)	Rat C6 glioma cells	*In vitro*: 0.1–10 μM	*C6 cells*: ↑ PREG in a dose-dependent manner	
Ravikumar *et al.* (2016)	Rat C6 glioma cells	*In vitro*:12.5, 25, 50 μM	*Cells*: ↑ PREG in a dose-dependent manner	
Ravikumar *et al.* (2016)	Rat primary astrocytes	*In vivo*: 50 mg/kg (i.p.)	*Rat brain*: No effect on all neurosteroids tested (PREG, PROG, ALLO, DHP, THP)	
FGIN(1–27) and other indoleacetamides				
Romeo *et al.* (1992)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*: 1 nM to 1 μM	↑ PREG 2 folds at nM concentrations	FGIN(1–27) and some other indoleacetamides showed increases in neurosteroid production.
Korneyev *et al.* (1993)	Adrenalectomized and castrated rats	*In vivo*: 7 μmol/kg (i.v.) or 200, 400, 800 μmol/kg (p.o.)	FGIN (all doses except 200 μmol/kg): ↑ PREG, no effect in DHEA in forebrain and cerebellum	

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Table 1 (*Continued*).

Reference	Model system(s)	Dose	Neurosteroids measured and drug effects	Inference
Kozikowski *et al.* (1993)	Isolated mitochondria from rat C6-2B glioma cells	Different indoleacetamides *in vitro*: 1 μM	A subset of compounds ↑ PREG in mitochondria	
Kozikowski *et al.* (1993)	Isolated mitochondria from rat brain	Different indoleacetamides *in vitro*: 1 μM	A subset of compounds ↑ PREG in mitochondria	
Romeo *et al.* (1993)	Isolated mitochondria from rat brain	*In vitro*: 10 nM to 10 μM	*Mitochondria*: ↑ PREG accumulation, max at 10 μM (5–6 folds)	
Romeo *et al.* (1993)	Adrenalectomized and castrated rats	*In vivo*: 800 μmol/kg (p.o.)	*Rat*: ↑ PREG 2.3 folds in the forebrain	
Bitran *et al.* (2000)	Rats	*In vivo*: 2.5 μg (intrahippocampal injections)	↑ ALLO in both hippocampus and plasma	
Petralia and Frye (2005)	Rats	*In vivo*: 5 μg (intracranial into ventral tegmental area)	↑ ALLO 2 folds in the midbrain	
XBD-173 (AC-5216, Emapunil)				
Wolf *et al.* (2015)	Mouse BV2 microglia cells	*In vitro*: 0.1–10 μM	*BV2 cells*: Mildly ↑ PREG	Effect of XBD-173 was stimulatory toward neurosteroid production. However, XBD-173 was not efficacious in EAE mouse model, and in the human clinical trial as an anxiolytic.
Wolf *et al.* (2015)	Rat C6 glioma cells	*In vitro*: 0.1–10 μM	*C6 cells*: Mildly ↑ PREG only at 10 μM	
Ravikumar *et al.* (2016)	Rat C6 glioma cells	*In vitro*: 12.5, 25, 50 μM	*Cells*: ↑ PREG in a dose-dependent manner	
Ravikumar *et al.* (2016)	Rat primary astrocytes	*In vivo*: 50 mg/kg (i.p.)	*Rat brain*: ↑ PREG, PROG, 5α-DHP, ALLO, 3β5α-THP, 5α20α-THP	
ONO-2952				
Mitsui *et al.* (2015)	Rats exposed to restraint stress	*In vivo*: 0.1, 1, 10 mg/kg (p.o.)	Blocked stress-induced increase in PREG, PROG, ALLO, THDOC in hippocampus	Effect of ONO-2952 was only tested using a stress model and observations could be subject to secondary effects. Results showed attenuated effects on neurosteroid production.
PIGAs (N,N-dialkyl-2-phenylindol-3-ylglyoxylamides) MPIGA (N,N-di-n-propyl-2-(4-methylphenyl)indol-3-ylglyoxylamide)				
Primofiore *et al.* (2004)	Rat C6 glioma cells	*In vitro*: 40 μM PIGAs	Selected compounds ↑ PREG by 40–166% while others had no effects on PREG	Selected compounds in this group could increase neurosteroid production but only at high concentrations. A few compounds that had high affinity for TSPO showed mild to no effect.
Da Settimo *et al.* (2008)	Rat C6 glioma cells	*In vitro*: 40 μM PIGAs	Some compounds ↑ PREG 60–171% while others had no effects on PREG	
Costa *et al.* (2011)	Human astrocytoma ADF cells	*In vitro*: 100 nM to 100 μM MPIGA	↑ PREG to 177% only at 40 μM; ↓ PREG to 50% at 100 μM	
Costa *et al.* (2011)	Human astrocytoma ADF cells	*In vitro*:100 nM to 100 μM MPIGA	↑ PROG to 167%, ↑ ALLO to 115% at 40 μM.	
Santoro *et al.* (2016)	Rat C6 glioma cells	*In vitro*: 10 μM PIGAs	↑ PREG by 97%	

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Table 1 (*Continued*).

Reference	Model system(s)	Dose	Neurosteroids measured and drug effects	Inference
Imidazopyridine- and Pyrazolopyrimidine- acetamides				
Serra *et al.* (1999)	Rats	*In vivo*: 3–50 mg/kg (i.p.) CB-34, CB-50 or CB-54	↑ PREG, PROG, ALLO, THDOC in plasma, brain of normal rats in a dose-dependent manner	A subset of compounds in this group that had high affinity for TSPO could stimulate synthesis of neurosteroids, whereas another subset *al*so with high affinity was without effects.
Serra *et al.* (1999)	Adrenalectomized and orchidectomized (ADX-ORX) rats	*In vivo*: 3–50 mg/kg (i.p.) CB-34	CB-34 at 25 mg/kg ↑ PREG, PROG, ALLO in ADX-ORX rats to a lesser extent	
Trapani *et al.* (1999)	Rats	*In vivo*: 25 mg/kg (i.p.) 2-phenylimidazo [1,2-α] pyridineacetamides	Selected compounds ↑ PREG, PROG, ALLO, THDOC in cerebral cortex and plasma, ↑ corticosterone in plasma. Some had mild or no effect	
Trapani *et al.* (2005)	Rats	*In vivo*: 25 mg/kg (i.p.) 2-phenylimidazo [1,2-α] pyridineacetamides	Selected compounds ↑ PROG, 3α,5α-THPROG, 3α,5α-THDOC in cerebral cortex and plasma. One compound with subnanomolar affinity for TSPO showed mild effect	
Zhang *et al.* (2014)	Rats after the time-dependent sensitization procedure in post-traumatic stress disorder (PTSD) model	*In vivo*: 0.3 mg/kg (p.o.) YL-IPA08	↑ ALLO in prefrontal cortex and serum	
Selleri *et al.* (2005)	Rat C6 glioma cells	*In vitro*: 40 μM2-arylpyrazolo[1,5-a] pyrimidin-3-yl acetamides	2 compounds ↑ PREG 70–90%, one had no effects on PREG	
Pyrrolobenzoxazepines				
Scarf *et al.* (2012)	Rat C6 glioma cells	*In vitro*: 40 μM	Some compounds ↑ PREG while others with high binding affinity to TSPO did not	This study demonstrated that neurosteroid production is not correlated to binding affinity of drugs to TSPO.
Pyridazinoindole (SSR180575)				
Ferzaz *et al.* (2002)	Rats	*In vivo*: 3 mg/kg (i.p.)	↑ PREG in brain, sciatic nerve (2 folds) but not in plasma	Effect of SSR180575 showed increase neurosteroid production, but not peripheral steroids.
PPIX				
Romeo *et al.* (1992)	Isolated mitochondria from rat C6-2B glioma cells	*In vitro*: 1 nM to 1 μM	No effect on PREG even at 1 μM	Effect of PPIX, considered an endogenous ligand with nanomolar affinity for TSPO showed no effect on neurosteroid production.

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absence of noise stimuli (Benavides *et al*. 1984). Despite the proposed specificity to TSPO, the effects of Ro5-4864 were also suggested to act via directly perturbing the $GABA_A$ receptor chloride ionophore complex (Weissman *et al*. 1984). The observation that Ro5-4864 was anxiogenic (File & Lister 1983) was also the case for PK11195 when provided at a higher dose (File & Pellow 1985). Therefore, discussions regarding the specificity of these compounds emerged even before putative effects for TSPO on steroid synthesis were proposed. Binding of PK11195 to other targets in the cell have been described: the ATP-binding cassette (ABC) transporters (Walter *et al*. 2005), constitutive androstane receptor (CAR) (Li *et al*. 2008, Anderson *et al*. 2011), pregnane X receptor (Anderson *et al*. 2011), oncoprotein B cell lymphoma 2 (Bcl-2) (Gonzalez-Polo *et al*. 2005) and the F_1F_0 ATP synthase (Seneviratne *et al*. 2012), all of which could confound the TSPO-mediated effect. Moreover, changes to membrane properties as a result of direct incorporation of PK11195 into the lipid bilayer have been suggested to contribute to TSPO-independent effects (Hatty *et al*. 2014).

Examination of neurosteroid production with diazepam that can bind to both $GABA_A$ receptor and TSPO has only been conducted using *in vitro* models with mild effects on neurosteroids observed (McCauley *et al*. 1995, Wolf *et al*. 2015). Effects of diazepam on TSPO are hard to delineate due to robust actions on GABAergic synaptic transmission. Results for etifoxine, commercially sold as an anxiolytic and anticonvulsant (Stresam), have been mixed. Etifoxine was demonstrated to be the most potent inducer of neurosteroid production *in vitro*, despite its lower binding affinity (Wolf *et al*. 2015). *In vivo* results have been conflicting, with contrasting results in the same rat model and dose showing induction (Verleye *et al*. 2005) or no effect (Ravikumar *et al*. 2016). Recent studies also indicate that induction of neurosteroids by etifoxine could occur independent of membrane receptors including TSPO (do Rego *et al*. 2015). Although there is literature correlating the anxiolytic effect of etifoxine and neurosteroid production (Ugale *et al*. 2007), early studies on etifoxine have indicated that it interacts with the chloride channel coupled to the $GABA_A$ receptor (Verleye *et al*. 1999), and can directly modulate GABAergic synaptic transmission (Schlichter *et al*. 2000). Moreover, *in vivo* effects for etifoxine that are anxiolytic in contrast to Ro5-4864 that appears anxiogenic indicate that interpretations of its mechanism of action may not be straightforward.

Reports for FGIN(1–27) and other indolacetamides demonstrate increases in varying subsets of neurosteroids. It must be noted that FGIN(1–27) was also investigated as an agent for inducing apoptosis in cancer cells by inducing oxidative stress (Maaser *et al*. 2001, 2005, Sutter *et al*. 2002, 2005). This distinct direction of FGIN(1–27) action introduces another layer of difficulty in interpreting a pharmacological relationship with TSPO.

The drug XBD173 (Emapunil) has been a topic of intense investigation for use as an anxiolytic (Kita *et al*. 2004). Optimistic outcomes were reported using a high dose of XBD173 in an induced model of panic disorder in rats (Rupprecht *et al*. 2009). Although neurosteroid production was proposed as a mechanism, it was not tested. Novartis Pharmaceuticals subsequently launched a human clinical trial (NCT00108836) for evaluating XBD173 efficacy in patients with generalized anxiety disorders. Although results from this trial were not published, it was reported that XBD173 treatment showed no reduction compared with placebo in a variety of anxiety measures. Although this failure was attributed to XBD173-binding affinity and the human TSPO polymorphism (A147T) (Owen *et al*. 2011), it remains merely a hypothetical proposition for drug efficacy (Wolf *et al*. 2015). Subsequently, XBD173 was removed from the drug development pipeline. Recent studies have shown moderate increases in neurosteroid production with XBD173 both *in vitro* and *in vivo* (Wolf *et al*. 2015, Ravikumar *et al*. 2016). Despite this neurosteroid effect, in rodent experimental autoimmune encephalitis (EAE) models, XBD173 was not efficacious (Ravikumar *et al*. 2016). As part of the same study, animals in a group that received etifoxine showed ameliorated EAE clinical signs, but etifoxine *in vivo* had no effect on neurosteroid production (Ravikumar *et al*. 2016).

Effect of ONO-2952 has been examined only in the context of a stress model (Mitsui *et al*. 2015). Only selected compounds of PIGAs, imidazopyridine acetamides, pyrazolopyridine acetamides and pyrrolobenzoxepines demonstrated a link to neurosteroid production, whereas some with high binding affinity to TSPO showed no effect on neurosteroidogenesis. Interestingly, for PPIX that is considered a high-affinity endogenous ligand, there are very few studies examining steroid production. PPIX could competitively displace PK11195 (Wendler *et al*. 2003) and PBR28 (Ozaki *et al*. 2010), suggesting that it binds to the same site on TSPO. In perhaps the only study testing this endogenous ligand, no steroidogenic effect was observed as a result of PPIX binding to TSPO (Romeo *et al*. 1992).

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Previously identified as a ligand for the $GABA_A$ receptor (Gray *et al*. 1986), DBI/ACBP was also observed to interact with TSPO (Garnier *et al*. 1994). Of the two ACBP-processed peptides (termed endozepines), triakontatetraneuropeptide (TTN; DBI(17–50)) and octadecaneuropeptide (ODN; DBI(33–50)), TTN could stimulate dose-dependent mitochondrial steroid synthesis similar to ACBP (Papadopoulos *et al*. 1991*b*). Examination of DBI and TTN in neuronal *in vitro* models also revealed mild to no effects with some inconsistencies. As described previously, ACBP function is linked to lipid metabolism (Mandrup *et al*. 1993, Gaigg *et al*. 2001). These observations were confirmed in ACBP-knockout mice that indicate no role in steroid synthesis (Neess *et al*. 2011, 2013, Bloksgaard *et al*. 2012). Moreover, studies by independent groups could not confirm the TSPO–DBI interaction (Bogan *et al*. 2007). In the long history of steroidogenesis research, there have been similar positive results using peptides that stimulated steroids through nonspecific mechanisms. A peptide termed the 'steroidogenesis activator peptide' (SAP) was initially linked to acute stimulation of steroid synthesis (Pedersen & Brownie 1983, 1987). However, subsequent studies demonstrated that the SAP was part of the glucose-regulated protein 78 (GRP78), with no involvement in steroidogenesis (Luo *et al*. 2006, Wang *et al*. 2009, Wisniewska *et al*. 2010, Wey *et al*. 2012).

There are some core problems with pharmacological approaches for targeting TSPO *in vivo*. (1) Effects of TSPO-binding drugs on systemic functions are not considered in neurosteroid studies. The use of ^{11}C-XBD173 identified high accumulation in the lungs, heart, adrenal glands and other organs rich in TSPO; accumulation observed in the CNS is relatively much lower (Zhang *et al*. 2007). Drug effects in these peripheral tissues/organs that express high levels of TSPO that could be more robust and distinct from nervous system responses are almost always ignored. (2) Physiological stimulation of steroid synthesis would involve factors beyond the mitochondrial cholesterol import mechanism. There needs to be concurrent activation of cholesterol uptake and synthesis, and activation of delivery systems that can move free cholesterol to the OMM. There is no evidence that TSPO-binding drugs are capable of activating these pathways. Moreover, there is also no correlation between the levels of TSPO expression and neurosteroid production capability within different regions of the brain. In support, some studies using *in vivo* models indicate that despite neurosteroid induction in the CNS, TSPO-binding drugs failed to induce steroid synthesis in high TSPO-expressing organs like the adrenal (Romeo *et al*. 1993, Ferzaz *et al*. 2002, Verleye *et al*. 2005). Therefore, the assumption that targeting TSPO could result in a robust cellular steroidogenic response might be a too simplistic view. One plausible explanation for neurosteroid production could be that induction of a form of cellular/organismal stress by these drugs could trigger a secondary steroidogenic response in the CNS unrelated to TSPO expression levels or TSPO function.

Despite the inconsistencies, lack of specificity and other confounding factors, pharmacological activation of TSPO and neurosteroid production continues to be extensively cited in the literature. Based on genetic studies, there is strong indication that the effects observed could be nonspecific or secondary responses unrelated to direct involvement of TSPO (Tu *et al*. 2015). Therefore, it is extremely important to reassess the physiological relevance of these pharmacological studies using genetic models that lack TSPO to delineate TSPO-mediated actions from off-target and secondary effects.

TSPO is not part of the mitochondrial permeability transition pore

Distinct from the purported role for TSPO in steroidogenesis, it was also thought that TSPO might play a role in the mitochondria-mediated cell death process called the mitochondrial permeability transition (MPT). The MPT refers to the opening of a nonspecific pore that permits any molecule <1.5 kDa through the inner mitochondrial membrane allowing equilibration of the mitochondrial matrix and the cytosol leading to loss of mitochondrial function and cell death (Haworth & Hunter 1979, Hunter & Haworth 1979*a*,*b*). Effects observed using TSPO-binding drugs (Kinnally *et al*. 1993) and copurification of TSPO with other proteins thought to be involved in MPT (McEnery *et al*. 1992), initially linked TSPO to this process. However, through development of conditional $Tspo^{c\Delta/\Delta}$ genetic models, it was recently demonstrated that TSPO plays no role in the regulation or structure of the MPT pore (Sileikyte *et al*. 2014). This was another surprising discovery because distinct from steroid synthesis, the validity of a potential alternate mechanism that linked TSPO and TSPO-binding drug action to a number of different neuropathologies was challenged. In order to keep this manuscript focused on neurosteroid production, we are not reviewing the different aspects of MPT regulation

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affected by this conclusion. Implications from this recent understanding of TSPO and advances toward explaining the MPT process have been critically assessed elsewhere (see review Bernardi *et al*. 2015).

Is TSPO2 an isofunctional or heterofunctional homolog?

Due to *Tspo* sequence conservation from bacteria to humans, there has been some interest in studying the functional evolution of this gene (Fan *et al*. 2012). TSPO2 (PBRL/Peripheral benzodiazepam-like) was identified as a gene that emerged from a duplication event preceding speciation of reptiles, birds and mammals (Nakazawa *et al*. 2009). Unlike TSPO expression that is observed at high levels in cells active in lipid storage/metabolism (Fig. 2), TSPO2 expression appears restricted to the bone marrow (Fan *et al*. 2009, Nakazawa *et al*. 2009), and has not been detected in steroidogenic tissues (confirmed in Morohaku *et al*. 2014, Tu *et al*. 2014). Subcellular localization of TSPO2 using specific antibodies has not been performed; localization based on overexpression studies using C-terminal GFP/DsRed-TSPO2 fusion proteins (chicken, mouse and human) in cell lines have led to conflicting proposals. The group who studied chicken TSPO2 demonstrated mitochondrial localization (Nakazawa *et al*. 2009), and the group who studied mouse and human TSPO2 proposed that TSPO2 is localized to the endoplasmic reticulum and the nuclear membrane (Fan *et al*. 2009). These studies have demonstrated functional disparities as well. Based on the study in the chicken, a strict coregulation of *Tspo2* expression but not *Tspo* expression with genes involved in hematopoiesis was demonstrated (Nakazawa *et al*. 2009). This led to the conclusion that TSPO2 may be involved in heme availability for the assembly of hemoglobin (Nakazawa *et al*. 2009). In contrast, the study with mouse and human TSPO2 was an extrapolation of previous work on TSPO and steroidogenesis from the same authors, and suggested that TSPO2 is involved in cholesterol uptake and trafficking in erythroid cell types (Fan *et al*. 2009). At the present time, there are no gene-knockout models to study loss-of-function resulting from TSPO2 deletion, and understanding of its function remains rudimentary. Nevertheless, what we do know is that distinct tissue localization patterns and regulation between TSPO and TSPO2 negates concerns of potential functional redundancy in studies that use *Tspo* gene-deleted models.

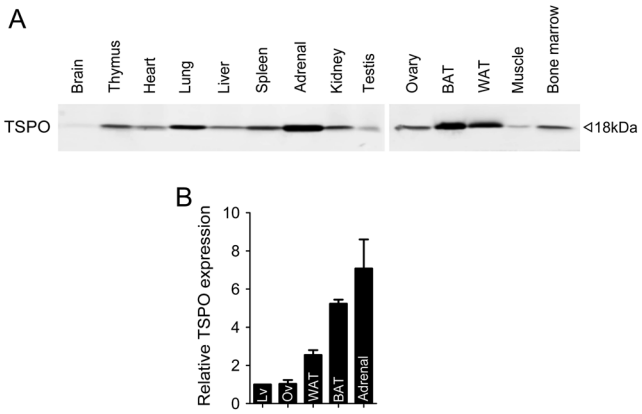

Figure 2
TSPO is highly expressed in tissues active in lipid metabolism. (A) Representative Western blotting showing TSPO expression in 14 different tissues (75 μg protein/sample). High expression of TSPO was observed in adrenal glands, followed by brown adipose tissue (BAT), white adipose tissue (WAT), lung and kidney. TSPO expression was weaker in other tissues and extremely low in the brain. (B) Compared with the liver (Lv), TSPO expression was similar in the ovary, 2.5-fold higher in the WAT, 5-fold higher in the BAT and 7-fold higher in adrenal glands (Adrenal). Republished with permission of the Endocrine Society, from *Endocrinology*, Tu LN, Zhao AH, Hussein M, Stocco DM & Selvaraj V, volume 157, issue 3, 2016; permission conveyed through Copyright Clearance Center, Inc.

Alternative perspectives for TSPO action in cells

As TSPO research spans multiple fields/disciplines, attempts to explain its function have resulted in rather divergent yet unique perspectives. Some of these perspectives lack coherence in that they do not describe a unifying function for TSPO that is applicable to all cell types and/or the organism as a whole. Nevertheless, to provide a balanced view, we present evidence published in support of the different perspectives (Fig. 3), and when appropriate, indicate limitations and areas that require further exploration to refine and accelerate understanding toward the precise function of TSPO.

1. TSPO as a regulator of redox homeostasis: There are seemingly disparate mechanisms that have been proposed for TSPO in redox homeostasis. First, a putative TSPO–VDAC association is believed to induce ROS production. It was demonstrated that TSPO overexpression in cells induces mitochondrial ROS production, with a reverse trend observed after TSPO knockdown (Gatliff *et al*. 2014). However, there exists contrasting evidence that TSPO overexpression could dampen mitochondrial ROS production through an identical VDAC-dependent mechanism (Joo *et al*. 2012, 2015). Second, without the need for any protein interactions, it has been suggested

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that TSPO could act to neutralize ROS. According to this hypothesis, the abundance of tryptophans in TSPO might react with ROS to generate tryptophan radicals (Guo *et al.* 2015). Third, a putative TSPO-NADPH oxidase 2 (NOX2) association has been proposed to play a role in ROS generation. In this model, TSPO is surmised to behave as a carrier or transporter for both cholesterol and heme (Guilarte *et al.* 2016). Fourth, it has been proposed that TSPO attenuates ROS signaling by modulating tetrapyrrole metabolism (Batoko *et al.* 2015).

Although all the above mechanisms are conceivable, they are attempts to explain TSPO function in cells based on effects that were initially observed using TSPO-binding drugs. Distinct from cells that consistently show high levels of TSPO expression (such as in the adrenal cortex and brown adipose tissue), these explanations almost always deal with cells that upregulate TSPO in response to different forms of stress. Therefore, it is possible that these interpretations may be confounded with cellular effects associated with the obvious link between stress and ROS production. The TSPO–VDAC relationship to ROS production has had contrasting results in similar TSPO overexpression studies (Gatliff *et al.* 2014, Joo *et al.* 2012, 2015). The TSPO–NOX2 association (Guilarte *et al.* 2016), remains a hypothesis. The finding that cardiac-specific $Tspo^{c\Delta/\Delta}$ mice did not show any difference in the extent of ischemia reperfusion injury (Sileikyte *et al.* 2014), a pathology that is partly directed by myocardial NOX2 (Braunersreuther *et al.* 2013), seems to suggest that TSPO and NOX2 may not be a primary mechanism. Perhaps via an interaction of multiple pathways, it is not too surprising that pharmacological evidence has linked TSPO to a variety of mechanisms related to cardioprotection after ischemia reperfusion injury: preventing mitochondrial permeability (Obame *et al.* 2007), increasing activities of mitochondrial oxidative enzymes (Xiao *et al.* 2010) or by reducing mitochondrial cholesterol transport (Paradis *et al.* 2013). In TSPO-deficient MA-10$^{Tspo\Delta/\Delta}$ Leydig cells, loss of TSPO resulted in modest increases in ROS production (Tu *et al.* 2016), an observation that was linked to a shift in cellular metabolism (discussed below). Therefore, future studies carefully examining *Tspo*$^{-/-}$ models to observe loss-of-function are absolutely essential before an effect for TSPO in redox homeostasis can be confirmed.

2. TSPO as an enzyme for protoporphyrin IX degradation: Distinct from studies involving steroid production, porphyrins are considered endogenous

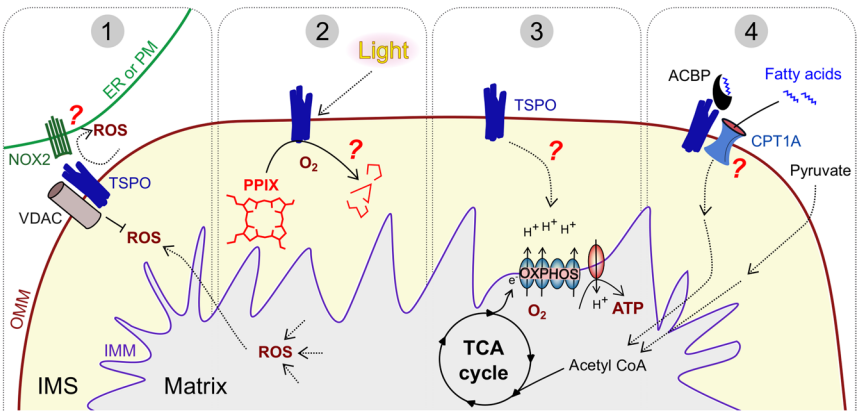

Figure 3
Alternative perspectives for TSPO action in cells. (1) TSPO as a regulator of redox homeostasis. It has been suggested that TSPO could interact with VDAC1 or NOX2 to induce production of ROS (Gatliff *et al.* 2014, Guilarte *et al.* 2016). On the contrary, the association of TSPO with VDAC1 (Joo *et al.* 2012, Joo *et al.* 2015), or TSPO by itself (Guo *et al.* 2015), could facilitate ROS neutralization. Although TSPO regulation of ROS is conceivable, at least as a secondary effect, functional evidence for the interactions and specific role of TSPO remains to be demonstrated. (2) TSPO as an enzyme for protoporphyrin IX degradation. Chemical catalysis of PPIX degradation by bacterial TSPO has been proposed as a function for TSPO (Ginter *et al.* 2013, Guo *et al.* 2015). The reaction rate was dependent on availability of light and oxygen. Lack of light in mammalian tissues that express high levels of TSPO like the adrenal glands raises question for the functional relevance and significance of this action. Nevertheless, conserved binding of TSPO to PPIX suggests a yet-to-be-determined action in cells. (3) TSPO as a regulator of oxygen consumption. Loss of TSPO had different effects in oxygen consumption rate in different cell types. Decreased oxygen consumption was observed in TSPO-deficient microglia and fibroblasts (Banati *et al.* 2014, Zhao *et al.* 2016), but no effects were observed in hepatocytes and Leydig cells (Sileikyte *et al.* 2014, Tu *et al.* 2016). These inconsistent effects are probably attributed to the diversity of mitochondrial types and energetic status of cells. Therefore, the direct mechanism remains unclear. (4) TSPO as a regulator of lipid metabolism. Several studies have correlated TSPO expression to functional changes in lipid metabolism (Wade *et al.* 2005, Leduc *et al.* 2011). It was identified that TSPO deletion could increase fatty acid oxidation in steroidogenic cells (Tu *et al.* 2016). Potential interaction of TSPO with ACBP or CPT1A could affects import of fatty acids into mitochondria for β-oxidation. All the above perspectives require additional investigation.

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ligands that bind TSPO (Verma *et al*. 1987). This porphyrin-binding property of TSPO appears highly conserved from bacteria to humans, but the function of this association continues to remain unclear. It was initially proposed that TSPO binds and transports protoporphyrin IX (PPIX), the precursor of heme, into the mitochondria (Wendler *et al*. 2003), a concept that emerged based on TSPO ligands being able to partially rescue cells from porphyrin-induced phototoxicity (Ratcliffe & Matthews 1995), and high levels of TSPO expression seen in the bone marrow (Taketani *et al*. 1994, Rampon *et al*. 2009). Although the property of PPIX binding to TSPO remains true, recent studies using *Tspo*$^{-/-}$ mice and cell lines have established that TSPO is not a porphyrin transporter for heme synthesis (Banati *et al*. 2014, Zhao *et al*. 2016). Using the bacterial *Chlorobium tepidum* TSPO purified in detergent, it was demonstrated that TSPO could induce rapid spectral changes to added PPIX indicative of chemical catalysis (Ginter *et al*. 2013). A similar observation has been reported for *Bacillus cereus* TSPO (Guo *et al*. 2015). However, the reaction rate was not proportional to TSPO concentration, but dependent on the availability of light and oxygen. Although this photo-oxidative PPIX degradation mediated by purified *C. tepidum* and *B. cereus* TSPO has been discussed in broad terms, there is no functional evidence that this occurs in any intact biological model systems. Moreover, the relevance of this light-dependent activity in deeper organs like the adrenal and brain remains questionable. Analysis of PPIX degradation/elimination as a time course in *Tspo*$^{-/-}$ mouse tissues and plasma suggests that this putative TSPO-mediated degradation, at least in mammalian systems, is not a critical regulator of PPIX levels (Zhao *et al*. 2016). Although this observation does not validate a role for TSPO in PPIX degradation, it certainly does not discount a physiological function for the highly conserved TSPO–PPIX association. Use of *Tspo*$^{-/-}$ models across different biological systems for investigating conserved properties in future studies may offer clues to uncovering specific functions for this association.

3. TSPO as a regulator of oxygen consumption: Evidence from lower organisms has indicated an oxygen-sensing function for TSPO (Yeliseev *et al*. 1997). In isolated primary microglia from *Tspo*$^{fl/fl}$ and *Tspo*$^{-/-}$ mice, measurement of oxygen consumption rate (OCR) indicated that basal OCR was significantly lower in *Tspo*$^{-/-}$ microglia (Banati *et al*. 2014). In an independent study, OCR compared between *Tspo*-deficient mitochondria isolated from liver-specific *Tspo*$^{c\Delta/\Delta}$ mice and control *Tspo*$^{fl/fl}$ mice showed no differences in OCR (Sileikyte *et al*. 2014). Although this contrast has been a topic of speculation with respect to the need for an intact cellular environment, and changes to metabolic demand in cells (reviewed in Gut 2015), it subsequently became clear that there was also no correlation between TSPO expression levels and OCR deficits observed in different cell types. Measurements of OCR in embryonic fibroblasts that express low levels of TSPO from *Tspo*$^{-/-}$ mice showed significantly diminished values compared with embryonic fibroblasts from *Tspo*$^{fl/fl}$ mice (Zhao *et al*. 2016). In contrast, measurements of OCR in steroidogenic MA-10 cells that express very high levels of TSPO showed no difference when compared with TSPO-deleted MA-10 cells (Tu *et al*. 2016). All these studies indicate that the change in OCR is a cell-type-dependent indirect effect, inconsistent with TSPO expression levels, perhaps reflecting on the diversity of mitochondrial types, metabolism and energetic status of cells, and therefore does not reveal a direct mechanism for TSPO in regulating OCR.

Tspo-knockout studies in another *in vivo* model, *Drosophila melanogaster*, indicated that *Tspo*$^{-/-}$ flies are without any abnormalities (Lin *et al*. 2014), similar to reports in mice (Banati *et al*. 2014, Tu *et al*. 2014). Interestingly, structures and cells in *Tspo*$^{-/-}$ flies showed increased survival after hydrogen peroxide exposure or γ-irradiation (Lin *et al*. 2014). In only male *Tspo*$^{-/-}$ flies, an extended lifespan was observed compared with male wild-type flies (Lin *et al*. 2014). Isolated *Tspo*-deficient mitochondria from these flies, irrespective of sex, showed a decreased rate of oxidative phosphorylation compared with *Tspo*$^{+/+}$ mitochondria (Lin *et al*. 2014). Continued studies in *Tspo*$^{-/-}$ D. *melanogaster* have linked TSPO function to ethanol sensitivity and tolerance (Lin *et al*. 2015). These seemingly disparate effects point to general mechanisms that surround mitochondrial function and do not pinpoint a specific indication for TSPO function.

Along the lines of cellular energy metabolism, a drug screen for identifying small molecules that induce expression of the gluconeogenic gene, phosphoenolpyruvate carboxykinase 1 (*Pck1*), using a transgenic zebrafish reporter line, PK11195 was identified as an agent that activates a state akin to fasting metabolism (Gut *et al*. 2013). Although this seems to be an exciting observation, it is unclear if the major effects observed are mediated through TSPO. This is because PK11195 is also known to bind the constitutive androstane receptor (CAR) (Li *et al*. 2008, Anderson *et al*. 2011), which can affect *Pck1* transcription, together with other genes that affect energy

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metabolism (Ueda *et al*. 2002). The overall functions of CAR could also be linked to fasting metabolism (Maglich *et al*. 2004, Ding *et al*. 2006). Therefore, interpretation of PK11195 or other drug effects in the context of TSPO needs further affirmation using genetic models that can delineate off-target effects from specific effects.

4. TSPO as a regulator of lipid metabolism: Two independent exploratory studies identified *Tspo* as a candidate gene that could influence lipid metabolism. First, *Tspo* was identified as one of the six novel transcripts that showed robust positive correlation with adipocyte differentiation in a differential display RT-PCR screen (Wade *et al*. 2005). Second, *Tspo* was identified as one of the five genes that could influence triglyceride metabolism in an examination of quantitative trait loci between inbred mouse strains (Leduc *et al*. 2011). In agreement, recent examination of TSPO expression levels in different murine tissues indicated that high TSPO expression correlated with tissues active in lipid storage/metabolism, and was not specific for steroidogenic cells (Fig. 2) (Tu *et al*. 2016). As support for this correlation, it must be noted that steroidogenic cells also have substantial presence of lipid droplets. Measurement of energy metabolism in TSPO-deficient MA-10$^{Tspo\Delta/\Delta}$ Leydig cells that do not show any deficits in steroid biosynthesis revealed a metabolic shift in substrate utilization from glucose to fatty acids compared with TSPO-expressing MA-10 cells. The MA-10$^{Tspo\Delta/\Delta}$ Leydig cells had higher levels of fatty acid oxidation and modest increases in ROS production compared with MA-10 controls (Tu *et al*. 2016).

Although the precise mechanism for this TSPO effect on lipid metabolism remains to be elucidated, there are a few plausible explanations: (1) Putative TSPO–ACBP interaction (Papadopoulos *et al*. 1991*b*) could be a direct link to fatty acid metabolism, perhaps affecting import at the OMM. ACBP is involved in fatty acid metabolism (Lee *et al*. 2007, Neess *et al*. 2011), and its expression mirrors TSPO in tissues (Tong *et al*. 1991, Toranzo *et al*. 1994). (2) Putative TSPO–VDAC1 interaction (McEnery *et al*. 1992). VDAC1 has been associated with the fatty acid transfer complex in the OMM (Lee *et al*. 2011*a*). (3) Physical changes to OMM properties due to loss of an abundant protein. There is evidence that the fatty acid transport protein CPT1A activity could be regulated by changes in OMM lipid composition and molecular order (Rao *et al*. 2011). Irrespective of the precise mechanism, a function for TSPO in fatty acid metabolism could explain its abundant presence in cells that are active in lipid storage. This function is also of particular interest in studying inflammatory cells and glia activation that result in TSPO upregulation, a mechanism that may be associated with the metabolic shift, as observed during immune cell activation (reviewed in Pearce & Pearce 2013). These clues provide a new direction for TSPO function that is indeed worthy of further investigation.

Neurosteroids and multiple sclerosis pathogenesis

Multiple sclerosis (MS) is an autoimmune disease characterized by inflammatory lesions and ensuing neurodegeneration in parts of the CNS. These pathologic lesions have prominent upregulation of TSPO (Harberts *et al*. 2013), and therefore, use of TSPO as a biomarker for detecting MS lesions is of significant interest. Numerous studies have demonstrated a link between neurosteroids and neuroinflammatory diseases like multiple sclerosis (see reviews El-Etr *et al*. 2005, Kipp & Beyer 2009, Noorbakhsh *et al*. 2014). Neurosteroid effects have been linked to promoting myelination (Melcangi *et al*. 1999, Ghoumari *et al*. 2003), inducing proliferation of neuroprogenitor cells (Ghoumari *et al*. 2005, Wang *et al*. 2005), reducing proinflammatory effects mediated by activated macrophages and glia (Ghezzi *et al*. 2000, Lee *et al*. 2011*b*) and reducing blood–brain barrier dysfunction (Ishrat *et al*. 2012). In human MS patients, neurosteroid levels were significantly reduced in the brain white matter, a finding that was consistent in EAE disease model in mice (Noorbakhsh *et al*. 2011). Therapeutic use of steroids has decreased the severity of EAE in animal models (Yates *et al*. 2010, Yu *et al*. 2010). TSPO upregulation is observed in microglia, infiltrating macrophages and astrocytes in response to injury/inflammatory insults (Cosenza-Nashat *et al*. 2009). The popular explanation for TSPO upregulation at MS lesions has been that it could have a protective effect by limiting inflammatory damage through promoting neurosteroid production. TSPO-binding drugs were considered to enhance this mechanism (Daugherty *et al*. 2013, Ravikumar *et al*. 2016). In a preliminary investigation using hGFAP-cre driven *Tspo*$^{c\Delta/\Delta}$ mice with recombination expected in astrocytes and postnatal progenitors leading to neurons and oligodendrocytes, it was demonstrated that TSPO deficiency could ameliorate severity of EAE (Daugherty *et al*. 2016). TSPO is upregulated in 'reactive' astrocytes at EAE lesions, albeit in a different spatiotemporal pattern compared with microglia/macrophages (Daugherty *et al*. 2013).

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Although neurosteroid production was not directly measured in these astrocyte and postnatal neuron-specific *Tspo*$^{c\Delta/\Delta}$ mice (Daugherty *et al*. 2016), the observation that TSPO presence is contributing to and not protective of the inflammatory pathology challenges the explanations linking TSPO and neurosteroid production. These emerging results from the use of TSPO-deficient mice in disease models not only confirm that TSPO is an important player in neuroinflammation, but also underscore the need to understand its core mechanism of action.

Concluding remarks

An early study that examined TSPO localization by autoradiography in whole-body rat sections concluded that expression was most concentrated in adrenals and skin, with substantial levels also evident in the heart, salivary glands, discrete regions of the kidney, epithelium of the lung, nasal and lingual epithelia, lining of the pulmonary artery, thymus, hair follicles, tooth buds and the bone marrow (Anholt *et al*. 1985*b*). We have come a long way from that initial observation, but functional interpretations for the past 30 years seem to be confounded by nonspecific outcomes and an apparent narrow focus toward determining TSPO function in divergent directions. This has also led to disparities in explaining TSPO function across different fields.

With initial links made to steroidogenesis, together with the increasing popularity of TSPO as a diagnostic marker and therapeutic target, the concept of its putative involvement in mitochondrial cholesterol import was perpetuated as a plausible explanation. In the field of neuroscience, this paradigm was adopted as the foundation for TSPO involvement in neurosteroid production. Recent definitive studies using both *in vitro* and *in vivo* genetic models have refuted the core basis of this explanation, and challenged the mechanisms underlying pharmacological effects observed using TSPO-binding drugs. Although the genetic evidence is clear, the recurrent use of steroid production as a mechanism to explain TSPO actions has made this paradigm shift difficult to comprehend, mainly because there exists no clear explanation for the precise function for TSPO.

Use of pharmacological agents to establish TSPO function is highly problematic due to the structural variations in compounds, differences in binding affinity, nuances of binding sites, complexities of *in vivo* systems and idiosyncratic off-target effects. Interpreting results from studies that use TSPO-binding drugs requires caution to avoid promulgating misconstrued TSPO functions. *In vivo* and *in vitro* *Tspo*$^{-/-}$ models could serve as a great system to test for ligand specificity and TSPO-mediated pharmacological effects (Middleton *et al*. 2015).

The alternate perspectives on TSPO action presented in this review are based on current information available on the attempts to explain its function. From what we know of these proposed mechanisms, it is evident that additional studies are necessary to decipher the primary action of TSPO. It must also be noted that these proposals may not be correct and do not preclude TSPO involvement in other distinct cellular functions. The workings sought for TSPO function need to be a unifying action relevant for explaining functional properties in different tissues, and the organism as a whole.

In summary, although the TSPO link to neurosteroid production remains to be directly tested using genetic models, it is becoming increasingly clear that the relationship may not be direct. At the present time, studies on TSPO with a focus on defining its physiological function, which would ultimately provide insight into its action in the nervous system, are necessary before pharmacological intervention for therapeutic benefits can be approached with confidence.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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