Translocator protein 18 kDa (TSPO): an old protein with new functions?

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ABBREVIATIONS

TSPO, Translocator Protein 18 kDa; PBR, peripheral-type benzodiazepine receptor; *Rs*TSPO, TSPO from *Rhodobacter sphaeroides*; mTSPO, TSPO from mouse; *Bc*TSPO, TSPO from *Bacillus cereus; At*TSPO, TSPO from *Arabidopsis thaliana;* MPTP, mitochondrial permeability transition pore; VDAC, voltage dependent anion channel; CRAC, Cholesterol Recognition/interaction Amino acid Consensus; DM, decyl maltoside; SDS, sodium dodecyl sulfate; PpIX, protoporphyrin IX; DPC, n-dodecylphosphocholine; WT, wild-type; PDB, Protein Data Bank; NMR, nuclear magnetic resonance.

ABSTRACT

The translocator protein 18 kDa (TSPO) was previously known as the peripheral benzodiazepine receptor (PBR) in eukaryotes, where it is mainly localized to the mitochondrial outer membrane. Considerable evidence indicates that it plays regulatory roles in steroidogenesis and apoptosis, as well as being involved in various human diseases, such as metastatic cancer, Alzheimer's and Parkinson's disease, inflammation and anxiety disorders. Ligands of TSPO are widely used as diagnostic tools and treatment options, despite there being no clear understanding of the function of TSPO. An ortholog in the photosynthetic bacterium, *Rhodobacter,* was independently discovered as the tryptophan rich sensory protein (TspO), and found to play a role in the response to changes in oxygen and light conditions that regulate photosynthesis and respiration. As part of this highly conserved protein family found in all three kingdoms, the rat TSPO is able to rescue the knock-out phenotype in *Rhodobacter*, indicating functional as well as structural conservation. Recently, a major breakthrough in the field was achieved: the determination of atomic resolution structures of TSPO from different species by several independent groups. This now allows us to reexamine the function of TSPO with a molecular perspective. In this review, we focus on recently determined structures of TSPO and their implications for potential functions of this ubiquitous multifaceted protein. We suggest that TSPO is an ancient bacterial receptor/stress sensor that has developed additional interactions, partners and roles in its mitochondrial outer membrane environment in eukaryotes.

Translocator protein 18 kDa (TSPO), also widely know as the peripheral benzodiazepine receptor (PBR), continues to be an intense research focus since its discovery in 1977[1]. In mitochondria, it was first identified as the secondary binding site for the widely used benzodiazepine anxiolytic drugs, but with a different ligand binding profile compared to the central nervous system binding site, the $GABA_A$ receptor[2]. TSPO is highly expressed in many tissues, especially those involved in steroidogenesis, and has been proposed to be an important player in cholesterol transport into mitochondria, the first and rate limiting step for steroid hormone synthesis[3, 4]. A considerable and still growing body of evidence supports TSPO's involvement in a number of other complex cellular processes, including porphyrin transport[5, 6], inflammation[7, 8], tumor progression[9-11], and Parkinson's and Alzheimer's diseases[12, 13]. Because of TSPO's reproducibly high expression in areas of inflammation, ligands for TSPO are widely used and actively developed as imaging agents and treatment options for brain damage[14-16].

Independent of its recognition in animal systems, TSPO was discovered in the carotenoid gene cluster[17] in the photosynthetic bacterium, *Rhodobacter,* a close living relative of mitochondria[18]. It was named the tryptophan-rich sensory protein (TspO), for its high tryptophan content and apparent role in regulation of the transition between photosynthesis and respiration induced by changes in levels of oxygen and light, a signaling process that may involve altered porphyrin transport[19, 20]. Importantly, the rat homolog of TSPO was able to rescue the TspO deletion phenotype in *R. sphaeroides*, indicating a conservation of functions through evolution[21]. TSPO was also identified in many other evolutionarily diverse species, including the plant *Arabidopsis thaliana* (*At*TSPO)[22], moss *Physcomitrella patens* (*Pp*TSPO)[23] and cyanobacteria *Fremyella diplosiphon* (*Fd*TSPO)[24], and *Synechocystis* sp. PCC 6803[25]. In these different species, it is a player in various stress responses, such as salt, oxidative stress and abscisic acid[22,

[23, 26, 27]. As a result of mounting evidence of its involvement in multiple cellular processes, TSPO was renamed as translocator protein 18 kDa (TSPO) in 2006 to recognize its diverse roles[28].

Despite a wealth of data in the literature since the 1970s, our understanding of TSPO function is far from clear. In fact, the essential role of TSPO in embryonic development and as the cholesterol transporter in mammals was recently challenged[29-31], making this protein even more of an enigma. Induction of TSPO under various stress conditions appears to be a common theme, as seen in inflammation in animals, altered light and O_2 conditions in bacteria and exposure to high salt in plants. However, its precise role in any of these processes has not been determined. The fundamental question of whether it acts as a receptor, a transporter/translocator, or possibly an enzyme, is the subject of much debate. Part of the difficulty in understanding TSPO function has related to the lack of atomic resolution structure and *in vitro* functional assay systems for biochemical characterization. Fortunately, some important breakthroughs have recently occurred. High resolution crystal structures of TSPO from two unrelated bacteria, as well as NMR structures of the mouse TSPO (mTSPO), were determined[32-34]. In addition, the ligand binding properties of purified TSPO proteins have been characterized[35, 36]. These new advances facilitate correlation of structure and function of TSPO in molecular detail.

This review will focus on the new TSPO structural information, areas of agreement as well as inconsistencies, and implications for TSPO function. A number of other recent reviews[37-43] illustrate the strong interest in the much-debated physiological functions and drug target potential of this multifaceted protein.

Crystal and NMR Structures – similarities and differences

Crystal structures of TSPO from two unrelated bacteria[32, 33] and NMR structures of the mouse protein[34, 44] were determined in the past two years, providing long awaited structural

information and the opportunity to compare and contrast the several structures.

In all structures of TSPO, the monomer of the protein adopts a helical bundle structure composed of five transmembrane helices (TM) (Figure 1A) as correctly predicted by various methods for identifying TM helices. A long loop (LP1) that connects TM1 and TM2 is also observed as predicted and shows well-defined structure. Despite the fact that the crystal structures are of proteins derived from two distinctly different bacteria, one gram negative (*R. sphaeroides*) and one gram positive (*B. cereus*) with only 23% sequence identity, they are remarkably similar, with or without ligand bound, suggesting a strong conservation of the structural fold during evolution. As shown in Figure 1B, not only the transmembrane helices of the *Rs*TSPO and *Bc*TSPO overlap very well, the LP1 also adopts a very similar structure with a signature short helix in the middle.

In contrast, the structure reported by Jaremko and coworkers, a rigorously analyzed NMR structure of the mouse homolog, mTSPO, shows distinct differences (Figure 1C). It is noteworthy that the protein used for these studies was disordered in the absence of ligand and only in the presence of excess concentrations of the ligand PK11195[34] could a stable tertiary structure be obtained. This contrasts markedly with the well defined biochemical behavior of *Rs*TSPO and *Bc*TSPO without ligand and their highly similar crystal structures (Figure 1B), as well as the virtually identical *Bc*TSPO structures with and without PK11195[33]. These data suggest that the disordered apo-structure of mTSPO results from the conditions of purification and refolding, and is not an example of a natively disordered protein as the investigators propose[45]. Considering that the mTSPO was purified in sodium dodecyl sulfate (SDS) before refolding and reconstitution into dodecylphosphocholine (DPC) for the NMR experiments, there are substantive concerns about both the apo- and ligand-induced conformations.

Indeed, the NMR structure of the mTSPO determined with PK11195 bound differs from the bacterial versions in important ways. Although mTSPO shows higher sequence conservation compared to *Rs*TSPO (32% identity) and *Bc*TSPO (27% identity) than they do to each other, the NMR structure is notably different from both (Figure 1C) with several of the transmembrane helices considerably shifted and side chains having opposite orientations[32, 33]. These alterations lead to substantial numbers of charged residues being exposed on the predicted membrane-embedded surface, unexpected for an integral membrane protein. It is reasonable to propose that the structural difference between mammalian and bacterial proteins, especially in the loop regions, are the result of evolutionary divergence, but at least the alignment of transmembrane regions of membrane proteins is expected to be maintained, as seen in the similarity between the structures of *Rs*TSPO and *Bc*TSPO. Rather than intrinsic differences, it seems more likely that conditions and detergents used in purification and NMR measurements contribute importantly to the differences in structure. Jaremko and colleagues do not report any binding studies to establish that the protein is in a native state under the conditions of their analysis, but others have shown that solubilization of *Rs*TSPO in the ionic detergent DPC results in ~ 20 fold lower binding affinity for PK11195 compared to the binding affinity in the non-ionic detergent decyl maltoside (DM)[46]. A similar detergent-induced alteration in ligand affinity was reported for the mTSPO, in which case PK11195 binding was abolished in the protein purified in SDS but recovered when the protein was reconstituted into liposomes[47]. These observations indicate a substantial detergent-induced effect on binding, perhaps accounting for some of the differences between the NMR structure of the mTSPO and the crystal structures of bacterial TSPO. Jaremko and colleagues have argued that the differences arise from crystal packing effects[44, 48]. However, the published crystal structures were determined from at least 5 different crystal forms, each with

unique differing packing arrangements and intermolecular contacts, and yet all yield the same overall tertiary structure. Thus, it is unlikely that "crystal packing" is the cause of these major discrepancies. It is relevant to note that the recently reported NMR structure of a mutant form of the mTSPO[44], containing the human polymorphism A147T, shows less alteration compared to WT than observed in the crystal structure of the same mutant created in *Rs*TSPO. The authors again suggest that the differences are due to crystal packing effects, but as discussed above, the disparity is more likely to be the result of detergent-induced effects and refolding issues. No data are provided to establish the nativeness of either the WT or the mutant mTSPO after purification or under the NMR conditions.

The first extra-membrane loop, LP1, is structurally conserved

LP1 has previously been proposed to play an important regulatory role in ligand binding to TSPO[49, 50]. Along with significant sequence identity, LP1 in *Rs*TSPO and *Bc*TSPO are almost identical in structure, including the signature short helix in the middle of the loop. Evolutionary co-variance analysis was previously used to identify important side chain interactions across evolution within TSPO and shows remarkable agreement with the crystal structures in predicting the interacting residues[35]. Interestingly, the interaction between W39 on LP1 and the G141 on TM5 (Figure 2), clearly seen in the crystal structure of *Rs*TSPO, was identified by this analysis as one of the most confident predictions of close interactions. Three other interacting pairs involving residues on the short helix within LP1 are also predicted and verified, including W30 with M97, W30 with K36, as well as D32 with R43 in TM2. Figure 2 shows that all predicted pairs are positioned to have favorable side chain interactions in the crystal structure of *Rs*TSPO. Four salt bridges involving predicted residue pairs are also observed, suggesting an evolutionarily stable structure of LP1. These co-variance predictions and structural observations

have two important implications regarding the function of LP1: first, despite limited conservation of LP1 sequence across species, it forms a rather stable structural motif instead of a random flexible loop, especially the second half starting from the highly conserved W30, which suggests an important functional role such as acting as a lid of the central cavity; and second, conserved interactions of LP1 with TM5 could play a role in regulating a lateral entrance to the central cavity for some hydrophobic ligands. Considering that the proposed cholesterol binding site is located on TM5, as is the A139T[51] mutation which has lower cholesterol affinity and shows a significantly narrower opening between TM5 and TM2, it is possible that TM5 and TM2 form a lateral gate in TSPO. The interaction of the second half of LP1 with TM5 (W39/G141) and TM2 (D32/R43) could contribute to its regulation. None of these interactions are seen in the NMR structures of mouse TSPO, where the conformation of LP1 is modeled quite differently, but several are conserved in the *Bc*TSPO structure.

Oligomeric states: is the *Rs*TSPO dimer significant?

Dimerization and oligomerization have been reported for TSPO in human, mouse and *R. sphaeroides in vivo* and *in vitro*[46, 52, 53]. The observation of the highly stable dimeric structure of purified *Rs*TSPO in solution[46] and in several different crystal forms[32, 54] emphasizes the likely importance of the dimer. However, the structure of the *Bc*TSPO was determined in both a dimeric and monomeric state while the NMR structure of the mTSPO was found to be a monomer. In addition, the dimer of *Bc*TSPO has a completely different interface compared to the *Rs*TSPO dimer (Figure 3). These observations bring up important questions that need to be addressed, namely, is the dimer interface conserved and is the dimer functionally significant. *Rs*TSPO forms a very tight and flat dimer interface and no monomer species is observed in solution or in the crystallized state[46]. These findings lend credence to the physiological

significance of the dimer form, also supported by the fact that the main interacting residues in the dimer interface (Figure 3A, cyan triangles), contributed mostly by TM3, are conserved between *Rs*TSPO and the mammalian proteins but not in the *Bc*TSPO. In contrast, the dimer interface in the *Bc*TSPO structure has a much smaller interface involving predominantly the top of TM2, with a sequence quite unique to *Bc*TSPO (cyan dots) (Figure 3). Considering that a monomeric form was used to grow these crystals and the *Bc*TSPO dimer is only observed in some of the crystal forms, this dimer may be a consequence of crystal packing. However, it could also be an alternative interface for higher order oligomerization, as observed in both *Rs*TSPO and the mammalian TSPO[53, 54]. The nature of any oligomeric state of mammalian TSPO cannot be ascertained, since the only structures currently available were obtained by NMR in a monomeric state that may be critical for obtaining high quality NMR data. Considering the evolutionary relationship between *R. sphaeroides* and mitochondria[18], as well as the sequence conservation in the TM3 region (Figure 3A), the same dimer interface is plausible for the mammalian proteins, but alternative and dynamic[53, 55] oligomeric forms involving different partners could be required to accomplish the complex functions of TSPO in mitochondria.

What have we learned about PK11195 and protoporphyrin IX binding sites?

TSPO can be fairly described as an "orphan receptor" since there is still no consensus as to which ligands are physiologically significant. Development of more specific ligands for TSPO continues to be an important focus, because TSPO ligands are widely used as a biomarker for brain inflammation as well as treatment for various diseases[16, 56]. More high resolution structures with different ligands bound will undoubtedly facilitate the drug development effort as well as provide new opportunities for better understanding TSPO function and its regulation.

The synthetic ligand, PK11195, often used as a diagnostic of TSPO involvement, was

resolved in the crystal structure of *Bc*TSPO (Figure 4A) as well as the NMR structure of mTSPO (Figure 4B), while a potential endogenous ligand, a porphyrin type molecule, was resolved in the crystal structure of *Rs*TSPO (Figure 4C,D). In all three structures, these ligands bind to the central cavity but in different positions. The PK11195 is observed in the middle of the cavity, interacting with residues from all five transmembrane helices (Figure 4A, B) whereas the porphyrin is less deeply inserted in a region between TM1 and TM2 (Figure 4C, D). While the central cavity is no doubt playing a major role in ligand binding, the residues involved remain to be further confirmed, since PK11195 interacts in a different orientation and with a different set of residues in the crystal structure of *Bc*TSPO (Figure 4A) compared to the NMR structure of mTSPO (Figure 4B). The altered positions of the side chains and charge due to the conditions required for the NMR structure determination is one likely source of differences. But the precise position is somewhat ambiguous in *Bc*TSPO as well, since it is found in a relatively low resolution crystal structure (3.5 Å) and thus not well resolved. Unambiguous identification of the interacting residues will therefore await additional structures and comparative binding analyses.

The other ligand found in the crystal structures was in the *Rs*TSPO, identified on the basis of a ring-shaped density that could be best fit by a porphyrin-like molecule (Figure 4C, D). This ligand identification is supported by spectra of the purified protein that suggest the presence of a porphyrin oxidation product[32]. An interesting additional clue comes from studies of the *Arabidopsis* homolog of TSPO in which a histidine residue (H91) has been identified to be critical for heme binding[57]. In the *Rs*TSPO structure a proline residue (P47) takes the place of that histidine, but if a heme were bound in the same place as the observed porphyrin, the heme iron would be located correctly to be ligated by a histidine at position 47 (Figure 4D). This observation supports the identification of a porphyrin in this site.

Although the current structures give many important and testable clues regarding the liganded states of TSPO, further studies will be required to establish precisely how PK11195, porphyrin, and many other ligands are bound and how their binding may influence the conformation or aggregation state of the protein.

New clues from structure regarding the cholesterol binding site

TSPO was initially proposed to play an important role in cholesterol metabolism because high expression of the protein was observed in steroidogenic tissue and TSPO ligands were observed to regulate steroidogenesis[3, 58, 59]. It's involvement in cholesterol regulation was further strengthened by findings that TSPO binds cholesterol with nanomolar affinity[47, 60]. However, the precise mechanism of TSPO-cholesterol interaction and the role of TSPO in cholesterol transport into mitochondria remain elusive. The embryonic lethality phenotype first observed in the TSPO knockout mouse[58] has recently been challenged[29-31, 61], emphasizing the need to further investigate the function of TSPO. Mounting evidence suggests that TSPO is not the transporter for cholesterol, but rather is involved in at least two dynamic complexes spanning both the outer and inner mitochondrial membranes, and potentially the ER, which play a role in cholesterol transport and processing[39, 55]. TSPO's unusually high affinity for cholesterol and the effects of its ligands on both transport and processing, make TSPO a likely player in, and regulator of, these processes, especially under stress conditions[62], as well as a promising drug target. Nevertheless, the binding site for cholesterol remains to be precisely defined.

A **C**holesterol **R**ecognition/interaction **A**mino acid **C**onsensus (CRAC) sequence was identified as the cholesterol binding site on the TM5 region of mTSPO by deletion mutational analysis[60]. Confirmation of this site from crystallographic studies is not yet available, but the existing structures of TSPO from the three different species all show that the CRAC sequence is

on the membrane exposed surface of the protein, rather than forming part of a central binding site or a channel at the dimer interface of TSPO. If confirmed, this location of the CRAC site would suggest that a complex of TSPO with other binding partners may be required for cholesterol transport[32, 39] and is consistent with other ligands binding independently and affecting cholesterol binding allosterically.

Insight into cholesterol binding from mutagenesis of *Rs*TSPO

We have taken advantage of the well-characterized *Rs*TSPO protein to further define the nature of cholesterol binding. This bacterial homolog has an intrinsically lower binding affinity for cholesterol, ~ 1000 fold lower than the nanomolar affinity of the human TSPO[46]. By comparative analysis of TSPO sequences, we identified a region one helix turn before the CRAC sequence that was distinctly less hydrophobic (ATA versus LAF) in the bacterial TSPO and much less conserved. When the mammalian version of the three amino acid sequence was substituted into the bacterial TSPO, the binding affinity of *Rs*TSPO for cholesterol was increased to a level similar to the human TSPO[35]. The result, defining a cholesterol binding enhancement motif[35], provides compelling evidence for the location of the cholesterol binding site. It also suggests a potential binding orientation for cholesterol in this region: the ring structure of the cholesterol could associate with the main CRAC sequence while the alkyl tail binds to the enhancement motif, consistent with the evolution of the enhancement motif in the mammalian TSPO family proteins[35] toward higher affinity for steroid-type molecules with a hydrophobic tail.

Computational prediction of a cholesterol binding site

To identify potential cholesterol binding sites, we applied the CholMine algorithm[63] to the *Rs*TSPO crystal structure. This method combines SimSite3D analysis (3-dimensional surface comparison and alignment) with knowledge of conserved interactions in known crystal structures

with cholesterol or cholate already bound, to search other protein structures for three-dimensional cholesterol/cholate binding sites. Compared to traditional sequence motif based prediction, the CholMine method works across diverse protein families and shows higher accuracy (~82%) in predicting true cholesterol or cholate binding sites for either soluble or membrane proteins.

The CholMine analysis predicted a cholesterol binding site on *Rs*TSPO close to the expected position involving TM5 (Figure 5). The most favorable position for cholesterol binding (black) traverses helices TM5 and TM4 (Figure 5A) and matches 8 out of 10 characteristic interactions for cholesterol determined from a series of unrelated cholesterol-bound PDB structures. This cholesterol site parallels the binding site for a monoolein lipid (cyan) found in the same position just below the cholesterol site in all our different crystal structures of TSPO (e.g., PDB#: 4UC1, 4UC2, 5DUO). An additional conserved lipid site is resolved in some structures just above the predicted cholesterol site, as seen in Figure 5B, which shows the location of the cholesterol site in a surface rendering of the TSPO dimer and includes more of the resolved lipids. The stacking arrangement of cholesterol with other lipids has been observed in structures of other membrane proteins, such as the β_2-adrenergic receptor (PDB: 2RH1)[64] where a palmitate (residue 415) is associated with cholesterol molecules (residues 412, 413 and 414). Interestingly, the predicted cholesterol binding site in TSPO is located in the vicinity of the previously defined CRAC site (yellow) and the enhancement motif (orange), shown as surface rendering in Figure 5B, but in the reverse orientation to what we predicted based on the mutagenesis analysis[35]. The tail is oriented toward the outside of the membrane plane and closer to the CRAC rather than the enhancement motif. As to why a bound cholesterol is not resolved in the crystal structure, it is noteworthy that monoolein is used at a high concentration in the lipidic

cubic phase crystallization condition and is very likely to out-compete other lipidic ligands on the protein surface, especially those with relatively low affinity such as cholesterol for *Rs*TSPO.

Binding of bilayer lipids

Determination of native lipid binding sites crystallographically is challenging, given the much higher concentration of non-native lipids or detergents required for the crystallization. Nevertheless, the location of an artificial lipid or detergent in a crystal structure is frequently the site where a native lipid is bound[65-67]. Notably, a number of alkyl chains identified in the crystal structures of *Rs*TSPO are seen consistently occupying the same positions in crystals grown under very different conditions and crystal packing, suggesting that the occupied locations may represent true lipid binding sites (Figure 6). The lipids that are the most consistently well resolved in all four dimeric structures are seen on the surface spanning the dimer interface (overlay of blue, light blue, orange and yellow), supporting the idea that the dimer is an important organizational unit providing the structural basis for strong lipid interactions of potential functional significance.

The functional puzzle of TSPO

The functions of TSPO remain elusive despite many studies in different systems and organisms. A variety of roles have been identified that often seem to be unrelated and sometimes conflicting. A general problem is recognized where conclusions are based on effects of TSPO ligands whose off-target interactions can be a concern. For instance, the benzodiazepine derivative Bz-423 was found to bind specifically to the F_1F_0 ATPase, but not TSPO, and induce the mitochondrial permeability transition[68, 69], raising questions about observations that other benzodiazepine-related ligands considered to be specific for TSPO, such as PK11195, may also induce off-target effects[70-72]. The seemingly definitive TSPO knockout experiments have also led

to conflicting results[31, 61, 62]. However, one consistent finding from knockout experiments appears to be altered mitochondrial energy metabolism, including lower oxygen consumption, membrane potential and ATP levels[40, 41, 61, 73]. This observation fits with the additional finding of increased fatty acid oxidation in the absence of TSPO[74]. But how these observations relate to another emerging common theme, sensing or responding to stress, and how they may be associated with oxygen radical production and regulatory phenomena, remains to be determined.

What is the role of TSPO in cholesterol transport or translocation?

Evidence for TSPO being a *bona fide* transporter is not strong. Binding of both cholesterol and porphyrin to TSPO have been demonstrated *in vitro* and *in vivo* but evidence for direct involvement in transport is still lacking, partially due to the difficulties of assaying the movement of neutral hydrophobic molecules across a membrane. Disappointingly, in none of the new structures of TSPO is cholesterol resolved, nor are there any obvious channels visible within the protein monomer or at the dimer interface[32, 33]. The only stable consistent dimer observed, the *Rs*TSPO dimer, has a remarkably tight interface, suggesting that this is not a transport pathway. In addition, the dimer interface does not involve any of the residues defined in the CRAC site or the enhancement motif.

In light of the structural information, we proposed an external pathway for cholesterol that would require a dimer of dimers or another binding partner for TSPO to facilitate movement[32]. In fact, experiments in mammalian cell culture systems provide evidence[55] of a 800 kDa protein complex being required to demonstrate the cholesterol side-chain cleavage activity that occurs in the mitochondrial matrix. In this complex TSPO is proposed to be the cholesterol binding/sequestering site, along with StAR, and the voltage dependent anion channel (VDAC) is proposed to be the direct binding partner of TSPO that facilitates transport. However, deleting

TSPO in at least one mouse model causes no obvious change in development or steroidogenic behavior, suggesting a regulatory role or possible functional redundancy, as well as the requirement for association with partner proteins[29-31]. Several other TSPO$^{-/-}$ mouse constructs have yielded conflicting reports regarding lethality and other characteristics[31, 41, 42, 58, 61]. These differences have been discussed in detail[39, 62, 75], clarifying some of the complex issues involved in the methodology that may explain the discrepancies. Recent results from conditional, cell-targeted TSPO deletion show altered development and hormone-driven steroid synthesis[62], in keeping with the role of TSPO as a stress response player in steroid metabolism.

TSPO and VDAC are consistently observed in the cholesterol transduceosome and metabolon complexes that bridge the mitochondrial inner and outer membrane and are responsible for hormone-induced steroidogenesis[39, 55, 76, 77]. Direct interaction of TSPO with VDAC has also been demonstrated with various methods, including blue native PAGE[55], immunoprecipitation and confocal microscopy[77], as well as co-purification[78]. However, the mode of interaction and the role in cholesterol transport by this complex at a molecular level are still unknown. With the availability of high resolution crystal structures of both TSPO[32, 33] and VDAC[79], these questions can now be investigated in more detail for the first time. Current knowledge of TSPO and VDAC suggests that a possible transport pathway could be through the interface of a VDAC and TSPO complex. This is supported by the fact that cholesterol binding sites on both TSPO and VDAC are predicted to be on the outside surface of the proteins[32, 35, 80]. Identifying the binding interface of TSPO and VDAC will be critical to understanding a transport mechanism. Computational docking (protein-protein, ligand-protein) and evolutionary co-variance analysis could provide some useful clues. Docking predictions of cholesterol on VDAC are already available[81]. However, the presence of TSPO is likely to change the energy landscape

significantly. Other components of the transduceosome and metabolon complexes need also be considered since they would be expected to have significant impact on the overall cholesterol transport activity.

High affinity cholesterol binding by TSPO appears to be a relatively new development during evolution, given the lack of cholesterol in bacteria and the increasing importance of cholesterol and steroid hormones in the physiology of mammals[35, 62, 76]. However, structural and biochemical analyses suggest that a binding site that can recognize cholesterol-like molecules already exists in bacterial TSPO, perhaps optimized for other related ligands such as hopanoids[35, 76]. The unusually high affinity of mammalian TSPO for cholesterol and the requirement for a dedicated complex for transport into mitochondria for steroidogenesis, suggests that the main function of TSPO may be to sequester cholesterol for further processing. The new structural evidence implies that cholesterol and porphyrin are bound at two distinct binding sites, but given the small size of the protein, they may nevertheless influence each other. The cholesterol binding function is expected to have evolved as a result of different pressures than the porphyrin binding ability. Yet it should be noted that cholesterol synthesis and further conversion to steroid hormones depends on heme-containing enzymes, suggesting a possible interplay between development of cholesterol binding and porphyrin binding capacities in TSPO.

An orphan receptor, a prototype transporter, a translocator?

Originally identified as the peripheral benzodiazepine receptor, TSPO binding of various synthetic and drug ligands and their effects on various pathological conditions have been extensively investigated[8, 82]. However, the endogenous ligand(s) and physiological functions of TSPO are still not established. Both cholesterol and porphyrins are proposed to be endogenous ligands for TSPO, and given their apparent binding at different sites on the protein, different

modes of action[32, 35, 46] are expected. Recent studies on the structure and function of G-protein coupled receptors (GPCRs) and various membrane transporters invite comparison with TSPO. Figure 7 A and B show that TSPO has a very similar shape to the inner helical bundle of the GPCR with the ligand binding site centrally located and facing out of the membrane towards the cytoplasm. The LP1 of TSPO is also similar in helical conformation to an extracellular loop of the GPCR β_2-adrenergic receptor, in which it acts as a lid of the ligand binding site, suggesting a similar regulatory role for LP1. Interestingly, the highly conserved WxPxF motif in the β_1 and β_2-adrenergic receptors[83-85], at the hinge of helix 6 where a large conformational change occurs during G-protein activation, is also found in TSPO as a highly conserved WtPvF motif on TM3.

To examine the possibility of conformational change in TSPO, we applied the ProFlex analysis to crystal structures of *Rs*TSPO. ProFlex is software designed to identify rigid *versus* less stable regions in protein structures[86], and therefore can identify likely regions for conformational change. This method of analysis of a crystal structure is able to correctly identify flexible main-chain regions of soluble as well as membrane proteins with functional significance[87, 88]. ProFlex analysis of the structure of the A139T mutant of *Rs*TSPO (PDB: 4UC1) indicates that the analogous WtPvF region in *Rs*TSPO (side chains shown as sticks in Figure 7C), like that in GPCRs (Figure 7A), also forms a helical hinge that can be independently flexible. In *Rs*TSPO, this hinge is adjacent to the proposed binding site for a porphyrin (green), suggesting that ligand binding to TSPO could influence conformation change in the helices, promoting a signaling event. However, given that the bottom (intermembrane) half of TSPO appears quite rigid in current structures (based on conserved conformation and low temperature factors), the signaling mechanism is likely to be very different than in GPCRs. Considering the location of the WxPxF motif at the dimer interface of *Rs*TSPO, one possibility is that ligand

binding would alter the oligomeric state of TSPO, with resultant modification of interactions with self or other partners.

On the other hand, TSPO can also be considered as half of a prototype transporter, in particular those with 10 transmembrane helices; for example, the LeuT family neurotransmitter sodium symporters (NSS). Interestingly, a "5+5 internal repeat" often exists[89] in these larger transporters with an inverted repeat topology. The equivalent in TSPO would be an anti-parallel dimer, as seen in EmrE[90, 91]. Arguing against this arrangement in TSPO, both the dimers seen in the crystal structures of *Rs*TSPO and *Bc*TSPO are parallel dimers.

As distinct from a transporter, a translocator may be considered a more general term for the activity of moving a substance from A to B, as opposed to specifically across a membrane. As discussed above, a translocator role for TSPO has been proposed[55, 92, 93], involving the sequestration of cholesterol (or porphyrin) and transfer to a protein partner. This appears consistent with current evidence and with the structural characteristics of the dimer of *Rs*TSPO, in which the predicted cholesterol binding site is found on the membrane exposed surface where a protein partner could associate (Fig 5).

TSPO induction under stress conditions across evolution

A diverse range of environmental stress conditions in different species and tissues affect TSPO expression levels, including salt and osmotic stress in cyanobacteria[24] and *Arabidopsis*[22, 26]. Oxidative stress appears to be a common theme and porphyrins have often been implicated as having a role in regulating it. Porphyrins bind to TSPO with micromolar affinity in all species so far characterized and are proposed to be endogenous ligands for TSPO and to play an important role in multiple processes in which TSPO is involved[5, 6, 46, 57, 73, 94]. In *R. sphaeroides*, TSPO is proposed to promote export of porphyrin-type molecules from the cell to regulate photosynthetic

gene expression[19]. Interestingly, TSPO is reported to play a similar role in regulating porphyrin accumulation in mammalian cell lines[95] when challenged with protoporphyrin IX, although recent studies in the TSPO knockout mouse do not confirm this role[61]. In plants[37, 57, 96], a role for *At*TSPO in scavenging of heme and porphyrins is reported, involving degradation of TSPO via an autophagy mechanism. Recent data in mouse also indicate that TSPO is involved in regulating mitochondrial ROS levels and mitophagy, dependent on the ratio of TSPO to VDAC1[73].

TSPO may also respond to, or augment, oxidative stress by directly facilitating the break down of porphyrin. This idea that TSPO is a porphyrin degrading enzyme was first suggested based on the observation of color change of porphyrin molecules when mixed with purified TSPO from the *Chlorobium tepidum*, an anaerobic phototrophic green sulfur bacteria[36] and further followed up with the TSPO isolated from gram positive bacteria *Bacillus cereus* and other sources[33]. However, the results show that this activity is relatively slow as well as light dependent, suggesting a limited applicability to most tissues in eukaryotic organisms. Nevertheless, it is possible that binding of porphyrin to TSPO leads to distortion of the ring structure, as suggested by crystals of *Rs*TSPO[32] and observed in several heme oxygenases[97, 98], which in turn could promote oxidation even in the absence of light. Considering the diverse physiology across kingdoms and the highly diverse forms of tetrapyrroles and other ligands, a variety of roles of TSPO in stress sensing or response in different organisms and tissues may have evolved.

Conclusion and outlook

The tryptophan-rich sensory protein or translocator protein, TSPO, is a member of a highly conserved and ancient protein family that exhibits apparently diverse functions across species in all kingdoms. Despite the fact that the mammalian ortholog was discovered over 30

years ago as a secondary binding site for benzodiazepine drugs, and a bacterial version has also been studied extensively, the precise functions of TSPO are still not understood. New structures of TSPO proteins from three different species provide some new clues regarding structure-function relationships. Studies of TSPO proteins from bacteria, plants, and mammals suggest that a common theme of stress sensing and response may underlie the apparent diversity of function and involve ligand induced conformational change and interaction with protein partners. However, many challenges still remain to developing a fuller understanding of TSPO function. One is the identification of the physiologically relevant protein partners of TSPO. Another is more rigorous and comprehensive *in vitro* characterization of function, ligand binding and conformational effects in prokaryotic and eukaryotic proteins. The growing appreciation for the role(s) TSPO plays in environmental stress and in human disease, as well as a new phase of structure-aided mutagenic studies, should augment efforts to understand the functions of this intriguing protein and its drug target potential.

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FIGURE LEGENDS

Figure 1. Comparison of TSPO monomers from different species.

The high resolution crystal structure of *Rs*TSPO A139T (PDB:4UC1) is colored in discrete rainbow and shown as cartoon in (A). TM1: blue; LP1: teal; TM2: green; TM3: wheat; TM4: orange; and TM5: red. (B): comparison of structure of *Rs*TSPO and *Bc*TSPO (PDB: 4RYQ). *Rs*TSPO is colored the same as in (A) and shown partially transparent, while the crystal structure of *Bc*TSPO is colored in magenta and shown as tube. (C): comparison of structure of *Rs*TSPO and mTSPO (PDB: 2MGY): *Rs*TSPO is colored the same as in (A) and shown partially transparent while the NMR structure of *m*TSPO is colored in black and shown as tube.

Figure 2. The external loop, LP1, has a defined, conserved structure across evolution and interacts with TM5 and TM2.

Residues on LP1 and interacting pairs predicted by co-variance analysis are shown in sticks and colored in matching colors with red having the highest confidence pairing across evolution, followed by magenta and orange. K36, D32 and R43 form predicted salt bridges (yellow dotted lines) with backbone and side-chains maintaining a defined structure for LP1. W39 and G141 also closely interact as predicted. Conservation of interacting pairs suggests the structure of LP1 and its interaction with TM5 and TM2 is an important structural element for TSPO across different species and may play an essential functional role. (Figure created in Pymol from PDB: 4UC1.)

Figure 3. *Rs*TSPO and *Bc*TSPO form different dimers.

Sequence alignment of TSPO from human, mouse, rat, *R. sphaeroides* and *B. cereus* is shown in (A) while the two different dimer assemblies for *Rs*TSPO (B,C; PDB:4UC1) and *Bc*TSPO (D,E;

PDB: 4RYJ) are shown in side views and top views. In (A), residues on the dimer interface of *Rs*TSPO are labeled as cyan triangles while residues for *Bc*TSPO interface are labeled as cyan dots.

Figure 4. Ligand binding sites for TSPO from different species.

Ligand binding sites in currently available structures of different species are positioned in the central cavity but have different interacting residues. TSPO proteins are shown in wheat while ligands (PK11195 and porphyrin) are shown in green. Side-chains interacting with the ligands are shown in orange sticks. (A): *Bc*TSPO with PK11195 bound (PDB: 4RYI); (B): mTSPO with PK11195 bound (PDB: 2MGY); (C): *Rs*TSPO with a representative porphyrin bound (PDB: 4UC1); (D): as in C but from the top to show the position of residue P47, which is a histidine and binds heme in plant TSPO.

Figure 5. Favored cholesterol binding site predicted in TSPO.

(A) A CholMine-predicted cholesterol binding position (black sticks) is shown on the crystal structure of *Rs*TSPO, where the A chain of *Rs*TSPO-A139T (PDB: 4UC1) is represented with main-chain ribbon and side chain sticks for the LAF and CRAC motif residues (A136, T137 and A138 in yellow and L142, F144, and R148 in orange, respectively). The main-chain of TSPO is colored according to crystal temperature factor values, with blue indicating low mobility, green, moderate mobility, and red, high mobility regions. (B) The AB dimer of *Rs*TSPO-A139T is shown in surface representation. The most favorable position for cholesterol binding is shown in black space-filling representation, located in the vicinity of the CRAC site (yellow) and LAF site (orange). Monoolein lipids are shown in cyan, a phospholipid is shown in blue, and a porphyrin-

type ligand is shown in red. In (A), one partial monoolein that sits parallel with the predicted cholesterol binding site is shown as sticks, while in (B), all crystallographically observed lipids are shown in space-filling representation

Figure 6. Observed lipid binding sites.

Lipids are consistently observed in similar positions in different crystal structures of *Rs*TSPO grown in different conditions (shown in sticks). Lipids observed in the 1.8 Å structure, (PDB: 4UC1), are shown in blue (AB dimer) and cyan (CC' dimer) while lipids observed in the 2.4 Å structure (PDB: 5DUO) are shown in orange (AB dimer) and yellow (CC' dimer). (CH note: Can these be reasonably called lipids? Or just alkyl chains?)

Figure 7. TSPO structure resembles GPCR structure with a similar "toggle switch".

TSPO resembles GPCRs in the overall architecture with the extracellular loop (teal) on top of the central ligand binding site. A highly conserved WxPxF motif was also found within a transmembrane helix in *Rs*TSPO (B), similar to that identified in the structure of β_2 adrenergic receptor (A). ProFlex analysis of *Rs*TSPO-A139T indicates that the WxPxF motif in *Rs*TSPO creates a flexible hinge, centered on the tryptophan, between two segments of TM3 (C). WxPxF motifs are shown in magenta in (A) and (B), while panel (C) is colored by main-chain flexibility based on ProFlex analysis[87, 88]. ProFlex analysis was performed on a ligand-free version of the A139T crystal structure (Chain B of PDB: 4UC1). It allows identification of regions of the TSPO structure with different degrees of stability: blue regions are highly constrained and mutually rigid, due to the presence of a dense network of hydrophobic interactions and hydrogen bonds; grey regions have borderline stability due to a weak network of hydrophobic and hydrogen bond

interactions; orange regions are more flexible; and red regions are highly flexible, with few stabilizing non-covalent interactions. Note that the relative flexibility of the helix 1-2 and 3-4 loops and the free C-terminal region of *Rs*TSPO-A139T (loops and short helix at top of figure) predicted by ProFlex are very similar to the flexibility of these regions indicated by crystallographic temperature factors in Figure 5A.

FIGURES

Figure 1

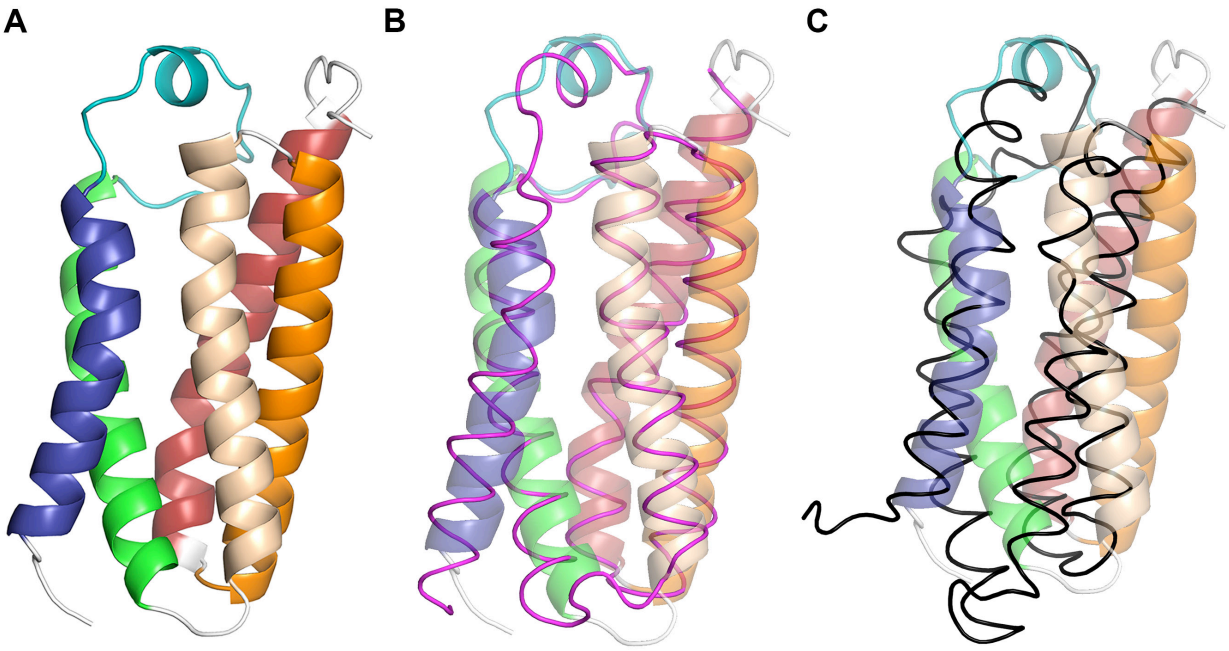


Figure 2

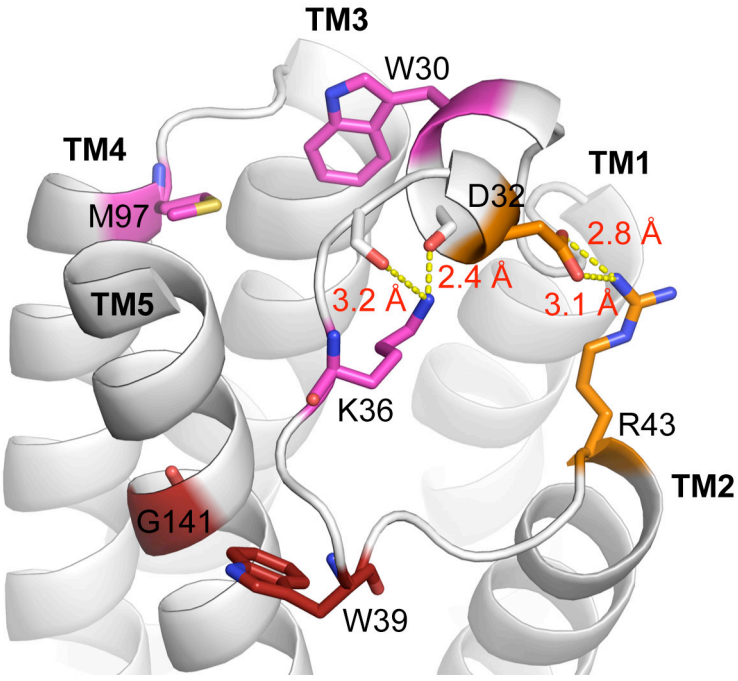


Figure 3

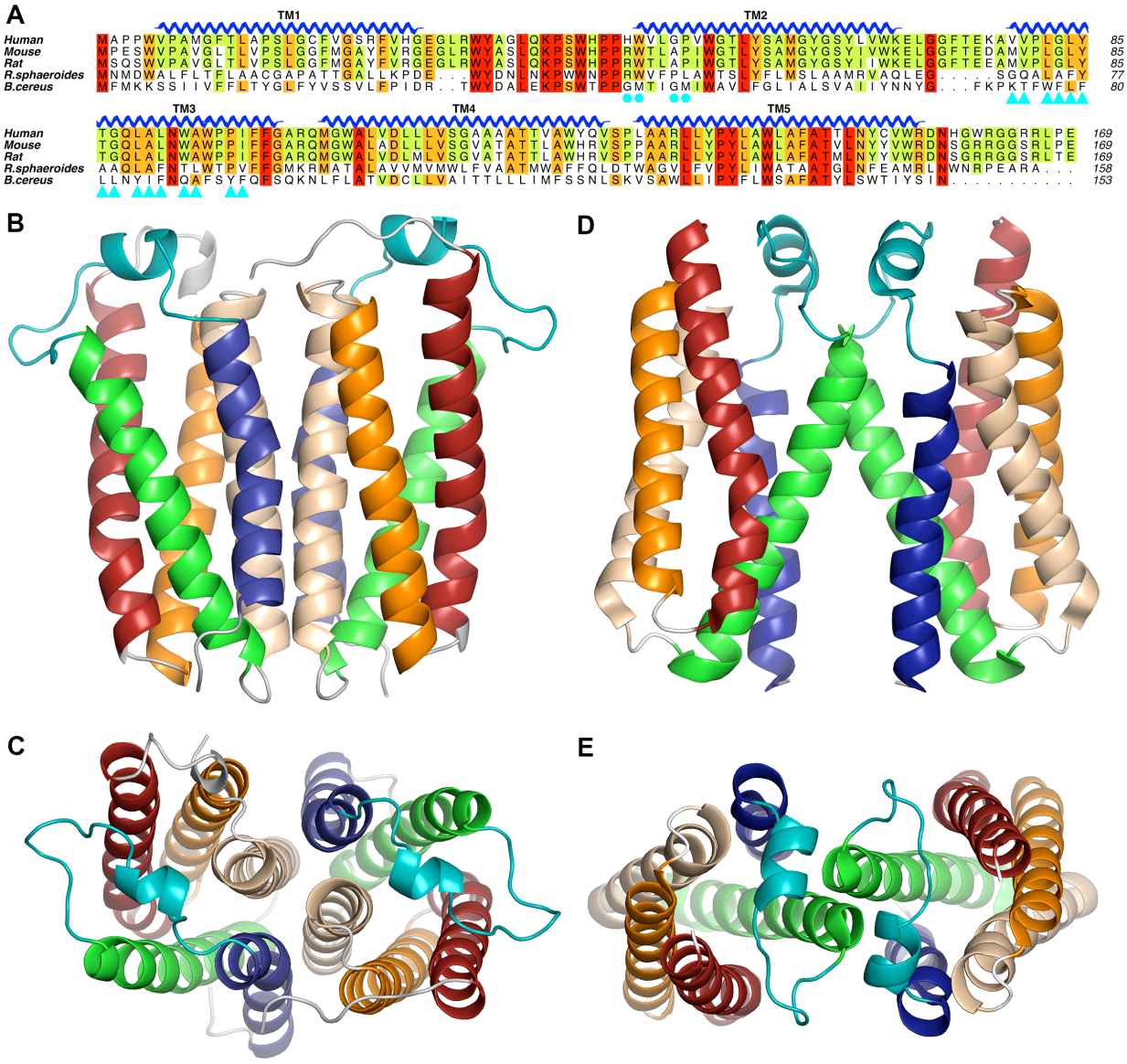

A
TM1
TM2
TM3
TM4
TM5
Human
Mouse
Rat
R.sphaeroides
B.cereus
B
C
D
E

Figure 4

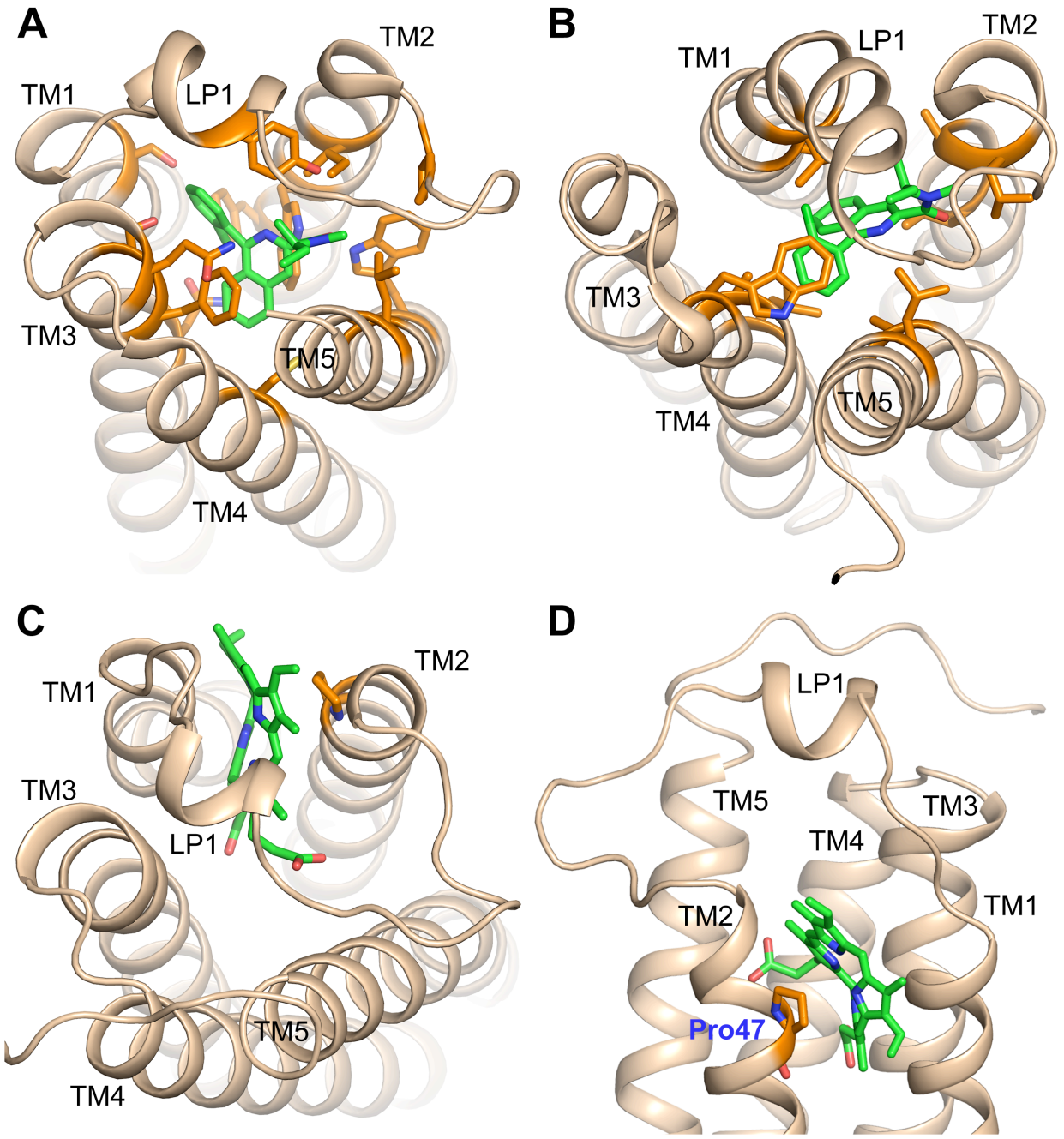

A
TM1
LP1
TM2
TM3
TM5
TM4
B
TM1
LP1
TM2
TM3
TM4
TM5
C
TM1
TM2
TM3
LP1
TM5
TM4
D
LP1
TM5
TM3
TM4
TM2
TM1
Pro47

Figure 5

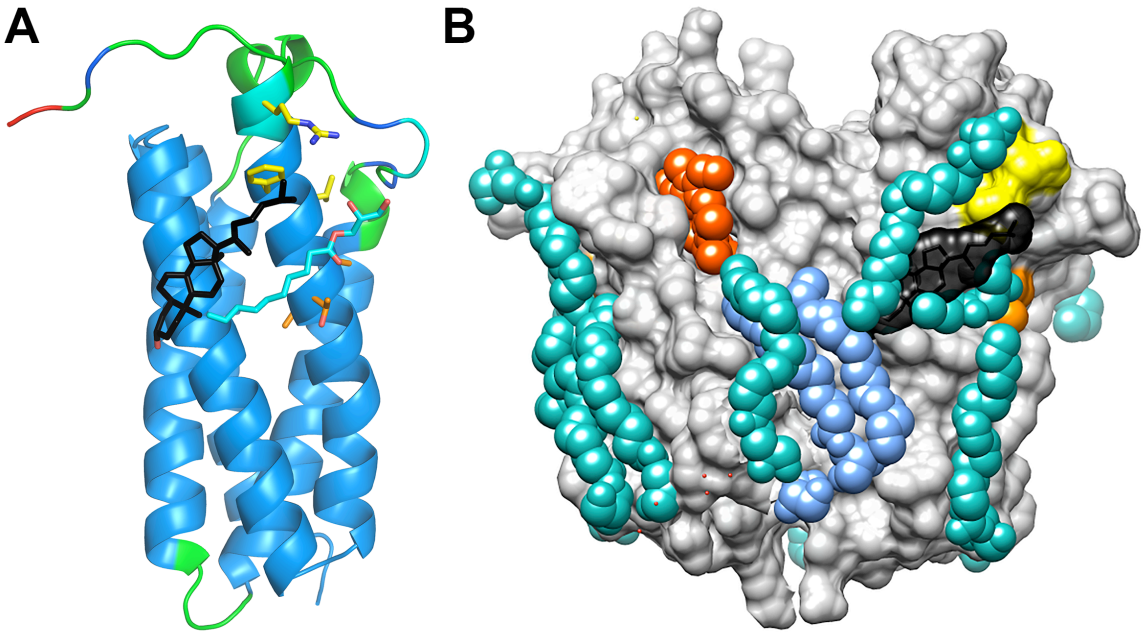


Figure 6

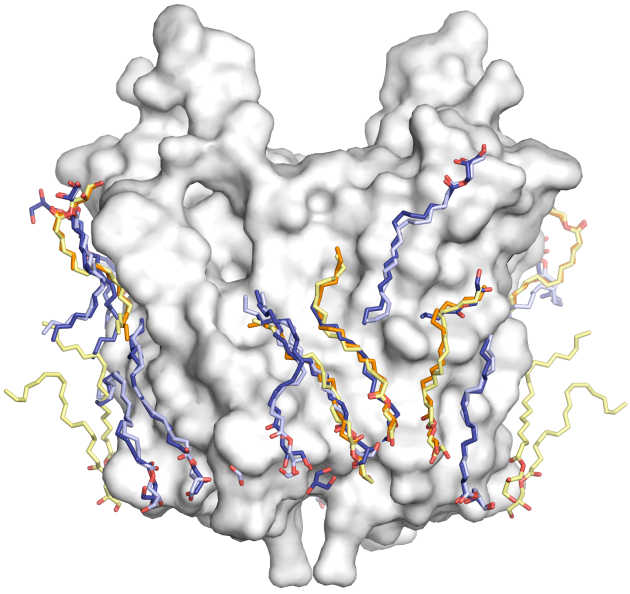
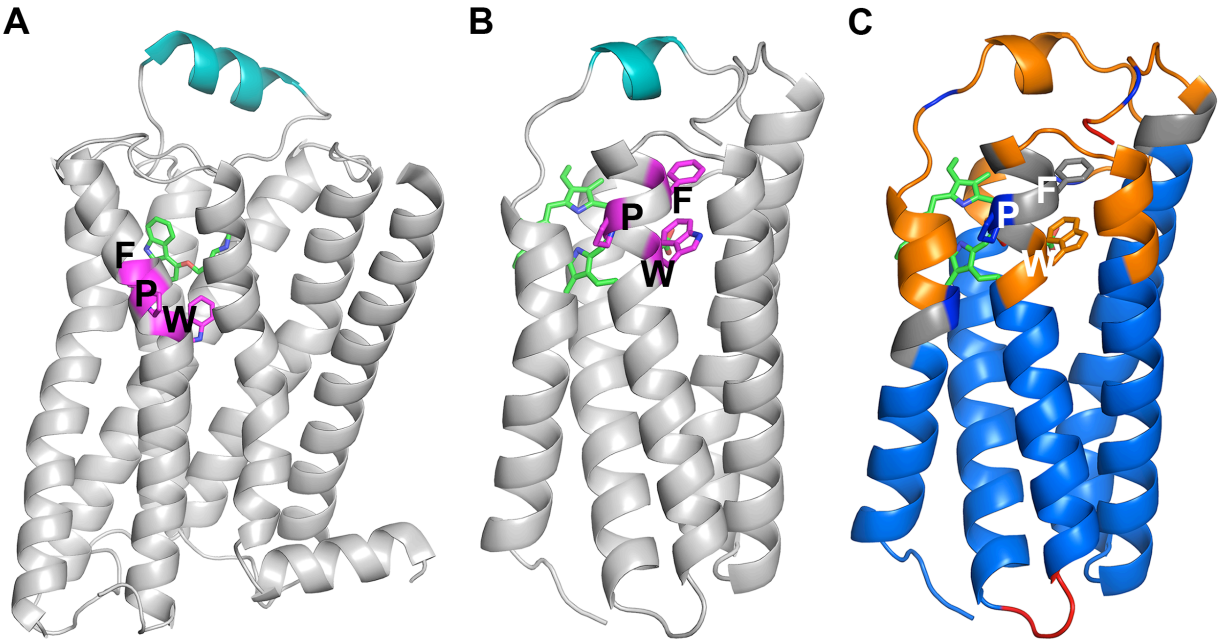


Figure 7



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Translocator protein 18 kDa (TSPO): an old protein with new functions?

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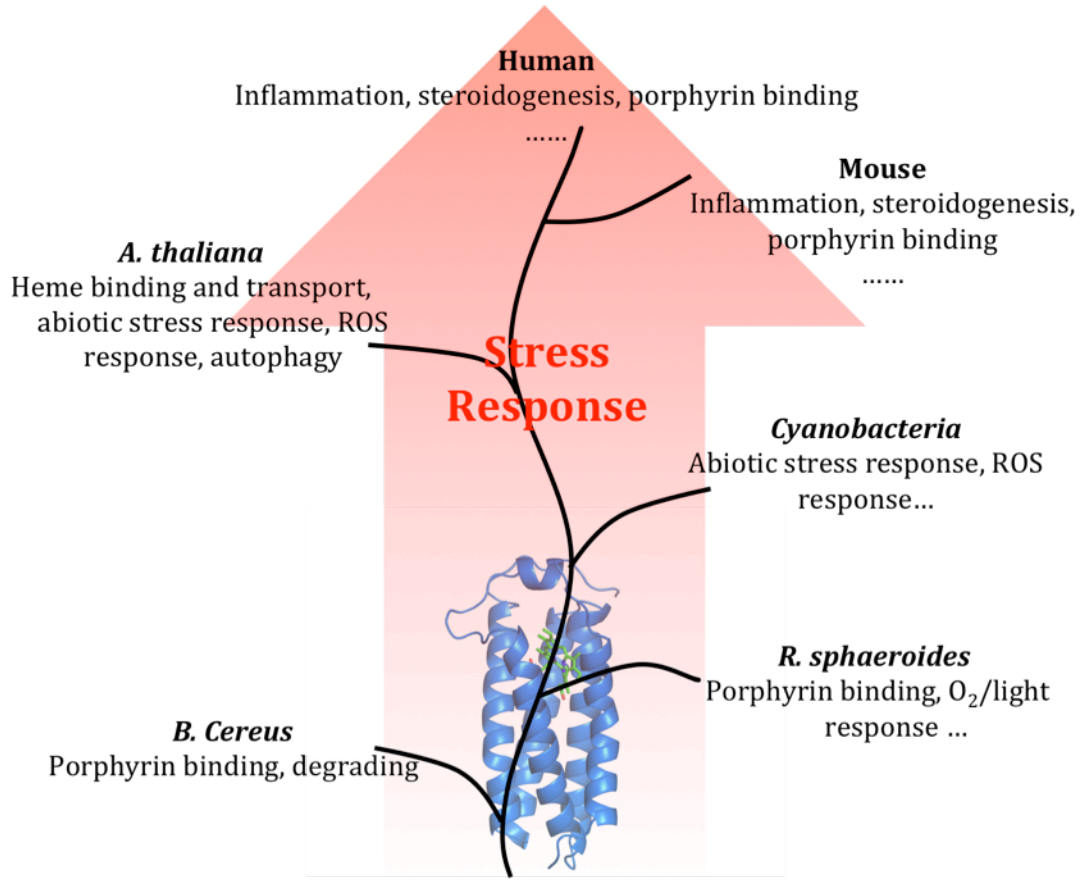
Human
Inflammation, steroidogenesis, porphyrin binding
.....
Mouse
Inflammation, steroidogenesis, porphyrin binding
.....
A. thaliana
Heme binding and transport, abiotic stress response, ROS response, autophagy
Stress Response
Cyanobacteria
Abiotic stress response, ROS response...
R. sphaeroides
Porphyrin binding, O_2/light response ...
B. Cereus
Porphyrin binding, degrading