This is the author's accepted manuscript of the following article:


The final publication is available at the journal website via the following link: http://dx.doi.org/10.1042/BJ20150899.

The full details of the published version of the article are as follows:

TITLE: TSPO: kaleidoscopic 18-kDa amid biochemical pharmacology, control and targeting of mitochondria

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JOURNAL TITLE: Biochemical Journal

VOLUME/EDITION: 473/2

PUBLICATION DATE: 5 January 2016

PUBLISHER: Portland Press

DOI: 10.1042/BJ20150899
TSPO: kaleidoscopic 18kDa amid biochemical pharmacology, control and targeting of mitochondria

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Abstract
The 18 kDa Translocator Protein (TSPO) localizes in the outer mitochondrial membrane of the cells and is readily up-regulated under various pathological conditions such as cancer, inflammation, mechanical lesions, and neurological diseases. Capable to bind with high affinity synthetic and endogenous ligands, its core biochemical function resides in the translocation of cholesterol into the mitochondria influencing the subsequent steps of (neuro-) steroid synthesis and systemic endocrine regulation.
Over the years however TSPO has also been linked to core cellular processes such as apoptosis and autophagy. It interacts and forms complexes with other mitochondrial proteins such as the voltage-dependent anion channel (VDAC) via which signaling and regulatory transduction of these core cellular events may be influenced. Despite nearly 40 years of study, the precise functional role of TSPO beyond cholesterol trafficking remains elusive even though the recent breakthroughs on its high-resolution crystal structure and contribution to quality control signaling of mitochondria. All this along with a captivating pharmacological profile naturally provides novel opportunities for investigating and understanding the significance of this highly conserved protein and the envisaged therapeutics development as here presented and discussed.

Summary statement
An alternative binding site for the benzodiazepines was discovered in the periphery of the central nervous system at the end of the seventies. Since then, the Peripheral Benzodiazepines Receptor (PBR), as it was initially named before the current nomenclature of Translocator Protein (TSPO), has exponentially gained interest from various disciplines of experimental and applied biomedicine. Inspired by the series of discoveries recorded in the past twelve months we here aim to review the most compelling aspects of TSPO science in order to inform on the biochemical and molecular pharmacology, homeostatic relevance and therapeutic potential of this mitochondrial stress-response pathway.

Keywords: TSPO, PBR, Steroids, Metabolism, Autophagy, Biochemistry and Mitochondrial Pharmacology

Abbreviations: Translocator Protein (TSPO); Peripheral Benzodiazepine Receptor (PBR); Voltage-Dependent Anion Channel (VDAC);

Introduction
The cross-disciplinary nature of TSPO has been recently remarked by prominent discoveries, which have pointed out this as an underlying regulatory pathway in cell homeostasis and signaling to fine-tune mitochondrial function and its quality control to acute and chronic stress conditions. This has
indeed unveiled a fundamental relevance in the molecular and biochemical physiopathology of mitochondria and their interplay with the intracellular environment.

TSPO was discovered in the 1977(1) and initially named as the peripheral benzodiazepine receptor (PBR) for the ability to bind benzodiazepine drugs outside of the central nervous system. It is an abundant, evolutionary conserved, protein found in every organs but with particularly high constitutive expression in steroidogenic tissue, including adrenal glands, gonads, placenta and activated brain microglia (2-4).

The pattern of TSPO expression and the early discovery that TSPO was an high-affinity binding protein of cholesterol resident in the outer mitochondrial membrane, established an exciting link between cholesterol transport and biosynthesis of steroids, including the neuronal kind of these (2, 5, 6). A large body of evidence has gradually defined TSPO as an essential component of cholesterol transport across the mitochondrial membrane and a rate-limiting step of steroid hormone production (7-10). This feature, in addition to the putative roles in protein import, porphyrins binding and transport, resulted in a new nomenclature as Translator Protein. Remarkably, this was introduced almost 30 years after its discovery and adopted to better describe TSPO function as cholesterol-translocator protein replacing the original name (11, 12).

TSPO is widely expressed throughout the body yet its density appears tissue-dependent. Steroidogenic tissues possess the majority of the protein whereas renal and myocardial tissues have reduced levels, and lower still, the brain and liver (13-15). Notably, distribution is not homogenous within a given organ. For instance, in the adrenal glands, the medulla is almost lacking in TSPO, while far greater levels of the protein are expressed in the cortex (16). It is localized to the outer mitochondrial membrane (2, 17, 18), and for this we call it a mitochondrial protein however it has been reported in the nucleus (19) and on the plasma membrane (20). Hitherto neither of these two alternative localizations has been properly validated.

TSPO expression rises readily in various cell types following exposure to pro-pathological stressors or physicochemical insults (10, 21, 22). Such a dynamic increase of expression is particularly evident in microglia cells of the brain (23), which has led TSPO to be exploited for diagnostic purposes as a biomarker of active disease or disease-related tissue remodeling through the generation of TSPO radioligands (12, 21).

The recurring association with cell and tissue pathology likewise its known druggability have made TSPO an attractive therapeutic target. Various are indeed the chemical entities with affinity for TSPO that have been reported with some of which entering clinical trials as either diagnostics or therapeutics (12).

Animal models of disease that were reported to benefit from treatments with TSPO ligands include those for Alzheimer’s Disease (AD), Multiple Sclerosis (MS), anxiety disorders, neuropathic pain, peripheral nerve injury, diabetes, rheumatoid arthritis, cancer and cardiac ischemia (21, 24-30). Among the therapeutic interventions exploiting TSPO the most promising one, hitherto, is based on
the prediction that neurosteroids could be beneficial in the treatment of inflammation and selective TSPO ligands would consequently hold a therapeutic potential by promoting the function of TSPO in steroidogenesis (21, 24, 31).

A role for TSPO in the formation of the mitochondrial membrane permeability transition pore (MPTP) has been long proposed, providing a rationale for the evidences linking TSPO with apoptosis, Ca^{2+} signaling and redox stress homeostasis. However, recent studies on gene-edited mice (knocked out for TSPO) have ruled this out and showed that MPTP is formed regardless of the presence of TSPO (32-36). Nevertheless, a regulatory role for TSPO and that of its drug ligands cannot be excluded. Several are the endogenous ligands for TSPO such as diazepam binding inhibitors (DBIs), porphyrins and cholesterol. There is substantial literature to date that describes cholesterol binding but much less about the physiological relevance of DBIs and porphyrins. DBIs exhibit micromolar affinity with both TSPO and the central benzodiazepine receptor, and are widely distributed in glial cells of the CNS and in steroidogenic peripheral tissues (37). They may be cleaved into other biologically active fragments and are shown to stimulate steroidogenesis via interactions with TSPO (38). Porphyrins, tetapyrrolic pigments important in heme biosynthesis pathways, may be scavenged by TSPO (39, 40). A number of interacting proteins (e.g. ACDB3, ACBD1, PRAX1, VDAC, 14-3-3, phospholipase A2) have been identified, which are involved in steroidogenesis and other TSPO-related functions including apoptosis and autophagy. Notably, the molecular link between TSPO and the Voltage Dependent Anion Channel (VDAC), a core element of the mitochondrial outer membrane, is restated by most of the recent literature, which highlights novel functional significance for this protein (41).

In this review we will outline the current knowledge and the implications of the most recent findings in the understanding of TSPO as core molecular determinant of mitochondrial biochemistry and pharmacology.

**TSPO and a bi-directional transcriptional signaling network**

The Tspo gene is composed of four exons, with a large intron that separates the first two. Multiple transcription start sites have been identified in the Tspo promoter in different species (42). The promoter lacks a TATA box or CCAAT elements but contains proximal GC-rich motifs and five tandem binding sites for specificity protein 1/ specificity protein 3 (Sp1/Sp3) factors. The promoter region also harbors putative binding sites that are conserved between mouse and human TSPO for a number of additional transcription factors including activator protein 1 (AP1), v-ets erythroblastosis virus E26 oncogene homolog (Ets) transcription factors, Myc and STAT members (42-46). It is likely that tissue-specific regulatory elements exist as in steroidogenic MA-10 and Y1 cells 585 base pairs of the promoter was required to maintain full activity compared to an extended 805 bp region in non-steroidogenic NIH-3T3 cells (42). Tspo is predominantly under the control of a PKC{\(\varepsilon\)}-dependent signal transduction pathway, which is constitutively active in steroidogenic and inducible in non-steroidogenic cells (46, 47). PKC{\(\varepsilon\)} activates
the Raf-MEK-ERK MAPK pathway and triggers Tspo transcription through cooperation with c-Jun, Ets, AP1 and STAT3 transcription factors (46-48). Basal Tspo transcription requires Ets and Sp1/Sp3 transcription factors (48), which are located in promoter regions 805-515 and 123-1 (47). Evidence has demonstrated that TSPO is directly related to changes in ROS generation and there are multiple ROS-sensitive elements that drive the expression of the Tspo gene, such as AP1, ERK1/2 and PKCε (46, 48). For example, increased ROS production in astrocytes results in nuclear accumulation of the Sp1 transcription factor, which increases TSPO mRNA (49). Upregulation of TSPO has also been observed after treatment with other ROS-producing compounds including TNFα (50, 51), phorbol-12-myristate 13-acetate (PMA) (46, 52) and the mitogen-activated protein kinase kinase (MEK) inhibitor, 2-(2'-amino-3'-methoxyphenyl)-oxanophthalen-4-one (PD98059) (48).

We have shown that overexpression of TSPO is associated with increased ROS production, which we have reported as a causative factor in TSPO-mediated mitophagy inhibition (41). The idea that overexpression of TSPO creates an oxidative cellular environment is very important as the expression of the Tspo is driven by downstream effectors of ROS (48). Under oxidative conditions, an increase in TSPO levels may be physiologically important to provide mitochondrial and cellular protection against initial ROS damage; should this be sustained, a positive feedback cycle in which the gene is re-expressed and mitophagy continuously impaired may manifest by leading to cumulative mitochondrial damage, which would impact cell and tissue health over time (53).

There are anyway reports showing, on the contrary, that sustained oxidative stress decreases TSPO expression contributing cell death (22, 48) although the transcriptional or protein degradation pathways in such conditions are ill-defined.

Numerous hormones including estradiol and aldosterone also regulate TSPO (48, 54, 55); the presence of hormones appears to be necessary for maintaining constitutive TSPO expression, since decrease in TSPO is observed in steroidogenic tissues following surgical removal of the pituitary gland (6) and the adrenal gland (54). Several studies have described that exposure to steroid hormones modifies TSPO levels, determined through TSPO binding studies (56-58). mRNA and protein levels do not always correlate, however, and the protein is reported to undergo conformational changes that result in increased ligand binding (55, 59). Hormones do cause a change in TSPO binding affinity to typical ligands and induce structural alterations including post-translation modifications such as cyclic adenosine monophosphate (cAMP, cyclic AMP, or 3’-5’-cyclic adenosine monophosphate)- dependent PKA-mediated phosphorylation (48, 60).

It is well established that hormones activate the cAMP pathway, being an important element in steroidogenesis contributing to lipid synthesis, cholesterol trafficking and protein phosphorylation. cAMP is a second messenger important in many biological processes including growth and differentiation, and is used for intracellular signal transduction in many different organisms.

Activation of the cAMP pathway leads to a redistribution of Acyl-coenzyme A binding domain containing 3 (ACBD3) from the Golgi to mitochondria, where it interacts with TSPO and recruits PKA
This has relevance in the activation of steroidogenesis and has been described in estradiol-induced neuroprogesterone synthesis in the hypothalamic astrocytes (63). Prolonged exposure to hormones including estradiol can lead to a decrease in TSPO levels, signifying a feedback mechanism (48, 55). A negative feedback loop has been described to regulate Tspo transcription following the identification of microtranscriptional regulation by cAMP dependent natural antisense transcripts (NAT). Antisense transcription was initially considered as transcriptional noise, however, it is increasingly becoming recognized as an important regulator of gene expression and may form self-regulatory circuits that allow genes to regulate their own expression [for review see (64)]. At the Tspo locus a short interspersed element (SINE) of the B2 family was identified that was able to drive the expression of long transcripts. Its extension overlapped exon 3 of the Tspo gene and formed a NAT specific for Tspo that was dependent on cAMP (65).

Alternatively, peroxisome proliferator-activated receptor alpha (PPARα) (66) has been linked to decreases in TSPO expression (48, 67-69). PPARα interferes with ROS-activated NFkB, STATs and AP-1 transcription signaling pathways (70), factors with binding sites in the Tspo promoter (47, 48). It has been also suggested that PPARα reduces the binding of AP-1 to its PKCζ-associated site on the Tspo promoter resulting in a decrease in TSPO mRNA (48) while previous studies have shown a positive correlation between PPARs and increased TSPO expression (52). Interestingly, activation of PPARα may also occur via a cAMP-PKA dependent mechanism creating a further compatible regulatory mechanism as activation of PPARα does bear anti-inflammatory effect (71-73), which may be logically linked to a decrease in TSPO expression (Figure 1).

What emerges from the existing literature is that TSPO represents an important element in facilitating cellular adaptation to various pathological and pro-pathological stimuli by integrating hormone and REDOX sensitive pathways.

Detailed identification of the transcriptional regulatory pathways and feedback loops could prove fundamental to our understanding of this protein’s function and responsiveness to stress conditions.
TSPO and its phylogenetic structure

TSPO has tryptophan-rich regions that are highly conserved between prokaryotes and eukaryotes (Figure 2). The structure of TSPO was first predicted based on its secondary structure and modelled using molecular dynamics simulation to give a two-dimensional membrane topological model comprising an intramitochondrial short amino-terminal region and five amphipathic α-helices linked by hydrophilic loops leading to an extramitochondrial carboxyl-terminal tail (74).

An early 3D structure of TSPO, built upon the 7 transmembrane rhodopsin structure, which was almost the only membrane protein structure available at the time, was modelled as only long enough to span one phospholipid layer rather than crossing the entire bilayer membrane (74). Later TSPO topological classification in the mitochondrial membranes of yeast supported a five-transmembrane structure but with extended α-helices that are in fact able to traverse the entire membrane bilayer (75). However, CD spectrum results from mouse TSPO (MmTSPO) demonstrated the presence of a mainly helical secondary structure while NMR data confirmed the presence of at least five helical transmembrane segments (76).

Bacterial homologues have also been used to provide detailed insights into the structure of TSPO: TSPO from Rhodobacter sphaeroides (RsTSPO), one of the closest ancestors of mitochondria, not only has a primary structure similar to that of human TSPO but is also reported to have functional similarities (77). Cryo-EM and image analysis studies of RsTSPO revealed a dimeric, five transmembrane domain architecture for TSPO at 10 Å resolution (78).

This structural information indicated monomeric TSPO could function as a channel and could therefore translocate cholesterol across the outer mitochondrial membrane, in line with its well-defined role in steroidogenesis (21, 79). Recently, a high-resolution structure of mammalian TSPO reconstituted in detergent micelles in complex with the ligand PK 11195 has been obtained, further supporting a five transmembrane alpha helical conformation (80).

Another powerful study has described crystal structures for Bacillus cereus TSPO (BcTSPO) at 1.7 Å resolution in complex with PK 11195. These authors demonstrated oxidative catalytic activity between TSPO and PpIX (protoporphyrin IX), a reaction also observed in TSPO from Xenopus tropicalis and Homo sapiens that leads to PpIX degradation. This has been attributed to ROS-mediated generation of tryptophan radicals at residues W53 (hs numbering; transmembrane domain 2) and W143 (hs numbering; transmembrane domain 5), which are both shown to participate in the stabilization and cleavage of PpIX. Two other conserved tryptophan residues are located in Loop 1 (W32 and W40; hs numbering) and in the cholesterol recognition amino acid consensus (CRAC) cytoplasmic domain located at the C-terminus (W155; hs numbering), which are considered to be important in other TSPO-ligand and –protein interfaces (77, 81-83). The identification of potential catalytic regions in TSPO is particularly interesting and it would be worth understanding how such biochemical activity influences TSPO molecular interactions and downstream processes under conditions of REDOX stress since
different experimental conditions are already known to affect $K_{d}$ values for PpIX binding; e.g. 0.3 μM (81) versus 8.6 μM (78).

Quite notably, TSPO has been characterized in monomeric, dimeric and multimeric states although the functional significance of its polymerization requires further clarification. Analysis of RsTSPO revealed a dimer (78, 81) while studies on MmTSPO portray a monomeric protein in complex with PK 11195 (80) although there is a possibility of oligomeric states (84). BcTSPO on the other hand has been extracted as monomeric, dimeric and as higher oligomeric species with a dimeric model in complex with PK 11195 (40). Interestingly, cellular REDOX status has been previously linked with TSPO oligomeric status in response to ROS increases TSPO preferentially forms covalent polymers (85).

TSPO can function both as a monomer and polymer. In its monomeric form TSPO binds PK 11195 and cholesterol with nanomolar affinity (79) although the translocation of cholesterol is preferentially mediated through polymeric forms or in complex with other protein binding partners (86).

A TSPO genetic polymorphism in humans Ala(147) → Thr(147) is associated with psychiatric disorders (87-89) and reduced pregnenolone production (90).

The polymorphism resides one helical turn before the CRAC cholesterol binding motif, and a reduction in cholesterol binding has been identified with a four- to fivefold lower affinity than wild-type (91, 92). Complications in the application of certain TSPO PET ligands, including [11C]-PBR28, [18F]-BPR111 and [(18)F]-FEPPA for imaging brain inflammation have been linked to this polymorphism (88, 93-96). However, structural analyses have shown that A147T TSPO is able to retain the same structural profile as the wild-type protein, and binds the first generation TSPO ligand, PK 11195, with comparable affinity (97). Contradictory to this study, which used mTSPO (97), RsTSPO A139T exhibited a lower binding affinity to PK 11195 as well as PpIX (92) and this is supported by crystal structures of the A139T mutant at 1.8 and 2.4 Å resolution in RsTSPO, which show conformational changes that alter the structural environment in both the CRAC domain and possible ligand binding domains. These conflicting reports however can be ascribed to the structural differences between mammalian and bacterial species. Nevertheless, they still raise the question as to how functional activity data obtained in bacterial systems can be applied to mammalian TSPO and therefore if this has been preserved intact in the phylogenesis. Additional studies conducted in mammalian TSPO systems will therefore be of essence particularly with regards to the apparent catalytic activity exhibited by TSPO in the degradation of PpIX as already pointed out (40).

Anyhow the tremendous effort by colleagues who endeavored, in series, to generate the actual structure of the protein is of invaluable importance not only to inform TSPO biochemistry but also to pave novel avenues of research to dissect and exploit the nature of TSPO-ligands binding as well as the design of novel chemicals.

**Tsopo** knockout mice: new insights of an old function
The most studied role assigned to TSPO is in the transport of cholesterol into mitochondria for hormone biosynthesis. Hormone-induced steroidogenesis in all tissues begins with the conversion of the precursor cholesterol to pregnenolone, a reaction catalysed by the enzyme P450 cholesterol side chain cleavage enzyme (CYP11A1), located in the IMM, facing the matrix [reviewed in (98)]. The rate-limiting step is the transport of cholesterol from the cellular stores across the mitochondrial membranes. In order for this step to be achieved, cholesterol must accumulate at the outer mitochondrial membrane (OMM) and then be transferred to the IMM.

The steroidogenic acute regulatory protein (StAR) is a hormone induced mitochondria-targeted protein that has been shown to initiate cholesterol transfer into mitochondria (99-101). It binds cholesterol (102) and is active at the OMM where it is proposed to interact with TSPO to form a complex that facilitates mitochondrial cholesterol import (62, 101, 103, 104). Other components of the complex include ACBD3 (Acyl-coenzyme A binding domain containing 3) and the regulatory subunit R1α of PKA (62, 83, 105). TSPO can bind cholesterol through its CRAC domain (106), and mutagenesis in this region interferes with cholesterol binding and transfer of cholesterol into the IMM, preventing steroidogenesis (107). TSPO gene silencing blocks cholesterol transport into the mitochondria, also reducing steroid production, while reintroduction of TSPO is able to restore steroidogenic capacity (83). Parallel studies conducted in a bacterial system expressing MmTSPO demonstrated time- and temperature-dependent uptake of radiolabeled cholesterol. A number of non-steroidal ligands that interact with the CRAC motif were recently identified through molecular modeling and in silico screening of chemical libraries and were shown to potently inhibit steroidogenesis (108).

Taken together there is a large body of evidence supporting a function for TSPO in cholesterol transport; although it has also been suggested that it stores cholesterol until later requirement since cholesterol loaded membranes release cholesterol in response to PK 11195 (79, 109).

Recently, however, a number of studies from independent groups have reported that TSPO global knock out mice are viable and that loss of the protein has no effect on basal steroid hormone biosynthesis (32, 35, 110, 111). These studies not only question the role TSPO holds in steroidogenesis but contrast early attempts to generate Tspo−/− knockout mice, which resulted in embryonic lethality (5). However, the viability of mice has been shown to vary depending on methodology: Nr5a1-driven conditional knock-outs were born at a normal Mendelian ratio while Amhr2-Cre driven conditional knock-outs were born at a ratio of 4.4% (111). Global Tspo knockout phenotypes can range from lethal, when whole gene deletion is performed, to no phenotype, as in the case of cre-loxP technique, thus presenting the possibility that methodological differences may be the cause of such discrepancy (112). Phenotypic characterization carried out on currently available global and conditional knock-out strains is summarized in Table 1. However, it is important to consider that compensatory mechanisms may play an important part in ensuring ongoing steroidogenesis in a TSPO null setting, and these may be different in the various strains, which may be attributed to methodology as highlighted already. In both global and conditional Tspo knock-out studies, several
genes involved in steroidogenic processes were found to be upregulated including \textit{Cyp21a2} (110), \textit{Abca2} (110), and \textit{Scarb1} (111) in the adrenal glands and \textit{Lhcgr} (111) in the testes (Table 2). The study by Fan et al describes how tissue-specific deletion of \textit{Tspo} in gonadal tissues had little effect on gonadal steroidogenesis although ACTH stimulation of corticosterone production in \textit{Tspo} depleted adrenals was severely impaired in both male and female animals (111). Interestingly, in \textit{Tspo}−/− mice, there is an accumulation of lipid droplets in the adrenal glands, implying decreased lipid metabolism, while instead lipid stores were depleted in testes and ovaries, implying excessive substrate consumption (111). These observations provide strong evidence that there is an important role for TSPO in hormone-mediated steroidogenesis and lipid and cholesterol metabolism.

TSPO is also attributed to immune regulation, with elevated expression observed in microglia and macrophages. Recently it was highlighted that TSPO is involved in cholesterol trafficking in macrophages, since its overexpression leads to increased transcription of proteins involved in cholesterol efflux, ACBA1 (ATP-binding cassette A1), PPARα (peroxisome-proliferator-activated receptor α) and LXRα (liver X receptor α), and corresponds with increased efflux of cholesterol to acceptors (113). This pathway is activated upon a moderate cholesterol load stress and it is proposed as a protective mechanism to reduce macrophage cholesterol mass (113). Other genes involved in immune regulation are similarly altered in \textit{Tspo}−/− mice (see Table 2) further validating a role for TSPO in the regulation of these processes. \textit{Tmem178} is proposed as a negative regulator of macrophage activation and \textit{Ca}2+ signaling at the ER level (114), and is reduced in \textit{Tspo}−/− mice. Conversely, there are increased levels of \textit{Zbtb7b}, which is associated with T-cell maturation (115) and regulates the development of Natural Killer T (NKT) cells (116) and ties in the Tu et al study (110) with the recent work by Banati et al (32), who observed an increase in NKT cells present during haematological analysis in female \textit{Tspo}−/− mice. TSPO has previously been reported as having antiretroviral activity by inhibiting Env protein expression (117). In support of this study, decreased levels of \textit{Trim12a}, coding for protein within the anti-retroviral TRIM family, and \textit{Pydc4}, coding for an AIM2-like receptor that activates STING-dependent interferon (IFN) production as part of the antiviral response (118), were observed in \textit{Tspo}−/− mice (110). The reduced levels suggest that TSPO is involved in an upstream transcriptional signaling cascade that influences the antiviral response.

Another interesting observation in \textit{Tspo}−/− mice is a disparity in body weight that occurs only in females, with an increase in weight in the knock-out strain (34), which the authors attribute to a hormone-dependent metabolic pathway. Previously the TSPO ligand, PK 11195, was shown to alter the expression of metabolic genes in white adipose tissue (22) and liver (29) of male mice although no study has examined the application of ligands in female mice yet to assess possible sexual disparity in ligand efficacy. Notably PPARα, regulated by hormones and cAMP, holds a key role in integrating the mammalian clock and energy metabolism (119) and notably influences \textit{Tspo} transcriptional regulation (48). Since gender based differences have been observed previously in metabolic patterns of circadian rhythms (120), this may be an explanation for the gender-based differences in body
weight seen in Tspo<sup>−/−</sup> mice. This is particularly interesting as it could reveal a therapeutic potential for TSPO ligands in the development of gender-based personalized medicine for human diseases relating to metabolic disorders, which are often linked to circadian disruption (121).

The overarching conclusions that are consistent between all the knock-out studies indicate that due to the relative lack of phenotype in Tspo<sup>−/−</sup> mice, TSPO is likely to be most involved in pathological and stress-related conditions. Future follow on studies could therefore focus on assessing susceptibility in standard stress models of diseases where TSPO has already been linked to, for example, LPS-induced inflammation, ischemia-reperfusion injury, radiation/chemical-induced cancers, overfeeding or bacterial/viral infection. Nonetheless, age-related studies should also be recommended based on the brain-imaging evidences lately produced (96).

**TSPO and its molecular partnership with VDAC1**

TSPO is included in a complex with the 32kDa voltage-dependent anion channel (VDAC) (17), of which there are three different isoforms. The relative ratio of these proteins is tissue and condition dependent (122). Early studies indicate that 4-6 molecules of TSPO associate with one molecule of VDAC to form a single mitochondrial pore (5, 123) but the precise biochemical properties governing the interaction remain unclear and require further investigation, which may be facilitated with the recent high-resolution characterization of its 3D structure (80).

There is a growing body of work that supports a functional interplay between TSPO and VDAC particularly in dictating the efficiency of mitochondrial metabolism and quality control.

The long standing role of TSPO in steroidogenesis appears to be linked to its interactions with VDAC (124, 125), and relative levels of each protein have comparative expression profiles in steroidogenic tissues (126). STAR, TSPO and VDAC all contain binding motifs for 14-3-3ε, a newly identified negative regulator of cAMP-mediated induction of steroidogenesis (124, 127). VDAC is a primary target of 14-3-3ε, with which forms a protein-protein scaffold that influences downstream TSPO interactions leading to reduced cholesterol import into mitochondria (124). In fish testis, tsps and vdas mRNA levels are both correlated with reproductive stage and gonadosomatic index (GSI) while in females, gonadal tsps and vdas expression are negatively correlated with GSI and levels of plasma testosterone and 17β-estradiol (126).

As mentioned above, TSPO has a regulatory metabolic role. For example the ligands, FGIN-1-27 and PPIX are both shown to reduce glycolytic activity and cellular ATP levels (128, 129) while PK 11195 increases ATP levels (130). TSPO overexpression is associated with reduced Ca<sup>2+</sup>-dependent activation of mitochondrial respiration (41), which has been linked to VDAC activity while the effect of PK 11195 on quinolinic acid-induced glucose metabolic disturbance is reported to involve VDAC (131).

TSPO is strongly associated with inflammation, and studies have demonstrated the importance of the TSPO-VDAC interplay in this response too. In vascular endothelial cells, TNFα and other
inflammatory cytokines induce mitochondrial ROS production and expression of vascular cell adhesion molecule-1 (VCAM-1) although TSPO overexpression interferes with this sequence of events (132). In contrast to the apparent parallel expression profiles in steroidogenic tissues, TSPO expression negatively correlates with VDAC1 expression in endothelial cells, an observation that the authors attribute to the reduced mitochondrial ROS and lower levels of VCAM-1 in overexpressing TSPO conditions (132).

The mitochondrial TSPO-VDAC complex is further involved downstream of PARK2-mediated activity (41, 133). We determined that increased TSPO expression in mouse embryonic fibroblast cells leads to a reduction in PARK2-mediated mitochondrial ubiquitination during FCCP-induced mitophagy (41). Mechanistically, VDAC1 was required for this TSPO-dependent activity and further attributed to a ROS-signaling pathway since mitophagy could be restored in over-expressing TSPO cells in the presence of the antioxidant, MnTBAP (Manganese (III) tetrakis (4-benzoic acid)porphyrin chloride) (41).

A further study has confirmed a role for TSPO-VDAC in regulating PARK2 function, showing that TSPO-VDAC is involved downstream of the PARK2-mediated immune response in Drosophila since PARK2 overexpression failed to rescue lethality caused by septic injury in TSPO-silenced larvae (133).

This evidence suggests that the interplay between VDAC and TSPO is important in all the major functions attributed to TSPO including steroidogenesis, hormone biosynthesis, immune/inflammatory regulation and energy metabolism, processes for which mitochondria are critically involved. The removal of different VDAC isoforms can yield mice without mitochondrial dysfunction or evident phenotype, which is also the case in certain strains of TSPO KO mice (please see section above). This may appear particularly confounding, as each of these proteins can comprise up to 5% of total outer mitochondrial membrane protein levels in certain tissues. However, some functional redundancy has been described between the various VDAC isoforms, which may explain the apparent lack of phenotype. Furthermore, in the case of the VDAC1\(^*\) strains, there was considerable variation in phenotype, which was notably dependent upon the methodology used to generate the line. VDAC1\(^*\) mice obtained from a mixed genetic background (C57BL6/129SvEv) were born at a lower than expected mendelian ratio whilst VDAC1\(^*\) mice, bred onto the C57B16 background, showed near complete lethality (134).

**TSPO in cellular and tissues pathology**

**Cancer**

TSPO is overexpressed in a variety of cancers including those affecting the brain (135). TSPO expression is elevated in human prostatic intraepithelial neoplasia, primary prostate cancer and metastases when evaluated against normal prostate tissue and benign prostatic hyperplasia (136). A
positive correlation with disease progression was observed too and immunohistochemical studies in oral cancer have yielded similar results. Interestingly, the five-year survival probability dropped from 65% in patients with TSPO negative tumors to just 7% in patients with high tumoral TSPO content (137). Results, from colorectal carcinomas in human patients, indicated that TSPO was overexpressed in 67% of tumors in comparison to corresponding normal mucosa in which expression was found to be significantly higher. And in this study, TSPO did not differ for expression between intermediate versus high-grade tumors or in lymph node-positive versus negative patients (138). Overexpression of TSPO has also been observed in breast cancer cell lines (139) and in clinical studies (140). In the former, estrogen receptor-negative breast cancer cell lines had significantly higher TSPO expression compared to ER-positive lines and the levels of TSPO were positively correlated with the proliferation marker, Ki-67 (139). This observation was mirrored in the clinical study in which TSPO expression was significantly increased in tumoral versus normal breast cells and TSPO was again correlated with Ki-67 levels. TSPO was anyway never associated with reduced prognosis in the whole patient sample, but in lymph-node negative group of patients, elevated TSPO was linked to a shorter disease-free survival period, indicating that TSPO could be used to identify a higher risk population in this category (140). TSPO is reported overexpressed in oesophageal cancer cells compared to normal oesophageal epithelium (141), as well as in human endometrial carcinoma, where significantly more mitochondrial TSPO was observed in contrast to normal endometrium (142). The link between TSPO and cancer is therefore consolidated even though the underlying pathogenic mechanism driving a primary oncogenic profile is still lacking.

**Inflammatory Brain conditions**

High levels of TSPO are recurrently associated with glial cell activation and inflammation (143, 144). Chronic inflammation is an early feature of many neurological conditions and other age-related conditions including atherosclerosis [reviewed in (145)]. Uptake of TSPO ligands in the brain is generally low in normal healthy adults, but overall uptake is increased with age (146). An increase in TSPO expression has been observed during the onset of disease in amyotrophic lateral sclerosis (ALS) patients (147). Similarly, brains of subjects with mild cognitive impairment (MCI), who are at an increased risk of developing Alzheimer’s disease (AD), or subjects with mild and early forms of AD, exhibit higher ligand binding compared to age-matched controls (148, 149) although other studies contradict this observation (150). Widespread glial activation is likewise associated with the pathological process that occurs in Parkinson’s disease (PD) and TSPO ligands have shown increased binding profiles in rat models mimicking the early stages the disease (151). Clinical studies show that compared to controls, PD patients have increased levels of TSPO ligand binding in the regions of the brain (152), although the degree of binding did not alter over a two-year period and was not associated with clinical severity demonstrating that TSPO overexpression and inflammation are likely early diagnostic factors (153). In stroke patients, TSPO expression and glial activation is...
increased in the peri-infarct zone for several weeks following insult (154) while bio-imaging has revealed elevated TSPO expression in inflamed atherosclerotic plaques of the vasculature (155, 156). The precise molecular mechanisms characterizing a functional role for TSPO in these diseases are unclear leaving a general understanding that the upregulation of TSPO is an adaptive consequence rather than a causative factor on which the mitochondrial dependent rewiring of cell signaling and metabolism of the cells, is affected. It is anyway known that factors playing part in the regulation of TSPO expression such as ROS, Interleukin-1 (IL-1) and TNF α, are clearly involved in this process suggesting that a molecular pro-inflammatory role is plausible (18, 157).

TSPO is also implicated in peripheral inflammatory diseases for example the Inflammatory Bowel Disease (IBD). In this it has been demonstrated an increase in TSPO expression in the colon likewise in human intestinal biopsies from IBD patients (18). TSPO ligands have been indeed developed or adapted along the years to illustrate areas of inflammation through in vivo imaging analysis, making this one of the most active fields for biomedical applications targeting the protein. Interestingly, almost unexplored is whether these same tools may be effective as therapeutic means against the diseases in which they are adopted. In this regard, some preliminary material seems to support this notion (21, 24, 158) but more is to be done.

The pharmacology of a natural mitochondrial target

The consolidated profile as a multi-drugs binding protein has made TSPO a topic of natural interest for pharmacologists. Numerous are indeed the publications reporting the use of TSPO targeting chemicals to dissect and/or correct biological processes associated with the protein. The archetype synthetic TSPO ligands, prevalently used are the 4’-chboro-derivative of diazepam, Ro 5-4864, and the isoquinoline carboxamide, PK11195 which respectively bind TSPO with nanomolar affinity even though this is greater for PK11195 (K<sub>D</sub> <20 nM) (159). Several endogenous ligands for the protein have been also identified among the porphyrins (cholesterol, hemin, protoporphyrin IX, mesoporphyrin IX, deuteroporphyrin IX) (160, 161).

In spite of the extensive use, the most recent evidences suggest that these may not be so reliable since Ro 5-4864 varies its efficacy across species whilst PK11195, exerts cellular effects independent of TSPO (162-165) soon as it is over-dosed (µM rather than nM).

This has consequently stimulated the search for alternative compounds based on different chemical structures. Another class of compounds generated and still binding TSPO with nM affinity was identified in the 2 aryl-3-indoleacetamides (FGIN-1) (166), which retains good cross-species efficacy. Furthermore this drug does hold anxiolytic-like effects (167) likewise the XBD173 (AC-5216; Emapusil). The mechanisms by which these two chemicals operate are univocal by inducing a boost of the production of neurosteroids. Once they are bound to TSPO they would in turn potentiate the GABAA receptor-based neurotransmission leading to a beneficial effect linked with the regulatory cascade depending on this receptor.
However, a series of highly specific synthetic ligands for TSPO have been successfully developed in the form of radiotracers for PET bio-imaging of inflammation, for example the 7-chloro-N,N,5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4H-pyridazino[4,5-b]indole-1-acetamide (SSR180575) (168) and 2-(5, 7-diethyl-2-(4-(2-fluoroethoxy)phenyl)pyrazolo[1,5-a]pyrimidin-3-yl)-N,N-diethylacetamide (VU11S1008) (169) and the 12a ([18]F)GE-180). Thus, the exploitation of TSPO upregulation for the in vivo diagnostic imaging of patients is probably the primary and mostly corroborated clinical value for TSPO and holds the potential to grow and extend across multiple fields by refinement in the chemistry of TSPO-binding ligands in order to overcome imaging complications. In spite of this the pharmacological regulation of TSPO does remain debated.

Very recently Selvaraj and Stocco have, very timely and elegantly, stimulated attention towards the word 'ligand' as loosely used in the TSPO literature in reference to all substances that bind TSPO at various distinct sites (170). This is quite appropriate, as findings on ligand-binding response have proved inconsistent along the years, ranging from steroid hormone production to cell proliferation, signaling and apoptosis. The word ligand thus describes a small-molecular substance that binds to a target biomolecule (or complex) without the need to elicit or modulate a specific biological response as required instead by an agonistic or antagonistic which directly associated with the function of the target. In the case of TSPO such function is -for now- represented solely by the translocation of cholesterol and consequent synthesis of steroids.

However, gene-edited animal models for TSPO do not reassemble, challenged with TSPO-binding chemicals, the pharmacological effects recorded in vitro experiments, implying these chemicals may have alternative biochemical/pharmacological relevance beyond TSPO or the TSPO dependent translocation of cholesterol may not represent per se the perfect assay to sample their activity. Although basal steroids could be still formed without TSPO being expressed a regulatory role for the protein cannot be excluded and thus TSPO ligands, which do mediate a robust effect on steroids as well as neurosteroids formation, may also be affected.

The manufacture of steroids might indeed entail concurrent limiting steps depending on alternative molecular elements and therefore likely to be too elaborated to allow fast discrimination of TSPO pharmacology. The effort should be therefore to outline alternative fast-response assays, which could insight, not only the binding capacity but also the agnostic/antagonistic profiles of the compounds.

In this the recent advancements in comprehending the structure and the functional role of TSPO in respect of cell signaling mitochondrial cell biology may represent the solution. Even without a direct etiological link, this quest for TSPO-binding therapies has been extensive in recent years and applied to various diseases prompted by the pressing need for drug discovery pipelines, being the upregulation of TSPO constant in pathological lesions.

Thus, TSPO ligands have still proven efficacious in ameliorating disease pathology in numerous preclinical models and most remarkably in models bearing phenotypes in the central nervous system.
Recently, Ro5-4864 was shown to amend Alzheimer’s disease associated phenotypes in vivo (25) by mediating positive effects in steroid hormones production (109) (Table 3).

Human clinical trials have been performed for Emapunil to treat anxiety disorders with promising outcomes reported in an induced model of panic disorder (31). However, the high TSPO binding variability across human subjects -recently observed- does render unlikely a drug development via this mechanism of targeting (operating by inducing a boost of the production of neurosteroids as recorded following challenge with TSPO ligands) which remains, anyhow the most promising one (174). On the other hand, the early assessment of mutations in TSPO (e.g. A147T SNP), via advanced imaging protocols, could lead to personalized therapies and consequent tangible benefit for patients.

What is known since long time is that TSPO-binding drugs can cause death of cancer cells and therefore considered potential anticancer therapies. This was prevalently associated with the putative role of TSPO in activating the Mitochondrial Permeability Transition Pore (MPTP) that was also hypothesized to be involved in the protection mediated by the TSPO ligands as heart protectors and tools to reduce infarct size after ischemia-reperfusion (175, 176). The recent advancements in the clarification of the MPTP molecular entity rule TSPO in its formation and regulation and therefore in the consequent protein-associated pharmacological mechanisms thus calling for these to be clarified if therapeutic uses are to be exploited.

This further highlights how structure and conformational adaptation of the protein are essential to better dissect the pharmacology of this pathway and in this way ease the comprehension and validation of current and future chemicals.

Concluding remarks

The aspects of TSPO above discussed report of a multifaceted mitochondrial pathway characterized by interdisciplinary interest. The evidences gathered along the years have enriched the landscape of TSPO science favoring its current exploitation by diagnostic and therapeutic protocols.

Being brought up to general attention, as an off-target binding site of the benzodiazepines TSPO is now a corroborated regulatory element in the mitochondrial and cellular pharmacology of lipids.

In the attempt to interpret the series of information homogenously it seems that the accumulation of TSPO, reconciled during inflammation and therefore targeted by advanced protocols of in vivo imaging, prevents the removal of mitochondria by the autophagy-operated quality control mechanisms leaving the organelles to be primed by the cells for steroids synthesis and undermining other core functions that are indeed compromised by the accumulation of TSPO.

The concerted cooperation among disciplines, a structured cooperation by experts and the advancements in the technology and experimental protocols will continue to improve and modernize TSPO science incrementing its significance in mammalian pathophysiology to obtain an informed understanding of this regulator and target of the mitochondrial function.
Declaration

Authors disclose no conflicting interests related to the matter of this publication.

Acknowledgements

The research activities on TSPO lead by MC are supported by: BBSRC, MRC, PPCT, The Umberto Veronesi Foundation, MarieCurie Actions and LAM-Bighi Grant to the all of which we are sincerely grateful.

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Legends to Figures

Figure 1: TSPO transcriptional regulation - crosstalk between hormone and REDOX sensitive signal transduction pathways

Hormonal stimuli that bind to G-protein coupled receptors (GPCR) in the plasma membrane results in cAMP synthesis from ATP via adenylate cyclases. cAMP is a signaling molecule that triggers the release of ACBD3 from the Golgi, which translocates to mitochondria, where it recruits PKA through its interaction with TSPO. PKA has been demonstrated to phosphorylate TSPO and VDAC and its kinase activity is dependent upon local concentrations of cAMP. cAMP also activates PPARα, which interferes with Tspo transcription factors including AP-1. TSPO is also sensitive to changes in REDOX status. Cytoplasmic or mitochondrial sources of ROS may activate the PKCε-Ral1-MEK1/2-ERK1/2 signaling cascade, which promotes Tspo transcription via the activity of c-Jun and STAT-3 transcription factors. An increase in TSPO may potentiate this response by maintaining an oxidative environment. ROS have also been demonstrated as promoting the formation of TSPO multimers.

VDAC – voltage dependent anion channel; TSPO – translocator protein; ACBD3 – Acyl-coenzyme A binding domain containing 3; PKA – cAMP dependent protein kinase A; cAMP - cyclic adenosine monophosphate; ROS – reactive oxygen species; PPARα - peroxisome proliferator-activated receptor alpha; STAT3 – signal transducer and activator of transcription 3; AP-1 – activator protein 1
Figure 2: TSPO domain architecture and cross-species sequence alignment

Sequence alignment of TSPO using ClustalX software, showing conserved regions including the cholesterol-binding domain (CRAC sequence) in rhodobacter sphaeroides TspO, Homo sapiens, Mus musculus and Canis lupus familiaris. Transmembrane (TM) domains are denoted by black lines (TM1: 6-26 a.a; TM2: 47-67 a.a; TM3: 80-100 a.a; TM4: 106-126 a.a; TM5: 135-155 a.a.). Loops 1 (6-26) and 3 are cytosolic; loops 2 and 4 are facing the mitochondrial intermembrane space. The longer cytosolic loop 1 is implicated in dimerization, possible VDAC interaction, and the binding of ligands. b) The default colour scheme used for sequence alignments generated in ClustalX, the graphical interface for the ClustalW multiple sequence alignment programs. Residues in the alignment are assigned a colour according to the amino acid profile. The table shown gives the criteria as clauses +X%, xx, y; where X is the minimum percentage presence of any of the xx or y residues accompanied by a functional profile. White regions are unconserved.
Table 1: Phenotypic characterization of global and conditional Tspo-/- mice

<table>
<thead>
<tr>
<th>Mitochondria phenotype</th>
<th>Global C57BL/6-Tspo&lt;sup&gt;tm1GuMu(GuwiyangWurra)&lt;/sup&gt;</th>
<th>Global (Tu et al) Mitochondrial volume in primary fibroblasts unchanged; Mitochondrial morphology unchanged in testis and ovary</th>
<th>Nr5a1-Cre Driven Tspo Conditional Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>No differences observed</td>
<td>Increased body weight in female mice (~1g, 1-5 weeks of age)</td>
<td>Depletion of lipid storage in testis; Increased accumulation of lipids in adrenals</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>No effect on lipid deposits in adrenals</td>
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<tr>
<td>Hormone-induced steroidogenesis</td>
<td>hCG-induced plasma testosterone unchanged Increased levels of plasma estradiol</td>
<td>Impaired ACTH stimulation of corticosterone production hCG-induced plasma testosterone unchanged Increased epinephrine production</td>
<td></td>
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<tr>
<td>Steroidogenesis</td>
<td>No change in basal steroidogenesis</td>
<td>No change in basal steroidogenesis</td>
<td>No change in basal steroidogenesis</td>
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<tr>
<td>Immune response</td>
<td>Microglial activation following neuronal injury normal; Increased levels of NKT cells in female mice</td>
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</table>

Table 2: Compensatory gene expression in Tspo-/– mice: focus on the immune response and hormone regulation

<table>
<thead>
<tr>
<th>TSPO Function</th>
<th>Gene</th>
<th>Tissue</th>
<th>Protein product function</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immune Response</strong></td>
<td>Tmem178</td>
<td>Adrenal</td>
<td>Negative regulator of macrophage activation&lt;sup&gt;109&lt;/sup&gt;</td>
<td>↓&lt;sup&gt;106&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Zbtb7b</td>
<td>Adrenal</td>
<td>T-cell maturation&lt;sup&gt;106&lt;/sup&gt;,&lt;sup&gt;107&lt;/sup&gt;</td>
<td>↑&lt;sup&gt;106&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Trim12a</td>
<td>Adrenal</td>
<td>Anti-viral response</td>
<td>↓&lt;sup&gt;106&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Pydc4</td>
<td>Adrenal</td>
<td>Anti-viral response&lt;sup&gt;112&lt;/sup&gt;</td>
<td>↓&lt;sup&gt;106&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Hormone Regulation</strong></td>
<td>Cyp21a2</td>
<td>Adrenal</td>
<td>P450 enzyme required for adrenal steroidogenesis&lt;sup&gt;117&lt;/sup&gt;</td>
<td>↑&lt;sup&gt;106&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Abca2</td>
<td>Adrenal</td>
<td>Regulates cholesterol efflux to ApoE3&lt;sup&gt;118&lt;/sup&gt;</td>
<td>↑&lt;sup&gt;106&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Scarb1</td>
<td>Adrenal</td>
<td>Mediates uptake of HDL-derived cholesterol and cholesteryl ester&lt;sup&gt;119&lt;/sup&gt;</td>
<td>↑&lt;sup&gt;107&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Lhcgr</td>
<td>Testes</td>
<td>Hormone receptor, required for reproduction</td>
<td>↑&lt;sup&gt;107&lt;/sup&gt;</td>
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<tr>
<td>Ligand</td>
<td>Chemical Class</td>
<td>Disease-relevant study</td>
<td>Outcome</td>
<td>Ref</td>
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<tr>
<td>ZBD-2</td>
<td>Phenylpurine acetamide</td>
<td>Mouse: Middle cerebral artery occlusion                                                   Neuroprotection</td>
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<td>Midazolam</td>
<td>Benzodiazepine</td>
<td>Rat: Single prolonged stress model                                                        Reversed PTSD-associated freezing and anxiety-like behaviour via neurosteroidogenesis</td>
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<td></td>
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<tr>
<td>YL-IPA08</td>
<td>Pyridazinoindole acetamide</td>
<td>Mouse: Inescapable electric foot shock induced model of PTSD                             Reduced anxiety and fear, mediated by allopregnanolone synthesis; antagonized by PK 11195</td>
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<tr>
<td>PK 11195</td>
<td>Isoquinoline carboxamide</td>
<td>Rat: Model of cortical contusion with hyperbaric oxygen therapy                           Reversed protective effects of hyperbaric hyperoxia in brain injury</td>
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<td>Etifoxine</td>
<td>Benzoxazine</td>
<td>Mouse: Experimental autoimmune encephalomyelitis (EAE) model                              Reduced inflammatory pathology in spinal cord, promoted oligodendoglial regeneration; promoted recovery when administered before and after development of symptoms</td>
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<tr>
<td>Ro-5-4864</td>
<td>Benzodiazepine</td>
<td>Rat: 30 min coronary occlusion/15 min reperfusion                                         Reduced infarct size and improved mitochondrial function post injury, reduced oxidative stress and oxysterol formation by lowering cholesterol accumulation</td>
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<td>MPIGA</td>
<td>Phenylindolygly oxalamide</td>
<td>Rat: Elevated maze test                                                                   Anxiolytic effects</td>
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<tr>
<td>FGIN-1-27</td>
<td>Arylindol acetamide</td>
<td>Mouse: Subcutaneous xenograft of HT29 cells in right thigh                               40 % reduction in the growth rate of grafted tumors</td>
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<tr>
<td>SSR 180575</td>
<td>Pyridazinoindole acetamide</td>
<td>Mouse: MRL/lpr model                                                                     TSPO ligands reduced pulmonary inflammation and alveolitis onset; similar effects seen with PK 11195 and Ro5-4864</td>
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<tr>
<td>Emapunil</td>
<td>Phenylpurine acetamide</td>
<td>Mouse: Vogel-type conflict test; light/dark box, social interaction tests                 Anti-anxiety effects</td>
<td>31</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rat: Forced swimming test                                                                  Anti-anxiety effects</td>
<td>186</td>
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Table 3: in vivo assessment of TSPO ligands in animal models of disease