PERSPECTIVE

Translocator protein 18 kDa (TSPO): old dogma, new mice, new structure, and new questions for neuroprotection

The normal development and optimal functioning of the brain requires a vigilant immune surveillance system to detect and remove potential risk factors and prevent infection and tissue damage. Microglia are the resident immune cells and the frontline defenders responsible for the immune response of the brain. Resting microglia possess a ramified morphology with numerous thin processes that continuously sample the environment. In response to inflammatory signals, microglia become activated and transform their morphology into a thick, amoeboid-like shape. Activated microglia proliferate, migrate to sites of injury, and mediate a cascade of immune responses which protect tissues and maintain homeostasis. However, sustained activation of microglia in the diseased areas leads to over-production of toxic, pro-inflammatory cytokines, which aggravate neuronal damage. An interplay between environmental and genetic factors seems to be important for the interaction between neurons and microglia. The accumulation of neurotoxins affects microglia as well as neurons, and danger signals released from the degenerating neurons induce the microglia to enter a reactive state and overly recruited activated microglia exacerbatethe neurodegeneration process. Many familial neurodegenerative genes whose mutations cause neuronal toxicity also enhance pro-inflammatory responses in microglia. Therefore, hyper-activation of the immune cells in the nervous system, termed "neuroinflammation", may not be the initiator of the neuropathologies, but rather may be considered as one of the major contributors to disease progression, intimately implicated in a variety of neuronal diseases. As such, the severity of neurodegeneration is often parallel to the level of neuroinflammation, and suppression of neuroinflammation yields neuroprotection in a broad spectrum of animal models of neurological disorders. These observations suggest that environment-gene interactions induce the vicious cycle of tissue damage through amplification of neuroinflammation. In addition to the characteristic and dynamic morphological changes, inflammatory activation of microglia induces a change in their gene expression profiles. Thus, immunological detection of marker proteins, such as IBA-1 or CD11b, has been commonly used to monitor microglia activation in the nervous system. However, the traditionally used immunohistochemical techniques have limitations and are not easily applicable to *in vivo* situations. Therefore, there is an imminent need for the development of biological markers and probes to monitor neuroinflammation *in vivo*. In this regard, high expression levels of translocator protein 18 kDa (TSPO) in the diseased state, which are especially predominant in activated microglia, have attracted increasing interest for its use as an imaging target for the visualization of injured brain areas and the monitoring of microglial activation.

TSPO, formerly known as the peripheral benzodiazepine receptor (PBR), is an integral membrane protein with five transmembrane-domains. It is located primarily on the outer mitochondrial membrane. As the old name indicates, TSPO was first reported as an alternative receptor for benzodiazepine drugs in peripheral tissues, distinguished from the central receptors found in brain tissue. Subsequently, it was found to bind other molecules, such as cholesterol and porphyrins. Although TSPO is expressed in all tissues, its expression is particularly high in the tissues that synthesize steroids, and in glial cells in the brain. Increased binding of PK11195, the prototype TSPO ligand, in microglia following CNS injury represents the up-regulation of TSPO in microglial activation (Banati, 2002). The prototype TSPO ligand PK11195 stimulated steroid synthesis in several tumor cell lines. TSPO also has a cholesterol recognition amino acid sequence at its C-terminus. The results from several *in vitro* studies have implicated TSPO in cholesterol transport into mitochondria, the late-limiting step in the steroid biosynthesis. Therefore, TSPO has been considered as a critical factor in steroidogenesis. Thus, the new name TSPO was suggested to more accurately represent the key molecular function of this molecule regardless of its subcellular or tissue distribution. Moreover, the ubiquitous expression and evolutionary conservation of TSPO from bacteria to mammals strongly suggested its essential role in cellular processes. This presumption was further supported by the results of an earlier study that claimed the embryonic lethality of TSPO whole body knockout (KO) mice, although the details of the methods for the design and generation of the KO mice were not provided (Papadopoulos et al., 1997).

More current studies, however, have been performed with the newly generated global or steroidogenic tissue-specific KO mice. Results from such studies have revealed unexpected results by showing that a genetic deficiency of TSPO had no effects on steroid biosynthesis or embryonic development. Furthermore, the KO mice were viable with no evident phenotypic abnormalities. A group of researchers led by Selvaraj developed conditional TSPO KO mice which had the gene product deficient only in testicular steroidogenic cells (Morohaku et al., 2014). This genetic ablation of TSPO in testicular steroidogenic cells did not affect normal testicular development or the synthesis of the hormone testosterone. The lack of an effect of TSPO gene deficiency on hormone synthesis could not be explained by compensatory changes in the expression of other steroidogenic genes, as the loss of TSPO did not affect the expression of any other steroidogenic genes. Prompted by these findings, which were contrary to the purported essential role of TSPO in steroid synthesis, a germ cell-specific deletion approach was adopted to generate TSPO global KO mice (Tu et al., 2014). Similar to previous recent findings, an analysis of the global KO mice revealed results that were in opposition to the established dogma of the indispensable role of TSPO in steroidogenesis. Mice with a TSPO deletion survived without abnormalities, and their adrenal and gonadal steroidogenesis showed no defects. Additionally, a reexamination of the effects of TSPO knockdown in several steroidogenic cell lines showed that TSPO knockdown did not affect steroid hormone biosynthesis.

An independent global KO mice study conducted by Banati et al. (2014) corroborated these findings. Banati's research group generated global TSPO KO mice and observed normal viability and development, and no defects in cholesterol transport or the level of pregnenolone, which is the first product generated in the mitochondria after cholesterol import. Microglial activation after neuronal injury seemed to be unaffected, although the microglial cell response was tested only in the facial nucleus after peripheral facial nerve lesion. It remains to be examined whether the loss of TSPO impairs microglial activation in the central nervous system in this KO mouse. Of interest, microglia from TSPO KO mice showed reduced metabolic activity with decreased ATP production. These recent findings challenged much of the early work focused on the PBR/TSPO as the essential regulator protein of steroid biosynthesis. Furthermore, an additional genetic study aimed at testing the role of TSPO in the regulation of the mitochondrial permeability transition pore (mPTP) disputed the previous model involving TSPO in the molecular composition of the mPTP (Sileikyte et al., 2014). Conditional KO mice in which the TSPO gene was ablated in the liver and heart revealed no differences from control mice in the regulation or structure of the mPTP. Additionally, there were no ultrastructural changes, and functional parameters of the mitochondria, such as calcium retention capacity, oxygen consumption rate, and porphyrin uptake, were the same irrespective of the presence or absence of the TSPO gene. mPTP is known to mediate the tissue damage caused by ischemia/reperfusion (I/R) injury. However, when the I/R injury protocol was performed on hearts isolated from mice in which the cardiac TSPO gene was conditionally deleted, the extent of tissue damage was the same as for hearts isolated from control mice. Interestingly, the prototypical TSPO ligands affected mitochondrial function equally in both control and TSPO-null mitochondria. As such, the results from these reports suggested that the notable effects of the TSPO ligands on a number of pathological conditions may occur through TSPO-independent mechanisms, at least under certain conditions.

Structural biologists also began to take a keen interest in the role of TSPO. Zweckstetter and his research group reported the three-dimensional structure of the mouse TSPO in complex with PK11195 using nuclear magnetic resonance spectroscopy (Jaremko et al., 2014). Ten months later, two additional publications revealed the high-resolution crystal structure of bacterial TSPO (Guo et al., 2015; Li et al., 2015). The detailed structure of TSPO at the atomic level may provide insight into the putative biological functions of TSPO and help resolve the current controversy surrounding its key roles and functions in the near future. Unfortunately, however, elucidation of the structures of TSPO initiated new disputes, as some of the key features differed between studies, such as monomer *vs.* dimer composition, and the exact location of the transmembrane helices.

The normal survival of the TSPO conditional or global KO mice and lack of obvious phenotypic abnormalities cast doubt on the purported essential roles of TSPO in steroidogenesis and other physiological processes. Therefore, the precise biological function of TSPO remains to be elucidated. In that regard, the role of TSPO in immune reactions and neuroinflammation may deserve more attention. We previously reported that TSPO negatively regulated microglial activation and suppressed neuroinflammation (Bae et al., 2014). TSPO ligands attenuated the production of pro-inflammatory cytokines in response to lipopolysaccharide treatment *in vitro* and *in vivo*. Furthermore, over-expression of TSPO yielded similar outcomes, while knockdown of TSPO elevated inflammatory responses. Despite the promise of the TSPO ligands as potential therapeutic tools, the exact role of TSPO in the regulation of neuroinflammation at the molecular level remained elusive. Our data demonstrated the negative role of TSPO in neuroinflammation and indicated that up-regulation of the TSPO level in activated microglia was an adaptive response to resolve inflammatory responses (**Figure 1**). Because we wanted to explore how TSPO modulated neuroinflammation in activated microglia, we focused on the link between TSPO and the different states of microglial activation; pro-inflammatory M1 polarization and anti-inflammatory M2 polarization. Our findings indicated that TSPO over-expression, or the TSPO ligands, promoted anti-inflammatory M2 polarization genes. However, this cannot entirely reflect *in vivo* characteristics of up-regulation of TSPO in neuroinflammation state and requires further investigation. In a global KO mouse study from Tu et al. (2014), the authors also noticed differential expression of the genes involved in immune activity, suggesting a potential change in immune function depending on the absence of the TSPO gene. An additional prospective future direction for the study of TSPO may be an examination of the role of TSPO in psychiatric disorders. The crystal structure of the bacterial TSPO mutant corresponding to the human A147T polymorphism associated with psychiatric disorders revealed a tilting of the transmembrane helices and a subsequent conformational change (Li et al., 2015). As many anti-depressant drugs also subdue neuroinflammation, it is emerging as a potential common etiology for depression and psychiatric disorders.

The unexpected outcomes from the recent genetic studies have cast doubt on the trademark role most often attributed to TSPO. Despite these controversies, as well as lack of clarity regarding the cellular function(s) of TSPO, the potent neuroprotective effects of the TSPO ligands implicate them as part of potential therapeutic strategies, rather than simple markers of neuroinflammation. Therefore, it will also be necessary to identify the endogenous targets of the TSPO ligands to understand their true biochemical and pharmacological actions, which may lead to better targeted design of therapeutic agents.

This Perspective article was particularly focused on introducing recent controversial findings concerning the traditionally accepted role of TSPO in cholesterol transport and steroid synthesis, and discussed potential directions for future study. As part of the challenge to this long-standing dogma, the exact physiological functions and molecular mechanisms underlying the activities of TSPO will need to be determined. Additionally, a more careful interpretation of findings obtained from genetic and structural studies will be warranted. However, TSPO is a highly conserved

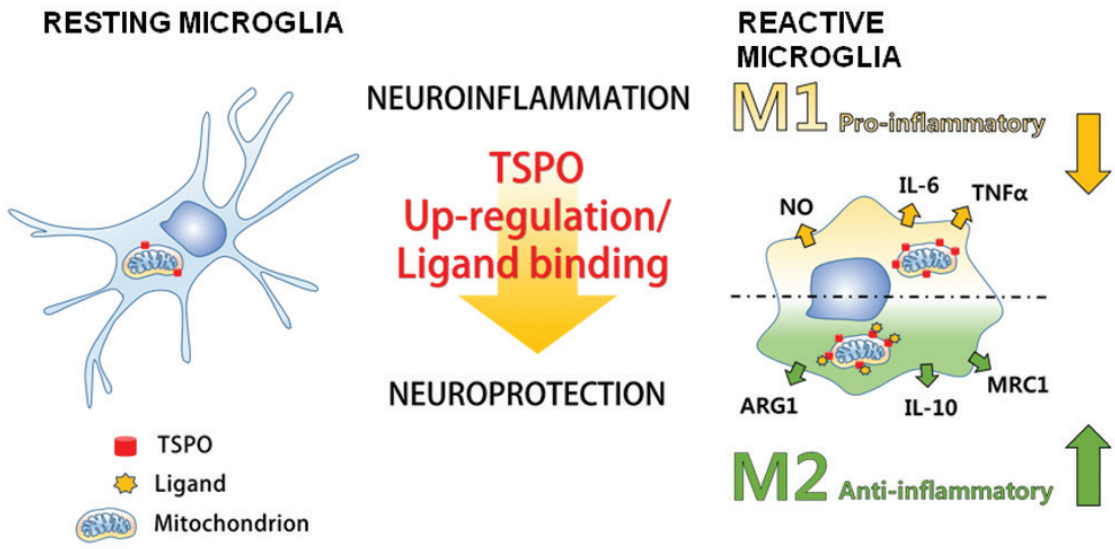

Figure 1 Two different types of microglial activation modulated by high expression levels of translocator protein 18 kDa (TSPO) or its ligand during neuroinflammation.
The TSPO level is elevated in activated microglia. Binding of TSPO to its ligand, or up-regulation of its expression level, enhances alternative M2 anti-inflammatory microglial activation. M2 polarization results in more phagocytic activity and the up-regulation of anti-inflammatory genes to resolve neuroinflammation and promote recovery from tissue damage. In contrast, M1 pro-inflammatory microglial activation seems to be reduced by the up-regulation of TSPO or its ligand.

and evolutionally ancient gene. Therefore, it may have many functions with multiple levels of regulation. Although it may not play a critical constitutive role at its basal state, certain pathological and stress-related conditions may reveal the true nature of TSPO.

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