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Perspective

The Translocator Protein (18 kDa): Central Nervous System Disease and Drug Design

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1. Discovery of the Translocator Protein (TSPO[a]) (18 kDa)

In humans, the benzodiazepine receptor exists in two different forms. Central benzodiazepine receptors (CBRs) are found in the brain and form an allosteric site on the $GABA_A$ receptor complex. Ligands acting at the CBR exert their pharmacological effects by allosterically modulating the effects of GABA and manipulating choride (Cl^-) influx at the $GABA_A$ receptor pore,[1,2] causing downstream effects on GABA-mediated inhibition. Behavioral studies have shown CBRs to be responsible for $GABA_A$-induced anxiolysis and sedation,[3] making ligands acting at this site of important therapeutic significance.

In 1977 Braestrup and Squires[4] discovered that centrally acting benzodiazepines such as diazepam also bound to sites that were not associated with the $GABA_A$ receptor complex in the peripheral nervous system (PNS) and central nervous system (CNS). Originally found in the rat kidney, these pharmacologically distinct receptors were named "peripheral benzodiazepine receptors" (PBRs). When first identified, the PBR was named according to its tissue distribution and according to the class of ligands by which it was discovered. Further studies with specific and high affinity ligands revealed that PBR is present primarily on mitochondrial membranes on all tissues assayed in the PNS and also in the CNS.[5,6] The function of the PBR is largely tissue specific, revolving primarily around steroid synthesis. Diazepam binding inhibitor (DBI) is a high affinity endogenous ligand for PBR whose interaction with the protein is thought to promote the translocation of cholesterol, thus promoting steroid synthesis.[7−9] Porphyrins such as protoporphyrin IX and heme are the most potent known endogenous PBR ligands,[10,11] each with a specific, nanomolar affinity.[11,12] However with the continuous discovery of structurally diverse synthetic PBR ligands[8] and the widespread distribution of the PBR in locations both central and peripheral prompted a change of nomenclature. In 2006 Papadopoulos and colleagues[13] proposed "translocator protein (TSPO) (18 kDa)" as a new name for the PBR, which better reflects the function and tissue distribution of this protein.

Both the TSPO and CBR bind the high-affinity ligand diazepam,[4] with a 3 nM affinity in rat brain and an affinity of 40 nM in rat kidney.[4] However, the two sites differ in their affinities for other drugs such as the isoquinoline carboxamide **1** (PK11195) (Figure 1), which has a nanomolar affinity for the TSPO (0.63−2 nM)[14,15] but a much lower affinity for the $GABA_A$ receptors in the CNS.[15] Similarly, **2** (Ro5-4864), the chlorinated analogue of diazepam (Figure 1), is a benzodiazepine with high affinity for the TSPO (7.3 nM)[14] but with very low affinity for $GABA_A$ receptors (163 μM in rat brain mem-

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[a] Abbreviations: TSPO, translocator protein; CBR, central benzodiazepine receptor; CNS, central nervous system; PBR, peripheral benzodiazepine receptor; PNS, peripheral nervous system; DBI, diazepam binding inhibitor; MPTP, mitochondrial permeability transition pore; VDAC, voltage-dependent ion channel; ANT, adenine nucleotide transporter; hCG, steroidogenesis stimulator; AIF, apoptosis inducing factor; ROS, reactive oxygen species; AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; MS, multiple sclerosis; PAR, planar aromatic region; LA, lipophilic area; FRA, freely rotating aromatic ring region; EPM, elevated plus maze; EAE, experimental autoimmune encephalomyelitis; PET, positron emission tomography; LPS, lipopolysachccaride; N_2O, nitrous oxide; MHC-II, major histocompatobility complex II; ODN, octadecaneuropeptide; DHEAS, dehydroepiandrosterone sulfate; PREGS, pregnenolone sulfate; AGP, α1-acid glycoprotein.

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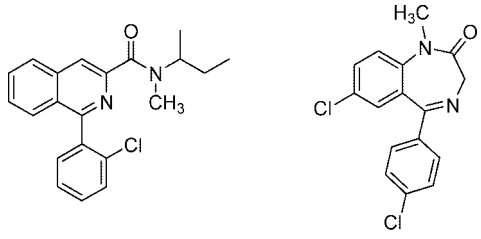


1 2

Figure 1. Prototypic TSPO ligands **1** (PK11195) and **2** (Ro5-4864).

branes[16]). **2** differs from diazepam only by a chloro substituent in the para-substituted position of the 5′ aromatic ring, a well-characterized 4′-substitution that dramatically reduces the activity of the compound and other benzodiazepines at the $GABA_A$ receptor.[17,18]

LeFur and co-workers[15] classified **1** as an antagonist and **2** as a TSPO agonist or partial agonist and suggested that the two compounds bound to a single binding site, with compound **2** being able to completely reverse the binding of compound **1**. However, there are various instances whereby these two compounds can have opposite or very similar actions,[9] prompting the hypothesis that each ligand binds to a separate but partially overlapping binding site. The ongoing synthesis of new TSPO-specific ligands, together with genetic methods, is needed in order to elicit more information about the structure and functional significance of the TSPO binding site(s).

2. Widespread Distribution and Cellular Localization of TSPO

The TSPO is primarily located on mitochondria throughout all tissue and is particularly highly expressed in tissues involved in steroid synthesis. Anholt and co-workers[19] demonstrated the localization of TSPO in the outer mitochondrial membrane, where the protein is particularly concentrated at outer/inner mitochondrial membrane contact sites.[20] In addition, TSPO is found in smaller concentrations in other subcellular compartments. TSPO is localized in the cell surface as a part of the plasma membrane,[21] and a small amount is localized to the nuclear fraction of cells.[17,19] The widespread distribution of TSPO in mitochondria suggests a role for the protein in regulating mitochondrial functions, with implications in steroidogenesis and apoptosis.

TSPO is also expressed in peripheral blood leukocytes, in particular in monocytes, polymorphonuclear cells, and lymphocytes.[22−24] TSPO is also expressed in small amounts in resting microglial cells, which are the immune cells of the CNS and are significantly up-regulated during microglial activation.[6,25]

3. Molecular Structure of TSPO

The structure of TSPO is highly conserved throughout species,[13] consisting of a tryptophan-rich 18 kDa protein with five transmembrane domains (Figure 2). Within these molecules, transmembrane domains consist of extended α-helices, long enough to span an entire membrane bilayer, with shorter loop and tail regions.[26] The α-helices are linked by hydrophobic loops, with a carboxyl-terminal tail located outside the mitochondria and a short amino terminal inside the mitochondria.[27] Murail and co-workers[28] were the first to provide experimental evidence for a five-helix fold of TSPO and show that each transmembrane domain constitutes an autonomous folding unit. TSPO forms complexes of four to six molecules whose organization is postulated to form a single pore,[29] reflecting the function of TSPO as a transporter protein in the mitochondrial membrane.[12] Furthermore, the tertiary structure of TSPO is relatively unstable at rest; however, it becomes strongly stabilized in response to cholesterol translocation.[28]

At a subcellular level, TSPO is present predominantly on the outer membrane of mitochondria and is particularly enriched at contact sites between the inner and outer membrane, sites also known as the mitochondrial permeability transition pore (MPTP). The 18 kDa TSPO protein interacts with various other proteins at these sites, including the 32 kDa voltage-dependent ion channel (VDAC) and the 30 kDa adenine nucleotide transporter (ANT).[30] TSPO interacts with VDAC at the interface of the inner and outer mitochondrial membranes and with ANT on the inner membrane (Figure 2). VDAC is concentrated at sites of contact between outer and inner mitochondrial membranes[31] and is thus thought to function as a channel for substances traveling into the mitochondria.

Specific TSPO ligand binding requires only the 18 kDa TSPO protein;[32] however, it is thought that TSPO requires VDAC and ANT in order to be a functional unit,[30] as binding without these associated proteins is of little functional significance. Thus, it is suggested that the 18 kDs TSPO protein itself carries specific ligand binding sites.

TSPO also interacts with proteins not located within mitochondrial membranes. PRAX-1 is a recently characterized 220−250 kDa protein that is found in the cell cytoplasm and is the only known cytoplasmic protein that partially colocalizes with TSPO in mitochondria.[33] PRAX-1 functions as an adaptor protein, recruiting effectors from the cytoplasm in order for them to interact with TSPO.[33]

Various studies have described TSPO in the form of a dimer, which may be responsible for the active form of the protein.[34,35] Porphyrins such as **3** (protoporphyrin IX) and **4** (heme) are endogenous high affinity ligands for TSPO,[10,11] whose plane of symmetry may result in binding to the dimerized form of the protein, with a single ligand binding to two TSPO molecules[34] (Figure 3). Furthermore, Scatchard analysis of porphyrin binding to TSPO postulated a two-binding site model,[12] consistent with the formation of a TSPO dimer.

4. Function of the Translocator Protein (18 kDa) in the CNS

4.1. Regulation of Steroidogenesis. Biosynthesis of tissue-specific steroids typically involves the conversion of cholesterol into pregnenolone, which occurs through cholesterol side chain cleavage by cytochrome P450scc and auxiliary electron transferring proteins, localized on the inner mitochondrial membrane.[36] Cholesterol transport from the outer to the inner mitochondrial membrane is the rate-determining step in steroid and bile acid syntheses.[20,36] Widespread studies on the location and function of TSPO have found its role to primarily involve cholesterol transport through mitochondrial membranes and thus steroid synthesis.[27,36,37]

Cholesterol transport from the outer to inner mitochondrial membrane is activated by specific ligand binding to TSPO, after which cholesterol undergoes metabolism and begins the steroidogenesis cascade.[13,37] Cholesterol may interact with a receptor binding site on the c-terminus of the TSPO[38] and may also have a channel-like interaction with cholesterol, suggested by its transport via TSPO from outer to inner mitochondrial membranes.[39] The role of TSPO in steroidogenesis has been demonstrated in knockout and antisense experiments,[13] whereby down-regulation of TSPO causes a decrease in steroid synthesis.

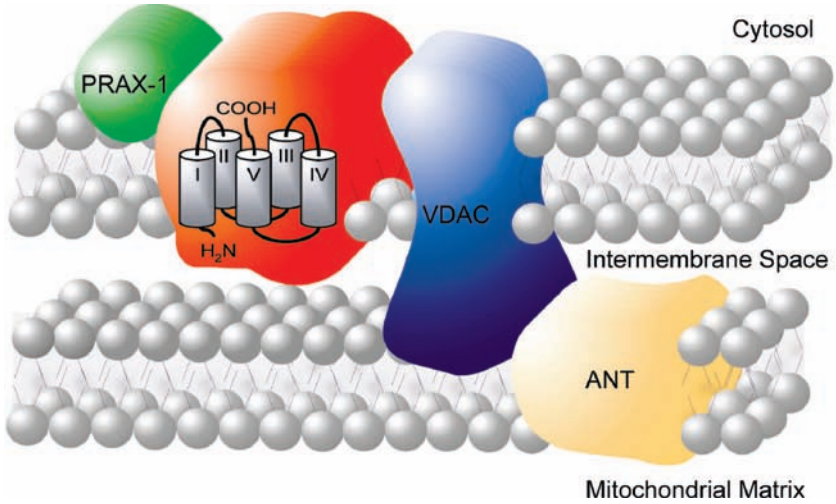
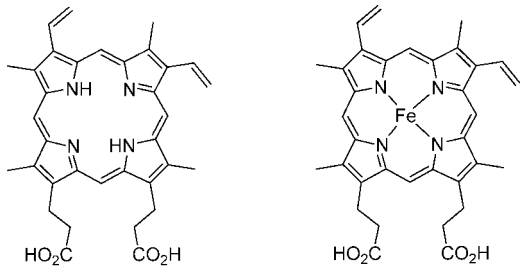


Figure 2. Molecular structure and location of 18 kDa TSPO and its associated proteins in mitochondrial membranes. With the 32 kDa VDAC and 30 kDa ANT, TSPO forms a complex at mitochondrial permeability transition pores (MPTP). Also shown is adaptor protein PRAX-1, which interacts with TSPO as an adaptor protein.



3 **4**

Figure 3. Chemical structures of endogenous TSPO ligands **3** (protoporphyrin IX) and **4** (heme).

In the same way, disruption of the TSPO gene in a R2C Leydig tumor cell line resulted in a dramatic decrease in steroid synthesis.[36,40]

TSPO is generally organized in clusters of four to six molecules; however, with the addition of a steroidogenesis stimulator (hCG) to Leydig cells, there is a rapid increase in TSPO ligand binding plus various morphological changes, such as the redistribution to larger clusters of over seven molecules and the rapid reorganization of the mitochondrial membrane.[29] Boujrad and co-workers[29] found that in the presence of steroid synthesis-inducing hormones, binding affinity of compound **2** was higher, suggesting a morphological change in the receptor and the uncovering of a higher affinity binding site (from 5.0 to 0.2 nM).

TSPO involvement in steroid synthesis may also contribute to host defense, as systemic steroid levels increase immediately after injury, pain, and fever, in response to the stimulation of corticotrophin-releasing hormone secretion by various cytokines.[39,41] Furthermore, the relationship between steroid levels and anxiety means TSPO may be a promising target for the treatment of psychiatric disorders that involve steroid synthesis dysfunction.

4.2. Regulation of Apoptosis. Mitochondria have multiple functions including the induction of lipid peroxidation of membranes, protein and enzyme oxidation, and DNA damage, all ultimately leading to apoptotic and necrotic cell death.[42] Mitochondria influence the induction of cell death because of their ability to regulate and change of the balance of antiapoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as Bax and cyclophilin D. MPTP plays an important role in the modulation of signaling pathways mediating apoptotic and necrotic cell death. The opening of the MPTP is influenced by pro- and antiapoptotic proteins such as Bcl-2 and Bax, respectively. This MPTP opening causes swelling and uncoupling of mitochondria,[43] resulting in the release on intermembrane proteins such as cytochrome *c* and apoptosis inducing factor (AIF) into the cytosol. Once in the cytosol, AIF moves to the cell nucleus, where it causes DNA fragmentation and other processes to induce cell death.[44] Cytochrome *c* acts in the cytosol to induce the caspase cascade resulting in the activation of caspase-3 and the ultimate destruction of the cell nucleus, cytoskeleton, and plasma membrane.[44] Apoptosis is defined as the transient opening of MPTP, as reclosure of the pore ensures this transient process does not result in necrosis and ATP levels are ultimately maintained inside the cell.[43]

Under physiological conditions, Ca^{2+} overloading within the mitochondrial matrix and reactive oxygen species (ROS) generation are the most important triggers of MPTP.[44,45] Such triggers cause the activation of cyclophilin D, which is bound to the mitochondrial matrix end of ANT, which in turn causes the opening of MPTP. However, activation of TSPO by specific ligands such as compound **1** can cause the opening of MPTP, a trigger that is independent of calcium concentrations within the mitochondrial matrix.

In 2007 Li and co-workers[45] showed that the specific TSPO ligand **1** induces mitochondrial release of cytochrome *c* and ultimately induced mitochondrial uncoupling. Compound **1** also facilitates the induction of apoptosis, reverses Bcl-2-mediated inhibition of apoptosis,[46] and facilitates TNF-α-induced necrosis,[47] therefore having a multicomponent effect on the induction of cell death. It is important to note, however, that the proapoptotic effects of **1** are only significant at concentrations 1000-fold higher that those required for specific binding to TSPO.[46−48]

Compound **2** also causes proapoptotic effects via its interaction with TSPO. Compound **2** reverses apoptosis resistance by inhibiting Bcl-2-mediated cytoprotection, causing the release of cytochrome *c* and activation of caspases.[48] Compound **2** also induces cell death via apoptosis at micromolar concentrations (10−100 μM), through caspase-3 cleavage and mitochondrial impairment, resulting in the release of cytochrome *c*.[49] Conversely, **2** also exhibits potent antiapoptotic activities.[50] In the human lymphoblastoid cell line U937, compound **2** demon-

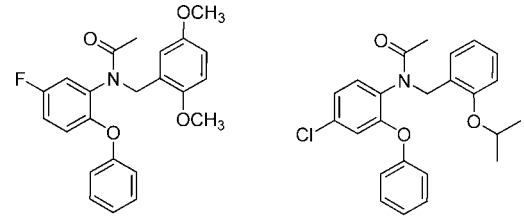
strated antiapoptotic behavior, protecting cells from TNF-α-induced apoptosis, however with no effect on these cells alone.[50] This effect was blocked by the TSPO "antagonist", compound **1**, an agent that also enhanced the proapoptotic effect of TNF-α on this cell line. The existence of conflicting effects caused by **2** on apoptosis may be explained by the use of different cell lines, as effects of **2** are largely species dependent. Additionally, there may exist both high and low affinity binding sites for **2**, each with different functional significance, as the antiapoptotic activity displayed by **2** was significant from a dose of 10 nM and reached a maximum at 10 μM, while proapoptotic effects occurred at doses higher than 10 μM. Further probing into the nature of the compound **2** binding site on TSPO needs to take place in order to properly characterize the functional significance of this ligand.

4.3. Immunomodulation. The presence of TSPO in a wide range of immunomodulatory cells such as microglia, blood monocytes, lymphocytes, and leukocytes implies TSPO involvement in immune response; however, the mechanism through which this occurs is largely unknown. Macrophages express high numbers of TSPO binding sites, and in mouse studies, TSPO ligands, specifically benzodiazepines, inhibit the capacity of macrophages to produce ROS and inflammatory cytokines such as IL-1, TNFα, and IL-6.[51] Furthermore, TSPO is involved in the regulation of phagocyte oxidative metabolism, a process that is normally required for inducing effective elimination of foreign antigens.[52] This immunosuppressive function of some TSPO ligands suggests an important role in host defense mechanisms and inflammatory response.

In the CNS TSPO is minimally expressed on microglial cells. However, upon the injection of excitotoxic compounds, there occurs a dose-dependent increase in the level of TSPO,[6,25] an up-regulation that is correlated closely with microglial activation. Inflammatory mechanisms initiated by microglia are implicated as part of the primary and secondary mechanisms of inflammatory neurodegenerative diseases such as Alzheimer's disease (AD),[53] whereby the activation of microglia initiates an inflammatory response that may excacerbate neuronal damage.[54] The inflammation that occurs in the brain during such neurodegenerative diseases is thought to involve TSPO through its increased presence in activated microglia, thus presenting the possibility for the use of specific TSPO ligands to prevent or limit neuroinflammation.

In response to brain injury and insult, microglia migrate to the site of damage and proliferate in order to contain and regulate any damage. Microglial cells also respond to any pathological event that directly or indirectly affects the CNS, through the production and release of potent neuroinflammatory cytokines such as TNF-α and interleukin-1β (IL-1β), arachidonic acid derivatives such as cyclooxygenase-2, excitatory amino acids, and ROS.[25,54−57] Activation of microglia is also associated with a change in their morphology, from a ramified resting state to an amoeboid-like structure, with a significantly increased expression of major histocompatibility complex molecules and complement receptors.[55,56]

Microglial activation is a nonspecific response to neuronal injury and is not disease-specific. However, the involvement of activated microglia in different CNS diseases differs with respect to its role in disease progression and severity. By use of TSPO as a marker for activated microglia, it is possible to determine exactly what role neuroinflammation plays in specific CNS disease states, opening doors for treatment or inhibition of disease progression.



5 **6**

Figure 4. Structures of phenoxyphenylacetamide derivatives **5** (DAA1106) and **6** (DAA1097).

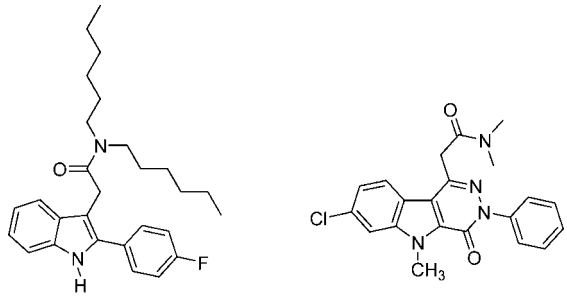
5. TSPO Ligands for Targeting Microglia

Considering the dramatic morphological changes that take place during microglial activation, it is plausible to suggest that dramatic changes also occur to the TSPO and to the conformation of TSPO binding site(s). In addition to the prototypic ligands **1** and **2**, there exists a range of synthetic TSPO ligands whose binding profile will potentially aid in the understanding of the TSPO binding site(s) and should help to determine any conformational changes in the TSPO protein that may occur in various CNS disease states. TPSO ligands have the potential to be used in in vivo and post-mortem analyses of a variety of CNS diseases including AD, Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), and mood disorders. However, it is important to determine which chemical motifs are needed for both high affinity and functional significance.

5.1. Phenoxyphenylacetamide Derivatives. Compounds **5** (DAA1106) and **6** (DAA1097) (Figure 4) are phenoxyphenylacetamide derivatives, derived by opening the diazepine ring of **2**.[8] These ligands are selective and potent TSPO ligands, with a higher lipophilicity and affinity than **1**, and largely inactive metabolites.[58,59] Compound **6** activates steroidogenesis in a similar fashion to **1**,[60] while **5** is unable to activate steroidogenesis alone. The functional differences between these two ligands, despite their closely related chemical structure, provide insight into the link between structure and function at the TSPO binding site. Furthermore, the ability of both compounds **5** and **6** to displace the binding of **1** at nanomolar concentrations[60] suggests that the two classes of compounds share a common binding domain. However, the functional difference between compounds **1** and **5** suggests that the latter compound possesses additional or dissimilar binding features not common to **1**.

5.2. Indoleacetamide Derivatives. FGIN-1 compounds are indoleacetamide derivatives that bind selectively with a nanomolar affinity to TSPO and increase steroidogenesis.[61] These compounds have no affinity for the $GABA_A$ receptor; however, they do display anxiolytic and anticonvulsant properties in experimental animals, possibly through indirect activation of $GABA_A$ receptors by neurosteroids. Compound **7** (FGIN-1-27) (Figure 5) is the compound with the highest affinity for TSPO. Compound **8** (SSR180575) (Figure 5) is another indoleacetamide derivative with nanomolar affinity for TSPO[8,62] and is similarly able to stimulate steroidogenesis.[63] However, indoleacetamide derivatives are both highly lipophilic, thus making their suitability as ligands for probing TSPO questionable.

5.3. Pyrazolopyrimidines. Pyrazolopyrimidines are used as ligand-binding probes for TSPO, with a range of structurally similar compounds established in order to determine structure−activity relationships (SARs) at TSPO binding sites (Figure 6). Selleri and co-workers[64] found that the addition of substituents to the R_1 and R_3 positions (Figure 7) of the pyrazolopyrimidine base



7 8

Figure 5. Structures of indoleacetamide derivatives **7** (FGIN-1-27) and **8** (SSR180575).

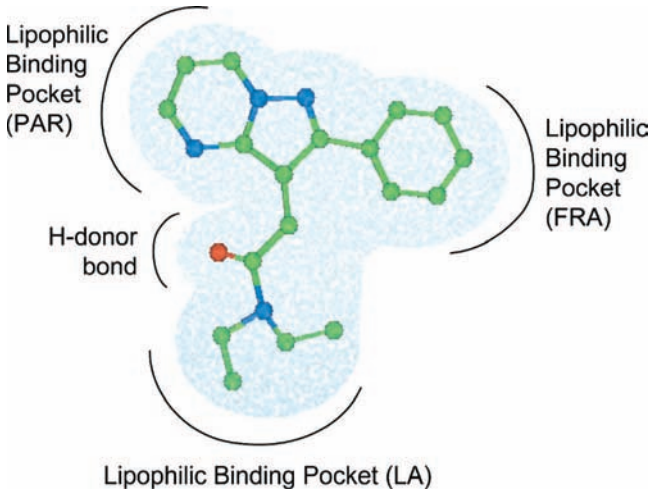


Figure 6. Postulated binding residues on a basic pyrazolopyrimidine, including PAR, FRA, LA, and a hydrogen-bond donating site.

structure aided in the development of high binding and highly selective compounds. However, there was no correlation between TSPO affinity and steroidogenic activity, and the relationship between structure and function of the compounds is yet to be elicited. It is possible that substitution at position R of the 2-phenyl ring (Figure 7) is implicated in increased binding affinity for the TSPO binding site, through higher lipophilicity of the compound. It is postulated that this substitution is required to maintain ligand affinity and specifity in the freely rotating aromatic ring region (FRA) of the binding pocket[64] (Figure 6). It is important to note, however, that no substitutions were made to the polar component of the molecule, as this substituent is thought to be paramount for the formation of a hydrogen bond at the binding site.[64,65] Other binding pockets, the planar aromatic region (PAR) and lipophilic area (LA) (Figure 6), are also thought to be essential for the interaction between ligands and protein.[64]

Pyrazolopyrimidines **10** (DPA-713) and **11** (DPA-714) (Figure 7) are two structurally similar pyrazolopyrimidines[8] but with very different functional effects on steroidogenesis. Compound **10** is a selective, high affinity pyrazolopyrimidine TSPO ligand with nanomolar affinity for TSPO (4.7 nM).[66] It has been ^{11}C-labeled and is rapidly taken up into the primate brain in vivo, with potent and selective activity at TSPO.[67] Compound **11** is a structurally similar compound with a slightly lower affinity (7 nM) than **10** when screened against [^{3}H]**1** in rat kidney.[66] While being structurally similar, **11** is able to stimulate steroid synthesis to levels 80% above baseline, with significantly higher potency than compound **1**, while **10** has no effect on steroid synthesis.[66] The disparity between binding affinity and functional effects suggests that subtle differences in the structure of TSPO ligands can have a great impact on their functional effects.

5.4. *N,N*-Dialkyl-2-phenylindol-3-ylglyoxyamides. Compounds derived from **12** (*N,N*-dialkyl-2-phenylindol-3-ylglyoxyamides) are a new class of structurally diverse TSPO ligands (Figure 8), a large number of which are able to displace [^{3}H]**1** from rat kidney membranes with nanomolar to subnanomolar affinities.[68] This class of compounds is also able to stimulate steroid biosynthesis at a similar or higher rate compared to **1** in rat C6 glioma cells.[68] Most marked was **13** (Figure 8), which increased pregnenolone production more effectively than **1** and showed clear anxiolytic effects in rats in the elevated plus maze[68] (EPM).

Information drawn from the structure−activity profile of these structurally related ligands provides more information about the possible pharmacophore of the TSPO binding site(s). Da Settimo and co-workers[68] suggest that substitutions at R_3 (Figure 8) should be electron-withdrawing for an interaction with the FRA binding pocket (Figure 6). Furthermore, optimal R_4 substitutions are electron-withdrawing and small in size while R_5 substitutions have little effect on increasing affinity.[68] Overall, however, the pharmacophore proposed for this class of ligands does not significantly differ from the model described by Selleri and co-workers[64] for pyrazolopyrimidines at the TSPO.

6. Evidence for the Involvement of TSPO in CNS Disease

Activated microglia are implicated in the pathology of many different neurodegenerative diseases, through distinct processes that involve immune response and inflammation. The increased density of TSPO in CNS disease seems to be an effective and reliable marker for neuroinflammation and gliosis with neuronal damage.[69] By determinination of the nature of changes in the distribution and structure of TSPO in health versus disease, it may be possible to target TSPO only on activated microglia and therefore with specific therapeutic effects. Before this occurs, however, the functional significance of TSPO ligands in CNS disease states must be determined, with new possibilities for more effective disease diagnosis, analysis of progression, and a new therapeutic target for the treatment of neuroinflammatory pathologies.

It seems that TSPO is preferentially up-regulated in areas of neuroinflammation that are characteristic of each CNS pathology. For example, TSPO up-regulation is most marked in the frontal temporal cortex in frontal temporal dementia,[70] in the frontal and mesotemporal cortex in AD,[53] in the cerebral cortex in ischemic stroke,[71,72] and in areas such as the dorsal hippocampal commisure and corpus callosum, both known to exhibit demyelination in a neurotoxic model of limbic system injury.[73,74] Furthermore, TSPO up-regulation is particularly marked in the striatum of HD patients,[75] a condition that is characterized by neuronal loss in this area.[73]

6.1. In Vitro Evidence. The development of specific tritiated (^{3}H) ligands for TSPO presents an avenue for the in vitro exploration into the causes and pathology of disease. In vitro studies can provide insight into the mechanism of TSPO up-regulation in neurodegenerative and neuroinflammatory diseases and can reveal the nature of TSPO involvement in disease, therefore providing possible avenues for potential treatments.

In vitro radioliand binding in an activated microglial cell line demonstrated a significant increase in the maximum [^{3}H]**1** binding sites,[76,77] compared with resting microglia. This increase in the number of binding sites was independent of any changes in TSPO binding affinity,[76] signified by no significant change

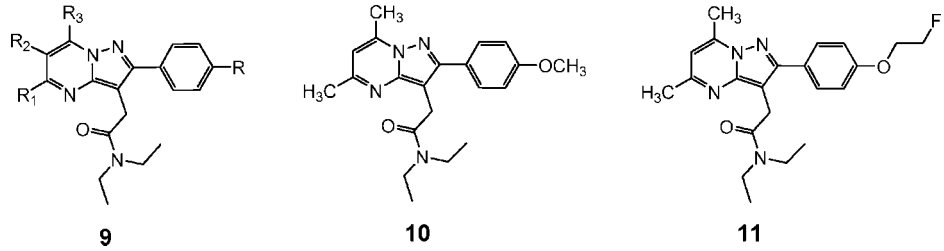


Figure 7. Chemical structures of various pyrazolopyrimidines depicting the parent structure of pyrazolopyrimidine derivatives **9** with substitutions possible at R, R_1, R_2, and R_3 positions: **10** (DPA-713) and **11** (DPA-714).

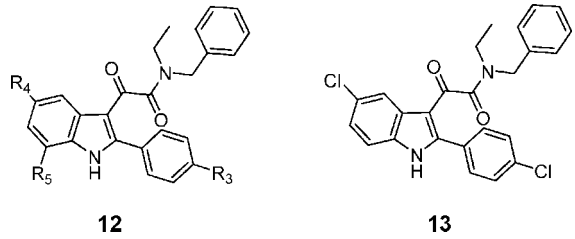


Figure 8. *N*,*N*-Dialkyl-2-phenylindol-3-ylglyoxyamide **12**, depicting base structure with possible substitutions at R_3, R_4, and R_5, and an analogue **13** from the series with an anxiolytic profile.

in the dissociation constant K_D (Table 2). Furthermore, [^{3}H]**2** binding is significantly increased following intrastriatal kainite injections in a rat model of neurodegeneration, with an increase in the total number of binding sites and a minimal change in K_D.[78] Furthermore, the pattern of [^{3}H]**1** binding in a kainite-lesioned animal brain was closely matched to the patterns of neuronal degeneration in autoradiography studies,[79] thus strengthening the notion that **1** is a valuable tool to potentially quantify the extent of neuronal degeneration.[79] Such findings suggest an increased density of TSPO in neuroinflammatory disease, without an apparent change in the structure of the binding site for compound **1**. However, further probing with other TSPO ligands needs to be performed in order to elicit the true nature of changes to TSPO structure and function in health and disease.

TSPO expression is elevated in experimental autoimmune encephalomyelitis (EAE), an immune-mediated demyelinating disease, as signified by an increase in [^{3}H]**1** binding in spinal cord and brain tissues,[80] particularly in inflamed white matter,[81] with a correlation between [^{3}H]**1** binding and activated microglial cells.[81] Furthermore, once clinical symptoms have disappeared, TSPO up-regulation persists, thus suggesting persistent microglial infiltration and activation, with a possible protective function in EAE.[80] TSPO ligand binding reflects the clinical severity of EAE[82] and is increased in CNS regions known to be most affected by the disease.

6.2. Human Post-Mortem Evidence. Post-mortem sources of information about neurodegenerative disease support the data from in vitro studies, indicating an increase in TSPO ligand binding in neuroinflammation and therefore a clear involvement in disease states (Table 1). The binding of [^{3}H]**1** is significantly up-regulated in the post-mortem HD brain,[83] the extent of which provides an approximate reflection of the severity of the disease, demonstrated most profoundly in the putamen and in cortical regions.[83] More interestingly, there is a difference in the binding affinity of compound **1** in different brain areas, possibly reflecting the different concentrations of endogenous ligands in various areas of the brain.[83] Furthermore, the binding of [^{3}H]**1** in the post-mortem HD brain is not correlated with the duration of the disease and is rather associated with a selective increase in ligand binding in the putamen, independent of the caudate nucleus and globus pallidus[78] (Table 1), suggesting a focal inflammation pattern concentrated at specific areas of CNS injury.

The involvement of TSPO in AD has recently been shown by autoradiography using radiolabeled compound **5** on human post-mortem tissue. Gulyás and collegues[84] demonstrate a significant up-regulation of **5** binding in parietal and temporal regions and in the thalamus and white matter of AD patients[84] (Table 1). Regions of increased **5** binding were later correlated with immunohistohemical studies showing microglial increment in these brain regions, particularly in AD brains,[84] which also correlated with the pathoanatomically preferred regions in the AD brain.[84]

The binding of [^{3}H]**1** is 3−4 times higher in white matter plaques of post-mortem MS patients, compared with white matter in normal controls,[76] as well as in normal-appearing MS white matter and central gray matter colocalized with activated microglia.[76] In plaque-containing post-mortem MS tissue, binding of [^{3}H]**1** is most concentrated at the edges of chronic active plaques and is correlated with immunoreactivity for activated microglia.[81] The absence of ligand binding at the center of the MS plaque suggests that TSPO correlates only with the presence of reactive microglia and macrophages rather than being nonspecifically associated with demyelination and gliosis.[81] The strong spatial correlation between increased TSPO density and the histological extent of the lesion suggests a focal reaction to brain injury, with implications to consider when treating various different CNS diseases.

6.3. Imaging Using Positron Emission Tomography (PET). PET imaging using specific TSPO ligands to label activated microglia may help to track the progression of neuroinflammation and may also aid in determining the effectiveness of therapies designed to treat neuroinflammatory diseases.[85] The density of TSPO is greatly increased in disease states, reflecting the inflammatory reaction to brain injury;[86] thus, TSPO ligands can be used as markers of microglial activaton, neuroinflammation, and neuronal damage.[76,81,87]

Radiolabeled **1** and other TSPO ligands have been used to image neurodegenerative pathologies such as AD,[53,88] PD,[89,90] HD,[75,91,92] and MS.[87] PET imaging using TSPO ligands to label activated microglia and therefore neuronal cell loss can help to understand the regional brain distribution and severity of neuroinflammation and can be a valuable tool to determine different approaches to take in the treatment of each individual disease. By determination of the localization of activated microglia, it may be possible to determine the involvement of activated microglia in particular stages of disease, with a role in initiation or progression, as a primary or secondary cause.

Table 1. Regional Distribution and Up-Regulation of TSPO in Normal Controls Compared to Patients with CNS Pathology[a]

CNS pathology	in vitro technique	affected region	control B_{max} (fmol/mg)	disease B_{max} (fmol/mg)	refs
Alzheimer's disease	autoradiography with [^{125}I]desfluoro-**5**	parietal lobe	0.32 ± 0.16	1.26 ± 0.10	84
		white matter	0.24 ± 0.02	1.12 ± 0.26	
		temporal lobe	0.22 ± 0.13	1.02 ± 0.29	
Huntington's disease	autoradiography with [^{3}H]**1**	frontal cortex	39.5 ± 6.9	49.6 ± 11.8	113
		putamen	46.6 ± 8.4	78.9 ± 30.6	
		temporal cortex	ns	ns	
Huntington's disease	autoradiography with [^{3}H]**2**	putamen	40.71 ± 5.04	ns	78
		caudate nucleus	ns	61.67 ± 4.99	
		globus pallidus	ns	ns	
schizophrenia					
aggressive	radioligand binding with [^{3}H]**1**	platelets	7191 ± 3299	5074 ± 1791	99
homicidal			6880 ± 2534		
nonaggressive			7035 ± 2421		
major depression	radioligand binding with [^{3}H]**1**	platelets	4410 ± 227	3393 ± 154	114

[a] B_{max} values are expressed as fmol/mg and indicate the maximum binding potential of each radioligand. "ns" denotes no significant difference between healthy controls and patients with CNS pathology.

7. TSPO as a Therapeutic Target for the Treatment of CNS Disease

While TSPO ligands can be used as markers of neuroinflammation, they can also alter the function of microglia. Wilms and co-workers[25] found that microglial proliferation and activation are decreased in the presence of various TSPO ligands, which have the potential to alter the pathogenesis of neuroinflammatory disease. In microglial cell cultures, activation of microglia by lipopolysachccaride (LPS) causes an increase in inflammatory mediators TNF-α and nitrous oxide (N_2O). However, treatment of activated cells with clonazepam, midazolam, diazepam, and compound **1** causes the increase in inflammatory cytokines to be inhibited, by inhibiting the synthesis and release of proinflammatory and neurotoxic molecules generated by activated microglia.[25] Compound **2** is also able to suppress LPS-induced TNF-α activity in mouse macrophages in a dose-dependent fashion.[93] Such effects are neuroprotective in nature, with these compounds presenting a promising avenue for the treatment of neuroinflammatory disease.

Veiga and co-workers[94] further demonstrated the ability of compound **1** to reduce microglial activation, through a mechanism independent of neuronal survival. Reactive gliosis induced in the rat hippocampus by LPS caused an increase in major histocompatobility complex II (MHC-II) and an increase in the number of proliferative microglia, without inducing cell death.[94] Treatment with **1**, however, resulted in a decrease in MHC-II immunoreactive cells and inhibited the proliferation of microglia,[94] while benzodiazepine compound **2** had no significant effect.

In PD, the degeneration of neurons in the substantia nigra leads to a loss of dopamine and a disruption in the neuronal circuitry that controls movement.[95] Therapies for the treatment of such neurodegenerative diseases are aimed at promoting neuronal survival and preserving neuronal function. The neuroprotective action of **1** was again shown in a model of HD, whereby compound **1** significantly reduced the level of microglial activation and the downstream release and expression of proinflammatory cytokines.[92] Therefore, neuroprotective agents such as **1** could be used to prevent neuronal cell loss in diseases such as AD, PD, and HD, whereby neurodegeneration as a result of inflammation is a key factor in disease initiation.

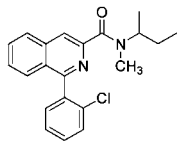
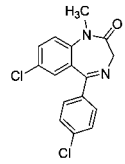
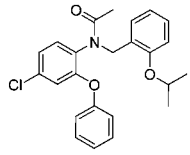
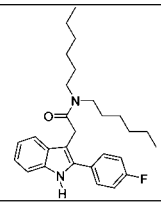
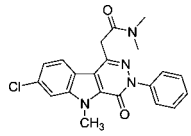
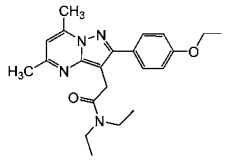
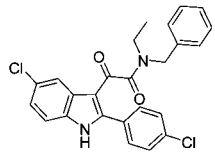
The loss of dopaminergic neurons in PD patients may also be caused by the defective regulation of apoptosis.[96] The regulatory function of TSPO in apoptosis and the ability of specific TSPO ligands such as compound **2** to inhibit apoptosis mean that TSPO as a therapeutic target may have the potential to inhibit the progression of such diseases by preventing cell death. The many different ways in which TSPO ligands have the potential to alter neurodegeneration make this a promising target for the treatment of a variety of CNS diseases.

TSPO and its ligands may also be important therapeutic targets in CNS disease because of their involvement in nerve regeneration. DBI-derived peptides are TSPO ligands and are present in Schwann cells. Octadecaneuropeptide (ODN) is one such ligand, which is believed to be involved in peripheral nerve regeneration in a rat model of sciatic nerve degeneration.[86,97] It is suggested that TSPO agonists such as ODN cause nerve regeneration by stimulating steroid synthesis.[86,97,98] Compound **2** locally increases pregnenolone synthesis in the sciatic nerve,[97] thus suggesting that this ligand and others with similar functional characteristics may have a similar effect on nerve regeneration, with therapeutic implications in the treatment of nerve degeneration.

In models of central and peripheral neurodegeneration, compound **8** increases neuronal survival and improves functional recovery in acrylamide-induced neuropathy in rats.[63] Compound **8** also increases pregnenolone accumulation in the brain and sciatic nerve,[63] possibly providing a mechanism for the nerve regeneration and neuroprotective effects of the compound. Thus, various TSPO ligands promote neuronal survival and repair in models of central and peripheral neuronal disease, with the potential to be used for the treatment of neurodegenerative diseases.[63]

Conversely, TSPO may be involved in CNS disorders such as schizophrenia through its role in steroid synthesis. Aggressive behavior is associated with the negative symptoms of schizophrenia and is correlated with alterations in neurosteroid levels in the brain.[99,100] Mandel and co-workers[101] showed that administration of steroid agonists at $GABA_A$ receptors decreased aggressive behavior;[100,101] thus, the regulatory role of TSPO in neurosteroid synthesis implies a role for the protein in indirect $GABA_A$ receptor modulation, as neurosteroids bind to an allosteric site on the $GABA_A$ receptor. By binding at this site, neurosteroids cause an increase in $GABA_A$-mediated Cl^- influx, ultimately leading to anxiolysis and sedation. Thus, TSPO ligands that manipulate neurosteroid synthesis have the potential to be used in CNS diseases such as schizophrenia. Compound **7** is one such ligand that increases steroid synthesis and has

Table 2. Structure, Binding Affinities, Functional Effects, and Possible CNS Applications of Functional TSPO Ligands[a]

Compound	Structure	Binding Affinity	Functional Effects	Refs	Implication for CNS disorders
1		K_D 0.6-2.0 nM	• Stimulates steroid synthesis • Induction of apoptosis • Inhibition of cell proliferation • Inhibition of immune response and inflammation	14, 15, 25	• Selective marker of microglial activation • Possible neuroprotective agent in CNS disease • Limited *in vivo* by low bioavailability
2		K_D 1.1-7.3 nM	• Biphasic effect on apoptosis • Inhibition of cell proliferation • Inconsistent effect on immunomodulation	14, 15, 78	• Selective marker of microglial activation • Possible neuroprotective agent in CNS disease • Study is limited by species and cell line differences
6		K_i 0.92-4.47 nM	• Stimulates steroid synthesis	59, 60	• Anxiolytic effects with potential to be used to treat anxiety • Limited *in vivo* by high lipophilicity
7		K_i 5.0 nM	• Induction of apoptosis • Stimulates steroid synthesis	8, 59, 61, 102	• Possible treatment of mood disorders and anxiety • Limited *in vivo* by high lipophilicity
8		K_D 3.0 nM	• Stimulates steroid synthesis • Promotes neuronal survival and repair	62, 63	• Possible neuroprotective agent in CNS disease • Limited *in vivo* by high lipophilicity
11		K_i 7.0 nM	• Stimulates steroid synthesis (much greater than PK11195)	66	• Possible treatment of mood disorders and anxiety • Possible effect on neuroregeneration through steroid synthesis
13		K_i 3.33 nM	• Stimulates steroid synthesis (much greater than PK11195)	68	• Possible treatment of mood disorders and anxiety • Possible effect on neuroregeneration through steroid synthesis

[a] K_D values were determined by the radiolabeling (^{3}H) of the individual ligand in membranes from rat cortex. Where radiolabeling was not possible, K_i values were determined using membranes from rat cortex (or rat kidney in the case of **13**) and screened against [^{3}H]**1**.

been shown to delay the onset of isoniazid and metrazol-induced convulsions and can elicit anxiolytic behavior.[61,102] All effects are both flumazenil-insensitive and able to be blocked by **1** and therefore mediated solely by an interaction with TSPO.[102] Conversely, compounds that are unable to produce any effect on steroid synthesis are also able to cause anxiolytic effects in laboratory animals. Compound **5** is one such ligand, which has a nanomolar affinity for TSPO, has no effects on steroid synthesis, but also has potent anxiolytic effects.[59] Such results suggest that the anxiolytic effects of TSPO ligands may be unrelated to their effects on steroid synthesis.[60]

However, the existence of a range of "excitatory" neurosteroids, such as dehydroepiandrosterone sulfate (DHEAS) and pregnenolone sulfate (PREGS),[100] could contradict the therapeutic anxiolysis produced from an increase in overall steroid synthesis, by negatively modulating the $GABA_A$ receptor and causing aggression.[100] Metabolites of these neurosteroids such as allogregnanolone, however, display $GABA_A$ agonist properties.[100] The multicomponent effects of an increase in steroid synthesis mean that targeting TSPO for the treatment of predominantly negative symptoms of schizophrenia needs to be specific to the type of neurosteroid produced. There is a need to more fully understand

the link between the 3D-structure of the TSPO binding site(s) and the relationship with the TSPO function in order to use this protein to target such psychological disorders.

TSPO is also involved in other mood disorders, whereby the regulatory function in steroidogenesis may also play a role. Patients with generalized anxiety disorders and post-traumatic disorders display significantly decreased platelet levels of TSPO.[59,103] However, TSPO binding sites on platelets in anxious patients are increased in response to diazepam treatment.[103] This differential effect may be attributed to an indirect down-regulatory effect of stress hormones that are released in higher concentrations during stress.[103] The same effect is observed on lymphocytes, whereby the density of TSPO is decreased in anxious patients but reversed after chronic diazepam treatment.[104] Stress and anxiety modify TSPO expression in both central and peripheral tissue. The down-regulation of TSPO binding sites can be explained by the fact that in stress disorders, there is an increased release of anxiogenic neuropeptides such as DBI acting on TSPO receptors.[104,105]

The clear involvement of TSPO in CNS disease states makes it an ideal target for the development of new treatments. The regulatory role of TSPO in apoptosis, steroidogenesis, and immunomodulation means that it can be targeted in diseases whereby dysregulation of these functions contributes to the cause and progression of the pathology.

8. Conclusion

The characterization of TSPO binding sites is vital in understanding which chemical motifs are necessary for both high affinity and functional significance. Le Fur and co-workers[15] originally characterized **1** as a TSPO antagonist and **2** as an agonist, each activating different conformational states of the same protein.[106] However, more recent evidence suggests that the binding sites for each ligand may be separate but allosterically coupled.[21,44] In support of this hypothesis, Skowronski and co-workers[107] found that the binding of [^{3}H]**1** and [^{3}H]**2** could be modified separately in rat kidney mitochondrial membranes. Furthermore, site-directed mutagenesis studies on TSPO suggest certain residues such as Val-154, Glu-29, Arg-32, and Lys-39, present in the first putative loop, are important for the binding of compound **2** but not **1**.[108] Further research into the characterization of TSPO binding sites needs to take place in order for the efficient synthesis of high-affinity TSPO ligands, with functional significance for the treatment of CNS diseases.

However, the use of TSPO ligands for therapeutic applications may be narrow because of the widespread distribution and multiple roles of this protein. Additionally, it is important to determine the structural and functional significance of TSPO ligands before applying them to treat CNS diseases. Often there is no correlation between affinity and functional activity at the TSPO.[64] When a range of structurally diverse compounds are established and screened, more information may be elicited about the structure−function relationships at the TSPO binding site, with the potential of using TSPO ligands to target specific processes with implications on specific diseases.

Further synthesis of structurally diverse compounds and continued refinement of SARs should also help to develop compounds with ideal properties to be used in the in vivo imaging of neuroinflammatory disease. Compound **1** has traditionally been used to study neuroinflammation by labeling TSPO on microglial cells in PET studies and also has a potential neuroprotective effect[25] by inhibiting inflammatory cytokines. In vivo, however, compound **1** is restricted by its highly variable kinetic behavior and low signal-to-noise ratio, due to its high lipophilicity and low bioavailability.[91,109,110] Additionally, **1** is a high-affinity substrate for glycoproteins such as α1-acid glycoprotein (AGP),[58,109] which are relatively stable in normal subjects but are significantly up-regulated in acute inflammation, with the potential to alter the free plasma concentrations of the ligand and the ability to alter levels of ligand binding, independent of microglial activation.[109] With regard to PET imaging, the potential therapeutic intervention of this neuroprotective ligand could cause slight changes in TSPO expression,[110] therefore manipulating the real and sometimes subtle changes in TSPO expression in disease. Additionally, compounds **7** and **8** are more lipophilic than **1**; thus, their suitability as ligands for the probing of TSPO in vivo is questionable.

The search for a more bioavailable TSPO ligand is important to improve the suitability and effectiveness of agents used in vivo to label and potentially treat CNS disease. Newer ligands, such as **5** and **10**, are emerging as more sensitive and reliable markers of neuroinflammation, producing a sufficient signal to allow for reliable quantitative analysis.[88,111] While [^{11}C]**5** displays a higher signal-to-noise ratio, partly due to the lower lipophilicity of the compound,[110] **5** and **10** have no functional effects on steroidogenesis,[60,66] thus making these ideal compounds for imaging, with no known conflicting therapeutic or functional effects.

Mounting evidence exists for the role of TSPO in CNS disease, thus increasing the potential for ligands at the TSPO binding site(s) to be used for neuroprotection. In vitro radioligand binding studies have shown that activation of microglia causes an increase in the number of TSPO receptor binding sites, signified by a significant increase in maximal binding, without a change in binding affinity.[76] However, considering the marked chemical and morphological changes that take place after microglial activation, it is plausible to suggest that similar dramatic changes occur to the TSPO binding site that is located on microglia. Such changes would unveil various possibilities for the treatment of neuroinflammatory disease, through the utilization of ligands specific to TSPO binding sites only present on activated microglia.

Thus, the development of new ligands with improved pharmacodynamic properties is important in order to gain an ability to discriminate between the different functional states of TSPO.[112] Current studies outlining the importance of TSPO in diseases are limited because of significant differences in binding domains, including affinity, selectivity, and structure.[112] It is important to address which components of the TSPO and its complex are associated with disease states to enable the design of specific molecules in order to fully harness the utility of the TSPO as a therapeutic target.

Biographies

Alana M. Scarf received her Bachelor of Science (Hons) degree in Pharmacology from The University of Sydney in 2006, where her work focused on the development of selective anxiolytic agents at the $GABA_A$ receptor. She is currently working at the Brain and Mind Research Institute, Sydney, Australia, in collaboration with both the Drug Discovery Research Unit and the Alzheimer's and Parkinson's Disease Laboratory. Her current interests are in the development of in vitro methodology for the evaluation of TSPO drug molecules and their potential usefulness for the treatment of CNS neuroinflammatory disease.

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