Translocator Protein as a Promising Target for Novel Anxiolytics

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Abstract: Neurosteroids are able to rapidly control the excitability of the central nervous system, acting as regulators of type A receptors for GABA. Over the last two decades, many authors have confirmed that neurosteroid level alterations occur in psychiatric disorders, including anxiety disorders. More recently, investigators have manipulated neurosteroidogenesis in an effort to correct neuronal excitation and inhibition imbalances, which may lie at the root of neuropsychiatric conditions. In line with this strategy, emerging data have demonstrated that a promising target for therapy of anxiety disorders is the Translocator Protein (TSPO). TSPO is a five transmembrane domain protein (18 kDa) that is expressed predominantly in steroid-synthesizing tissues. At the subcellular level, TSPO is localized at contact sites between the outer and inner mitochondrial membranes and mediates the rate-limiting step of neurosteroidogenesis. Brain concentrations of neurosteroids can be affected by selective TSPO activation. Indeed, TSPO drug ligands are able to stimulate the primary neurosteroid formations that enhance $GABA_A$ receptor activity, pregnenolone and allopregnenalone, both in *in vitro* steroidogenic cells and *in vivo* animal models. A spectrum of TSPO ligands has been shown to exert anxiolytic actions when administered in rodents. Some TSPO drug ligands could potentially reach clinical development. For example, recent evidence has shown that the selective TSPO ligand, XBD173 (AC-5216, Emapunil), exerts anxiolytic effects not only in animal models, but also in human volunteers. Herein, we review the current literature regarding the central nervous system biology of TSPO, a promising molecular target, in combination with its available ligands.

Keywords: Translocator protein (TSPO), TSPO drug ligands; neurosteroids, type A receptors for GABA ($GABA_AR$ receptors); anxiety disorders, steroidogenesis, glial cells, anxiolytic effects.

ANXIETY AND THE $GABA_A$ RECEPTOR

Anxiety is a normal reaction to stress that helps one deal with a tense situation. When anxiety becomes excessive and irrational, it becomes a disabling disorder. Anxiety disorders are the most common type of psychiatric disorders (incidence of 18.1%, lifetime prevalence of 28.8%) [1, 2]. As such, these disorders account for a $42.3 billion annual cost in the United States, with over 50% of the total sum directed towards non-psychiatric medical treatment expenses [3]. European studies have provided information about the prevalence of generalized anxiety disorders in the general population and clinical samples; approximately 5% of the population develops such disorders at least once in their life [4].

The past decades have observed a massive growth in the knowledge of the anxiety neurobiology through detailed examinations of fear response, behavioral components. Several studies have demonstrated that the major components of cerebral circuits that coordinate defensive/adversive response to fear and stress are the amygdala and GABAergic pathways. GABAergic pathways are able to reduce the release of many neurotransmitters that are involved in anxiogenic responses. Accordingly, interruption of GABAergic transmission can generate anxiety in animal models [5-11]. Consistent with a role in controlling anxious states, the levels and binding properties of $GABA_A$ receptors can be altered in stress conditions and in anxiety [12-20].

A variety of psychiatric disorders exhibit perturbations of GABAergic transmission. Notably, some of these conditions have been associated with abnormal levels of certain neurosteroids. Such steroids are able to selectively enhance the function of $GABA_A$ receptors by their own site on the GABA complex [21-24].

$GABA_A$ RECEPTOR MODULATION

Benzodiazepines and $GABA_A$ Receptor Activity Modulation

$GABA_A$ receptors are pentameric hetero-oligomers, and their subunits share a conserved structure. Sixteen distinct $GABA_A$ receptor subunit genes have been identified to date, which are classified by sequence identity into the following seven subunit classes: α1-6, β1-3, γ1-3, δ, ε, π and θ [25]. However, a range of experimental approaches has suggested that the majority of $GABA_A$ receptor subtypes in the brain are composed of heteropentameric assemblies of α, β and γ subunits [26]. Benzodiazepines (Bdz) are commonly used in therapy as anxiolytic, anticonvulsant, myorelaxants and hypnotic agents. Bdz are able to modulate GABA transmission through their binding at Bdz site on $GABA_A$ receptor complex. The Bdz binding site is localized at the interface between the subunits α and γ. Once bound to the Bdz binding site, the Bdz locks the GABA receptor complex into a structural conformation that is able to bind natural agonist GABA

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with high affinity. This event increases the opening frequency of the associated chloride ion channel, subsequently hyperpolarizing the membrane of neurons.

Neurosteroid Metabolism and $GABA_A$ Receptor Activity Modulation

Steroids, such as glucocorticoids, mineralocorticoids and sex hormones, bind to intracellular receptors, which interact with distinct nucleotide sequences to alter gene transcription and protein synthesis on timescales of greater than tens of minutes [27]. Additionally, steroids possess rapid, nongenomic effects, particularly in the brain. These non-genomic activities are characterized by extremely rapid effects that range from milliseconds to minutes and do not require interaction with steroid hormone receptors. In the central nervous system (CNS), these effects are thought to involve steroid modulation of membrane-bound neurotransmitter receptors [28]. Arguably, the most important and widespread effect of steroid molecules may be due to GABA receptors, notably, the $GABA_A$ receptor [29].

Because of their lipophilic structure, steroids can easily diffuse across the blood-brain barrier if produced peripherally from the adrenal glands and gonads. Additionally, the brain exhibits the ability to synthesize its own steroids *in situ* independently of the peripheral source formation. Because the steroid molecules can undergo synthesis *de novo* in the CNS from cholesterol or peripheral steroid precursors and easily cross the blood-brain barrier, they are termed "neurosteroids." Some steroids are considered both neurosteroids - and neuroactive steroids because they are produced in the brain and in the adrenals, respectively, which exhibit effects on the brain [30, 31]. Although a great deal is known about the metabolism of various neurosteroids, much remains unknown, and the investigation of these various processes continues. Neurosteroids are synthesized *de novo* from cholesterol in the CNS through a series of enzymatic processes that are controlled by cytochrome P_{450} and non-P_{450} enzymes. Translocation of the cholesterol substrate from the outer to the inner mitochondrial membrane is the rate-limiting step of neurosteroidogenesis and appears to regulate all neurosteroid levels in the CNS [32]. Steroidogenesis is initiated at the inner mitochondrial membrane, whereas the cytochrome P_{450} cholesterol side chain cleavage enzyme (P_{450}ssc or CYP11A1) catalyzes the conversion of cholesterol to pregnenolone [33]. The biosynthetic pathway for neurosteroids is shown in Fig. (**1**).

Pregnenolone is the precursor of progesterone (P). Subsequent catalysis with 21β-hydroxylase (21β-HSD) converts P into deoxycorticosterone (DOC). 5α-reductase catalyzes the reduction of P and DOC into the 5α-pregnane steroids, 5α-dihydroprogesterone (5α-DHP) and 5α-dihydrodeoxycorticosterone (5α-DHDOC), respectively, and 5β-reductase reduces P and DOC to 5β-dihydroprogesterone (5β-DHP) and 5β-dihydrodeoxycorticosterone (5β-DHDOC), respectively. These pregnane steroids may be further reduced to the neurosteroids 3α,5α-tetrahydroprogesterone (3α,5α-THP), tetrahydrodeoxycorticosterone (3α,5α-THDOC), 3α,5β-THP and 3α,5β-THDOC by 3α-hydroxysteroid oxidoreductase (3α-HSD). These latter neurosteroids are the most potent positive modulators of $GABA_A$ receptor activity. In Fig. (**1**), the neurosteroids that potentiate $GABA_A$ receptor function (positive modulators) are inserted in rectangular boxes, and the neurosteroids that inhibit $GABA_A$ receptor function are inserted in circular boxes (negative modulators). Like many GABA-receptor potentiators, including barbiturates, etomidate, and propofol, the neurosteroid positive modulators enhance the interaction of GABA with $GABA_A$ receptors, an effect shared by low nanomolar concentrations of specific endogenous metabolites of progesterone and deoxycorticosterone. These steroids exhibit no effect on $GABA_A$ receptor single-channel conductance but greatly facilitate the open state of the GABA-gated ion channel. Furthermore, at modestly excessive concentrations (i.e., submicromolar to micromolar) of those required for enhancement of GABA-evoked responses, the steroids can directly activate (i.e., in the absence of GABA) the $GABA_A$ receptor channel complex. Channel activation occurs even though this action takes place through a site that is independent of the GABA-binding site and is also likely distinct from the site through which steroid potentiation is mediated [34].

In contrast to the positive modulatory steroids, the key determinants that mediate steroid inhibition of the $GABA_A$ receptors are unknown. Pregnenolone sulphate (PS) and dihydroepyandrosterone sulphate (DHEAS) act as non-competitive antagonists at the $GABA_A$ receptors and are less potent than the enhancing steroids.

$GABA_A$ Receptor Neurosteroid Binding Sites

One outstanding question in the field of inhibitory GABA receptors for at least two decades is whether neurosteroids bind to a definite site on a receptor protein to exert their effects or merely affect receptor function by perturbing the membrane lipid. Many studies have been performed to determine how neurosteroids interact with the $GABA_A$ receptor, as this aspect is considered a prerequisite for understanding their physiological and pathophysiological roles in the brain. The following points clearly demonstrate beyond reasonable doubt that specific neurosteroid binding sites do exist on ionotropic $GABA_A$ receptors. The interaction of a steroid with the $GABA_A$ receptor is critically dependent upon the steroid structure and is enantioselective [35, 36]. The discrete effect of receptor subunit mutations on steroid activity [37, 41] and crystallographic studies indicate that the steroid can indeed dock with many proteins [42, 43] and testify to the likelihood of defined $GABA_A$ receptor binding sites. Recent results have shown evidence for two neurosteroids action sites on $GABA_A$ receptors. One site spans the M1 and M4 transmembrane domains of the α subunit and accounts for the increasing action of some steroids. The other site exists between the M1 transmembrane domain of the α subunit and the M3 domain of the β subunit and is responsible for the direct channel gating via the steroid [41].

TRANSLOCATOR PROTEIN (TSPO) (18 KDA)

A protein was identified in 1977 as a binding site for the benzodiazepine diazepam in peripheral tissues [44]. This protein was named peripheral-type benzodiazepine receptor (PBR), to be distinguished from the central benzodiazepine receptor (CBR), which is associated to the chloride channel/$GABA_A$ receptor complex. Although the term 'PBR' is a

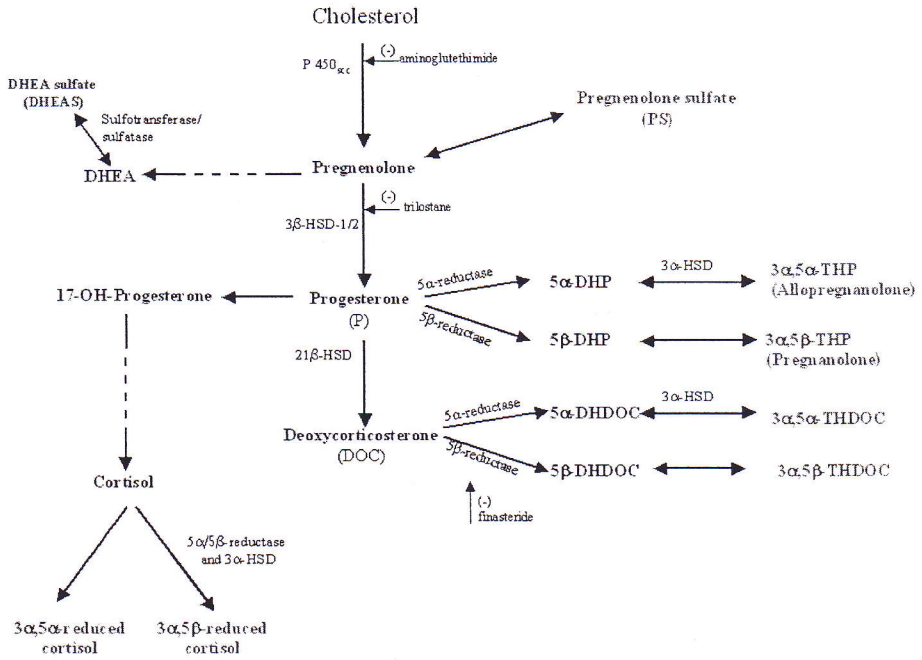

Fig. (1). Metabolism of neurosteroids. Most biosynthetic and metabolic reactions cited in the text are indicated with the corresponding enzymes and some of their pharmacological blockers (marked -). The neurosteroids that potentiate $GABA_A$ receptor function (positive modulators) are inserted in rectangular boxes, and the neurosteroids that inhibit $GABA_A$ receptor function are inserted in circular boxes (negative modulators). P_{450}ssc= cytochrome P_{450} cholesterol side chain cleavage enzyme; 3b-HSD-1/2= 3b-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; 21b-HSD= 21b-hydroxylase; 5a-DHP= 5a-dihydroprogesterone; 5b-DHP= 5b-dihydroprogesterone; 3a-HSD= 3a-hydroxysteroid oxidoreductase; 5a-DHDOC= 5a-dihydrodeoxycorticosterone; 5b-DHDOC= 5b-dihydrodeoxycorticosterone; 3a,5a-THDOC= tetrahydrodeoxycorticosterone; DHEA= dihydroepyandrosterone.

widely accepted name in the scientific community primarily for historical reasons, multiple other names have been used to refer to this protein, including mitochondrial benzodiazepine receptor, mitochondrial diazepam-binding inhibitor (DBI) receptor complex, PK-111195-binding sites, isoquinoline-binding protein (IBP), pk18 and ω3 receptor. Recent data have increasingly supported its renaming with the aim to represent its subcellular role and putative tissue-specific functions more accurately. Recently, a team of scientists has proposed the following new name for this protein: translocator protein (TSPO) (18 kDa) [45].

Functional inactivation of TSPO induces an early embryonic lethal phenotype in mice [46]. Together with the observation that TSPO is well conserved throughout evolution [47], this finding highlights the significance of TSPO in tissue development and function.

TSPO is widely expressed throughout the body but is particularly enriched (20- to 50-fold) in tissues in which steroids are synthesized, such as adrenal, gonad and brain cells [45]. In the CNS, TSPO is usually expressed in ependymal and glial cells. However, TSPO expression has also been shown in some neuronal cell types, such as cells of the olfactory bulb, neuroblastoma cells, cultured cortical neurons and rat dorsal ganglia sensory neurons [48, 52]. At the subcellular level, TSPO is primarily localized to the outer mitochondrial membrane (OMM), especially at OMM-IMM (inner mitochondrial membrane) contact sites. Hydropathy profile analysis of the 169-amino acid TSPO sequence suggested a putative five transmembrane structure that has since been experimentally confirmed [53]. As a major component of the outer mitochondrial membrane, TSPO mediates various mitochondrial functions, including cholesterol transport and steroid hormone and bile salt syntheses, mitochondrial respiration, mitochondrial permeability transition pore (mPTP) opening, apoptosis and cell proliferation [47, 54-57]. Notably, the role of TSPO in most of these functions was discovered using TSPO drug ligands; a direct role for this protein has been demonstrated in only a few of these functions (e.g., steroid biosynthesis and cell proliferation).

TSPO and Cholesterol Binding

Among endogenous ligands, cholesterol binds to TSPO with nanomolar affinity. Once cholesterol is bound to TSPO, it is committed to its use in steroidogenesis. TSPO is the gatekeeper of this process and controls the cholesterol transport rate from the OMM to the IMM, which is the first step in steroids synthesis [58, 59].

Expression of TSPO, using an inducible mouse cDNA TSPO vector in *E. coli* DE3 cells, which have no TSPO, no cholesterol, and do not synthesize steroids, induced the ability to take up cholesterol in a time-dependent, temperature-sensitive and energy-independent manner. Moreover, it has been confirmed that TSPO functions as a cholesterol translocator, suggesting that TSPO might also function as a cholesterol sink, holding cholesterol until it is released by the binding of another ligand [60, 61].

The role of TSPO in steroidogenesis has also been investigated with a molecular approach based on the disruption of the TSPO gene in constitutive steroid-producing R2C rat Leydig cells via homologous recombination. Without TSPO proteins, the generated G418/ganciclovir-resistant cell line produced small amounts (10%) of steroids when compared to normal R2C cells. Transfection of these cells with TSPO cDNA restored high levels of steroid production by the TSPO-negative R2C cells, indicating that the cholesterol transport mechanism was impaired due to the absence of the TSPO protein [46].

To identify the potential binding sites of the TSPO-cholesterol interaction, molecular modeling and site-directed mutagenesis techniques have been employed. A cholesterol-binding site at the cytoplasmic C-terminus region of TSPO has been reported and confirmed after the TSPO C-terminus deletion (Δ153-169), which reduced cholesterol uptake [61, 62]. Additionally, a cholesterol recognition amino acid consensus (CRAC) sequence has been identified (-L/V-$(X)_{1-5}$-Y-) in the TSPO C-terminus [61-65]. This CRAC pattern has been observed in several other proteins that are known to interact with cholesterol. Li and Papadopoulos (1998) [61] have hypothesized that leucine or valine interacted with the hydrophobic side chain of cholesterol and tyrosine interacted with the polar 3'OH-group of cholesterol. They tested this hypothesis and replaced the Y153 with a serine; this amino acid replacement completely abolished the ability of TSPO to take up radiolabeled cholesterol. Although the substitution of A147 with threonine in the mouse TSPO amino acid sequence did not affect cholesterol uptake, we have recently found that the spontaneous mutation of Ala147Thr (rs6971) within the C-terminus of the human TSPO affected the pregnenolone production in lymphomonocytes of healthy individuals [66]. Specifically, the lymphomonocytes of Thr147 homozygous or heterozygous individuals produced significantly lower levels of pregnenolone when compared with Ala147 homozygous individuals. These results suggested that the presence of a single copy of the Thr147 allelic variant can influence the cholesterol translocation efficacy of human TSPO.

Studies on three-dimensional (3-D) TSPO models suggested the five α helices form a channel with an interior hydrophilic and uncharged surface. The channel interior was shown to bind cholesterol [60]. The topography and organization of TSPO have been investigated by transmission electron and atomic force microscopy, performed on cell mitochondrial preparations [54]. The images showed that the TSPO formed clusters containing four to six molecules [67]. Such topography changes are blocked by selective inhibitors of PKA (H-89) and TSPO ligands [68] and associated with hormonal treatment [69]), facilitating cholesterol transfer.

Notably, the antisera for distinct TSPO regions have identified diverse immunoreactive proteins (18, 40, 56, 72, 90 and 110 kDa) that correspond to TSPO polymers [70]. Such polymers have been correlated to reactive oxygen species (ROS) levels because the ROS were shown to induce polymer formation of soluble proteins. To further characterize their nature, spectroscopic analyses were performed, revealing covalent dityrosine cross-links between TSPO monomers. Moreover, cholesterol binds to TSPO monomers/polymers, and the addition of TSPO ligands to the polymers increased the cholesterol-binding rate [70]. The monomer is able to bind cholesterol with high affinity but not TSPO ligands. The polymer binds the TSPO ligands with high affinity, which induces rapid cholesterol binding.

Regarding the functional mechanism of cholesterol transport, TSPO has been proposed to serve as a cholesterol exchanger; in steroidogenesis, cholesterol would be exchanged with either pregnenolone or progesterone, steroids that are formed in the mitochondria and compete with cholesterol, inhibiting both ligand- and cholesterol-binding to TSPO [54]. Various studies have been aimed to determine if other proteins were assisting TSPO in cholesterol mitochondrial translocation. Because TSPO is primarily located at the mitochondrial contact sites, it has been suggested that TSPO does not function alone in the OMM [71].

TSPO has been described to be associated with other proteins located either in the OMM and IMM or in the cytosol. A photolabeled TSPO was shown to be associated with two proteins of 32 and 30 kDa [72], the voltage-dependent anion channel (VDAC) and the adenine nucleotide transporter (ANT).

Modeling has shown that VDAC binds cholesterol and influences its distribution in the mitochondria [73-75]. In addition to these mitochondrial membrane proteins, some cytosolic proteins regulate the function of mitochondrial TSPO [76-87]. A two-hybrid technique and cDNA library screening using TSPO as bait identified several TSPO-associated proteins. PBR (TSPO)-associated protein 7 (PAP7) showed high affinity for TSPO [87], and PAP7 is known to bind the regulatory subunit RIa of PKA. PAP7 has been suggested to recruit the PKA holoenzyme into proximity of the cholesterol-mediating transport proteins, playing a regulatory role in their activity via phosphorylation. PKA might phosphorylate TSPO [88-91], triggering the reorganization of TSPO topography and function and leading to cholesterol uptake and transport to the inner mitochondrial membrane.

Additionally, PBR (TSPO)-associated protein 1 (PRAX-1) has been reported to specifically interact with the C-terminus of TSPO, but its presence is restricted to brain and thymus [92].

The steroidogenic acute regulatory-related lipid transfer (StART) protein is also related to TSPO. Steroidogenic cholesterol is targeted to the mitochondria though proteins containing the StART domain [93]. The 210-amino acid sequence forms a hydrophobic channel that can hold a sterol-molecule [94]. The structure becomes stable upon cholesterol binding [95]. Transfection of StAR expression vectors in both mouse Leydig MA-10 and COS F2 cells, which contain the components of CYP11A1, was found to increase steroidogenesis [96, 97]. StAR has been suggested to assist with cholesterol transfer to the mitochondria. When StAR expression was reduced, the hCG-stimulated MA-10 Leydig cells stopped producing progesterone after twenty minutes, while in TSPO-depleted cells, steroidogenesis was inhibited after ten minutes [98]. This arrested time difference was attributed to the presence of OMM cholesterol available to be used for steroidogenesis.

In summary, the hypothetical mechanism of cholesterol transport to mitochondria involves many proteins [59, 99]. The StAR protein may bind cholesterol in the cytoplasm and then transport it to the mitochondria. At the OMM, phosphorylation of the StAR protein by PKA [100] is facilitated by the presence of the adapter protein, PAP7 [87]. Cholesterol is then transferred from StAR to TSPO [72]. StAR has been proposed to function in the hormone-induced transport of cholesterol to the OMM, whereas TSPO regulates the translocation of cholesterol into the IMM.

ENDOGENOUS AND SYNTHETIC TSPO LIGANDS

Endozepines, a family of neuropeptides that were originally isolated from rat brain extracts on the basis of their ability to displace benzodiazepines from their binding site at the $GABA_A$ receptor are endogenous TSPO ligands [101]. Endozepines are derived through naturally occurring proteolytic processes from a common polypeptide precursor, DBI, which is widely expressed in the nervous system [102]. The major biologically active peptide fragments are the octadecaneuropeptide, DBI33-50 (ODN), and the triakontatetraneuropeptide, DBI17-50 (TTN) [103]. DBI has been shown to bind long-chain (C12-C22) acyl-CoA esters with high affinity, and is thus known as the acyl-CoA binding protein [104]. Recently, DBI was classified as a member of the acyl-CoA binding domain containing ACBD proteins and was renamed ACBD3 [105]. Furthermore, the endogenous ligands of TSPO are cholesterol and porphyrins, which exhibit nanomolar and micromolar affinities for TSPO, respectively [61, 106]. Endozepines are synthesized by peripheral nerve Schwann cells. In the CNS, the DBI gene is primarily expressed in glial cells. Classical drug ligands include isoquinoline 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) and benzodiazepine 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (Ro5-4864). PK 11195 binds exclusively to TSPO, whereas the benzodiazepines (Ro5-4864, AHN-086) require other mitochondrial protein components for full binding capacity. Isoquinolines became important drug ligands for the presence and function of TSPO in various tissues and cells, and their discovery was critical for further isolation and characterization of the TSPO protein. Over the last two decades, a spectrum of additional TSPO ligands has been developed and can be subdivided into different chemical classes. These classes include, the imidazopyridines, such as alpidem, which also binds to the central $GABA_A$/benzodiazepine receptors and related molecules, indole derivatives, FGIN-1-27 and SSR180575, pyrrolobenzoxazepines, phenoxyphenyl acetamide derivatives, DAA1106, and many others.

TSPO LIGANDS AND STEROID PRODUCTION: *IN VITRO* CELL MODELS

TSPO was proposed to play a role in steroidogenesis after ligand-binding studies revealed increased expression levels of TSPO in steroidogenic tissues and subcellular localization examinations indicated that TSPO was primarily localized to the OMM.

Mukhin and collaborators were among the first to investigate the potential role of TSPO in steroidogenesis [107]. These authors evaluated whether TSPO drug ligands affected steroid production in the peripheral steroidogenic cells, Y-1 mouse adrenal tumor cell line. The tested TSPO drug ligands stimulated the production of the primary steroid product of Y-1 cells, 20α-hydroxyprogesterone, in a concentration-dependent manner (TSPO drug ligand concentrations ranged from 1 nM to 50 μM). The potencies of each TSPO ligand that stimulated Y-1 cell steroidogenesis correlated very closely with their binding affinities to TSPO, suggesting that the effect of these drugs on steroidogenesis were consequent to their binding to TSPO. These findings represented a starting point, and researchers then began to better characterize such TSPO function using multiple steroidogenic cell systems and endogenous and drug ligands of TSPO (Table **1**).

The studies that followed soon after the initial study were performed using the available TSPO drug ligands, including isoquinolines, PK11195 and PK14067, and the benzodiazepine, 4'-Cl-diazepam (Ro5-4864). The binding parameters of such ligands have been previously characterized and demonstrated specific and selective binding to TSPO. Additionally, the benzodiazepine, diazepam, which binds to TSPO and central $GABA_A$/benzodiazepine receptors, has been investigated regarding TSPO steroidogenic function.

Papadopoulos and collaborators [108], using MA-10 mouse Leydig cells, have demonstrated that PK11195 and PK14067 were the most potent TSPO ligands, stimulating progesterone production by 3- to 4-fold. Among the benzodiazepines, Ro5-4864 was the most potent, inducing a 3.5-fold increase of steroidogenesis, whereas diazepam only attained a 2-fold stimulation. A correlation between the ligand-binding affinity to TSPO and their steroid stimulatory effect was also shown in this study. In addition, the authors studied whether the TSPO ligands, PK11195 (1μM) and Ro5-4864 (10 μM), could exert an additive stimulatory effect on steroid production with respect to other regulators of MA-10 Leydig cell steroidogenesis, chorionic gonadotropin (CG) or epidermal growth factor (EGF). The TSPO ligand actions did not enhance the stimulation CG-induced, but did enhance the stimulation EGF-induced. Using isolated mitochondria in the presence of exogenous cholesterol and trilostane, an inhibitor of further pregnenolone metabolism, the authors demonstrated that TSPO ligands stimulated production of pregnenolone. This effect was not observed with mitochondria devoid of its outer membrane (mitoplasts), which is in agreement with the localization of TSPO in the OMM. To identify the exact step in the mitochondrial pregnenolone formation that was activated by the TSPO ligands, the cholesterol level present in the OMM and IMM before and after treatment with the TSPO ligands was quantified. The obtained results demonstrated that the TSPO ligands stimulated pregnenolone formation by inducing the TSPO-mediated translocation of cholesterol from the outer to the inner mitochondrial membrane.

In the same year, Krueger and Papadopoulos [109] elucidated the potential mechanism underlying the stimulation of steroid production that was induced by the hormonal action and TSPO ligands, using Y-1 adrenal tumor cells and isolated mitochondria. These authors discovered a relationship between the hormone-induced steroid production and TSPO in Y-1 adrenal tumor cells, which was previously observed

Table 1. ***In Vitro*** **Effects of TSPO Ligands**

TSPO Ligand	*In Vitro* Model	*In Vitro* Effects	References
Ro5-4864 PK11195 PK14067 PK14068	Y1 mouse adrenal tumoral cell line	Stimulation of 20-α-hydroxyprogesterone formation, the main steroid product of these cells. -The potencies of these TSPO ligands to stimulate steroidogenesis correlated very closely with their binding affinity to TSPO.	[a]
Ro5-4864 PK11195 PK14067	MA-10 mouse Leydig tumoral cell line	Stimulation of progesterone production. -The action of TSPO ligands was not additive to stimulation by CG, but was additive to EGF.	[b]
	Isolated mitochondria	-TSPO ligands stimulated pregnenolone biosynthesis when supplied with exogenous cholesterol.	
PK11195	Y1 mouse adrenal tumoral cell lines -isolated mitochondria	Stimulation of 20α-OH-Progesterone in cells. Stimulation of pregnenolone independent of exogenously supplied cholesterol in isolated mitochondria.	[c]
Ro5-4864	C6-2B rat glioma cell line -intact cells	Stimulation of pregnenolone formation following incubation with Ro5-4864 nanomolar concentrations. -This effect was dose-dependent.	[d]
DBI TNN, DBI-(17-50) ODN, DBI-(33-50)	Mitochondria of C6-2B	Stimulation of pregnenolone production by DBI and TNN, DBI-(17-50).	[e]
arylindole-acetamide derivatives (collectively termed FGIN-1)	Mitochondria of rat C6-2B glioma cell line	2b, 2f, 2o, 2s, 2t, 2v, 2z stimulated pregnenolone formation. -A good correlation was found between binding affinity and the ability of the ligand to stimulate pregnenolone synthesis. -2z accumulated pregnmolone in rat brain mitochondria.	[f]
FGIN-1-27, FGIN-1-44	Isolated rat brain mitochondria	Stimulation of the rate of pregnenolone synthesis. -This effect was inhibited by PK11195.	[g]
pyrrolobenzoxazepine derivatives	Y-1 mouse adrenal cell line	5a, 17e, 17 h, 17i, 17j, 17l stimulated pregnenolone production as PK11195.	[h]
pyridopyrrolo- and pyrrolobenzoxazepine derivatives	MA-10 mouse Leydig cell line	11a, 11g, 11i, 11j and 11p stimulated progesterone production as PK11195.	[i]
phenylindolyl-glyoxylamide derivatives (PIGA)	C6 rat glioma cells	5-7, 13-21, 24-27 stimulated pregnenolone production. -15, 19, 26 and 27 were more effective than PK11195.	[l]
phenylindolyl-glyoxylamide derivatives (PIGA)	C6 rat glioma cells	1-21, 25-34, 45-49, 52-56 stimulated pregnenolone production. -8, 9, 21, 25, 30, 32, 34 were more effective than PK11195.	[m]
arylpyrazolo-pyrimidin-acetamide derivatives	C6 rat glioma cells	3j, 3o, 3q, 3r, 3t stimulated pregnenolone production as PK11195.	[n]
N,N-di-n-propyl-2-(4-methylphenyl)indol-3-ylglyoxylamide (MPIGA)	ADF human glioblastoma multiforme cell line	Stimulation of allopregnanolone and progesterone production.	[o]
	Conditioned medium from MPIGA-treated ADF cells	Potentiates the ^{36}Cl- uptake into rat cerebral cortical synaptoneurosomes.	
phenylimidazo-pyridine derivatives	Xenopus oocytes expressing human cloned $GABA_A$ receptor	-A subset of compounds potentiates the GABA-evoked Cl- currents.	[p]
emapunil (AC-5216/XBD173)	Mouse neocortical slices	Potentiates GABAergic neurotransmission.	[q]

[a]= data from reference [107]; [b]= data from [108]; [c]= data from [109]; [d]= data from [110]; [e]= data from [111]; [f]= data from [117]; [g]= data from [130]; [h]= data from [119]; [i]= data from [120]; [l]= data from reference [125]; [m]= data from [126]; [n]= data from [123]; [o]= data from [127]; [p]= data from [128]; [q]= data from [138].

in MA-10 cells. Indeed, the stimulation of 20α-hydroxyprogesterone production by adrenocorticotropin (ACTH) was nonadditive with that of the stimulation by PK11195. When the cells were treated with the protein synthesis inhibitor, cycloheximide, the steroid synthesis blocking induced by ACTH was shown, which is an effect characterized by an accumulation of cholesterol in the OMM. In contrast, PK11195-stimulated steroidogenesis was not inhibited by cycloheximide. When the isolated mitochondria were used, the stimulation of pregnenolone production by PK11195 was independent of the exogenously supplied cholesterol, indicating that TSPO acted on the cholesterol that was already situated within mitochondrial membranes.

To investigate steroid biosynthetic TSPO function in the CNS, Guarneri [110] and Papadopoulos [111] selected the C6-2B subclone of the rat glioma cell line as a steroidogenic model. The experimental model used by Guarnieri and collaborators [110] consisted of intact C6-2B cells that were incubated with precursor, [^{3}H]mavalonolactone ([^{3}H]MVA), to detect the [^{3}H]MVA incorporation in pregnenolone and thus, quantify the formation of this steroid. The authors measured the time course of pregnenolone formation in untreated and Ro5-4864-treated C6-2B cells. Under these conditions they showed a biphasic incorporation of [^{3}H]MVA into cholesterol and pregnenolone, with an initial rapid phase (within 1 min), followed by a slower phase. The pregnenolone formation was stimulated by nanomolar concentrations of Ro5-4864 after 5 min of incubation with [^{3}H]MVA. The stimulatory effect was dependent on the drug concentration, and the maximal effect was achieved at 10 nM. The experimental model used by Papadopoulos and collaborators [111] consisted of mitochondria isolated by C6-2B cells. In the isolated mitochondria, the effect exerted by the natural TSPO ligand, DBI, on pregnenolone production was investigated. The occupancy of TSPO with nanomolar concentrations of DBI and its naturally occurring processing product, DBI-(17-50), increased pregnenolone formation. DBI(33-50), which exhibits a higher affinity for $GABA_A$ receptors but a low affinity for TSPO, was ineffective in stimulating pregnenolone synthesis.

Studies conducted by different laboratories have corroborated the observation that the synthesized classic and natural TSPO ligands, Ro5-4864 and PK11195, and DBI and DBI-(17-50), respectively, stimulated steroidogenesis in ovarian granulosa [112], placenta [113], sciatic nerve Schwann steroid-synthesizing [114] and brain [115] cells and in human testicular fragments [116].

Together, these findings have prompted researchers to synthesize novel TSPO ligands and evaluate their ability to promote the steroid biosynthesis. First, the novel ligands with various chemical structures were evaluated for specific binding to TSPO using a [^{3}H]PK11195 radioligand competitive binding assay. To ensure the TSPO-binding selectivity of such new ligands and exclude their binding to the CBR, the new ligands were tested for binding to CBR using the CBR-selective radioligand, [^{3}H]Ro151788, in cerebral cortex cell membranes. A subset of ligands with the best binding affinity to TSPO were then tested for their potential activity on steroid production in well-validated steroidogenic cell systems. The structurally different ligand classes included indolacetamides [117, 118], benzoxazepines [119, 120], imidazopyridineacetamides [121], phenoxyphenylacetamides [122], pyrazolopyrimidineacetamides [123, 124] and indol-3-ylglyoxylamides [125, 126]. The effects of novel synthesized TSPO ligands on steroid production are summarized in Table **1**. The compound names are listed as reported by the authors in published manuscripts. The new compounds that were more effective than the classic TSPO ligand, PK11195, are also listed in Table **1**.

In 1993, Kozikowski and collaborators [117] developed and studied a new class of compounds that bind with high affinity and specificity to TSPO. These compounds were indoleacetamide derivatives, collectively termed FGIN-1. A subset of arylindole-acetamide derivatives with high TSPO binding affinity were observed to stimulate pregnenolone production from the mitochondria of C6-2B glioma cells [117]. A good correlation was found between TSPO binding affinity and the ability of these ligands to stimulate pregnenolone synthesis.

Novel pyridopyrrolo- and pyrrolo-benzoxazepine derivatives stimulated progesterone production with similar potency as the classic TSPO ligand, PK11195, in the Y-1 adrenal and MA-10 Leydig tumoral cells [119, 120]. Arylpyrazolo-pyrimidin-acetamide derivatives also stimulated pregnenolone production using PK11195 in C6 rat glioma cells [123, 124].

A series of highly potent and selective TSPO ligands, which represent conformationally constrained analogues of FGIN-1 derivatives, has been developed and are known as N,N-dialkyl-2-phenylindol-3-ylglyoxylamides (PIGA). The new PIGA derivatives were effective in stimulating pregnenolone production in C6 rat glioma cells, and a number of these ligands were more effective than PK11195 [125, 126]. Of this class of TSPO ligands, N,N-di-n-propyl-2-(4-methylphenyl)indol-3-ylglyoxylamide (MPIGA), the derivative that presented theoretically calculated physicochemical properties and fulfilled the requirements for adequate distribution into the CNS, was selected and used in *in vitro* investigations. As such, MPIGA was found to stimulate the production of pregnenolone from ADF human glioma cells when trilostane, an inhibitor of pregnenolone metabolism, was added to salt culture medium [127]. Without the addition of trilostane, MPIGA increased the formation of allopregnanolone, the primary positive steroid modulator of $GABA_A$ receptor activity. Following cell treatment with MPIGA, the amount of the primary negative steroid modulator of $GABA_A$ receptor activity, DHEAS, was not detectable in the salt culture medium. The salt culture medium that was derived from the MPIGA-treated ADF cells was evaluated and shown to affect the $GABA_A$ receptor activity in a well-validated *in vitro* model, consisting of synaptoneurosomes obtained from the rat cerebral cortex. The culture medium obtained from the MPIGA-treated ADF cells increased the uptake of $^{36}Cl^-$ into the synaptoneurosomes. The MPIGA-treated cells were then incubated with aminoglutethimide, the first enzyme inhibitor in the steroidogenic pathway (cytochrome P450scc) to demonstrate that the observed effect was mediated by the steroid molecules that were released from the cells following treatment with MPIGA. Under these experimental conditions, an increase of $^{36}Cl^-$ uptake into the

synaptoneurosomes was not observed. Other authors have previously evaluated whether TSPO ligands affected the $^{36}Cl^-$ uptake [128]. Because they found that these TSPO ligands possessed some affinity for the CBR, the authors investigated whether a subset of new phenylimidazo-pyridine derivatives directly modulated the opening of the Cl^- channel in Xenopus oocytes expressing cloned $GABA_A$ receptors.

TSPO LIGANDS, STEROID PRODUCTION AND ANXIOLYTIC EFFECTS: ANIMAL MODELS

Ligand interactions with TSPO stimulates cell steroid production *in vitro*. This discovery has prompted several authors to investigate whether the TSPO ligands affected the steroid production in animal models and exerted anxiolytic effects (Table **2**).

To this aim, authors have performed studies in rodents deprived of gonads and adrenal glands to eliminate the peripheral steroid sources. The first synthetic molecules that were administered *in vivo* included the classic TSPO ligands, such as the benzodiazepine derivative, Ro5-4864 [129], isoquinoline derivative, PK11195 [129-131]; and the arylindol acetamide derivatives, FGIN-1-27 and FGIN-1-44 [129, 130]. In these initial studies, the authors studied the effect of TSPO ligand administration on steroid production, measuring the pregnenolone content in various animal brain regions. The animals were pre-treated with the drug trilostane.

The *in vivo* administration of Ro5-4864, FGIN-1-27 or FGIN-1-44 increased pregnenolone levels in the brain of these animal models. For example, the effect of Ro5-4864 on the brain pregnenolone content was maximal (70-100%) at a dose of 18 mmol/kg, 5-10 min after intravenous injection. The effect of FGIN-1-27 was maximal (80-150%) at doses ranging from 400 to 800 mmol/kg, after oral administration [129]. In contrast, the administration of PK11195 did not change the pregnenolone levels in the brain of rats [132]. This result is evidence that the administration of PK11195 prevented the Ro5-4864- or FGIN-1-27-induced pregnenolone increase, suggesting that PK11195 may be an antagonist of TSPO [129, 130]. Romeo and collaborators have reported that both FGIN-1-27 and FGIN-1-44 reduced the fear of novelty during an elevated plus maze test when administrated orally to rats [130]. This group of researchers showed that FGIN-1-27 delayed the onset of isoniazid- and metrazol-induced convulsions [118, 131]. Moreover, FGIN-1-27 elicited anxiolytic-like effects in two anxiety animal models, the neophobic and conflict-punishment behaviors that were induced during an elevated plus maze test and Vogel conflict test, respectively [131]. The *in vivo* effect of FGIN-1-27 on brain steroid production has also been studied after microinjecting FGIN-1-27 into the dorsal hippocampus of rats [132]. Bitran and collaborators selected the hippocampus as a site for investigation because their previous study demonstrated that microinfusions of pregnenolone into the dorsal hippocampus elicited anxiolytic-like effects [133]. These authors found that intrahippocampal injections of FGIN-1-27 (2.5 μg) increased hippocampal allopregnanolone levels. Additionally, intrahippocampal injections of FGIN-1-27 produced anxiolytic-like effects during plus-maze and shock-probe burying tests [132].

The potential stimulatory steroidogenic *in vivo* effect exerted by the intraperitoneal administration of a representative subset of novel synthesized phenylimidazo-pyridine derivatives (compounds 17, 20, 26, 34 and 35) [128] and imidazopyridine derivatives (compounds CB 34, 50 and 54) [134] have been separately explored in intact rats that were deprived of their steroidogenic endocrine glands. The progenitor of these TSPO ligands has to be considered Alpidem, which is known to bind both TSPO and $GABA_A$/ benzodiazepine receptors with high affinity.

Trapani and collaborators have found that almost all representative ligands (i.e., compounds 17, 20, 26 and 34), with the exception of compound 35, markedly increased the levels of pregnenolone, progesterone, allopregnanolone and THDOC in the plasma and brain of rats [128]. A number of these ligands also possessed some affinity for the central $GABA_A$/benzodiazepine receptors, which prompted the authors to investigate whether this subset of derivatives directly modulated the Cl^- channel opening in Xenopus oocytes expressing cloned $GABA_A$/benzodiazepine receptors. Their results indicated that compounds 34 and 35, in good accordance with their ability to bind to the GABA/ benzodiazepine receptor, positively modulated the GABA-induced Cl^- channel opening. In contrast, compounds 17, 20 and 26, which showed almost no affinity for the GABA/ benzodiazepine receptor, did not modulate this response to GABA.

Serra and collaborators [134] have demonstrated that the CB compounds (3-50 mg/kg) induced a dose-dependent increase in the concentrations of pregnenolone, progesterone, allopregnanolone and THDOC in the plasma and brain. CB34 also stimulated the brain concentrations of neuroactive steroids in adrenalectomized-orchidectomized rats, suggesting that this compound stimulated brain steroidogenesis independently of its effect on peripheral tissues. The increase in brain neurosteroid content that was induced by CB34 has been associated with marked anti-conflict behavior during the Vogel test [134]. At doses of 25 and 50 mg/kg, the number of licking periods during punishment increased 3 and 4 fold, respectively.

Okuyama and collaborators have examined the behavioral profiles of the two TSPO phenoxyphenylacetamide derivatives, DAA1097 and DAA1106, in the mouse light/dark exploration test and in the elevated plus-maze test [122]. In these *in vivo* studies, both these TSPO ligands showed potent anxiolytic properties. Oral administration of DAA1097 and DAA1106 significantly increased the time spent in the light area at doses of 0.03 and 0.1 mg/kg, p.o., and 0.1 and 0.3 mg/kg p.o., respectively. Analogous results were obtained from the elevated plus maze test, where the time spent in the open arms was significantly enhanced by DAA1097 and DAA1106 doses of 3.0 mg/kg, p.o. These TSPO ligands were also tested for their potential to produce typical side effects of anxiolytic drugs. In contrast to diazepam (30 mg/kg) and buspirone (100 mg/kg), both compounds, in doses up to 100 mg/kg, p.o., produced no effect on spontaneous locomotor activity. The effects observed in the test of hexobarbital-induced anesthesia potentiation in mice were different; DAA1106, diazepam and buspirone significantly increased sleeping time, and DAA1097 did not.

Table 2. ***In Vivo*** **Effects of TSPO Ligands on Neurosteroid Levels and Psychopharmacological Effects**

Animals	TSPO Ligand	*In Vivo* Effect on Neurosteroid Levels	Psychopharmacological Effects	References
Rats deprived of steroidogenic endocrine glands.	FGIN-1-27 Ro5-4864	-Increased brain pregnenolone levels; -no effect on plasma pregnenolone levels. Maximal effect at the dose of 18 mumol/kg for Ro5-4864 and 400-800 mumol/kg for FGIN-1-27.	Not tested.	r
	PK11195	-No effect on brain pregnenolone levels; -prevents brain pregnenolone accumulation induced by FGIN-1-27.		
Rats deprived of steroidogenic endocrine glands and pre-treated with trilostane.	FGIN-1-27 FGIN-1-44	-Increased brain pregnenolone levels. (FGIN-1-44 was rapidly converted to FGIN-1-27 in the rat brain).	-Reduced fear and novelty in elevated plus maze test.	g
	PK11195	-No effect on brain pregnenolone levels; -prevented brain pregnenolone accumulation induced by FGIN-1-27.		
Intact rats	FGIN-1-27	Not tested.	-Exerted anxiolytic effects in Vogel conflict and in plus maze tests; -delayed the onset of isoniazid and metrazol-induced convulsions.	s
Intact rats	FGIN-1-27	Not tested.	-delayed the onset of isoniazid-induced convulsions; -inhibits neophobia in elevated plus maze test.	t
Intact rats	2-arylindole-3-acetamides	Not tested.	-2r, 2s, 2z, 2aa, 2cc, 8c, 11 (about 50 μmol/kg, os administration) exerted antineophobic effect in elevated plus maze test; -2z (1.1 μmol/kg intravenous administration) exerted antineophobic effect.	f
Rats deprived of steroidogenic endocrine glands			-2z (225 μmol/kg, os administration) exerted antineophobic effect.	
Intact female rats	PK11195	-No effect on ovarian and adrenal hormone levels.	Not tested.	u
Intact male rats	derivatives 17, 20, 26, 34	-Increased brain and plasma levels of pregnenolone, progesterone, allopregnanolone and THDOC (at 25 mg/kg, i.p.).	Not tested.	p
Intact rats and rats deprived of their steroidogenic endocrine glands	CB34, CB50 CB54	-Increased brain and plasma levels of pregnenolone, progesterone, allopregnanolone and THDOC(from 3 to 50 mg/kg i.p.).	-CB34 (25 and 50 mg/kg, intraperitoneal injection) elicited anticonflict effect in Vogel test.	v
Intact rats	DAA1097 DAA1106	Not tested.	-DAA1097 and DAA1106 exerted antianxiety effects in mouse light/dark exploration test (0.1mg/kg, os administration) and elevated plus-maze test (3mg/kg, os administration); they showed no effect on spontaneous locomotor activity; -DAA1106 (100 mg/kg, os administration) increased sleeping time in hexobarbital-induced anesthesia.	z

(Table 2) contd....

Animals	TSPO Ligand	*In Vivo* Effect on Neurosteroid Levels	Psychopharmacological Effects	References
Intact rats (ligand microinjected into dorsal hippocampus)	FGIN-1-27	-Increased hippocampal and blood allopregnanolone levels.	-Produced anxiolytic effects in plus-maze and shock-probe burying test.	x
Intact rats	emapunil (AC-5216/XBD173)	Not tested.	-Produced anti-anxiety effects in Vogel test in rats (0.1-3 mg/kg, os administration), in light/dark box (0.003-0.01 mg/kg, os administration), and social interaction tests in mice (0.01-0.3 mg/kg, os administration); -no myorelaxant effects; -did not affect the memory; -did not prolong hexobarbitone-induced sleep; -produced no distinct change in the electroencephalogram.	w
Intact rats and rats deprived of steroidogenic endocrine glands	etifoxine	-Increased brain levels of pregnenolone, progesterone, allopregnanolone and THDOC (at 500 mg/kg, i.p.); -finasteride reduced drastically the brain allopregnanolone levels.	-Produced anxyolitic effects in Vogel test; -finasteride attenuated the anticonflict effect of etifoxine.	j
Intact rats	PIGA 32	Not tested.	-Showed anxiolytic effects in elevated plus maze test (at 30 mg/kg, intraperitoneal injection).	m
Intact mice Intact rats	emapunil	-Induction of neurosteroidogenesis (mice).	-Produced anti-anxiety effects in social exploration test and elevated plus maze test (rats); -did not affect spontaneous locomotor activity (rats); -prevented experimentally-induced panic by lactate- or cholecystokinin tetrapeptide (CCK4) (rats).	q
Human healthy volunteers			-Exerted anxiolytic effects using the CCK4 challenge (at 90 mg/day).	
Intact mice	emapunil	Not tested.	-Produced anxiolytic effects in mice (at 0.1 mg/kg, os administration). -did not produced anxiogenic-like effects or body weight loss upon treatment withdrawal at any of the doses tested (0.1, 1 or 10 mg/kg, os administration; twice daily).	y
Intact rats	MPIGA	Not tested.	-Exerted anxiolytic effects in elevated plus maze test (30 mg/kg, intraperitoneal injection)	o

[r]= data from reference [129]; [g]= data from [130]; [s]= data from [131]; [t]= data from [118]; [f]= data from [117]; [u]= data from [150]; [p]= data from [128]; [v]= data from [134]; [z]= data from [122]; [x]= data from reference [132]; [w]= data from [136]; [j]= data from [135]; [m]= data from [126]; [q]= data from [138]; [y]= data from [137]; [o]= data from [127].

Etifoxine (6-chloro-2-ethylamino-4-methyl-4-phenyl-4H3,1-benzoxazine hydrochloride, trade name StresamR) is a non-benzodiazepine drug that is registered in France for psychosomatic manifestations of anxiety. Etifoxine acts as an anticonvulsant and anxiolytic in rodents, and in humans, it is effective for treating adjustment disorders with anxiety. The etifoxine action mechanism is not fully understood. Initial studies established that etifoxine binds directly to the $GABA_A$ receptor and potentiates GABA-evoked chloride currents. Specifically, the β subunit was shown to play a major role in determining the effect of etifoxine on the $GABA_A$ receptor. Furthermore, etifoxine does not target the high-affinity binding site of benzodiazepines, which is located in the extracellular domain at the αγ interface. Eti-

Table 3. Change of TSPO Expression Levels and TSPO Genetic Associations in Psychiatric Disorders

Psychiatric Diagnosis	TSPO Parameter	References
Bipolar depression with adult separation anxiety.	- Decreased platelet TSPO protein levels.	[aa]
Unipolar depression with adult separation anxiety.	- Decreased platelet TSPO protein levels.	[ab]
Bipolar or unipolar depression with adult separation anxiety.	- Positive association with the functional polymorphism rs6971.	[ac]
Panic and adult separation anxiety.	- Decreased platelet TSPO protein levels.	[ad]
Generalized social phobia.	- Decreased platelet TSPO protein levels.	[ae]
Posttraumatic stress disorder.	- Decreased platelet TSPO protein levels.	[af]
Posttraumatic stress disorder.	- Decreased lymphocyte TSPO protein levels.	[ag]
Suicidal adolescent population.	- Decreased platelet TSPO protein levels.	[ah]
Schizophrenia.	- Decreased platelet TSPO protein levels.	[ai]
Chronic schizophrenia.	[^{11}C]DAA1106 PET imaging: positive correlation with symptoms.	[al]
Panic disorder.	- No variation in lymphocyte TSPO protein levels.	[am]
Generalized anxiety disorder.	- Decreased lymphocyte TSPO protein levels.	
Obsessive-compulsive disorder.	- Decreased lymphocyte TSPO protein levels.	
Generalized anxiety disorder.	- Decreased TSPO mRNA in peripheral blood mononuclear cells.	[an]
Panic disorder.	- Decreased platelet TSPO protein levels.	[ao]
Obsessive-compulsive disorder.	- No variation in platelet TSPO protein levels.	

[aa]= data from reference [139]; [ab]= data from [140]; [ac]= data from [141]; [ad]= data from [142]; [ae]= data from [143]; [af]= data from [145]; [ag]= data from [146]; [ah]= data from [147]; [ai]= data from [148]; [al]= data from reference [149]; [am]= data from [151]; [an]= data from [144]; [ao]= data from [152].

foxine inhibited the binding of the selective TSPO radioligand [^{3}H]PK11195, which prompted researchers to investigate whether this ligand could increase GABAergic neurotransmission by an indirect mechanism involving TSPO activation and the resulting neurosteroid biosynthesis enhancement. The *in vivo* administration of etifoxine (50 mg/kg) was associated with increased concentrations of pregnenolone, progesterone, 5α-dihydroprogesterone and allopregnanolone in the plasma and brain of rats [135]. Because these steroids also increased in rats that were deprived of gonads and adrenal glands, the authors have concluded that the anti-anxiety effect exerted by etifoxine was independent of peripheral steroidogenic sources. In particular, the authors suggested that the primary involved neurosteroid in the anxiolytic effect of this drug might be allopregnanolone. Notably, the conversion inhibition of progesterone into its 5α-reduced metabolites, including allopregnanolone, by finasteride attenuated the anti-conflict effect of etifoxine.

Da Settimo and collaborators [126] have evaluated the best performing phenylindolyl-glyoxylamide compounds, PIGA 21 and PIGA 32, in terms of pregnenolone production for their potential *in vivo* anxiolytic effects after conducting the elevated plus-maze test in rats. Increases in the number of entries and time spent in open arms were observed following the administration of PIGA 32 (30 mg/kg, i.p.). In a subsequent study, increases in the entries and time spent in open arms were observed when a ligand belonging to this class of derivatives, MPIGA, was administered at a dose of 30 mg/kg [127]. An anxiolytic profile of MPIGA was also observed when the compound was administered at a higher dose of 50 mg/ kg. Additionally, at this dose, MPIGA exhibited no effect on the total arm entries, thus indicating that even at high doses, MPIGA does not exert non-specific effects on rat behavior in the elevated plus maze. As described above, MPIGA was effective in inducing the human steroidogenic cell line ADF to produce neurosteroids, which exerted an overall positive modulation of $GABA_AR$ activity. Together, these findings suggest that the MPIGA-induced stimulation of neurosteroid production, which in turn induced the positive modulation of $GABA_AR$ activity, could be the action mechanism by which MPIGA exerts its anxiolytic activity.

Kita and colleagues [136] have investigated the ability of the TSPO ligand, Emapunil (AC-5216, XBD173), to exert anti-anxiety- and anti-depressive-like effects in various animal models. The authors found that Emapunil produces anti-anxiety effects during Vogel, light/dark box and social interaction tests. Moreover, Emapunil exhibited no myorelaxant effects and did not affect the memory and prolonged hexobarbitone-induced sleep even at doses as high as 1000 mg/kg. Although it did slightly prolong the ethanol-induced sleep time at 1000 mg/kg, Emapunil produced no distinct changes in the rats' electroencephalograms. In a more recent study, the same authors [137] demonstrated that Emapunil, when repeatedly administrated, did not induce tolerance to its anxiolytic-like effects or withdrawal symptoms. Recently, Rupprecht and collaborators [138] have shown that

Emapunil did not enhance GABA-evoked chloride currents via its interaction with the $GABA_A$ receptor. In mouse neocortical slices, Emapunil was able to potentiate GABA-mediated neurotransmission, which was prevented by the 5α-reductase inhibitor, finasteride. This result suggested that the enhancement of GABAergic neurotransmission by Emapunil is mediated indirectly through the generation of GABAergic neurosteroids. This ligand exerted anxiolytic effects in rats during the social exploration and elevated plus maze tests. Moreover, Emapunil counteracted the lactate- or cholecystokinin tetrapeptide (CCK4)-induced panic in rodent paradigms. Emapunil was the first TSPO ligand to be administrated to human healthy volunteers using the CCK4 challenge. Seventy-one healthy subjects were randomized for a 7-day treatment with the placebo, Emapunil doses of 10, 30 or 90 mg/day or alprazolam doses of 2 mg/day before undergoing a CCKA challenge. Differences in attenuating CCK4-induced anxiety were observed regarding the placebo, alprazolam and the highest Emapunil dose. Additionally, 57% of the subjects treated with alprazolam complained about withdrawal symptoms, such as sleep disturbances or restlessness; these effects were almost absent in the Emapunil-treated groups. Thus, in humans, this placebo-controlled parallel group study indicated that Emapunil exhibits anxiolytic properties and fewer side effects, as compared with benzodiazepine derivatives.

TSPO AND PSYCHIATRIC DISORDERS

The critical role of neurosteroids in the regulation of CNS function has suggested to investigate the potential TSPO involvement in psychiatric disorders that are characterized by altered neurosteroid levels (Table **3**) [66, 139-149]. The studies were primarily based on the quantitative assessment of mRNA and protein levels in peripheral models, such as platelets and lymphocytes. Most of the authors have focused on anxiety. As such, significantly low levels of TSPO have been found to be associated with generalized social phobia [143, 144] and the intermediate anxiety phenotype, separation anxiety (i.e., adult separation anxiety disorder (ASAD)) [139, 140, 142]. In this last case, a negative correlation between the TSPO levels and ASAD diagnosis was shown, irrespective of the principle psychiatric diagnosis (i.e., panic disorder, bipolar and unipolar depression). An association between a TSPO polymorphism that influences the production of pregnenolone, Ala147Thr, and an intermediate anxiety phenotype has also been demonstrated [66, 141]. Reduced TSPO levels have also been found in post-traumatic stress disorder [145, 146] and schizophrenia [148]. Moreover, a recent study of Positron Emission Tomography has shown a positive correlation between TSPO levels and symptoms in persistently violent schizophrenia patients [149].

ABBREVIATIONS

TSPO	=	Translocator protein
Bdz	=	Benzodiazepines
CNS	=	Central nervous system
P_{450}ssc or CYP450scc	=	Cytochrome P_{450} cholesterol side chain cleavage enzyme
$GABA_AR$	=	Type A receptors for GABA
P	=	Progesterone
21β-HSD	=	21β-Hydroxylase
DOC	=	Deoxycorticosterone
5α-DHP	=	5α-dihydroprogesterone
5α-DHDOC	=	5α-Dihydrodeoxycorticosterone
5β-DHP	=	5β-Dihydroprogesterone
5β-DHDOC	=	5β-Dihydrodeoxycorticosterone
3α,5α-THP	=	3α,5α-Tetrahydroprogesterone
3α,5α-THDOC	=	Tetrahydrodeoxycorticosterone
3α-HSD	=	3α-Hydroxysteroid oxidoreductase
PS	=	Pregnenolone sulphate
DHEAS	=	dihydroepyandrosterone sulphate
PBR	=	Peripheral-type benzodiazepine receptor
CBR	=	Central benzodiazepine receptor
DBI	=	Diazepam-binding inhibitor
IBP	=	Isoquinoline-binding protein
OMM	=	Outer mitochondrial membrane
IMM	=	Inner mitochondrial membrane
mPTP	=	Mitochondrial permeability transition pore
CRAC	=	Cholesterol recognition amino acid consensus
VDAC	=	Voltage-dependent anion channel
ANT	=	Adenine nucleotide transporter
PAP7	=	PBR (TSPO)-associated protein 7
StART	=	Steroidogenic acute regulatory-related lipid transfer
PRAX-1	=	PBR (TSPO)-associated protein 1
ODN	=	Octadecaneuropeptide
TTN	=	Triakontatetraneuropeptide
PK11195	=	1-(2-Chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinolinecarboxamide
Ro5-4864	=	7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one
CG	=	Chorionic gonadotropin
EGF	=	Epidermal growth factor
ACTH	=	Adrenocorticotropin
[^{3}H]MVA	=	[^{3}H]mavalonolactone
PIGA	=	N,N-Dialkyl-2-phenylindol-3-ylglyoxylamides
Etifoxine	=	6-Chloro-2-ethylamino-4-methyl-4-phenyl-4H3,1-benzoxazine hydrochloride

CCK4	=	Cholecystokinin tetrapeptide
ASAD	=	Adult separation anxiety disorder
FGIN-1	=	Arylindole-acetamide derivatives
MPIGA	=	N,N-Di-n-propyl-2-(4-methylphenyl)indol-3-ylglyoxylamide.

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