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## Imaging neuroinflammation with TSPO: A new perspective on the cellular sources and subcellular localization

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### Abstract

Translocator Protein 18 kDa (TSPO), previously named Peripheral Benzodiazepine Receptor, is a well-validated and widely used biomarker of neuroinflammation to assess diverse central nervous system (CNS) pathologies in preclinical and clinical studies. Many studies have shown that in animal models of human neurological and neurodegenerative disease and in the human condition, TSPO levels increase in the brain neuropil, and this increase is driven by infiltration of peripheral inflammatory cells and activation of glial cells. Therefore, a clear understanding of the dynamics of the cellular sources of the TSPO response is critically important in the interpretation of Positron Emission Tomography (PET) studies and for understanding the pathophysiology of CNS diseases.

Within the normal brain compartment, there are tissues and cells such as the choroid plexus, ependymal cells of the lining of the ventricles, and vascular endothelial cells that also express TSPO at even higher levels than in glial cells. However, there is a paucity of knowledge if these cell types respond and increase TSPO in the diseased brain. These cells do provide a background signal that needs to be accounted for in TSPO-PET imaging studies. More recently, there are reports that TSPO may be expressed in neurons of the adult brain and TSPO expression may be increased by neuronal activity. Therefore, it is essential to study this topic with a great deal of detail, methodological rigor, and rule out alternative interpretations and imaging artifacts.

High levels of TSPO are present in the outer mitochondrial membrane. Recent studies have provided evidence of its localization in other cellular compartments including the plasma membrane and perinuclear regions which may define functions that are different from that in mitochondria. A greater understanding of the TSPO subcellular localization in glial cells and infiltrating peripheral immune cells and associated function(s) may provide an additional layer of information to the understanding of TSPO neurobiology. This review is an effort to outline recent advances in understanding the cellular sources and subcellular localization of TSPO in brain cells and to examine remaining questions that require rigorous investigation.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pharmthera.2021.108048>.

## Keywords

Translocator protein 18 kDa (TSPO); Microglia; Astrocytes; Neuroinflammation; Positron emission tomography – immunofluorescence; Confocal imaging

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## 1. Introduction

For more than three decades, Translocator Protein 18 kDa (TSPO) has been interrogated as a biomarker of neuroinflammation mediated by infiltrating peripheral inflammatory cells and activation of resident microglia and astrocytes, the brain cell types that mount an inflammatory response to a variety of brain insults and pathologies (Kreisl et al., 2020; Kwon & Koh, 2020; Lahooti, Chhibber, Bagchi, Varahachalam, & Jayant, 2021). In this review, whenever we indicate microglia in brain disease it also includes the possibility of infiltrating peripheral inflammatory cells into the brain that contribute to the TSPO signal (Kreisl et al., 2020). Today, many neurological, neurodegenerative, and psychiatric diseases have been examined by TSPO Positron Emission Tomography (PET) imaging and there are many reviews on this topic (Chen & Guilarte, 2008; Guilarte, 2019; Kreisl et al., 2020; Meyer et al., 2020). Interestingly, the early TSPO-PET imaging studies were described in brain tumors such as glioblastoma that express high TSPO levels and can be easily detected from the surrounding brain neuropil with low TSPO expression (Junck et al., 1989; Pappata et al., 1991). This is one of the advantages of TSPO as a biomarker in that TSPO levels are low in the normal brain neuropil and increase specifically at primary and secondary sites of disease based on the degree of injury, making it relatively easy to detect following an acute or chronic pathological event or in brain cancer. Another advantage has been the significant advances in the development of new classes of radioligands with improved pharmacokinetics, with low levels of non-specific binding (Alam, Lee, & Lee, 2017; Werry et al., 2019; Zhang et al., 2021), and even the recent possibility of new ligands with low sensitivity to the TSPO polymorphism known to alter the affinity of TSPO to second-generation radioligands (Kim et al., 2020; Mattner et al., 2021; Vo et al., 2020). Recent studies also provide the prospect of new TSPO radioligands that are selective for glial cells with no sensitivity to vascular endothelial cells (Ji et al., 2021). As a biomarker of neuroinflammation, TSPO has been studied and validated in a multitude of preclinical animal models of diverse CNS pathology and neurodegenerative disorders (Chen & Guilarte, 2008; Guilarte, 2019; Kreisl et al., 2020). Furthermore, it can be used to track neuroinflammation prospectively at multiple time points in the life course or the evolution of a disease (Focke et al., 2019; Kang, Pandya, Zinger, Michaelson, & Gauthier, 2021; Sacher et al., 2019; Sucksdorff et al., 2020).

One of the perceived disadvantages of TSPO-PET imaging is that there is still a lack of knowledge on the temporal profile of the cell types responsible for the TSPO response in many brain diseases. While an increasing number of preclinical studies are beginning to provide this vital information, less is known in human studies. Furthermore, there is emerging evidence that the TSPO response in preclinical animal models may differ from the human condition (Owen et al., 2017). This too requires further investigation and see Table 1. Other disadvantages of TSPO-PET neuroimaging studies are related to the second-

generation radioligands that are sensitive to a TSPO polymorphism (rs6971) which alters the affinity of the protein for the radioligand (Owen et al., 2012). While genotyping provides a suitable solution to this problem for comparison of normal versus disease subjects, the role of the polymorphism on the function of TSPO in a specific glial cell type or peripheral immune cell is not known. Previous and recent studies are beginning to provide novel information on the impact of the polymorphism on function and disease outcome (Colasanti et al., 2013; Costa et al., 2009; Costa et al., 2009; Owen et al., 2012; Troike et al., 2021).

Another aspect that has been noted as a disadvantage of TSPO-PET studies is that TSPO is widely expressed throughout the brain and there is no brain region with a complete lack of the protein, making the modeling of PET studies more complicated since there is no reference region (Kreisl et al., 2020). In fact, as it will be shown in this review, the expression of TSPO in blood vessels is significant throughout the neuropil in the normal brain. Lastly, the fact that the TSPO response to brain injury and disease originates from microglia and astrocytes is sometimes perceived as a problem because it is not specific for microglia only nor for astrocytes only. However, the TSPO response does follow the brain cells (microglia and astrocytes) that are responsible for the neuroinflammatory response, therefore, a biomarker of neuroinflammation. In summary, TSPO is a robust and well-validated biomarker of neuroinflammation for use in preclinical and clinical neuroimaging studies.

## 2. The evolution of studies on the cellular sources of the TSPO response in brain pathologies

There are several extensive and critical reviews on the cellular sources of the TSPO response to diverse CNS pathologies (Chen & Guilarte, 2008; Guilarte, 2019; Kreisl et al., 2020; Nutma et al., 2019; Rupprecht et al., 2010; Tournier, Tsartsalis, Ceyzeriat, Garibotto, & Millet, 2020). While the early dogma that TSPO is solely a “microglia response” rather than a glial response (microglia and astrocytes) has been proven scientifically invalid in the last few years, new uncertainties are appearing in the literature including neurons of the adult brain being a source of TSPO that could potentially influence TSPO-PET imaging studies. Therefore, rather than provide a repetition of what has been published in the past, this review will focus on new studies that are providing valuable information and methodological approaches to study TSPO.

Microglia and astrocytes were one of the first brain cell types that were implicated to express TSPO and to contribute to the TSPO response to brain injury and disease in preclinical animal models (reviewed in Chen & Guilarte, 2008; Guilarte, 2019). While early in the history of TSPO it was thought that the origin of the signal in CNS pathologies was exclusively from microglia (reviewed in Chen & Guilarte, 2008; Guilarte, 2019), contemporary studies that have rigorously examined this question have shown that the TSPO signal in CNS pathologies is derived from both microglia and astrocytes (see Table 1 and (Guilarte, 2019)). What is also known is that the contribution of microglia and astrocytes to the TSPO signal is not possible to define in the continuum of any disease from a PET scan. Furthermore, the temporal profile of a microglia and/or astrocytic TSPO response

is brain region- and disease-specific (Chen & Guilarte, 2008; Guilarte, 2019). Therefore, preclinical animal models of human brain disease are relevant to address these important questions. Nevertheless. It should be noted that studies focused in understanding the cellular sources of the TSPO response in human brain tissue have been limited. A greater effort to validate the experimental animal data and its relevance to the human disease condition is needed (See Table 1). A review of the literature since 2019, that at a minimum examined the cellular sources of the TSPO response in microglia and astrocytes, in preclinical studies or human postmortem brain tissue are provided in Table 1. Using these criteria there were a total of 9 studies using experimental animals and/or human tissue. These studies examined animal models of Alzheimer's disease (AD) and human AD tissue ( $n = 4$ ), multiple sclerosis (MS) based on cuprizone treatment ( $n = 2$ ), MS human tissue ( $n = 1$ ), lipopolysaccharide (LPS)-induced inflammation ( $n = 1$ ), and injection of Tumor Necrosis Factor (TNF) ( $n = 1$ ).

In different animal models of AD, there is evidence that TSPO upregulation is derived from microglia and astrocytes. A study that examined TSPO and amyloid deposits in sub-regions of the hippocampus in the 3xTgAD mouse model showed that increased TSPO levels appeared prior to amyloid deposits in different regions of the hippocampus (Tournier et al., 2019). Increases in TSPO expression and amyloid were observed first in the subiculum, then dorsal hippocampus, and later in the ventral hippocampus. Furthermore, immunofluorescence imaging of TSPO with glial cell markers indicates that TSPO was primarily associated with microglia and exhibited a lower level of expression in astrocytes. However, the TSPO cellular source component of the study was only performed in animals at 21 months of age. This is important because the study was performed with 3xTgAD mice at 1, 6, 12, and 21 months of age (Tournier et al., 2019) and it is known that the cellular sources of the TSPO response change dynamically at different stages of the disease (Guilarte, 2019). Relevant to this review, in this same publication, the authors state the lack of TSPO immunostaining in pyramidal neurons of the hippocampus (Tournier et al., 2019). In the context of comparing the cellular sources of TSPO in animal models and the human condition, it was shown that TSPO is highly expressed in human choroid plexus as has been shown in mice (Tournier et al., 2019)(Also see Fig. 1 demonstrating high TSPO levels in the choroid plexus of mice).

Using a different AD animal model, in this case the TgF344-AD rat model and a quantitative new approach to assess the cellular sources of the TSPO response, the authors reach a different conclusion (Tournier et al., 2020). TSPO was increased in the hippocampus at 12 and 24 months of age in TgF344-AD rats compared to wildtype rats. Importantly, at 12 months of age, the TSPO signal was exclusively from astrocytes while at 24 months it was from both astrocytes and microglia providing a different perspective than in their previous study. They also found that vascular endothelial cells expressed significant radioligand binding, but they do not contribute to the increased TSPO ligand binding in the TgF344-AD rat model. In the same study, the authors also performed analysis of temporal cortex tissue from AD patients. Consistent with the animal model of AD astrocytes and microglia, but not endothelial cells, were the main source of the increased TSPO binding in the temporal cortex tissue from AD subjects relative to controls.

Another study by Gui and colleagues (Gui et al., 2020) using postmortem brain tissue from normal and AD brains found that TSPO is expressed not just in microglia but also in astrocytes, endothelial cells, and vascular smooth muscle cells. In this human study, there was also no TSPO labeling of neurons. Finally, Ji and colleagues found that TSPO binding increased in the brain of PS19 (tau AD mice) mice, and the increase was driven by microglia and astrocytes (Ji et al., 2021). They further show that TSPO appears to be more prominent in microglia from AD tissue than astrocytes. From these studies in preclinical animal models when compared to human AD tissue, it suggests that the sources of the TSPO response in AD animal models and human tissue are from microglia and astrocytes.

Studies using animal models of MS also examined the cellular sources of the TSPO response. In a study by Nack and colleagues, cuprizone-induced demyelination produced an increase in TSPO levels associated with microglia and astrocyte activation and axonal injury (Nack et al., 2019). Most of the microglia and approximately one-third to one-half of the astrocytes expressed TSPO based on the temporal profile of the injury. Another study by Zinnhardt and colleagues (Zinnhardt et al., 2019) also used a cuprizone model of MS. In the latter study, peak levels of TSPO radioligand binding occurred at 4 weeks of cuprizone treatment in the corpus callosum, hippocampus, and thalamus and declined after 6 weeks of cuprizone treatment. Cuprizone-induced demyelination corresponded with the increase in TSPO levels in these brain regions. In agreement with previous studies from our laboratory (Chen, Baidoo, Verina, & Guilarte, 2004; Chen & Guilarte, 2008), the number of microglia increased after 4 weeks of cuprizone treatment but then decreased at 6 weeks relative to control tissue. On the other hand, there was a steady increase in astrocytes at 4 and 6 weeks of cuprizone treatment. Therefore, the TSPO response was mainly associated with microglia at 4 weeks of cuprizone treatment, while at 6 weeks of treatment TSPO expressed in microglia declines with a greater contribution of astrocytes to the TSPO signal, indicating the temporal dynamics of the cellular sources of the TSPO response.

Finally, a study by Nutma and colleagues (Nutma et al., 2019), using brain samples from MS patients and age-matched controls examined markers for microglia, astrocytes, and lymphocytes to assess the sources of the TSPO response. Cells double-labeled for TSPO and the microglia marker HLA-DR increased 20-fold in the active core and the rim of chronic active lesions relative to normal white matter. TSPO was increased 20-fold in astrocytes labeled with glial fibrillary protein (GFAP) across all lesions in active and inactive lesions relative to white matter from control subjects and this counted for 25% of the TSPO signal in these lesions. They also found that TSPO was in vascular endothelial cells (<5%) and in lymphocytes (<1%), but their total contribution was a small fraction of the total TSPO signal. Again, in MS, the animal data is consistent with the human data, microglia and astrocytes are the main contributors to the TSPO signal.

Other studies examining TSPO expression in the neuroinflammatory response associated with LPS and cytokine administration provide a similar picture. Pannell and colleagues (Pannell et al., 2020) injected TNF-inducing adenovirus (Ad-TNF) or IL-4 in mouse striatum followed by TSPO-PET imaging 48 h (IL-4) or 5 days (Ad-TNF) after injection. Using immunohistochemical staining, they found that TSPO expression was significantly increased in the injected hemisphere relative to the contralateral side after Ad-TNF but

not IL-4. A significant increase in astrocytes was observed at 3 days and microglia increased significantly at 5 days after Ad-TNF injection. The authors also showed that TSPO expression increased in both astrocytes and microglia after Ad-TNF injection. Consistent with previous studies noted above in AD and in MS, vascular endothelial cells isolated from Ad-TNF injected animals showed no increase in TSPO expression concluding that the contribution of vascular endothelial cells to the TSPO signal in the injured brain is not increased.

Another study examined the cellular sources of the TSPO response following injections of LPS. Vicente-Rodriguez and colleagues (Vicente-Rodriguez et al., 2021) used adult rats with intracerebral (striatum) and intraperitoneal LPS injections. Four days after intracerebral or 24 h after systemic LPS administration, TSPO-PET studies were performed. In the intracerebral LPS injections, there was a significant TSPO increase in the ipsilateral versus the contralateral injected site in the TSPO-PET which was confirmed using ex-vivo TSPO autoradiography. A similar effect was found in multiple brain regions of animals injected with LPS systemically. Examining the brain of the animals for the cellular sources of the TSPO response using double-label immunofluorescence confocal imaging they found that microglia together with astrocytes are the major contributors to the increased TSPO signal in the PET and autoradiography studies (Table 1). In this study, they also examined the TSPO signal in neurons and vascular endothelial cells using triple-label immunofluorescence. Although they describe the presence of TSPO in neurons and vascular endothelial cells, these cells did not increase TSPO levels after LPS administration. They conclude that although TSPO is expressed in various brain cell types, microglia and astrocytes are the only cells that increase the TSPO signal after LPS administration.

In conclusion, the most recently published studies in which the cellular sources of the TSPO response were examined in diverse CNS disease conditions indicates that while TSPO is expressed in multiple brain cell types, microglia and astrocytes appear to be the only cells that increase TSPO in pathological conditions. Importantly, the expression of neuronal TSPO remains controversial, with studies indicating TSPO expression in adult neurons (Gong et al., 2019; Notter et al., 2020; Nutma et al., 2019; Tournier, Tsartsalis, Ceyzeriat, Garibotto, & Millet, 2020; Wang, Fan, & Papadopoulos, 2012) and other studies indicating no TSPO expression in adult neurons (Betlazar, HarrisonBrown, Middleton, Banati, & Liu, 2018; Gui et al., 2020; Tournier et al., 2019), including findings from our own laboratory provided in this review.

### **3. A new approach to assess the cellular sources of the TSPO response in CNS pathologies**

Defining the cell types that are responsible for the TSPO signal in the healthy and diseased brain is an important undertaking for the interpretation of PET brain imaging studies and to understand the pathophysiology of a given brain disease. Until recently, the typical approach has been to perform double- or triple-label immunofluorescence confocal imaging with cell-specific markers and TSPO. Tournier and colleagues have described an important methodological advancement that can provide quantitative information not only about the

cellular sources of TSPO, but also which cell types increase TSPO in the diseased brain (Tournier et al., 2020). The authors used antibody-based fluorescence-activated cell sorting to select specific cell types from dissociated brain tissue using flow cytometry combined with TSPO radioligand binding. Based on the combination of these two techniques, they were able to determine the level of [<sup>125</sup>I]-CLINDE binding to TSPO that originates from a specific brain cell type isolated using flow sorting (Tournier, Tsartsalis, Ceyzeriat, Medina, et al., 2020). This can be done in two ways. First, one can administer a dose of a radioactive TSPO ligand to the animal, excise the radioactive brain, dissociate cells, select the different cell populations using antibody-based cell sorting and then count the radioactivity associated with each cell type. The second approach is to extract the brain, dissociate the cells, exposed them to a radioactive TSPO ligand in vitro, and then sort the cell types with an antibody-based cell sorting and count the radioactivity associated with each cell type. Overall, this is an important quantitative approach to provide disease-specific information about the cellular sources of the TSPO response.

Tournier and colleagues used this approach to examine the cellular sources of the TSPO response in an animal model of AD, the TgF344AD rat model, and in brain tissue from AD subjects (Tournier, Tsartsalis, Ceyzeriat, Fraser, et al., 2020). Based on TSPO radioligand counting, there was a significant TSPO increase in the hippocampus of the TgF344-AD rat at 12 months while at 24 months of age increases were measured in the hippocampus, frontal cortex, striatum, cerebellum, and residual brain tissue. Importantly, there was also increased TSPO binding in the eyes at 24 months but not in any other peripheral organs analyzed. Identification of the cell types in brain tissues from the TgF344-AD rat and wildtype was performed using fluorescence-activated cell sorting of radioligand treated tissue. It was found that the increased level of [<sup>125</sup>I]-CLINDE binding to TSPO in the hippocampus of 12-month TgF344-AD rat was associated with astrocytes and not with microglia and this was followed by astrocytes and microglia in the 24-months old TgF344-AD rat brain. Although TSPO binding was measured in endothelial cells, there was no increase in the TSPO binding to endothelial cells in the TgF344-AD rat relative to control indicative of a lack of contribution of endothelial cells to the increase in radioligand binding in the TgF344-AD rat brain. Therefore, the increase in TSPO binding in the TgF344-AD rat was associated with an astrocytic response followed by a “glial response” (microglia and astrocytes) in which there is a distinct temporal profile and an upregulation in the number of TSPO binding sites per cell without an expansion of the number of glial cells (Tournier, Tsartsalis, Ceyzeriat, Fraser, et al., 2020).

In the same study, they also examined temporal cortex tissue from AD and control subjects. There was a significant increase in TSPO binding that was higher in astrocytes than in microglia with no change in endothelial cells (Tournier, Tsartsalis, Ceyzeriat, Fraser, et al., 2020). In this study, there was no apparent increase in the number of astrocytes or endothelial cells but there was an expansion of microglia. Combined, these studies show that in the AD temporal cortex, the increase in TSPO is associate with astrocytes and microglia with no involvement of endothelial cells. It supports the notion that endothelial cells provide a “background” of TSPO binding that needs to be considered for in vivo PET imaging studies but the increase in TSPO in the diseased brain is a “glial response” in which the contribution of each glial cell type needs to be confirmed under each disease condition

and brain region. That is, the glial response at any time point in the disease progression could be solely due to astrocytes, microglia, or a mixed astrocyte/microglia response and it is dependent upon the disease, the disease stage, and the brain region involved (Chen & Guilarte, 2008; Guilarte, 2019).

#### 4. Is TSPO expressed in adult neurons?

There are reports indicating that TSPO is expressed in neurons in the normal rodent adult brain (Gong et al., 2019; Notter et al., 2020; Wang et al., 2012). These studies indicate that TSPO is expressed in Purkinje cells of the cerebellum (Betlazar et al., 2018; Nutma et al., 2019; Tournier, Tsartsalis, Ceyzeriat, Garibotto, & Millet, 2020; Wang et al., 2012), in pyramidal and dentate granule cells of the hippocampus (Notter et al., 2020; Wang et al., 2012) and in dopaminergic neurons of the substantia nigra (Gong et al., 2019). On the other hand, other studies provide no evidence of TSPO expression in neurons of the normal rodent brain (Betlazar et al., 2018; Tournier et al., 2019). Therefore, there is uncertainty in the published literature about the presence of TSPO in adult neurons and their potential contribution to PET imaging. Furthermore, a recent report suggests a selective increase in the putative TSPO neuronal signal following different neuronal activating paradigms in brain regions of the normal mouse brain (Notter et al., 2020). The latter findings put forth the possibility that a TSPO neuronal signal may contribute to the overall signal in TSPO-PET imaging studies. The contribution of neurons to the TSPO signal has not been considered previously because, although neuronal progenitor stem cells express high levels of TSPO, there is a marked decrease in TSPO expression with neuronal differentiation with no detectable TSPO expression in postmitotic, differentiated adult neurons (Tanimoto et al., 2020; Varga et al., 2009). Nevertheless, the fact that TSPO has been described to be expressed in adult neurons and its expression can be induced exclusively in neurons and not in glial cells by different neuronal activating paradigms requires significant attention and rigorous validation. It suggests that normal variations in daily human activities may modulate neuronal TSPO expression and thus be a source of variation in TSPO-PET imaging studies in normal subjects.

#### 5. Neuronal activating paradigms selectively increase neuronal and not glial TSPO: a critical analysis

The study by Notter and colleagues describing significant TSPO expression in neurons of the normal adult mouse brain used single-cell RNA sequencing (scRNA-seq) from normal adult mouse hippocampus (Notter et al., 2020). This was done to generate gene sets to identify specific brain cell type in the hippocampus. The degree of TSPO gene expression was then attributed to each individual cell type based on the scRNA-seq results. It was found that in the normal adult mouse hippocampus, 3% of neurons express TSPO relative to 40% of ependymal cells, 20% of endothelial cells, 15% of microglia, and 3% of astrocytes. Based on this information, approximately 60% of the TSPO signal in the normal adult mouse hippocampus is provided by ependymal and endothelial cells. Importantly, the authors further characterize neurons into four subclusters and show that amongst these neuronal subclusters, subcluster 3 accounts for most of the TSPO relative to subclusters 1/2/4.



These neuronal subclusters were not identified, but it is possible that neuronal subcluster 3 is associated with neural stem/progenitor cells that are Nestin-positive and reside in the hippocampus, a brain region with a very active adult neurogenesis niche in the subgranular zone of the dentate gyrus (Niklison-Chirou, Agostini, Amelio, & Melino, 2020; Vicidomini, Guo, & Sahay, 2020). These neuronal progenitor stem cells are known to express high levels of TSPO (Betlazar et al., 2018; Varga et al., 2009).

One of the goals of the study was to assess the source of the TSPO signal following different neuronal activating paradigms (Notter et al., 2020). To this aim, a critical experiment using scRNA-seq was not performed. That is, the authors did not compare cells from the brain of animals that experienced the neuronal activating paradigms to sham-controlled animals using the same scRNA-seq approach. This critical experiment would have provided a direct comparison of the TSPO expression attributed to each cell type using the same gene sets defined by scRNA-seq and would have been able to determine the cell type (s) or neuronal subcluster(s) from which the putative increase in the TSPO signal originated following the different neuronal activating paradigms relative to sham-control animals.

For the neuronal activating studies, the authors used TSPO immunofluorescence confocal imaging of phenotypically defined cells using cell-specific markers and provided images of apparent TSPO-positive puncta in neuronal perikarya and in glial cells from normal mouse adult brain tissue (Notter et al., 2020). Based on the images provided, it appears that neurons from the normal adult hippocampus express more TSPO-positive puncta per cell than microglia or astrocytes. However, this is difficult to assess because the magnification of the images provided in the different panels are not the same. That is, the neuronal panels have the highest magnification compared to the other cell types. Importantly, the signal intensity settings in the confocal images were not provided and if these are increased to improve image quality, it can introduce imaging artifacts.

An alternative interpretation of the images of TSPO-positive puncta in neurons that was not interrogated in the study is the possibility that TSPO-positive puncta that appear to be localized within neuronal cell bodies based on the double-label study may be localized to glial processes or blood vessels contacting or in near proximity of neuronal soma and processes (see sections 10 and 11). Finally, an important experiment that was not performed to verify that the TSPO-positive puncta in neurons is a specific signal, was to perform the same study in global TSPO knockout (KO) mice. The latter would have provided an important additional layer of confirmation that the putative TSPO signal originating from adult neurons is specific for TSPO and not an imaging artifact or antibody non-specific binding (see Fig. 1).

In the same study, for the neuronal activating paradigms, the authors used DREADD-based chemo-activation of neuronal activity in the medial prefrontal cortex and in the CA1 region of the hippocampus. The results seem to suggest a specific increase in TSPO gene and protein expression in neurons with no change in glial cells (Notter et al., 2020). The same neuronal selective increases in TSPO gene and protein expression were observed in the hippocampus when adult mice were briefly exposed to a novel environment (15 min) and in neurons of the nucleus accumbens following D-amphetamine administration (single

injection 2 mg/kg). In this part of the study, the authors used the expression of the activity-regulated genes *cFos*, *Arc*, and *Zif268* measured by qRT-PCR as an indication that the increase in TSPO following the neuronal activation paradigms is neuronal in nature (Notter et al., 2020). However, *cFos*, *Arc*, and *Zif268* are not selectively expressed nor selectively activated in neurons since these activity-regulated genes are also present in astrocytes and these same genes are upregulated in astrocytes following neuronal activation (Adamsky et al., 2018; Edling, Ingelman-Sundberg, & Simi, 2007; Hung et al., 2000; Rodriguez et al., 2005; Rodriguez et al., 2008). Therefore, the designation of the origin of the TSPO mRNA signal measured by qRT-PCR to be selective for neurons based on the increased expression of these activity-regulated genes is confounded by a putative contribution from astrocytes.

The authors also point out the possibility that neuronal activation or decreased neuronal activity may alter neuron-specific TSPO expression and this signal may contribute to changes in TSPO levels in PET studies of psychiatric disorders (Notter et al., 2020). The putative presence of TSPO protein in neurons based on immunofluorescence imaging does not equate with the level of functional TSPO radioligand binding that would ultimately determine if the putative neuronal TSPO signal is detectable using sensitive ex vivo imaging methods such as emulsion- or film-autoradiography or in PET imaging studies. In other words, is the putative neuronal TSPO signal sufficiently robust to be detected in imaging studies? While film or emulsion autoradiography was not performed in this study (Notter et al., 2020), the same group of investigators did compare TSPO immunofluorescence confocal imaging with TSPO autoradiography in a previous study using a different experimental paradigm (Notter et al., 2018). In the latter study, the activating paradigm was immune activation in a mouse model of schizophrenia to determine if there was a decrease in TSPO binding as has been observed in PET studies of schizophrenia subjects (Notter et al., 2020). They found that there was no change in TSPO radioligand binding using autoradiography in the brain of immune-activated animals relative to controls, although they described a decrease in TSPO using immunofluorescence in the immune-activated animals relative to controls. The lack of confirmation of the decrease in TSPO immunofluorescence by TSPO autoradiography questions the interpretation of the immunofluorescence results as immunofluorescence can be influenced by a variety of different methodological variables including imaging artifacts.

## 6. A critical examination of TSPO immunostaining studies in adult neurons: real or artifacts?

One of the first publications describing the putative presence of TSPO in neurons of the hippocampus and in Purkinje cells using TSPO immunohistochemistry with diaminobenzidine (DAB) visualization was related to the generation of a transgenic mouse model expressing green fluorescent protein (GFP) under the control of the *Tspo* promoter (*Tspo*-AcGFP) (Wang et al., 2012). Examination of the TSPO-DAB immunohistochemistry images from the dentate gyrus and pyramidal cell layer of the hippocampus as well as the Purkinje cell layer in the cerebellum shows that all neurons in the dentate gyrus and pyramidal cells of the hippocampus stain strongly for TSPO (Fig. 4 in (Wang et al., 2012)). Further, they also show TSPO immunostaining of presumed Purkinje cells in the cerebellum.

The fact that every single neuron in the hippocampus strongly labels with TSPO suggests nonspecific TSPO staining or an artifact of the staining procedure (Fig. 1). The possibility that the putative TSPO neuronal staining is an artifact in the study conducted by Wang and colleagues (Betlazar et al., 2018; Wang et al., 2012) can be surmised from the fact that the TSPO-DAB signal is not congruent with the *Tspo*-AcGFP signal. In Fig. 4 of their publication, panels a,d,g are Nissl stain to identify the neuronal population in the dentate gyrus (panel a), in pyramidal cells (panel d) and the Purkinje cells of the cerebellum (panel g). In the next row, there are representative images of TSPO-DAB immunostaining (Panels b,e,h). Lastly, panels c,f,i are from the same regions of the hippocampus and cerebellum but now providing the green fluorescence signal in the *Tspo*-AcGFP mouse hippocampus and cerebellum. From these images, the TSPO-GFP signal is not congruent to the TSPO-DAB signal, and very few cell express that express TSPO-DAB express TSPO-GFP suggesting that the “apparent” TSPO-DAB signal in panels b,e,h are highly likely to be nonspecific staining (see Fig. 1 for confirmation of non-specific immunohistochemistry staining in hippocampal neurons). In this study, there was no double-label immunostaining of TSPO with neuronal or glial markers to confirm the cellular sources of the TSPO signal. The authors compared the pattern of staining and presumed that it originated from neurons. However, the TSPO labeling pattern did not match between TSPO-DAB staining and *Tspo*-AcGFP. In fact, most of the *Tspo*-AcGFP signal is present outside of the granule cell neurons in the dentate gyrus (panel b), and outside of the pyramidal cell layer (panel e). Furthermore, the presumed *Tspo*-AcGFP signal from Purkinje cells (panel i) could be from Bergmann glia whose cell bodies are located within the Purkinje cell layer and have an intimate physical relationship with Purkinje cells or even from the significant amount of TSPO found in blood vessels.

## 7. Assessment of the specificity of TSPO immunostaining

Studies using TSPO-DAB immunohistochemistry in the hippocampus of TSPO wildtype (WT) or TSPO global KO mice with a TSPO antibody that has been well validated in our lab using a variety of different approaches including TSPO-KO mice (Loth et al., 2020 and supplementary materials within) shows that there is prominent TSPO immunostaining in blood vessels, in neurons of the pyramidal cell layer of the hippocampus, as well as in the choroid plexus and ependymal cells of the lining of the ventricles in WT mice (Fig. 1). To confirm the specificity of the staining protocol and the TSPO antibody used, TSPO staining was also performed in TSPO-KO brain tissue at the same time and under the same experimental conditions. Fig. 1 shows that in blood vessels, choroid plexus, and ependymal cells of the lining of the ventricles there is a complete loss of TSPO staining in the TSPO-KO mice relative to WT. However, the TSPO staining in neurons of the hippocampus remains in the TSPO-KO mice (compare panel A to B, C to D, and E to F in Fig. 1). The latter is a clear indication that the TSPO staining in neurons of the hippocampus is an artifact of the staining procedure and/or antibody cross reactivity. Therefore, even with well-validated TSPO antibodies, there is the possibility of imaging artifacts generated by the immunostaining procedure and validation of the staining method in TSPO-KO mice provides an important layer of confirmation to the specificity of the signal. Therefore,

previous studies using TSPO-DAB (Wang et al., 2012) indicative of neuronal TSPO staining are most likely due to non-specific staining or antibody cross-reactivity.

## 8. TSPO immunofluorescence confocal imaging in Purkinje cells of the cerebellum

Purkinje cells of the cerebellum have been described to express TSPO (Betlazar et al., 2018; Wang et al., 2012). As noted, the original observation was presented in the *Tspo*-AcGFP transgenic mouse study noted above (Wang et al., 2012). In this communication, they observed TSPO-positive staining in what appears to be Purkinje cell soma based on morphological criteria. However, we provide evidence in Fig. 1 that the TSPO-DAB staining generates non-specific labeling of neurons. On the other hand, TSPO immunofluorescence studies do not appear to have the same problem. Nevertheless, with either method, it is important to perform validation studies on the localization of the TSPO signal with cell specific markers. This is due to the fact that Purkinje cells have a very close relationship with Bergmann glia which are unipolar astrocytes that are positive for the astrocyte marker GFAP (Yamada & Watanabe, 2002) (Fig. 2). In fact, multiple Bergmann glia cell bodies are located alongside in intimate contact with Purkinje cell bodies in the Purkinje cell layer (Fig. 2) (Reeber, Arancillo, & Sillitoe, 2018; Yamada & Watanabe, 2002). Bergmann glia cell bodies wrap around Purkinje cell soma and processes (Fig. 2). They can be identified with GFAP (Reeber et al., 2018). GFAP-positive staining in Bergmann glia occurs from the cell bodies in the Purkinje cell layer, all the way to the pial surface (Reeber et al., 2018)(Fig. 2). Thus, Bergmann glia could easily be misidentified as Purkinje cells if a single staining with TSPO is performed. Therefore, a general concept that needs to be considered in the interpretation of TSPO immunostaining studies is the possibility that TSPO is localized with glia processes or cell bodies or even with blood vessels that are intimately contacting neuronal soma and processes. Furthermore, TSPO immunostaining by itself is highly unlikely to provide the identification of the cell type that is providing the signal.

Another study in normal mouse brain tissue did perform double label immunofluorescence confocal imaging of the Purkinje cell marker calbindin with TSPO (Betlazar et al., 2018). They show an apparent colocalization of TSPO with calbindin in Purkinje cell soma. However, even though in this study it may look like TSPO colocalization is with Purkinje cells, the possibility that TSPO cellular localization is in Bergmann glia, microglia, or blood vessels that are closely contacting Purkinje cell soma and processes was not ruled out. This possibility can offer an alternative explanation to the cellular origin of the TSPO signal and requires close investigation (see section 11 and 12). Considering that TSPO is localized in glial and vascular endothelial cells, and they have a close relationship with neurons, endothelial cell and glia staining should always be incorporated in studies that examine the putative labeling of TSPO in neurons.

## 9. Microglia and astrocyte processes contact neuronal cell bodies:

Several publications suggest that TSPO is expressed in neurons of the normal mouse brain. However, an alternative explanation that needs to be ruled out is the possibility that the

apparent TSPO neuronal signal may be due to the presence of TSPO in glia (microglia and/or astrocytes) processes contacting the neuronal cell body. What is the evidence for this possibility? In the cerebellum, there is evidence that cerebellar microglia interact in a dynamic way with the dendritic arbors and cell bodies of Purkinje cells (Stowell et al., 2018). In vivo two-photon imaging shows that in the Purkinje cell layer, microglia cell soma and processes are interspersed between Purkinje neuron cell bodies and could be observed moving around the Purkinje cell body and wrap around it (Stowell et al., 2018). Microglia arbors can contact 4–8 Purkinje cell bodies and sometimes they create a webbing over the cell body covering the entire side of the Purkinje cell (Stowell et al., 2018). These findings demonstrate that besides Bergmann glia, microglia in the Purkinje cell layer are closely associated with Purkinje cells and make contacts with their cell bodies and dendrites (Stowell et al., 2018). Similarly, another study (Refaeli et al., 2021) using large number of astrocytes made transparent by CLARITY and fluorescence labeling of astrocytes and pyramidal cells via viral vectors and two-photon imaging was able to determine the elaborate structure, distribution, and neuronal content of astrocytic domains in the CA1 region of the hippocampus. This study shows a large but varying number of pyramidal neurons in astrocytic domains in the CA1 pyramidal cell region. That is, pyramidal cell soma in the hippocampus, are highly innervated by astrocytic processes. Therefore, there is significant scientific evidence of the close interactions between glial cell bodies and processes with neurons. Therefore, the TSPO-positive puncta described in hippocampal and cerebellar neurons may be directly attributed to glial processes, either from microglia, astrocytes, or both and this possibility needs to be ruled out.

## **10. Immunofluorescent confocal imaging demonstrates TSPO colocalization with glial processes and blood vessels but not with neurons in the normal adult mouse brain**

To understand the sources of TSPO immunofluorescent staining in the Purkinje cell layer in the normal mouse brain, and to provide a picture of the complex interaction between Purkinje cells, Bergmann glia, and microglia, we performed quadruple-label immunofluorescent confocal imaging in the Purkinje cell layer of the cerebellum to visualize the relationship between Purkinje cells (stained with calbindin), astrocytes (stained with GFAP - Bergmann glia), microglia (stained with Iba1) and TSPO. Panel A in Fig. 3 shows a top-down view of a 3D condensed z-stack image of quadruple labeling of Purkinje cells (gray), Bergmann glia (green), microglia (blue) and TSPO (red). This image was generated using an experimental staining and image acquisition protocol described in the experimental protocol in the supplementary materials. In the image in Fig. 3 panel A, it appears that TSPO (red) positive puncta may be localized in Purkinje cells (gray). The areas of apparent colocalization of TSPO with Purkinje cells are circled in yellow for ease of identification. Because of the complexity of the composite image with all four signals merged in panel A, and to provide a clear picture of the TSPO staining (red) in the presence of Purkinje cells (this time in green), we removed the astrocyte (GFAP) and microglia (Iba-1) signals to generate panel B. Thus, panel B shows the same image as in panel A but this time only representing TSPO (red) and Purkinje cell (green) under the same imaging acquisition



using TSPO immunofluorescent confocal imaging that TSPO is present in neurons of the cerebellum or hippocampus. Overall, the above studies provide important information on the careful interpretation of TSPO immunostaining studies and the necessity to perform rigorous experimental protocols to eliminate potential artifacts that can confound the interpretation of the cellular sources of TSPO.

## 11. TSPO in blood vessels of the normal adult brain

To confirm that that tubular structures in which TSPO is localized in the normal Purkinje cell layer of the adult cerebellum and in the hippocampus are blood vessels (see Figs. 1, 3, and 4), we performed quadruple label immunofluorescence confocal imaging using the vascular endothelial cell marker CD31 (blue), TSPO (red), NeuN or calbindin (cyan), and GFAP (green). Fig. 5, Panel A shows that nearly all of the TSPO label is within the bounds of the CD31 positive blood vessel with no TSPO in the granule cells of the dentate gyrus of the hippocampus. The side view of the image in panel A shows that the blood vessel is in a different plane than the NeuN-positive granule cells. Panel B of the same figure shows the different individual signals. Fig. 5, panel C depicts the same quadruple label in the Purkinje cell layer. In panel C, the Purkinje cell is represented by the cyan color, TSPO is red, and CD31 in blue. The lower images in panel C indicate the close association of TSPO with CD31-positive blood vessels. These findings indicate that TSPO in blood vessels represent a significant percentage of the total TSPO signal in the normal adult brain neuropil.

## 12. TSPO subcellular localization: does location define function?

Understanding the cellular sources of the TSPO response in diverse CNS pathologies is an important scientific endeavor. However, there is a paucity of knowledge on the function(s) of TSPO in glial and peripheral immune cells that contribute to the TSPO signal in PET studies of CNS pathologies. Furthermore, the scientific literature has attributed a variety of different functions to TSPO, many of which have not been confirmed, and some of which have been recently questioned with the advent of global and conditional TSPO-KO mice. Based on the studies provided in the previous sections of this review, it is evident that microglia and astrocytes are the principal cells that upregulate TSPO in diverse pathologies. What is not fully understood are the function(s) of TSPO in microglia or astrocytes. In fact, there is very little data on the function and the subcellular localization of TSPO in glial cells in the normal and disease brain. A greater understanding of these parameters could provide useful information for understanding disease pathophysiology. Furthermore, while TSPO is clearly increased in diverse CNS pathologies, it is not known whether this increase in microglia and/or astrocyte is beneficial or detrimental to the disease condition. Here, we will examine what is known about the localization of TSPO in glial cells to understand its function and potentially advance the development of therapeutic strategies.

### 12.1. TSPO localization in the Outer Mitochondrial Membrane (OMM)

Early studies on the subcellular fractionation and detection of TSPO using radioligand binding showed that TSPO is highly enriched in the outer mitochondrial membrane (OMM) (Anholt, Pedersen, De Souza, & Snyder, 1986). One of the first reported biological roles for TSPO was as a cholesterol transporter in steroidogenesis and the rate-limiting step in

the transfer of cholesterol from the outer to the inner mitochondria membrane (Lacapere et al., 2001; Mukhin, Papadopoulos, Costa, & Krueger, 1989; Papadopoulos et al., 1997; Veenman, Papadopoulos, & Gavish, 2007). However, this function has come into question as studies utilizing TSPO-KO mice and cell-specific conditional TSPO knockdown have shown that cholesterol transport, as well as cholesterol-dependent steroid synthesis, are largely unaffected in TSPO-KO tissues, arguing against TSPO being necessary for those functions (Morohaku et al., 2014; Tu et al., 2014). Other studies have suggested a biological function of TSPO in the generation of cellular reactive oxygen species (ROS) (Choi, Ifuku, Noda, & Guilarte, 2011; Veenman et al., 2007). This is primarily due to direct evidence of subcellular localization of TSPO with cellular components involved in ROS production, such as NADPH oxidases (Loth et al., 2020), as well as extra-mitochondrial spaces such as vesicles and peroxisomes (Boujrad, Vidic, Gazouli, Culty, & Papadopoulos, 2000; Gatliff et al., 2017; Guilarte, Loth, & Guariglia, 2016). This is particularly important for microglia and infiltrating macrophages, which are a significant source of ROS production in a variety of different neurodegenerative conditions.

### 12.2. Mitochondrial associated ER membrane (MAM)

TSPO immune-gold electron microscopy in primary microglia has confirmed that TSPO is present in the OMM (Loth et al., 2020). This same study found that TSPO is also present in the endoplasmic reticulum (ER), mitochondria-associated ER membrane (MAM), and the plasma membrane (Loth et al., 2020). Since the MAM is a site of ER-mitochondria communication, we hypothesize (Guilarte et al., 2016) that it may be a subcellular site where TSPO binds heme and associates with NADPH oxidase (NOX) subunits (Paillusson et al., 2016; Taketani, Kohno, Furukawa, & Tokunaga, 1995; Verma, Nye, & Snyder, 1987). NOX2 is a major source of ROS in the central nervous system and our recent finding of a TSPO-NOX2 interaction in primary microglia supports the hypothesis that TSPO could be modulating ROS generation in microglia, particularly following an inflammatory stimulus (Loth et al., 2020). Evidence for a putative TSPO localization at the MAM also comes from a study in hormone-treated steroidogenic cells. Hormone treatment of MA-10 (clonal strain of mouse Leydig tumor cells) cells increased the formation of MAM contact sites (Issop et al., 2015). High concentrations of cholesterol in MAM's lipid rafts could also serve as a platform for the transfer of cholesterol to mitochondria and facilitate the efflux of mitochondria-synthesized steroids products and possibly heme out of mitochondria to the ER (Fujimoto & Hayashi, 2011). Therefore, MAMs are subcellular sites in which TSPO, cholesterol, and heme may be found in lipid rafts that could serve as an assembly platform.

### 12.3. Plasma membrane

Although cell fractionation studies determined that TSPO was mostly associated with the OMM (Krueger & Papadopoulos, 1990), there are reports of TSPO also present in the plasma membrane, albeit at lower levels than in mitochondria (Olson, Ciliax, Mancini, & Young, 1988; Woods & Williams, 1996). Plasma membrane TSPO has been described in the heart, liver, adrenal, and testis, and on hematopoietic cells (Batoko, Veljanovski, & Jurkiewicz, 2015; Oke, Suarez-Quian, Riond, Ferrara, & Papadopoulos, 1992). TSPO has also been observed in the plasma membrane of erythrocytes (Olson et al., 1988). Recently, TSPO was shown to be ubiquitously expressed on the cell surface of various



immune cell types using flow cytometry, with monocytes and neutrophils having the highest frequency of TSPO surface expression (Blevins, Crawford, Azzam, Guilarte, & Kaminski, 2020). Since these cell types can infiltrate the brain and contribute to the TSPO signal, they are an essential cell type to consider. Furthermore, activating monocytes with pro-inflammatory LPS increased the frequency of TSPO surface expression in the absence of altered TSPO gene expression. The authors also observed an increased frequency of TSPO surface localization in monocytes from a chronic inflammatory disease such as chronic HIV infection. This novel finding in myeloid lineage cells (Blevins et al., 2020) has also been confirmed in primary microglia using flow cytometry (Loth et al., 2020). In addition, BV2 cells, a microglia cell line, express plasma membrane TSPO that can be increased by LPS activation (Shimoyama et al., 2019). In that study, LPS treatment increased TSPO expression in the mitochondrial and plasma membrane fractions in BV2 cells using subcellular fractionation. TSPO was localized in the plasma membrane fraction in the control group and was almost 2-fold higher in the LPS-treated group (Shimoyama et al., 2019). These studies confirm that TSPO is found on the surface of virtually all immune cells including microglia and can be modulated by LPS, however, the biological implications of this subcellular localization are still unknown. The potential role of TSPO in cholesterol transport could have biological implications for immune cells with TSPO on their plasma membrane. Given the crucial role of cholesterol in immune cell function, it has been speculated a role of TSPO in the plasma membrane (Blevins et al., 2020). Further studies are needed to gain a better understanding of the role of TSPO in the plasma membrane.

#### 12.4. Perinuclear region

Evidence that TSPO is located in the perinuclear region in microglia and astrocytes were shown after trimethyltin (TMT) exposure to induce brain injury in rats (Kuhlmann & Guilarte, 2000). Using double-labeling fluorescence histochemistry of GSI-B<sub>4</sub> to detect activated microglia or GFAP for activated astrocytes in combination with TSPO labeling, TSPO expression was found to be abundant not only in the cytoplasm but also in the perinuclear area in both glial cell types following TMT-induced neurotoxicity (Kuhlmann & Guilarte, 2000). Thus, the perinuclear localization of the TSPO in microglia and astrocytes might be associated with the differentiation, proliferation, and migration potential of these cells following a brain insult. However, the functional role of TSPO in the perinuclear compartment has yet to be determined. Previous evidence of perinuclear TSPO localization in cultured astrocytes has also been shown in primary cultures of enriched astrocytes obtained from newborn rat cerebella (Alho, Varga, & Krueger, 1994). Using electron microscopy, the authors observed localization of TSPO around the mitochondria and enlarged endoplasmic reticulum, nuclear membrane, cell membrane, and centrioles. TSPO was also located in cell division structures in dividing cells, indicating that TSPO may play a role in cell growth and division. However, no functional experiments were performed.

Similarly, a prominent location of TSPO in the perinuclear region was also shown primarily on microglia/macrophages following intracerebral hemorrhage (ICH) in brain sections of male mice (Bonsack, Alleyne Jr., & Sukumari-Ramesh, 2016). Using double immunohistochemical labeling of proliferating cell nuclear antigen (PCNA) combined with TSPO, the authors showed a marked perinuclear expression in activated microglia.

The authors suggest a role of TSPO in microglial proliferation after ICH, which can be associated with neurodegenerative conditions. However, the functional role of TSPO was also not validated in this study. Consistent with this observation, *in vitro* studies have shown that TSPO is acutely upregulated in retinal microglia in mouse models of retinal inflammation and injury at mRNA and protein levels and shows a perinuclear distribution (Wang et al., 2014). Double immunohistochemical analyses of CD11b, as a marker of activated microglia, and TSPO, showed a concentrated subcellular localization of TSPO in the perinuclear region in cultured retinal microglia after LPS exposure. On the contrary, GFAP-labeled astrocytes and GS-labeled Muller cells did not show TSPO expression after LPS activation. Cultured retinal microglia showed that TSPO expression is increased after LPS activation at mRNA and protein levels in activated microglia and is concentrated in the perinuclear region, using immunohistochemical analyses. Again, the authors did not evaluate the functional role that TSPO may play in cultured activated retinal microglia in the perinuclear region. Finally, a newly reported function of TSPO in nucleus associated mitochondria (NAM) has been identified where TSPO links mitochondria and nucleus during the mitochondrial-retrograde response, allowing for redistribution of cholesterol that deacetylates NF-KB and promotes a pro-survival response (Desai et al., 2020; Fan & Papadopoulos, 2020; Yasin et al., 2017). This study suggests that TSPO may play a role in regulating nuclear gene expression through intracellular signaling. However, the mediators of mitochondrial to nuclear signaling induced by TSPO and its ligands are still unknown.

Overall, the studies reviewed here evaluate the subcellular localization of TSPO in glial and other cells using microscopy-based approaches, which could have potential limitations such as off-target binding of antibodies due to cross-reactivity to other proteins, and potential artifacts due to fixation and permeabilization of the cells that can potentially affect subcellular localization. Moreover, it is also worth noting that even using high-quality reagents; it may be challenging to determine precisely whether a signal represents all or a subset of the protein of interest because of the relative accessibility of the anti body to different cellular compartments and the tendency to focus on fluorescence signal that have higher intensities and punctate pattern, versus lower intensities but diffuse patterns. Therefore, to appropriately determine the subcellular localization of a protein, imaging techniques should also be combined with subcellular fractionation methods that can separate cellular structures, typically by centrifugation based on their sedimentation coefficients, for further separation and confirmation of the subcellular localization.

### 13. TSPO oligomerization: does it alter function?

TSPO has been reported in several studies to exist as a monomer, dimer, or higher-order oligomeric form (Korkhov, Sachse, Short, & Tate, 2010; Li et al., 2016). The oligomerization may vary between species and tissues, so the oligomeric forms of TSPO may not be conserved between species but may be associated with different functions (Hiser, Montgomery, & Ferguson-Miller, 2021; Li, Liu, Garavito, & FergusonMiller, 2015). *In vivo* studies have shown that TSPO exists in multimers up to six units (Boujrad, Vidic, & Papadopoulos, 1996; Delavoie et al., 2003). Cholesterol is shown to bind better to TSPO monomers than polymers, whereas TSPO drug ligands exhibit higher binding to TSPO polymers (Jaipuria et al., 2017; Rone et al., 2012; Si Chaib et al., 2020). TSPO monomer

to polymer transition may also be driven by hormones and ROS (Lacapere & Papadopoulos, 2003). In response to ROS, TSPO forms covalent polymers in Leydig and breast cancer cells both in vitro and in vivo (Delavoie et al., 2003). A study using colonic cells showed that the TSPO ligand PK11195 could induce TSPO polymerization by stabilizing the dimeric form (Issop et al., 2016). High molecular weight TSPO was also observed in the retina after laser injury in vivo (Wolf, Herb, Schramm, & Langmann, 2020). Thus, evidence so far show that the oligomerization state and ligand binding specificity for mammalian TSPO may be affected by different binding partners (Hiser et al., 2021). However, more work remains to be done to understand the functional significance of the different oligomeric forms of TSPO. Furthermore, most of these studies were not performed with glial cells, thus, their relevance to glial cell biology remains to be discovered.

## 14. Summary

The recent peer-reviewed literature has described significant advances in understanding the cellular sources of the TSPO response in neuroinflammation and the development of methodologies to study the function of this ancient and biologically important protein. There is yet much more to be done from many different perspectives including the development of better and more cell selective radioligands, improved mathematical modeling to quantify TSPO in PET studies, and a greater understanding of its function in the brain cells that express TSPO. While TSPO is highly expressed in mitochondria, and this has been the most widely studied subcellular compartment associated with function, other subcellular compartments also expressed TSPO although at lower levels, but not necessarily of a lesser functional significance to a specific cell type. From a methodological perspective, more quantitative and better approaches are being developed to study the sources and the degree of induction of TSPO in glial cells. When in vitro immunostaining methods are used for this purpose, careful validation of the techniques are essential to eliminate imaging artifacts which are common using this type of methodology. Finally, an important validation of TSPO imaging is provided by the use of readily available global or conditional TSPO knockout cells, tissues, and animals.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

<b>3xTgAD</b>	Triple transgenic mouse model of Alzheimer's disease
<b>AD</b>	Alzheimer's disease
<b>Ad-TNF</b>	Tumor Necrosis Factor-inducing adenovirus

<b>BV2</b>	Microglia cell line
<b>CD11b</b>	Cluster of Differentiation Molecule 11B, microglia marker
<b>CD31</b>	Cluster of Differentiation 31, blood vessel marker
<b>CLARITY</b>	Clear Lipid-Exchanged Acrylamide-Hybridized Rigid Imaging/ Immunostaining/In Situ-Hybridization Compatible Tissue Hydrogel
<b>CNS</b>	Central Nervous System
<b>DAB</b>	Diaminobenzidine
<b>DREADD</b>	Designer Receptors Exclusively Activated by Designer Drugs
<b>ER</b>	Endoplasmic reticulum
<b>GFAP</b>	Glial Fibrillary Acidic Protein, astrocyte marker
<b>GFP</b>	Green Fluorescent Protein
<b>GSI-B<sub>4</sub></b>	Griffonia Simplicifolia 1 Isolectin B <sub>4</sub> , microglia marker
<b>HLA-DR</b>	Human Leukocyte Antigen-DR isotype, microglia marker
<b>Iba1</b>	Allograft Inflammatory Factor 1, microglia marker
<b>ICH</b>	Intracerebral hemorrhage
<b>IL-4</b>	Interleukin 4
<b>KO</b>	Knockout
<b>LPS</b>	Lipopolysaccharide
<b>MA-10</b>	Clonal strain of mouse Leydig tumor cells
<b>MAM</b>	Mitochondria-associated ER membrane
<b>MS</b>	Multiple sclerosis
<b>NADPH</b>	Nicotinamide Adenine Dinucleotide Phosphate
<b>NAM</b>	Nucleus associated mitochondria
<b>NeuN</b>	Neuronal Nuclei, neuron marker
<b>NF-KB</b>	Nuclear factor kappa-light-chain-enhancer of activated B cells
<b>NOX</b>	NADPH oxidase
<b>OMM</b>	Outer mitochondrial membrane
<b>PCNA</b>	Proliferating cell nuclear antigen
<b>PET</b>	Positron Emission Tomography

<b>PS19</b>	Transgenic Tau mouse model of Alzheimer's disease
<b>qRT-PCR</b>	Real time Quantitative Reverse Transcription Polymerase Chain Reaction
<b>ROS</b>	Reactive oxygen species
<b>scRNA-seq</b>	Single-cell RNA sequencing
<b>TgF344-AD</b>	Double transgenic rat model of Alzheimer's disease
<b>TMT</b>	Trimethyltin
<b>TNF</b>	Tumor Necrosis Factor
<b>TSPO</b>	Translocator Protein 18 kDa
<b>WT</b>	Wildtype

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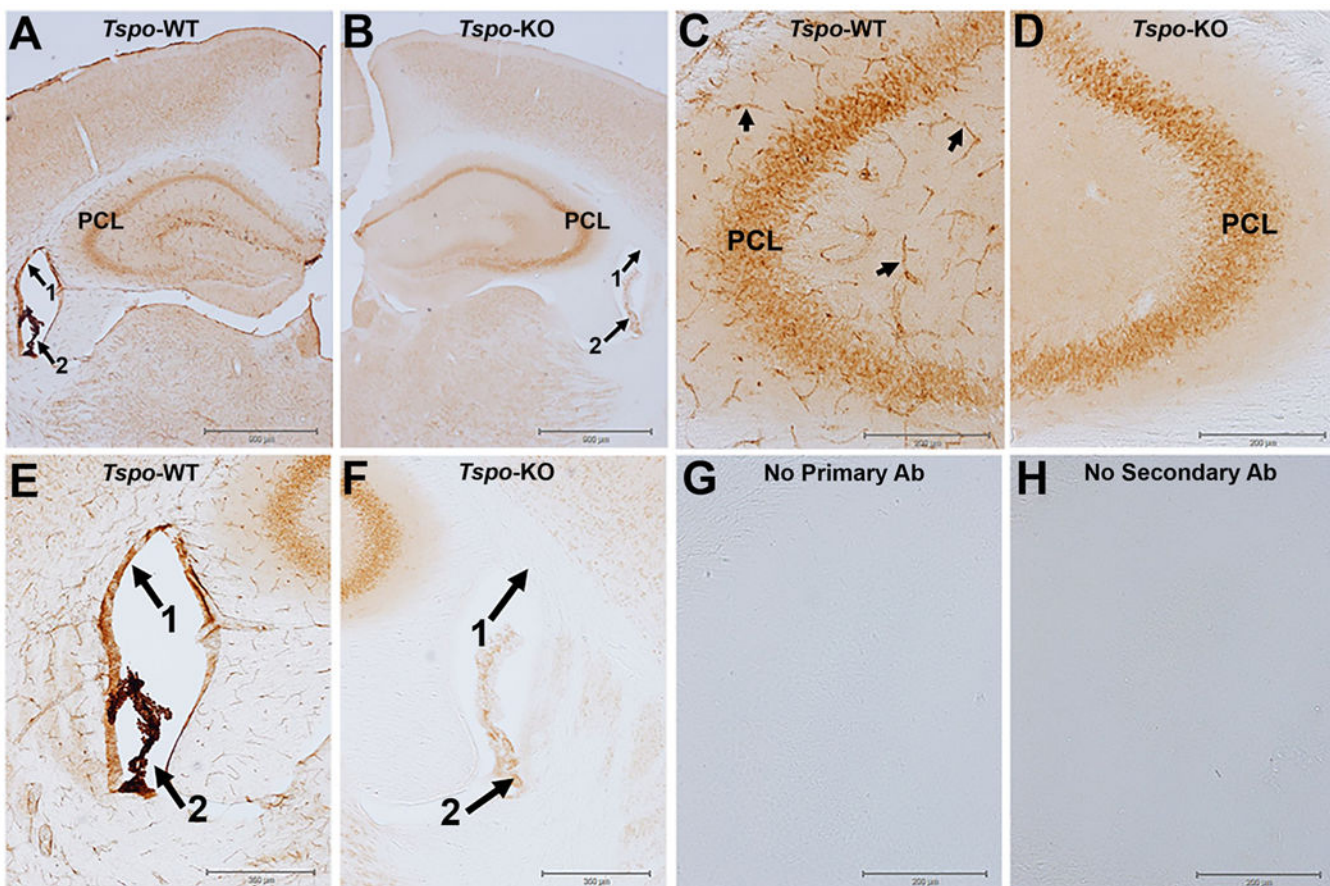
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**Fig. 1.** TSPO immunohistochemistry with diaminobenzidine (DAB) visualization in the hippocampus of wildtype and TSPO global knockout mice. A) TSPO immunostaining in a normal wildtype (WT) animal at the level of the hippocampus with high expression of TSPO in the ependymal cells of the lining of the ventricles (arrow 1), in the choroid plexus (arrow 2), in blood vessels throughout the brain neuropil, and in pyramidal neurons (PCL) of the hippocampus. B) Image of TSPO immunostaining at the same level of the hippocampus as in panel A but the brain tissue is from a global TSPO-KO mouse that was processed at the same time and under the same experimental conditions as in panel A. The image in the TSPO-KO tissue shows the complete loss of TSPO staining in the ependymal cells of the ventricles, blood vessels, and a nearly complete loss in the choroid plexus. However, there was essentially no loss in pyramidal neurons (PCL) of the hippocampus indicative of non-specific TSPO staining since the tissue is from a TSPO-KO mouse. C and D) are higher magnification images at the level of the CA3 region of the hippocampus. Arrows in Panel C point to TSPO staining of blood vessels in the WT tissue with the TSPO staining disappearing in blood vessels of the TSPO-KO mouse. E and F) are higher magnification images of the lateral ventricles. G and H) are images from the no primary or no secondary antibody controls. Brain slices from wildtype (WT) and TSPO knockout (KO) mice were processed using standard methods with the TSPO primary antibody (ab109497 Abcam) which has been

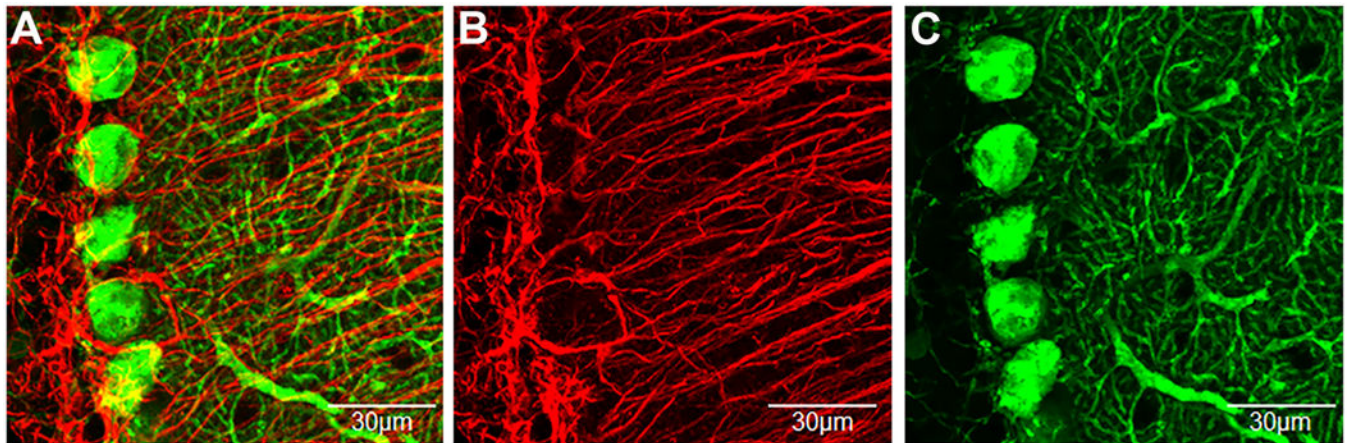
extensively validated using multiple approaches including TSPO-KO cells and tissue (Loth et al., 2020 and supplementary materials within). Experimental methods are described in supplementary materials section.

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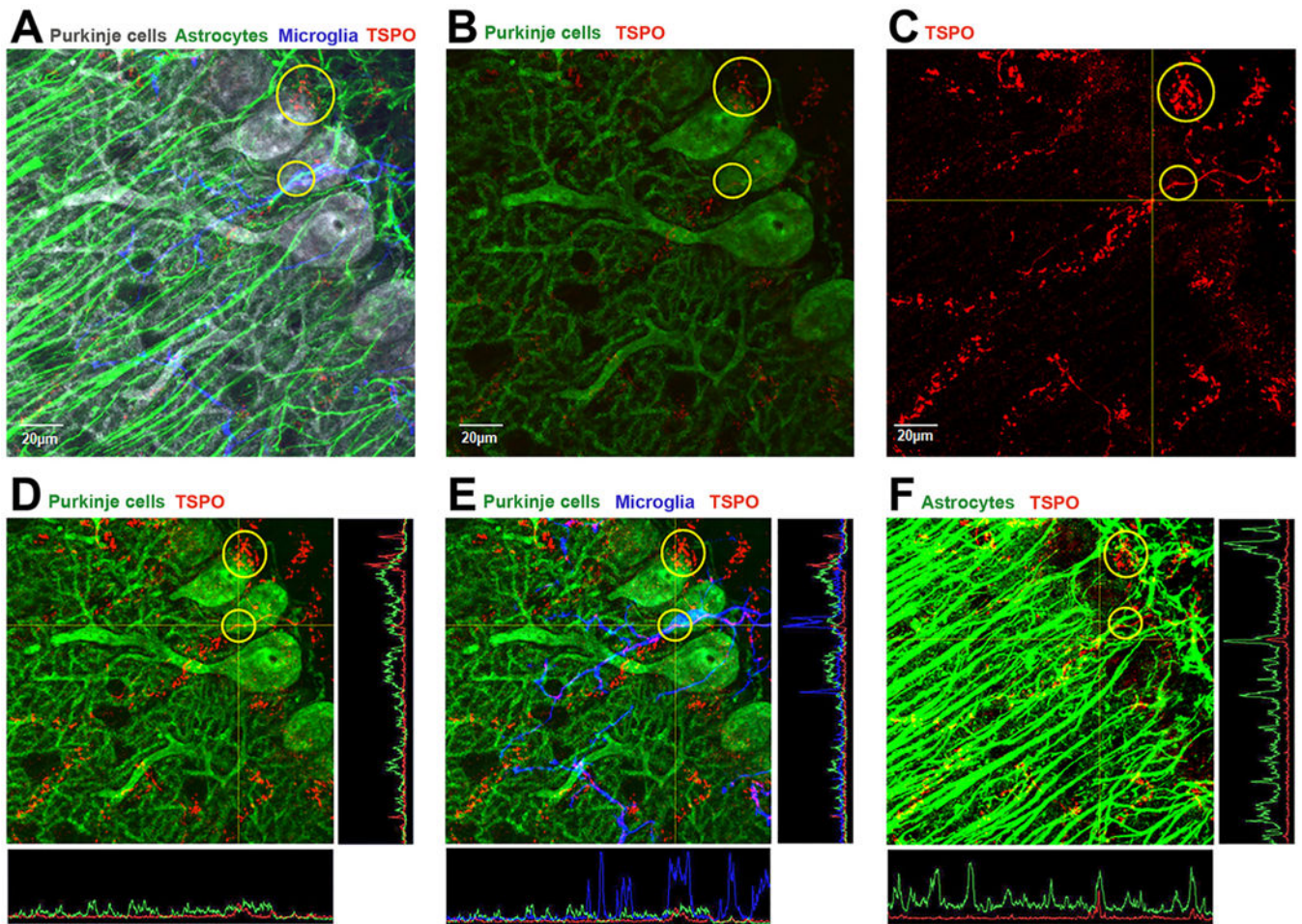
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**Fig. 2.** Immunofluorescent confocal imaging of Purkinje cells and Bergmann glia in the cerebellar cortex.

A) Top-view of a 3D condensed z-stack of double label immunofluorescence confocal imaging of Purkinje cells (calbindin-green) and Bergmann glia (GFAP-red). B and C are the presentation of the individual channels. Bergmann glia cell bodies closely contact and wrap Purkinje cell soma and processes. One distinguishing anatomical feature of Bergmann glia is that they have processes that originate at their soma in the Purkinje cell layer and have a relatively straight and lengthy trajectory to the pial surface of the molecular layer (Reeber et al., 2018). On the other hand, Purkinje cell processes are shorter and radiate at all angles throughout the molecular layer. Green = Purkinje cell; Red = Bergmann glia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.**  
 TSPO expression in the Purkinje cell layer of the cerebellum.  
 A) Quadruple immunofluorescence confocal imaging represented in a top-down view of a 3D condensed z-stack of Purkinje cells (calbindin-gray color), Bergmann glia (GFAP-green color), microglia (Iba1-blue color) and TSPO (red color). The image shows the complex network of glial processes and cell bodies intimately touching the Purkinje cell soma and processes. B) Same image as panel A but the Bergmann glia and microglia channels are not presented to show the relationship of TSPO (red) with Purkinje cells (calbindin-green). Based on this view, in the yellow circles it appears that TSPO may be localized in Purkinje cells. C) In this image, only the TSPO signal is presented and enhanced to be able to visualize since TSPO levels are low in the normal brain [see LUT settings in supplementary Fig. 1]. D) This image again shows the Purkinje cell (calbindin-green) and TSPO (red) but in this image both signals are enhanced [see LUT settings in supplementary Fig. 1]. With signal enhancement one can see a perceived colocalization of TSPO with the Purkinje cell. However, analysis of the TSPO and Purkinje cell signals in the yellow circle using line intensity profiles indicates they do not colocalize as the peaks do not correspond with each other. E) The image provided is the same as D, but the microglia (blue – Iba1) signal is also added. One can see based on the intensity profile that TSPO colocalizes with microglia and not with the Purkinje cell. F) This image represents GFAP (green) staining of Bergmann

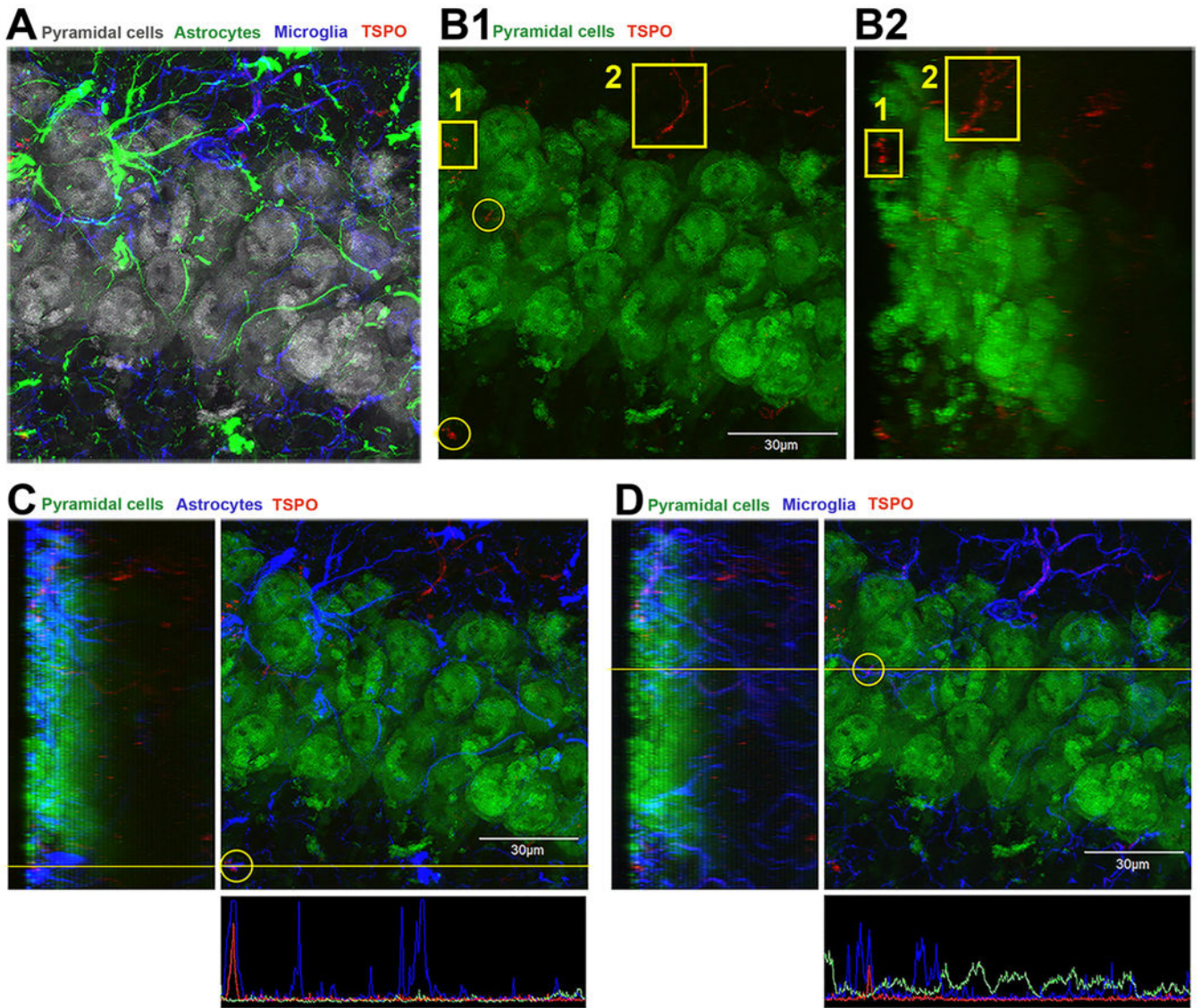
glia process which in different areas colocalize with TSPO (red) generating a yellow color. Intensity profiles indicate that TSPO (red) colocalizes with Bergmann glia processes (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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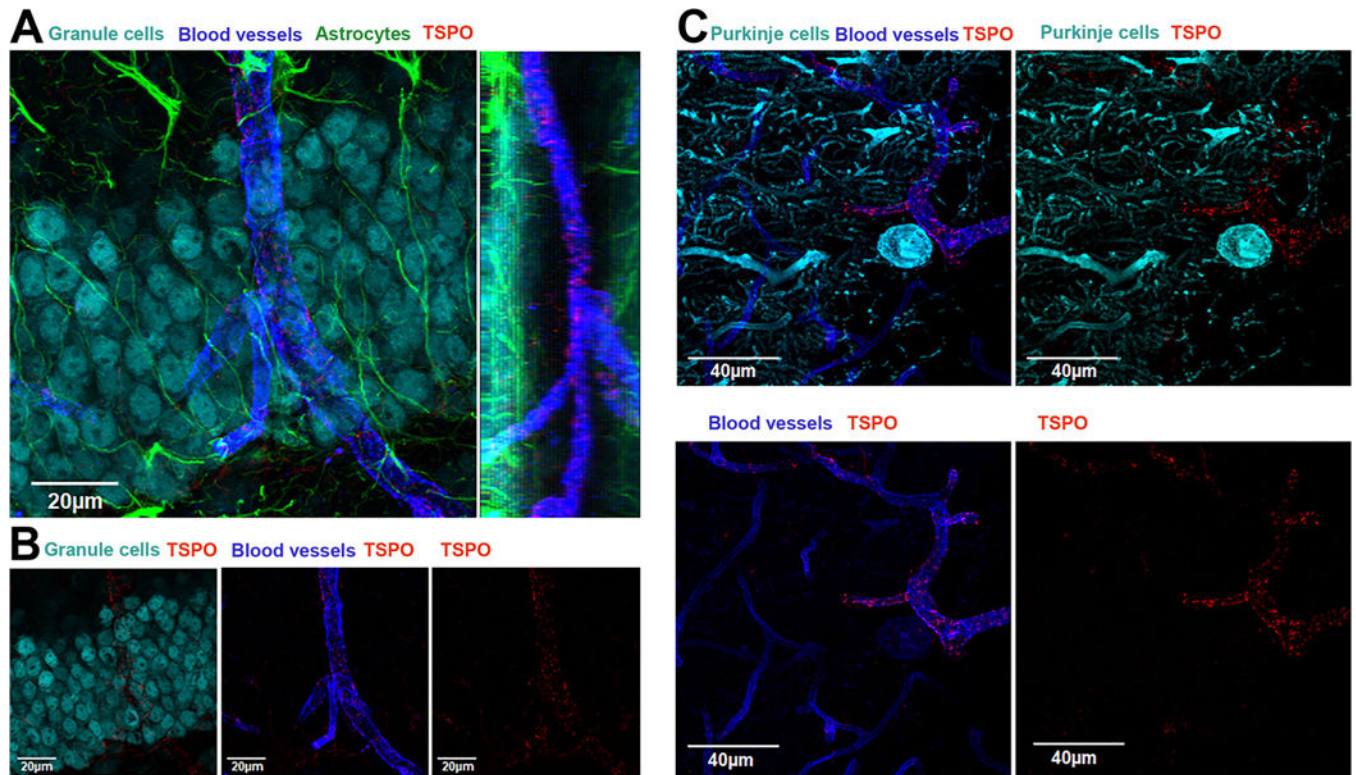
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**Fig. 4.** TSPO expression in the CA1 region of the hippocampus is not associated with neurons. A) Quadruple immunofluorescence confocal imaging represented in a top-down view of a 3D condensed z-stack of pyramidal neurons (NeuN-gray color), astrocytes (GFAP-green color), microglia (Iba1-blue color) and TSPO (red color). The image shows the complex network of glial processes and cell bodies intimately touching pyramidal neurons and processes. B1) This image is the same as in panel A, but now pyramidal neurons labeled with NeuN are displayed in green and TSPO in red. The microglia and astrocyte signal were removed to visualize the spatial relationship between TSPO and neurons. Areas of TSPO are noted in the yellow boxes and circles. There is a low level of TSPO expression that is outside or interspersed between pyramidal neurons. B2) This image is the same as in panel B1, but it represents a 67.5 degree rotation of the z-stack showing that the TSPO staining is not localized with neurons. C) Same image as B1 but now the astrocyte (blue signal) is added and the colocalization of TSPO with astrocyte in the yellow circle is analyzed. The



left side of the image is the side view of the zstack and the bottom is the intensity profile (yellow line) indicating signal colocalization of TSPO and astrocyte. D) A similar approach was used in panel D as in panel C, but in this case, the TSPO signal inside the yellow circle appears to colocalized with the microglia marker Iba1 (blue). Again, the side view of the z-stack shows that the TSPO signal colocalizes with microglia which is confirmed in the intensity profile (yellow line) of the signals in the bottom of panel D. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 5.** TSPO is highly expressed in blood vessels in the hippocampus and cerebellum of the normal mouse brain. A) Quadruple immunofluorescence confocal imaging represented in a top-down view of a 3D condensed z-stack of neurons in the dentate gyrus in the hippocampus (NeuN-cyan), blood vessels (CD31-blue), astrocytes (GFAP-green), and TSPO (red). The image shows the presence TSPO in a blood vessel in close contact with neurons in the granule cell layer. To the right is the side view of the z-stack showing the blood vessel with several TSPO positive puncta. B) the different panels show the individual signals showing that TSPO is present in blood vessels. C) Triple immunofluorescence confocal imaging represented in a top-down view of a 3D condensed z-stack of Purkinje cells (calbindin – green), blood vessels (CD31 – blue), and TSPO (red). Left panel shows the 3 signals and the right panel shows Purkinje cell and TSPO. Lower left panel shows the presence of TSPO in the blood vessel and the lower right panel is TSPO only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**

Studies examining the cellular localization of TSPO in brain cells.

Reference	Disease Model	Age/Time Point	Brain Regions	TSPO Cellular Source
Tournier et al. (2019)	<u>Alzheimer's Disease</u> 3xTgAD mouse	1, 6, 12, 21 months	Anterior dorsal hippocampus Subiculum Dorsal hippocampus Ventral hippocampus	21 month mice: Microglia (main source) Astrocytes (less)
Tournier et al. (2020)	Human tissue  <u>Alzheimer's Disease</u> TgF334-AD rat	Human tissue  12 months 24 months	Choroid plexus  Hippocampus Cortex Striatum Cerebellum Rest of brain	Epithelium Endothelial cells (some)  12 months: — Microglia ↑ Astrocytes — Endothelial cells 24 months: ↑ Microglia ↑ Astrocytes — Endothelial cells
Gui, Marks, Das, Hyman, and Serrano-Pozo (2020)	Human tissue	Avg age: Control 85.6 years AD 89.1 years	Temporal Cortex	↑ Microglia ↑ Astrocytes — Endothelial cells
Ji et al. (2021)	<u>Alzheimer's Disease</u> PS19 mouse  Human tissue	Multiple Ages	Temporal Pole Temporal Association Cortex Frontal Association Cortex Cerebellum  Hippocampus  Frontal Cortex	↑ Microglia ↑ Astrocytes — Endothelial Cells — Vascular Smooth Muscle Cells  ↑ Microglia ↑ Astrocytes ↑ Microglia ↑ Astrocytes
Nackett et al. (2019)	<u>Multiple Sclerosis</u> Cuprizone mouse	1, 3, 5 wks Cuprizone	Corpus Callosum Cortex	Microglia (most) Astrocytes (1/3)

Reference	Disease Model	Age/Time Point	Brain Regions	TSPO Cellular Source
Zinnhardt et al. (2019)	Multiple Sclerosis Cuprizone mouse	4 wks Cuprizone -demyelination 6 wks Cuprizone -remyelination (spontaneous)	Corpus Callosum Hippocampus Thalamus	Microglia: ↑ 4 wks; ↓ 6 wks Astrocytes: ↑ 4 wks; ↑↑ 6 wks
Nutma et al. (2019)	Multiple Sclerosis Human tissue	Multiple Ages	Multiple (areas with lesions) Normal appearing white matter (NAWM)	Microglia: 11–14 fold ↑ in active lesion/chronic active rim; 16–21 fold ↑ in number from NAWM
Pannell et al. (2020)	Neuroinflammation AdTNF Injection - mouse	Day 3, Day 5 post intracranial injection	Striatum Cortex	Astrocytes: 15–20 fold ↑ in all lesion subtypes; 5–7 fold ↑ in number from NAWM  Total TSPO+ cells in active lesions/chronic active rims: 40% microglia/macrophages 25% astrocytes <5% vascular endothelial cells <1% lymphocytes  TSPO+ cells in center of chronic active and in active lesions: 65% TSPO+GFAP+ astrocytes
Vicente-Rodriguez et al. (2021)	Neuroinflammation LPS Injection - rat	Adult	Hippocampus Substantia Nigra	Microglia: ↑ Day 5 Astrocytes: ↑ Day 3 Endothelial cells: No +TSPO ↑ Microglia ↑ Astrocytes