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THE CHANGING LANDSCAPE IN TRANSLOCATOR PROTEIN (TSPO) FUNCTION

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Abstract

Translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), is an outer mitochondrial membrane protein. TSPO has been shown to cooperate with the steroidogenic acute regulatory protein (StAR) and function in the transport of cholesterol into mitochondria. TSPO has also been considered as a structural component of the mitochondrial permeability transition pore. However, recent advances have changed these views of TSPO functions and have prompted a re-evaluation of established concepts. This review summarizes the history of TSPO, key elements of the debate, and functional experiments that have changed our understanding. Moving forward, we examine how this fundamental change impacts understanding of TSPO and affects the future of TSPO as a therapeutic and diagnostic target.

Keywords

mitochondria; benzodiazepine; cholesterol; steroid hormone; permeability transition; therapy

TSPO: a protein with a long history

Function of the translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR, see glossary), has been a topic of active research for the past 25 years. It was initially described in 1977 as a peripheral receptor for benzodiazepines, distinct from the central nervous system benzodiazepine receptor (γ-aminobutyric acid type A receptor/$GABA_A$ receptor), and its pharmacological characterization has been extensive ever since [1, 2]. Specificity of chemicals like PK11195 (an isoquinoline carboxamide derivative) and Ro5–4864 (4'-chlorodiazepam) that could bind to TSPO with high affinity, but not to the $GABA_A$ receptor, were exploited to study potential cellular actions mediated by TSPO such as in steroidogenesis and apoptosis [3]. Protein sequence for TSPO is fairly

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conserved from bacteria to humans (Figure 1), suggesting a possible evolutionarily conserved fundamental role for TSPO in cellular and organismal physiology [4].

Early studies highlighted predominant TSPO localization to the mitochondrial outer membrane. Although TSPO was widely detected in multiple organs, its expression appeared particularly high in steroid hormone producing cells of the adrenal glands, testes and ovaries. Two key studies linked TSPO function to steroid hormone biosynthesis: First, TSPO binding by PK11195 and Ro5–4864 could induce steroid hormone production in both adrenocortical and Leydig tumor cell lines [5, 6]. Second, TSPO knockdown or disruption by homologous recombination in rat Leydig tumor cells could decrease steroid hormone production [7, 8]. These studies were deemed highly significant because they demonstrated that TSPO effects were independent of the steroidogenic acute regulatory protein (StAR), a key player in mitochondrial cholesterol transport required for steroidogenesis (Box 1). As a result, TSPO was thought to function as the channel that receives cholesterol from StAR and mediates its transport to the mitochondrial inner membrane. This steroidogenic function for TSPO was the most studied among all its implicated properties. Given this putative link, up- or downregulation of TSPO expression in different tissues has been considered a direct indication of steroid hormone production in numerous studies [9–11]. Results that describe TSPO and steroidogenesis have been highlighted in recent literature reviews on this topic [12, 13].

Co-purification of TSPO with key molecular components previously implicated to form the mitochondrial permeability transition pore (MPTP) resulted in its inclusion as a regulator of this process [14]. Mitochondrial permeability transition (MPT) refers to a sudden increase in permeability of the inner mitochondrial membrane, a mechanism that has been associated with cell death seen in different human pathologies (Box 2). Early biochemical studies seeking core MPTP components identified the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane, the adenine nucleotide transporter (ANT) in the inner mitochondrial membrane, and Cyclophilin D (CyPD) in the matrix [15]. Demonstration that TSPO could be co-purified with VDAC and ANT resulted in attempts to dissect the effects of TSPO binding chemicals as means to regulate MPT. The chemicals PK11195 and Ro5–4864 were found to affect MPTP opening [16, 17]. The endogenous protoporphyrin IX, a heme precursor that binds with high affinity to TSPO could also mediate MPTP opening [18]. As a result, the VDAC/ANT/TSPO model became widely accepted as the structure of the MPTP.

Subsequent genetic analysis of the putative core components of the MPTP systematically excluded a role for all genes encoding VDAC [19] and ANT [20] in MPT. These findings set the field on a new course that led to the recent definition of the molecular nature of the MPTP, in that it is formed by dimers of F_0F_1 ATP synthase [21]. Although a role for TSPO was not discussed in the new model, it remains to be explained how TSPO binding chemicals could modulate cell death processes. The pharmacological evidence supporting a TSPO link to MPTP appears well documented [22–25]. Developments in the field of MPT have been extensively described in a recent literature review on this topic [26].

TSPO as a biomarker in human disease pathology

TSPO expression in the central nervous system is very low under normal physiological conditions and restricted to astrocytes and microglia. However, in response to brain injury and inflammation, TSPO levels dramatically increase in these glial cells [27]. First identified for its pharmacological binding profile, use of selective and high-affinity TSPO binding chemicals like PK11195 labeled with radioisotope tracers aided in visualization of affected brain regions in several neurodegenerative diseases [2, 28]. This prospect led to extensive efforts to develop novel synthetic chemicals that bind with high affinity and specificity to TSPO for diagnostic imaging *in vivo* [28]. Use of TSPO as a diagnostic marker has been reliable, and several TSPO binding chemicals as positron emission tomography (PET) tracers have progressed from preclinical studies to human clinical trials. Clinical trials using TSPO binding chemicals were focused on diagnosis of a variety of pathologies including: traumatic brain injury (NCT01547780), Alzheimer's disease (NCT01209156; NCT01028209), Parkinson's disease (NCT01527695; NCT01028209), multiple sclerosis (NCT01428505; NCT00432900; NCT01028209), encephalopathy (NCT00459693), autism (NCT0132255), neuroinflammation (NCT02062099; NCT02233868; NCT02009826; NCT01881646), neurodegeneration (NCT02086240), dementia (NCT00613119) and neurocysticercosis (NCT00526916) [Source: www.clinicaltrials.gov].

Identical to inflammation and injury seen in the brain, TSPO overexpression is also observed in cardiac pathologies [29, 30], and in different cancers [31]. TSPO binding chemicals for use as diagnostic PET tracers to locate cardiac conditions and track cancer development are also under development. Human clinical trials to detect cardiac sarcoidosis (NCT02017522), carotid atherosclerosis (NCT00547976), and squamous and basal cell carcinomas (NCT01265472) have either been completed or are currently underway.

Exploiting TSPO upregulation in pathologies for diagnostic imaging as described above has potential to grow and extend across multiple fields. Although imaging complications have been encountered as a result of *in vivo* metabolism of these TSPO binding PET tracers and aberrant signals contributing to non-specific noise in some cases, new synthetic TSPO binding chemicals are being developed to tackle these drawbacks [32]. Therefore, diagnostic imaging is probably the primary clinical value that TSPO research has to offer at this present time.

What is the therapeutic potential of TSPO binding chemicals?

Upregulation of TSPO expression at sites of inflammation and injury has often resulted in questions regarding its context in disease etiology. Effects observed as a result of TSPO binding have in fact led to several seemingly disparate biological interpretations of TSPO function [1, 3]. Nevertheless, treatment with TSPO binding drugs has proven efficacious in ameliorating disease pathology in numerous preclinical models. Examples in the central nervous system include: multiple sclerosis, traumatic injury, excitotoxicity, contusion, neuropathy, neuroinflammatory pain, and anxiety disorders (reviewed in [2]). In a recent study it was demonstrated that the TSPO binding drug Ro5–4864 could not only attenuate, but also reverse the pathology associated with Alzheimer's disease *in vivo* [33]. A large part

of these positive effects were attributed to the function for TSPO in steroid hormone production [34]. Human clinical trials were performed for a TSPO "agonist" XBD173 (AC-5216, Emapunil) for treatment of anxiety disorders by Novartis and Dainippon Sumitomo Pharma (NCT00108836). According to the proof of concept: XBD173 can bind to TSPO and induce production of neurosteroids, which in turn can potentiate $GABA_A$ receptor-based neurotransmission and bring about anxiolytic effects. Promising outcomes were reported using a high dose of XBD173 in an induced model of panic disorder, but at lower doses XBD173 effects showed no efficacy and were not different from placebo [35]. High TSPO binding variability across human subjects was observed on subsequent testing of XBD173 [36]. Currently, XBD173 is not in the drug development pipeline of either of these companies.

TSPO binding drugs have also been demonstrated to cause death of cancer cells, and as a result they are also considered as potential anti-cancer therapeutics (reviewed in [31]). This cell death mediated by TSPO binding drugs in cancer cells has been linked to their putative role in activating MPT. Linked to a similar mechanism, use of TSPO binding drugs after ischemia-reperfusion injury in the heart offered protection to cardiomyocytes and reduced infarct size [37, 38].

Even without a direct etiological link, this quest for TSPO binding therapeutics has been quite extensive in recent years and applied to a variety of diseases. These investigations, prompted by the pressing need for drug discovery, were based on the established mitochondrial function of TSPO and its upregulation in pathological lesions. However, as the landscape in TSPO physiology is rapidly changing, these TSPO attributes need to be carefully re-evaluated.

Is there a role for TSPO in steroidogenesis?

Recent studies examining TSPO function using genetic approaches have changed our views of its involvement in steroidogenesis. Using a conditional knockout mouse with TSPO deletion in Leydig cells, it was demonstrated that lack of TSPO did not compromise testosterone production *in vivo* [39]. This was a surprising result [40], and it raised the question of whether findings in Leydig cells could be extrapolated to other steroidogenic cell types [41]. Subsequently, another study also demonstrated that global TSPO deletion ($TSPO^{-/-}$) in mice did not affect viability, fertility and the ability to generate steroid hormones [42]. $TSPO^{-/-}$ mice did not show any apparent abnormalities and produced both gonadal and adrenal steroid hormones at levels similar to control mice. These results were contradictory to results from a previous report showing that TSPO knockout in mice was early embryonic lethal [43]. In order to understand these conflicting findings, additional experiments were performed including knockdown of TSPO expression using siRNA in different steroidogenic cell lines (Mouse Leydig: MA-10 and MLTC; Mouse adrenal: Y1; Rat Leydig: R2C) to test for steroid hormone production. In this experimental setting, steroidogenesis was not affected [42], contradictory to a previous result suggesting that TSPO knockdown inhibited steroidogenic capacity in MA-10 cells [8]. In fact, it was observed that in one of the cell lines utilized, the human adrenal H295R cell line, there was no expression of TSPO mRNA or protein, yet this cell line can produce levels of steroid hormones equivalent to wild type

cells, clearly supporting the notion that TSPO is not involved in steroidogenesis [42]. Another discordant finding was with regards to the impact of TSPO on cell viability. It was previously shown that cells with TSPO knockdown of >70% cannot survive [44, 45], however, in later studies, *in vitro* TSPO knockdown of >80% in different steroidogenic cell lines showed no effect on viability [42]. In strong corroboration of evidence against TSPO in steroidogenesis, a subsequent recent study described an independently generated $TSPO^{-/-}$ mouse to have no defects in steroid hormone biosynthesis [46]. This study also showed that isolated steroidogenic mitochondria devoid of TSPO did not have any deficits in cholesterol transfer suggesting that TSPO was not involved in this mitochondrial process [46].

In addition, another recent study demonstrated that complete disruption of TSPO in the MA-10 cell line (MA-10: *Tspo*Δ/Δ) using CRISPR/Cas9-mediated mutagenesis did not have any effect on viability of the cells or steroidogenesis [47]. This result was again not consistent with another early report that mono-allelic disruption of TSPO in the R2C Leydig cell line obliterated its steroidogenic potential and caused phenotypic abnormality [7]. However, changes in phenotype or issues with viability were not observed in three independent clones of the MA-10: *Tspo*Δ/Δ cells examined in the recent study [47], suggesting that TSPO deletion does not affect essential cellular functions. Moreover, when MA-10: *Tspo*Δ/Δ cell clones were used to examine the steroidogenic ability of the TSPO binding chemical PK11195, both control and TSPO deficient cells showed identical increases in steroid hormone production [47]. This finding indicated that the effect of PK11195 on steroidogenesis in MA-10 cells is not mediated through TSPO, suggesting that previous studies reporting the pharmacological link between TSPO and steroidogenesis in this cell line and at the same range of concentrations [5, 6], were in all probability, off-target effects.

It has been recently proposed that alteration of physical membrane properties due to incorporation of TSPO binding chemicals into the membrane bilayer could contribute to some TSPO-independent effects [48]. Other studies have also associated effects of TSPO binding chemicals to membrane cholesterol accumulation/modulation [49, 50] and steroidogenesis [51]; but in the light of recent shift in understanding, whether these observations are indeed mediated through TSPO is a question that needs confirmation.

Collectively, recent results uncouple TSPO from steroidogenesis and cell viability thus opening the question of what is the true function of the protein.

TSPO and the mitochondrial permeability transition

A recent study using liver and heart specific conditional TSPO knockout mice concluded that TSPO is not involved in the regulation of MPT, or structure of the MPTP [52]. Results from this study showed that MPT could occur in the complete absence of TSPO. The link between TSPO and MPT was established by tests using TSPO binding chemicals that demonstrated an effect on MPT. However, these results published in numerous manuscripts over a period of 20 years indicate that effects mediated by the TSPO binding chemicals including PK11195 and Ro5–4864 were highly variable, and depend on experimental setting as well as the concentration used (nanomolar to micromole), and in some cases could even

be biphasic [16, 17, 45, 53]. In addition, different classes of TSPO binding chemicals bind to different regions in the TSPO structure with variable specificity. Nevertheless, these effects were considered to be specific, as it was demonstrated that anti-TSPO antibodies could block MPT [23] induced by the endogenous TSPO binding heme precursor, protoporphyrin IX [18, 54]. But, recent experiments using $TSPO^{-/-}$ cells indicated that the effects of both the endogenous protoporphyrin IX and the synthetic PK11195 and Ro5–4864 TSPO ligands were not mediated through TSPO [52], as shown previously [54], and that the mechanism by which outer mitochondrial membrane regulates MPT did not involve TSPO [52]. As a result, these observations have challenged previous findings implicating TSPO in MPT opening for a variety of pathological processes.

The mechanism underlying how MPT could still be activated by TSPO binding chemicals remains an interesting question. The new knowledge that the F_0F_1 ATP synthase forms the MPTP [21], and that TSPO binding chemicals like PK11195 can interact with, and inhibit F_0F_1 ATP synthase [55–57], suggests that the effects observed with these chemicals on MPT may be independent of TSPO [26].

Seeking a function for TSPO

The experimental evidence linking TSPO function to steroidogenesis and MPT over the past 25 years is substantial, but it should be noted that most studies were performed using TSPO binding chemicals. The finding that TSPO might not be involved in steroidogenesis was surprising. Similarly, excluding TSPO involvement in MPT was also unexpected. In both cases, genetic deletion of TSPO showed no effect on the purported functions [39, 42, 46, 52], and that the effects of TSPO binding chemicals on both steroidogenesis and MPT are not mediated through TSPO [47, 52]. These results refute a function for TSPO in both steroidogenesis and MPT and the core pharmacological basis for both models (Fig 2). Although the outcome for TSPO in these contexts can be considered negative, these developments are a substantial leap for research progress in these fields.

At the present time, we propose that mammalian TSPO is left without a known function. Although studies have elaborated TSPO's structural, biochemical and pharmacological properties, we believe that its precise physiological and pathological functions remain unknown. Future research will need to focus on examining mitochondrial functions in a comprehensive way including respiration, bioenergetics, metabolism, and stress responses, and also cautiously take into account distinct yet unexplained pharmacological observations [58]. Recent observations correlated with TSPO knockdown in different cells like activation of endoplasmic reticulum-associated protein degradation [59], inhibition of autophagy [60], decrease of cholesterol efflux [50], and increase pro-inflammatory cytokine production [61], also need careful consideration to identify the core structure-function relationship to TSPO (Fig 2). In addition, information from TSPO in lower organisms that include functions like oxygen sensing in photosynthetic bacteria [62], stress regulation in plants [63], apoptosis mediation and longevity in drosophila [64], and erythropoiesis regulation in zebrafish [65], should be evaluated for an evolutionary link when studying mammalian systems. However, it should also be taken into account that TSPO homology and organelle localization in these organisms may vary. Understanding the exact physiological mechanism of mammalian

TSPO action is imperative to delineating its organismal function(s), which will lead to better comprehension of its pharmacology (Box 3), and will unlock its potential as a diagnostic/therapeutic target.

Concluding remarks and future perspectives

In recent years, poor and frequently questionable validation of targets under investigation has led to drop in success rates of Phase II clinical drug trials from 28% to 18% [66]. A majority of these cases have resulted from the irreproducibility of published results that describe key findings [67]. This emphasizes the need to thoroughly investigate the pathways being targeted for drug discovery. With regards to TSPO, key findings supporting the TSPO-steroidogenesis model by knockdown in cell lines [8], gene deletion in cell lines [7], and gene deletion in mice [43] have not been reproducible in the same homologous systems [39, 46, 68], raising the question of whether mechanisms of functional redundancy are at play. We believe that if redundant or alternative mechanisms exist to accomplish TSPO function as recently suggested [69], they should have become evident in earlier studies. Thus, one must be open-minded to the possibility that TSPO function is yet to be identified. Previous studies using TSPO binding chemicals have indeed reported non-TSPO mediated effects [70–72]. We can only speculate that these effects represent non-specific integration of the chemicals perturbing cellular membrane bilayers [48] and/or unknown off-target effects in the case of steroidogenesis, or binding of TSPO ligands to F_0F_1 ATP synthase [55–57] in the case of MPT. It is also possible that these observations may be due to unknown effects due to complex crosstalk across multiple distinct pathways [73, 74]. So without understanding physiological function, it might never be possible to determine the exact pharmacological effects of specific TSPO binding chemicals (Box 4). We believe and hope that the emergence of viable $TSPO^{-/-}$ animals and $TSPO^{-/-}$ cell lines will inject new enthusiasm into the field and will offer an opportunity to re-evaluate the physiology of TSPO. These TSPO null models represent a new experimental approach to delineate the specific TSPO-based effects mediated by TSPO binding chemicals that will allow for a more focused understanding of its therapeutic potential.

As we look into the future, several important questions for TSPO remain (Box 5). Should investment in drug discovery based on TSPO continue? As a major part of TSPO research involves diagnostic *in vivo* imaging for changes in TSPO expression as indicators of inflammation and injury, it seems reasonable that even without a functional definition for TSPO, clinical trials assessing the specificity and diagnostic capabilities of TSPO binding chemicals should proceed. However, for meaningful therapeutic targeting of TSPO, mechanistic understanding of the protein function followed by designing of true (specific) ligands that act as agonists or antagonists to TSPO's yet to be uncovered function is warranted. Deciphering complex off-target effects of TSPO binding chemicals from existing literature/datasets to glean meaningful information may not be a pragmatic approach to address this problem. But as a practical twist, alternative targets that may mediate some of these beneficial effects could be identified to open new areas of exploration in drug discovery. Conclusively, understanding the precise physiological function for TSPO will be necessary to fully realize its potential as a therapeutic target.

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Glossary

Benzodiazepines
Refers to a class of drugs that are clinically used as muscle relaxants, anticonvulsants and sedative-hypnotics. Pharmacology of benzodiazepines is primarily mediated by its binding to the 'central' benzodiazepine receptor (γ-aminobutyric acid type A receptor/$GABA_A$ receptor) located in the central nervous system and potentiating inhibitory neurotransmission.

Mitochondrial cholesterol transport
Mitochondria are cellular organelles that consist of a double membrane creating two distinct internal compartments. For steroid hormone production, cholesterol must be transported through the outer mitochondrial membrane across the aqueous inter-membrane space to the matrix side of the inner mitochondrial membrane before it can be converted to pregnenolone. This is an essential and rate-limiting step in steroid hormone biosynthesis.

Mitochondrial Permeability Transition
Refers to an increase in permeability of the inner mitochondrial membrane to any molecule <1.5 kDa due to opening of a non-specific pore. Mitochondrial permeability transition (MPT) has been associated with cell death seen in numerous pathological conditions.

Mitochondrial Permeability Transition Pore
Refers to the structural components that form the pore in the inner mitochondrial membrane during MPT. Recent evidence suggests that this is formed by dimers of the F_0F_1 ATP synthase.

Peripheral benzodiazepine receptor
Benzodiazepines were observed to also occupy 'peripheral' sites distinct from the 'central' $GABA_A$ receptor on the outer mitochondrial membrane. The peripheral benzodiazepine receptor (PBR) was the protein characterized based on this distinct pharmacology, which was subsequently renamed as the translocator protein (TSPO).

Steroidogenesis
Refers to the biosynthesis of steroid hormones. In this process, steroid hormone producing cells convert cholesterol into pregnenolone in an enzymatic step catalyzed by CYP11A1/P450scc that occurs in the mitochondrial matrix. Pregnenolone is then converted into different classes of steroid hormones by subsequent enzymatic steps that happen within the endoplasmic reticulum and the mitochondria.

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Box 1.

Mitochondrial cholesterol import and steroidogenesis

The first enzymatic step in the biosynthesis of steroid hormones involves the cleavage of the side chain of cholesterol to form pregnenolone. The cytochrome P450 side chain cleavage (CYP11A1) that carries out this reaction resides on the matrix side of the inner mitochondrial membrane. To produce steroid hormones in response to trophic hormone stimulation, it is essential that cholesterol cross the outer mitochondrial membrane, the aqueous intermembrane space, and the inner mitochondrial membrane. This cholesterol import mechanism also forms a regulatory step that is rate limiting for steroid hormone production. It was discovered that rapid *de novo* synthesis of the steroidogenic acute regulatory protein (StAR) plays an indispensable role in mitochondrial cholesterol import required for adrenal and gonadal steroidogenesis (reviewed in [75]). Mutations in StAR were found to be the basis of lipoid congenital adrenal hyperplasia, and similar to this human condition, StAR gene-deletion in mice revealed an almost complete inability to synthesize steroid hormones. Nevertheless, the gap in understanding the precise mechanism by which StAR mediates mitochondrial cholesterol import, and the fact that StAR is not expressed in steroidogenic cells of the human placenta, led to speculations that another player that works at the level of the outer mitochondrial membrane in cooperation with StAR may be essential. TSPO filled the void in this model with overwhelming pharmacological indicators, but without direct evidence. Recent demonstration that TSPO may not be involved in steroidogenesis has reopened this informational gap in the cholesterol import model.

Box 2.

Mitochondrial permeability transition (MPT)

The MPT refers to an increase in permeability of the inner mitochondrial membrane to any molecule <1.5 kDa due to opening of a non-specific pore [76–78]. Based on recent evidence, the MPT pore (MPTP) is formed by dimers of F_0F_1 ATP synthase. Opening of this MPTP results in equilibration of the mitochondrial matrix and the cytosol leading to uncoupling, osmotic swelling of the matrix, cristae unfolding and rupture of the outer mitochondrial membrane. The ensuing traumatic release of intermembrane space proteins such as cytochrome *c* and apoptosis inducing factor can trigger apoptosis in these cells. Matrix Ca^{2+} is essential for this process, and MPTP opening is triggered by elevated matrix Ca^{2+} concentration. Although transient opening of the MPTP may exist under physiological conditions, persistent opening of the MPTP has been associated with cell death seen in numerous pathological conditions.

Box 3.

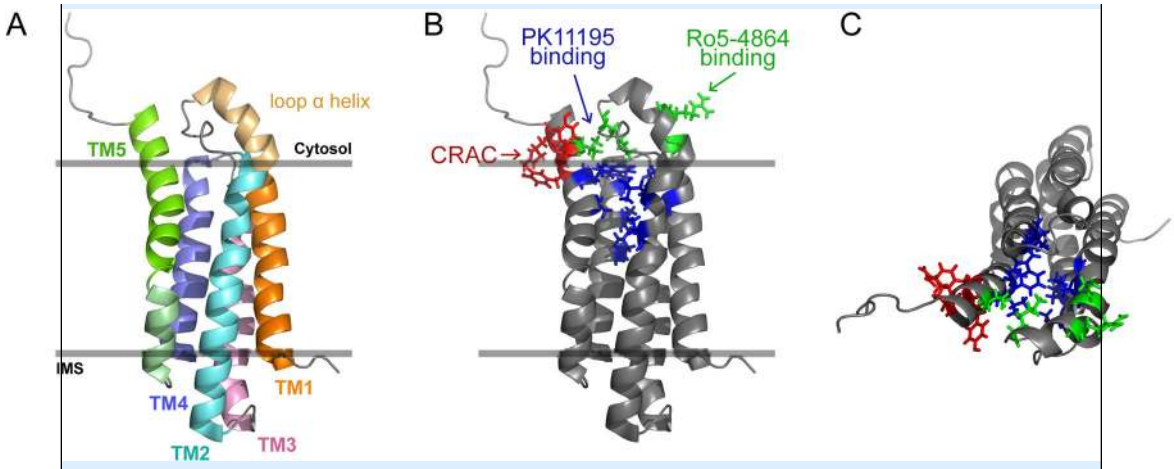
Are TSPO binding chemicals true ligands?

The word ligand used loosely in TSPO literature refers to all substances that bind to TSPO at different distinct sites; the findings about responses to ligand binding have also been inconsistent and diverse in the TSPO literature ranging from steroid hormone production to cell proliferation and apoptosis. By definition, ligand in biochemistry and pharmacology refers to a small molecular substance that binds to a target biomolecule (or a complex) and elicits/modulates a specific biological response (agonistic or antagonistic) that is directly associated with the target's function. With genetic models showing that physiological TSPO function does not correspond to pharmacological effects recorded in experiments using TSPO binding chemicals, it is reasonable to propose that these chemicals are not acting as ligands. Moving forward, it will be prudent to interpret binding results carefully and tie their pharmacologic effects to physiologic ones.

Box 4.

What does TSPO translocate?

In 2006, appeal from a scientific consortium (supported by Novartis Pharmaceuticals, Basel, Switzerland) [79], resulted in renaming of the peripheral benzodiazepine receptor (PBR) to the translocator protein (TSPO). The basis for this change in nomenclature was prompted by a desire to better reflect the structure and molecular "function" of this protein [79]. At that time, experimental homology modeling of the structure of mammalian TSPO was interpreted as a channel-like structure with a hydrophobic interior core lined by the cholesterol recognition amino acid consensus (CRAC) motif for putative translocation of lipophilic substrates. The key substrate in question was cholesterol, but translocation of other substances like porphyrins, and translocation of a variety of molecules during mitochondrial permeability transition (MPT) was also considered a possibility. Although cholesterol transport was considered the preeminent function for TSPO, direct physiological proof for such a function does not exist. A more recent high-resolution NMR structure of TSPO showed that the side chains of the CRAC motif (Figure I), considered central for cholesterol translocation, is located on the outside pointing towards the membrane environment [80] suggesting that previous homology models are inaccurate. In this study, structural evidence for TSPO homo-oligomerization, considered to be an important feature for cholesterol transport, was not observed. This supported other observations in the literature that challenged this concept [39, 68]. Moreover, recent physiological data show that TSPO is neither involved in mitochondrial cholesterol import required for steroidogenesis, nor in the regulation of mitochondrial permeability transition [39, 42, 52]. Besides steroidogenesis and MPT, it remains to be confirmed if mammalian TSPO plays a role in porphyrin transport and heme synthesis. Therefore, despite the name change, potential 'translocator' functions for TSPO still remain as unproven hypotheses.



BOX 4, Figure I. TSPO structure showing distinct cholesterol and PK11195 binding sites

(A) Structure of TSPO in the outer mitochondrial membrane (side view) showing the five α-helix transmembrane structure (TM1–5; IMS - inter-membrane space).

(B) TSPO structure showing: (1) location of the cholesterol recognition amino acid consensus (CRAC) motif at the C terminus (residues 147–159); side chains Tyr^{152}, Tyr^{153} and Arg^{156} (in red), essential for cholesterol binding are located on the outside of the TSPO structure and point toward the membrane environment. (2) Side chains of PK11195 binding pocket formed by residues Ala^{23}, Val^{26}, Leu^{49}, Ala^{50}, Ile^{52}, Trp^{107}, Ala^{110}, Leu^{114}, Ala^{147} and Leu^{150} (in blue), that do not involve side chains of CRAC motif amino acids. (3) Binding site for Ro5–4864 is distinct from that of PK11195 and was identified to include residues Glu^{29}, Arg^{32}, Lys^{39} and Val^{154} (in green) [81].

(C) Top view: Cholesterol binding side chains of the CRAC motif pointing outside of the TSPO structure and do not interfere with internal PK11195 binding site. Images were constructed from the high-resolution NMR structure PDB ID: 2MGY [80].

Box 5.

Outstanding questions

- What is the precise function of TSPO?
- In steroidogenesis, what is the mechanism that cooperates with StAR for mitochondrial cholesterol import?
- In MPTP, does TSPO pharmacology still have a case for regulation of MPT?
- What is the pathologic basis for TSPO upregulation in different inflammatory pathologies?
- Without a defined physiological function for TSPO can we consider TSPO a valid therapeutic target? Are the beneficial effects of TSPO binding chemicals specific? Can candidate TSPO binding drugs enter human clinical trials?

Highlights

1. Recent data question the role of TSPO in steroidogenesis and mitochondrial permeability transition and suggest that TSPO is not involved.
2. Exploiting TSPO upregulation in pathologies for diagnostic imaging has potential to grow and extend across multiple fields.
3. The physiological function of TSPO remains undefined and its future as a therapeutic target needs to be carefully reevaluated.

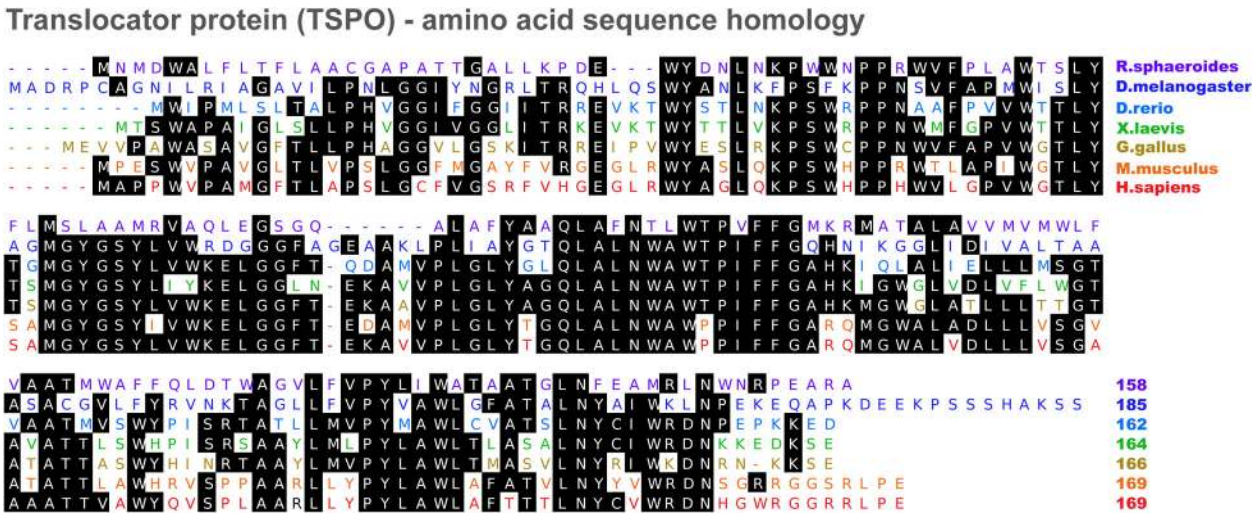


Figure 1. Translocator protein amino acid sequence homology

TSPO amino acid sequence comparisons showing fairly conserved consensus sequences (shaded) in different model organisms. Percent identity to *Homo sapiens*: *Rhodobacter sphaeroides* (33.5%), *Drosophila melanogaster* (42.6%), *Danio rerio* (54.3%), *Xenopus laevis* (57.3%), *Gallus gallus* (60.4%), and *Mus musculus* (81.1%).

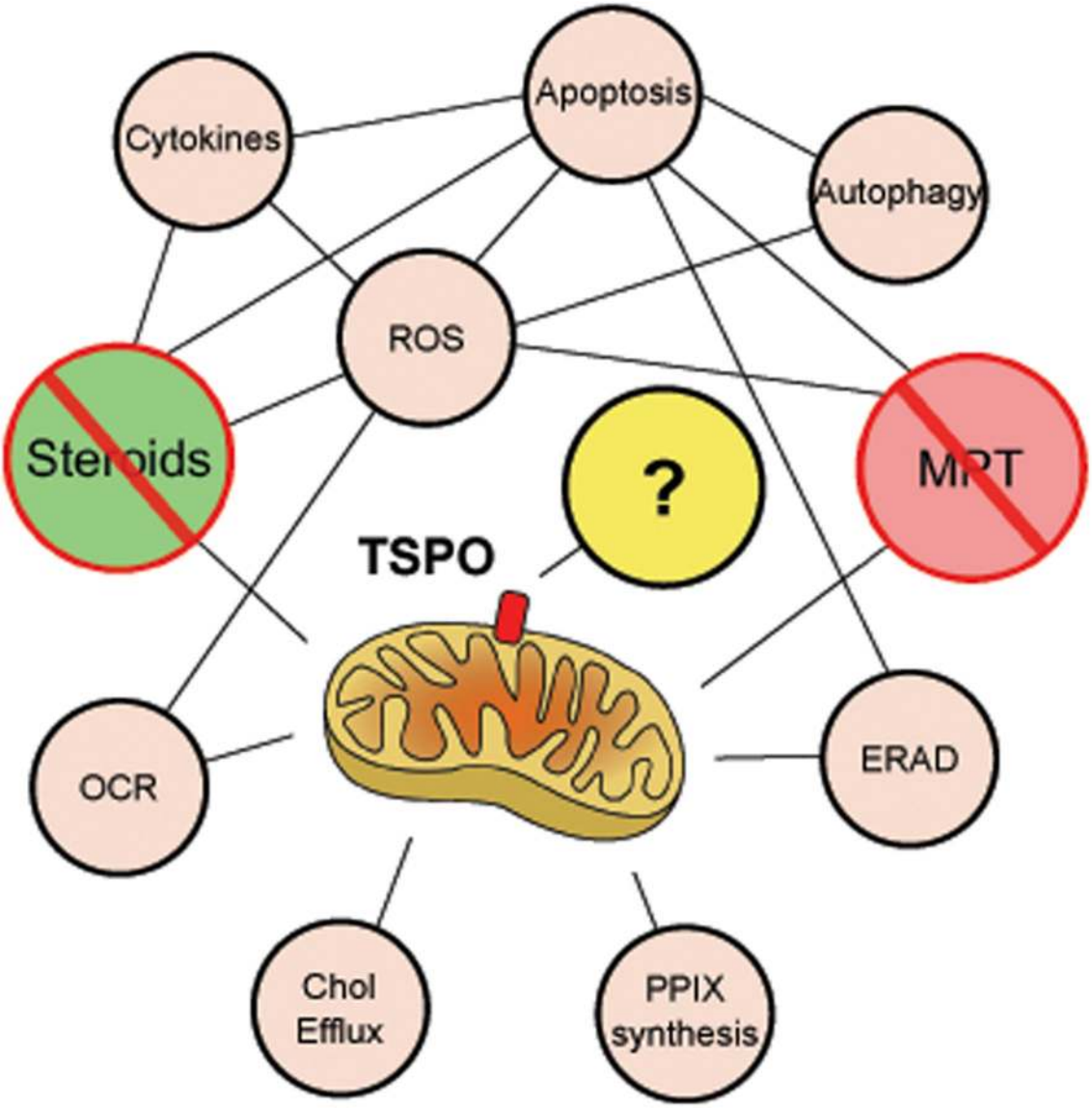


Figure 2. Reexamining the functional network of TSPO

Steroidogenesis and mitochondrial permeability transition, the two major putative TSPO functions associated with its structural properties, and linked to multifarious downstream effects have been refuted. Responses to TSPO knockdown or knockout in cells have recorded changes in reactive oxygen species (ROS) production, cytokine production, apoptosis, autophagy, endoplasmic reticulum associated protein degradation (ERAD), protoporphyrin IX (PPIX) synthesis, cholesterol efflux and mitochondrial oxygen consumption rate (OCR). However, precise function of TSPO in these observations remain to be elucidated.