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TSPO Finds NOX2 in Microglia for Redox Homeostasis

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Abstract

During the past decade, Translocator Protein 18 kDa (TSPO), previously named peripheral benzodiazepine receptor, has gained a great deal of attention based on its use as a clinical biomarker of neuroinflammation with therapeutic potential. However, there is a paucity of knowledge on the function(s) of TSPO in glial cells. Here, we identify a novel function of TSPO in microglia that is not associated with steroidogenesis. We propose a TSPO interaction with NADPH Oxidase linking the generation of reactive oxygen species (ROS) signaling to the induction of an antioxidant response to maintain redox homeostasis. This line of investigation may provide a greater understanding of TSPO glial cell biology and the knowledge gained may prove beneficial in devising therapeutic strategies.

Keywords

TSPO; peripheral benzodiazepine receptor; NADPH Oxidase (NOX2); redox homeostasis; microglia

TSPO - a biomarker of neuroinflammation

During the past decade, several studies have validated TSPO as a biomarker of neuroinflammation that is able to detect a wide range of brain pathologies [1–3]. The ability to visualize and measure TSPO levels in the living brain using non-invasive neuroimaging techniques such as Positron Emission Tomography (PET) provides a virtual window to view ongoing neuroinflammation in preclinical and clinical studies.

The cellular framework for TSPO as a biomarker of neuroinflammation and injury is derived from the activation of microglia and astrocytes, the glial cell types that express and upregulate TSPO levels [3, 4]. In the normal brain neuropil, TSPO is expressed at low levels, but TSPO levels increase markedly as a result of any type of brain injury [1–3]. Notably, the TSPO response to brain injury has distinct temporal profiles in microglia and astrocytes [4–

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6]. In most injury models, elevated levels of TSPO are initially associated with microglia activation, which is typically followed by a TSPO response in activated astrocytes [4–8]. Based on these observed differences in the glial cell TSPO response, it has been noted that TSPO may serve different functions in microglia and astrocytes [3, 9, 10]. To maximize the potential of TSPO as a biomarker and therapeutic target for neuroinflammation, there is a need to better understand the function(s) of TSPO in glial cells. Here, we identify a novel function of TSPO in microglia that is not associated with steroidogenesis. We propose a TSPO interaction with NADPH Oxidase linking the generation of ROS with the induction of an antioxidant response to maintain redox homeostasis. This line of investigation may provide a greater understanding of TSPO glial cell biology and the knowledge gained may prove beneficial in devising therapeutic strategies.

TSPO and NADPH Oxidase (NOX2) in microglia

Microglia are the immune-competent cells in the brain [11], and they upregulate TSPO levels following brain injury [2, 3]. However, there is paucity of knowledge on the function(s) of TSPO in both microglia and astrocytes. The current view is that TSPO is the rate-limiting step in the synthesis of neurosteroids [1]. TSPO is thought to transport cholesterol from the outer to the inner mitochondrial membrane for conversion to pregnenolone, the parent compound for the synthesis of all neurosteroids [1]. However, recent studies using conditional and global TSPO knockout mice have questioned the essentiality of TSPO in steroidogenesis. Further, unlike earlier attempts to generate *Tspo* $-/-$ mice that were embryo lethal, current studies have shown that *Tspo* $-/-$ mice are viable, and the absence of the TSPO protein does not alter basal steroid synthesis [12–14]. More recently, however, another study has shown that the viability of *Tspo* $-/-$ mice may depend on the methodology used [15]. The same study also showed that, although no significant differences in basal circulating steroids were observed in conditional knockout mice, hormone-stimulated steroidogenesis was compromised, indicating a role of TSPO in hormone-stimulated steroid synthesis. Therefore, the putative cellular function of TSPO in steroid synthesis appears to be in constant flux and definitive studies are needed in order to discover the function(s) of this fascinating protein.

In this background of uncertainty, a study has provided evidence for a novel function of TSPO in microglia that does not appear to be associated with steroid synthesis. Choi *et al.* [9] showed that exposure of primary microglia to the prototypical TSPO drug ligands *R*-PK11195 and Ro5-4864, at concentrations consistent with the TSPO affinity constant (K_d = 1–10 nM), increased microglia proliferation, phagocytosis and production of ROS [9]. Importantly, the TSPO ligand induced ROS production was abrogated by NADPH oxidase (NOX2) inhibitors providing the first evidence of a putative TSPO-NOX2 interaction [9].

Like TSPO, phagocytic NOX2 is a highly enriched protein in microglia and is a major source of ROS production in the CNS [16, 17]. NOX2 is a multi-subunit enzyme composed of the membrane subunits gp91^{phox} and p22^{phox}, the cytosolic subunits p40^{phox}, p47^{phox}, p67^{phox}, and the small G-protein Rac [16]. In the endoplasmic reticulum (ER), gp91^{phox} and p22^{phox} dimerize via two hemes to form flavocytochrome b₅₅₈ (Cy_{b558}) [16]. In phagocytic cells such as neutrophils and macrophages, activation of NOX2 occurs when a stimulus

promotes the translocation of the cytosolic subunits to the plasma membrane where they assemble with Cytb₅₅₈ to form a membrane-bound complex that generates superoxide radicals from NADPH and molecular oxygen [16]. NOX2 is expressed in the plasma membrane at the cell surface and at other intracellular sites such as lipid rafts and even in mitochondria [16]. Thus, the putative TSPO-NOX2 interaction raises important questions not only about the function of TSPO in microglia, but also about its subcellular localization. It is possible that in microglia, TSPO may have function(s) other than neurosteroid synthesis.

Cholesterol and Heme: a molecular basis for a TSPO-NOX2 interaction

A functional association between TSPO and NOX2 may be possible due to the ability of TSPO to bind both cholesterol [17, 18] and heme [19, 20]. As noted above, the NOX2 subunits gp91^{phox} and p22^{phox} are synthesized as separate polypeptides in the ER. p65, the precursor of gp91^{phox}, accepts two hemes in the ER and this allows assembly with p22^{phox} for the formation, maturation and stability of the Cytb₅₅₈ heterodimer [21–24]. This heterodimer is processed in the Golgi and traffics to the plasma membrane and other subcellular sites. In the absence of heme, gp91^{phox} and p22^{phox} do not associate, are retained in the ER, and are eventually degraded by the proteasome system [24]. Other studies have shown that cholesterol is needed for the translocation of the NOX2 cytosolic subunits to the plasma membrane or to lipid rafts to form an active NOX2 complex [25, 26]. Therefore, it is possible that TSPO participates in the generation of a functional NOX2 complex where TSPO behaves as a carrier or transporter for both cholesterol and heme [17–20].

Earlier studies in neutrophils have noted an unidentified 18kDa polypeptide in the membrane fraction that was also the antigen recognized by NOX2 inhibiting antibodies [27, 28]. Further, a monoclonal antibody raised against TSPO was able to dose-dependently and specifically enhance NOX2 activity (i.e., the oxidative burst) in neutrophils [29]. These studies suggested a TSPO-NOX2 interaction but this interaction was not investigated further. From a clinical perspective, there is also data that supports a putative TSPO-NOX2 interaction. Neutrophils from X-linked chronic granulomatous disease patients (X-CGD), an inherited phagocyte disease characterized by a deficiency in gp91^{phox} with impaired superoxide production and bactericidal activity, expressed decreased TSPO levels [30]. However, neutrophils from autosomal recessive CGD patients, who express normal levels of gp91^{phox}, have normal levels of TSPO suggesting that gp91^{phox} may modulate TSPO levels [30]. Consistent with a putative TSPO interaction with NOX2, interferon- γ therapy used for the treatment of X-CGD patients, not only partially restored Cytb₅₅₈ expression, but it also selectively restored TSPO levels in neutrophils [31]. These studies provide additional support for a putative TSPO-NOX2 functional interaction in phagocytic cells including microglia. Consistent with this hypothesis, studies from our laboratory using triple-label immunofluorescent confocal imaging of TSPO with gp91^{phox} and the microglia marker Mac-1 indicate that TSPO and gp91^{phox} colocalize in primary microglia providing additional support for a TSPO-NOX2 association [Figure 1].

The mitochondria associated ER membrane (MAM): a subcellular site for a TSPO-NOX2 interaction?

It is now well recognized that there are points of communication between mitochondria and the ER. Specifically, the MAM in which the ER membrane is closely apposed to the outer mitochondria membrane [32–34]. MAM functions include lipid metabolism, calcium signaling and the formation of the inflammasome involved in innate immunity, amongst several other functions [32–34]. MAM is a protein complex consisting of the voltage-dependent anion channel (VDAC), the ER calcium channel inositol (1,4,5) triphosphate receptor (IP3R), growth receptor protein 75 (GRP75), mitofusin 2, and cargo-sorting protein PACS2 [33–36]. TSPO is known to co-immunoprecipitate with VDAC and like VDAC, TSPO is highly expressed in the outer mitochondrial membrane [35]. Thus, it is possible that TSPO may be associated with the MAM and chaperone or transfer heme from mitochondria to the p65 precursor of gp91^{phox} in the ER for further processing with p22^{phox} leading to Cytb₅₅₈ formation [Figure 2]. In fact, studies have shown that the MAM is a subcellular site where heme transport occurs from mitochondria to the ER [36].

Evidence for a putative TSPO localization at the MAM also comes from a study in hormone-treated steroidogenic cells [37]. This study showed that hormone treatment of MA-10 cells results in a rapid and dramatic increase in the number of MAM contact sites formed. Other studies have shown the presence of high concentrations of cholesterol in MAM's lipid rafts microdomains [38] that could serve as a platform for the transfer of cholesterol to mitochondria for steroidogenesis and facilitate the efflux of mitochondria-synthesized steroids products and possibly heme out of mitochondria to the ER. Therefore, MAMs are subcellular sites in which TSPO, cholesterol and heme may be found in lipid rafts microdomains and lipid rafts could serve as an assembly platform for a TSPO-NOX2 interaction.

Precedence for TSPO functioning as a membrane-bound protein for the transfer of heme/porphyrins comes from studies in the gram negative bacterium *Rhodobacter spheroides* in which TspO, the TSPO homolog in *Rhodobacter spheroides*, has been shown to control the efflux of tetrapyrrole intermediates of the heme/bacteriochlorophyll biosynthetic pathway [39]. This is relevant to the function of the mammalian TSPO because the ability of RsTSPO to efflux tetrapyrroles can be replaced by TSPO from rats, indicating a highly conserved function [40].

Additional support for a heme transfer function for TSPO comes from the highly conserved tryptophan residues in the first periplasmic loop between the first and second membrane spanning domains [41, 42]. These tryptophan residues resemble a WWD heme-binding motif found in proteins involved in transmembrane heme delivery [43]. It has been hypothesized that the WWD domain binds heme to present it to an acceptor protein such that the heme vinyl groups attach to two reduced cysteinyl residues in the acceptor hemoprotein [43, 44]. This process prevents heme oxidation at the site of the transmembrane heme transfer.

TSPO is a stress-response protein involved in redox homeostasis in microglia

TSPO levels are known to increase following brain injury and inflammation [2, 3] and studies have shown that TSPO ligands induce microglia functions (e.g. proliferation, phagocytosis and ROS production) similar to those associated with microglia activation [9]. Other studies have shown that increased TSPO expression is associated with resistance against ROS and hydrogen peroxide cytotoxicity [45]. Conversely, knockdown of TSPO increases ROS production [46] suggesting that TSPO may participate in an antioxidant response pathway. The increase in TSPO levels that has been documented in microglia as a result of brain injury and inflammation may be associated with a microglial response for cell-autonomous protection from the high levels of ROS produced when microglia are activated. Further, increased TSPO may be needed to bind free heme released from hemoproteins as a result of oxidative stress [47].

The TSPO ligand-induced increase in NOX2-derived ROS documented in activated microglia [9] may serve as a cellular signal for the induction of redox sensitive transcription factors in order for microglia to maintain redox homeostasis. A transcription factor that is activated via redox signaling is Nrf2, which regulates the induction of a battery of over 600 cytoprotective genes involved in redox homeostasis [48–51]. Under basal conditions, Nrf2 is bound to Keap1 in the cytosol and this complex has a short half-life because Nrf2 is ubiquitinated and degraded by the proteasome system [51, 52]. Keap1 contains a large number of highly reactive cysteine residues and oxidative stimuli can modify the cysteine residues in Keap1 releasing Nrf2 for translocation to the nucleus. Nrf2 together with Maf proteins is able to bind at the antioxidant response element (ARE) in the promoter region of target genes such as heme oxygenase-1 (*HMOX1*) [53]. Heme oxygenase-1 (HO-1) is a particularly relevant enzyme because its function is to metabolize heme to carbon monoxide and biliverdin, which is further metabolized to bilirubin by biliverdin reductase [54]. Carbon monoxide and bilirubin have antioxidant properties and reduce inflammation in microglia [54].

Figure 3 indicates that Nrf2 is activated by ROS produced in microglia as a result of the TSPO-NOX2 interaction. We hypothesize that HO-1, one of the many Nrf2-dependent antioxidative genes, may be of importance for microglia since it degrades heme. Consistent with Figure 3, a previous study has shown that astrocytes release an active compound that is heat labile, smaller than 3 kDa, and induces HO-1 expression in microglia to prevent excessive inflammation [55]. The characteristics of this yet to be identified anti-inflammatory peptide points to the endogenous TSPO ligand triakontatetrapeptide (TTN), a small peptide derived from diazepam binding inhibitor (DBI) that is upregulated and released from astrocytes following injury [46]. Besides the induction of the HO-1 gene, other relevant antioxidant Nrf2-target genes, are those involved in glutathione synthesis; NADPH production; iron sequestration; quinone detoxification; and thioredoxin production, regeneration, and utilization [49–52]. Studies in primary microglia from Nrf2 knockout mice will be critical to understand the role of Nrf2 as a transducer of the putative TSPO-NOX2

interaction for cell-autonomous protection from ROS and other toxic products generated by activated microglia.

Lessons learned from *TspO* in plant: a sensor of redox homeostasis and free heme

In the plant *Arabidopsis thaliana*, TSPO is a tryptophan-rich sensory membrane protein that regulates abiotic stress [56]. Oxidative stress in plant cells is increased by treatment with the stress phytohormone abscisic acid (ABA). Abiotic stress regulates the expression of ABA-responsive genes, including *TspO* [56]. ABA-induced expression of *At*-TSPO in vegetative tissue is transient, and the degradation of TSPO is enhanced by tetrapyrrole synthesis [56]. *At*-TSPO is a heme-binding protein whose degradation requires heme binding and a functional macroautophagy pathway [56].

Other studies show that the moss *Physcomitrella patens* has a gene encoding a homologous TSPO protein (*PpTSPO1*) that plays an important role in redox homeostasis following exposure to the fungal elicitor chitin [57]. *PpTSPO1* knockout mutants have higher levels of ROS production than wild-type and constitutively higher levels of oxidative stress genes. These studies provide value insights on the putative TSPO-NOX2 interaction in microglia. The studies mentioned above and others provide strong evidence of a conserved stress response function of TSPO from bacteria to plants and in mammals [58].

Heme transporters generally belong to the ATP binding cassette protein family [59, 60]. However, some membrane proteins, similar to *At*-TSPO, can function as heme chaperones, as exemplified by CcmE in bacteria and *Arabidopsis* that can transfer heme to apocytochrome c [61]. Similarly, we hypothesize that TSPO in microglia may be able to transfer heme from mitochondria to the p65 precursor of gp91^{phox} in the ER by forming a functional protein-protein complex at the MAM [Figure 2]. This would provide a mechanism for TSPO to modulate the expression of plasma membrane proteins such as Cyt_b558. This putative TSPO function in microglia is consistent with studies in *Arabidopsis* where abiotic-stress induced TSPO is able to regulate the cell surface expression of PIP2;7 (aquaporin) by interacting with PIP2;7 at the ER and Golgi membranes [62]. Based on this literature and our own studies, we hypothesize that in primary microglia, stress induces the activation of TSPO for the transfer of TSPO bound heme from mitochondria to p65 in the ER, possibly via the MAM, in order for gp91^{phox} to bind p22^{phox} and form Cyt_b558. This complex is then transported to the plasma membrane, lipid rafts, or potentially even to the MAM. In this way, TSPO is able to modulate the expression of NOX2 in a similar fashion that has been shown for *At*-TSPO and PIP2;7 in plant.

Concluding Remarks

TSPO is a highly conserved and phylogenetically ancient protein that likely functions through conserved mechanism(s) to regulate protein expression and free heme involved in cellular stress responses. The proteins that TSPO interacts with and regulates may differ depending on the organism or cell system in which it is expressed. TSPO in microglia may have a protective role from the cell-autonomous generation of ROS and other toxic products

that activated microglia generate. Thus, in microglia, endogenous TSPO ligands such as TTN that are released from activated astrocytes [46] may activate TSPO and promote NOX2-dependent ROS signaling for the induction of Nrf2-dependent antioxidant genes to maintain ROS homeostasis and prolong their lifespan. The induction of Nrf2-target genes such as HO-1 provides a negative feedback loop to modulate NOX2-induced ROS by regulating the level of heme available for Cyt_{b558} formation or by degrading the heme in Cyt_{b558}, thus controlling Cyt_{b558} levels and ROS production. The HO-1 degradation of heme in Cyt_{b558} facilitates the degradation of gp91^{phox} and p22^{phox} by the proteasome system, thereby lowering Cyt_{b558} and ROS levels. It is possible that when microglia are chronically activated, as often occurs in neurodegenerative diseases, the balance of the TSPO-NOX2-Nrf2 interaction breaks down and the defense mechanisms are no longer able to regulate the level of ROS production. This would result in a chronic “toxic state” in which microglia-induced ROS exceeds antioxidant defenses resulting in injury not only to microglia, but also to surrounding neurons, thereby causing neurodegeneration.

This Opinion article introduces a novel concept on the function of TSPO in redox homeostasis in microglia that is not associated with neurosteroid synthesis. Since the role of TSPO in steroid synthesis has been recently challenged, an understanding of TSPO function(s) in specific glial cell types and their interactions demands greater attention (see Outstanding Questions). An increased understanding of this putative TSPO-NOX2 interaction in microglia is likely to prove beneficial for the development of novel therapeutic strategies to mitigate neuroinflammation.

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Glossary

TSPO	Translocator Protein 18 kDa; previously called the peripheral benzodiazepine receptor. The TSPO gene belongs to the tryptophan-rich sensory proteins and has been implicated in cholesterol transport and heme metabolism.
PET	Positron Emission Tomography is a non-invasive imaging technique that uses gamma ray emissions from positron-emitting radioisotopes to reconstruct the distribution of a ligand-bound radiotracer. The radioligand specifically binds to a receptor or enzyme protein and provides a spatial map of protein levels and distribution.
R-PK11195	The enantiomerically active form of the synthetic TSPO specific ligand <i>N</i> -butan-2-yl-1-(2-chlorophenyl)- <i>N</i> -methylisoquinoline-3-carboxamide. This TSPO ligand has

	been the most widely used for clinical and preclinical TSPO neuroimaging and physiological studies.
Ro5-4864	7-Chloro-5-(4-chlorophenyl)-1-methyl-3H-1,4-benzodiazepin-2-one. The 4''-chloro derivative of diazepam is another synthetic TSPO specific ligand which lacks affinity for the GABA _A receptor.
NOX2	A general term for Nicotinamide Adenine Dinucleotide Phosphate Oxidase or NADPH Oxidase. A membrane bound enzyme complex that is present in the plasma membrane and other subcellular sites such as lipid rafts and phagosomes and serves to generate superoxide radicals for bactericidal activity in phagocytic cells. NOX has several isoforms including NOX1, NOX2, NOX3, and NOX4.
Nrf2	Nuclear Factor Erythroid 2 Related Factor 2 is a basic leucine zipper transcription factor protein that regulates the expression of cytotoxic and antioxidative genes that are induced by neuroinflammation. Nrf2 is a regulator of cellular resistance to reactive oxygen species that can be induced by a variety of naturally occurring compounds such as sulforaphane, an isothiocyanate found in cruciferous vegetables.
Neuroinflammation	Microglia, the immune-competent cells of the brain are resident macrophages that exist in a resting state and survey the brain neuropil for disruption of cellular homeostasis. In response to brain injury, they rapidly transform into activated microglia and increase their proliferative capacity, phagocytic properties, express major histocompatibility complex antigens, and release reactive oxygen species, cytokines, nitrogen species, and chemokines. Neuroinflammation is believed to play a critical role in many neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.

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Outstanding Questions Box

- What are the function(s) of TSPO in microglia versus astrocytes under physiological and disease conditions?
- Is a TSPO-NOX2 interaction unique to microglia?
- What are the subcellular sites for a TSPO-NOX2 interaction?
- What are the signaling pathways and antioxidative genes that are activated by a TSPO-NOX2 interaction to maintain redox homeostasis in microglia?
- How can the new knowledge of the TSPO-NOX2 association in microglia be used to develop new therapeutic strategies? In which disease context is this information useful and/or applicable?
- Does knowledge of a TSPO-NOX2 interaction have implications for current neuroimaging techniques?

Trends Box

- The role of TSPO in steroidogenesis is a hotly debated topic based on recent studies in conditional and global TSPO knockout mice.
- A novel interaction of TSPO with NADPH Oxidase (NOX2) has been identified in microglia.
- TSPO interacts with gp91^{phox}, the principal NOX2 subunit.
- The TSPO-NOX2 interaction in microglia is associated with redox homeostasis.

Figure 1.

TSPO interacts with the gp91^{phox} subunit of NOX2 in primary microglia. Triple-label immunofluorescence confocal imaging of TSPO, gp91^{phox}, and the microglia marker Mac-1. There is a significant degree of co-localization of TSPO with the gp91^{phox} the principal heme-containing subunit of NOX2 in the cell cytoplasm and in filopodia. The boxed cell in panel A is amplified in panel B.

Figure 2. The mitochondria associated endoplasmic reticulum membrane (MAM): a putative subcellular site for a TSPO-NOX2 association

We propose that the MAM is a subcellular site in which TSPO and NOX2 can interact by TSPO acting as a chaperone for the transfer of heme from mitochondria to the p65 precursor of gp91^{phox} in order to facilitate Cyt_{b558} formation in the endoplasmic reticulum (ER). The Cyt_{b558} is further processed in the Golgi for transport to subcellular sites such as the plasma membrane, lipid rafts, and even potentially the MAM itself in which the formation of a NOX2 complex is active. This way, TSPO is able to modulate NOX2 levels under normal and stressed conditions.

Figure 3. Working model on the function of TSPO in microglia

Injured neurons release soluble factors (1) that activate microglia and astrocytes. When activated, microglia generate and release ROS, NO and pro-inflammatory cytokines that could be damaging to neurons and to themselves (2*). We propose that signals originating from activated astrocytes (2) binds to TSPO in microglia and in astrocytes for different cellular functions. A signaling molecule known to be synthesized, and released by astrocytes is the Diazepam Binding Inhibitor (DBI) peptide fragment trikontatetrapeptide (TTN) that has pM affinity for TSPO [63]. TTN can act in an autocrine fashion in astrocytes to

activate TSPO and potentially produce neurosteroids that may be helpful with repair mechanisms in injured neurons. On the other hand, in microglia, TSPO activation (3) may form a functional complex with NOX2 by providing heme and cholesterol to form an active Cyt_b558 complex (4) and generate ROS (5). The ROS can serve as an intracellular signal to translocate Nrf2 into the nucleus (6) for transcription of a battery of Nrf2-dependent antioxidant genes; for example heme oxygenase-1 (HO-1), in order to maintain redox homeostasis during periods of stress. Relevant to this working model recent studies have shown NADPH oxidase as a novel upstream regulator of Nrf2/Keap1 signaling [64]. The induction of HO-1 can act as a negative feedback loop to decrease NOX2 activity by degrading heme to CO and biliverdin (7). The inhibition of heme synthesis has been shown to decrease NOX2 activity and to promote the degradation of gp91^{phox} and p22^{phox} proteins [65]. Further, CO and biliverdin have antioxidant properties [54], which could help mitigate inflammation in the brain.