Increased Expression of Translocator Protein (TSPO) Marks Pro-inflammatory Microglia but Does Not Predict Neurodegeneration.

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Abstract:	Activation of the innate immune system plays a significant role in pathologies of the central nervous system (CNS). In order to follow disease progression and evaluate effectiveness of potential treatments involved in neuroinflammation, it is important to track neuroinflammatory markers in vivo longitudinally. The translocator protein (TSPO) is used as a target to image neuroinflammation as its expression is upregulated in reactive glial cells during CNS pathologies. However, it remains unclear in which microglial phenotypes TSPO levels are upregulated, as microglia can display a plethora of activation states that can be protective or detrimental to the CNS. Here, we polarized microglia into two extreme states in vitro and found that TSPO expression is increased in so-called classically activated or M1 microglia but not in alternatively activated or M2 microglia. In addition, we used a mouse model of peroxisomal multifunctional protein-2 (MFP2) deficiency that develops progressive and widespread neuroinflammation with no neuronal loss and in which the microglial phenotype has been previously characterized. We found that both transcript and protein levels of TSPO are significantly increased in the brain of Mfp2-/- compared to control mice and TSPO immunoreactivity colocalized predominantly with microglia in Mfp2-/- brain. In vitro and ex vivo autoradiography in Mfp2-/- mice using the TSPO radiotracer [18F]DPA-714, confirmed increased expression of TSPO. These data demonstrate that microgliosis can be revealed in non-neurodegenerative brain pathologies by tracking TSPO. Taken together, we show that induced TSPO expression marks a pro-inflammatory brain environment that is not necessarily accompanied by neuronal loss.
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Author Comments:	Dear editor, Herewith we would like to submit a paper entitled: "Increased expression of Translocator protein (TSPO) marks pro-inflammatory microglia even in the absence of neurodegeneration" for publication in Molecular imaging and biology. TSPO is the most common target for imaging neuroinflammation that is currently used. Coincident with the submission of our manuscript, a comprehensive review was published on 'Microglia in physiology and disease'. The authors state "…., correlating TSPO binding with microglia activation should be reviewed critically. We emphasize that TSPO expression should be analyzed within specific disease contexts rather than merely equated with the reified concept of neuroinflammation,..."(Wolf, Boddeke and Kettenman, Annual review of Physiology, in press). This is precisely the message of our manuscript. Indeed, we first show using an in vitro polarization protocol of microglia into extreme pro- and anti-inflammatory states that TSPO is only upregulated in pro-inflammatory microglia but not in anti-inflammatory. In addition, we examined TSPO expression in the inflamed brain of Mfp2-/- mice using biochemical and imaging approaches. We previously showed that these mice develop progressive and widespread neuroinflammation but no neuronal loss. By immunohistological and gene expression studies and by in vitro, ex vivo and in vivo TSPO imaging using the TSPO specific radiotracer [18F]DPA-714, we found that TSPO is significantly increased in the brain of Mfp2-/- compared to control mice colocalizing predominantly with microglia. Taken together, we show that induced TSPO expression marks a pro-inflammatory brain environment that is not necessarily accompanied by neuronal loss. The manuscript has been revised and approved by all authors. We acknowledge that this manuscript is our original, unpublished work and that it has not been submitted to any other journal for review. Looking forward to your response, Yours sincerely, Prof Myriam Baes
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Increased expression of Translocator protein (TSPO) marks pro-inflammatory microglia even in the absence of neurodegeneration

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Short title: TSPO in pro-inflammatory non-neurodegenerative microglia

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ABSTRACT

Purpose: Activation of the innate immune system plays a significant role in pathologies of the central nervous system (CNS). In order to follow disease progression and evaluate effectiveness of potential treatments involved in neuroinflammation, it is important to track neuroinflammatory markers *in vivo* longitudinally. The translocator protein (TSPO) is used as a target to image neuroinflammation as its expression is upregulated in reactive glial cells during CNS pathologies. However, it remains unclear in which microglial phenotypes TSPO levels are upregulated, as microglia can display a plethora of activation states that can be protective or detrimental to the CNS.

Procedures: We polarized microglia into two extreme states *in vitro* and found that TSPO expression is increased in so-called classically activated or M1 microglia but not in alternatively activated or M2 microglia. In addition, we used a mouse model of peroxisomal multifunctional protein-2 (MFP2) deficiency that develops progressive and widespread neuroinflammation with no neuronal loss and in which the microglial phenotype has been previously characterized.

Results: We found that both transcript and protein levels of TSPO are significantly increased in the brain of *Mfp2*$^{-/-}$ compared to control mice and TSPO immunoreactivity colocalized predominantly with microglia in *Mfp2*$^{-/-}$ brain. *In vitro* and *ex vivo* autoradiography in *Mfp2*$^{-/-}$ mice using the TSPO radiotracer [^{18}F]DPA-714, confirmed increased expression of TSPO. These data demonstrate that microgliosis can be revealed in non-neurodegenerative brain pathologies by tracking TSPO.

Conclusions: We show that induced TSPO expression marks a pro-inflammatory brain environment that is not necessarily accompanied by neuronal loss.

Keywords

TSPO, neuro-inflammation, microglia, PET tracer, peroxisomes, mouse model

INTRODUCTION

The innate immune system is implicated in all neurodegenerative disorders. Key players in neuroinflammatory processes are microglia and astrocytes. They become reactive, typically change their morphology and acquire modified functions [1, 2]. Neuroinflammation often precedes neuronal loss in neurodegenerative disorders such as in Alzheimer's disease (AD) and Parkinson's disease (PD) [3, 4]. Therefore, it is important to detect neuroinflammatory processes in presymptomatic stages as they mark early stages of neuropathology. The most studied biomarker for imaging of neuroinflammation is the 18 kDa translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), which is primarily expressed on the outer mitochondrial membrane of microglia and macrophages [5, 6]. TSPO is important for brain development and homeostasis during adulthood as it plays a crucial role in neurosteroidogenesis [7, 8]. TSPO expression in the CNS is low under healthy circumstances, but increases mainly in microglia in response to neuronal insults such as in the neurodegenerative diseases PD, AD, Amyotrophic lateral sclerosis (ALS), and Huntington disease (HD) [9, 10]. Reactive astrocytes can also induce TSPO expression in addition to reactive microglia [11].

It is still a matter of debate whether neuroinflammation accelerates or delays disease progression in neurological disorders. Reactive microglia play Janus-like roles in neurological disorders. During pathology, activated microglia can promote tissue injury (denoted as an M1 phenotype) but also tissue repair and remodelling (denoted as an M2 phenotype), dependent on type of disease and stage of disease progression [12, 13]. Therefore, it is important to precisely characterise neuroinflammation *in vivo* in order to apply anti-inflammatory therapies only during harmful functioning of the innate immune system. Although TSPO is generally accepted as an appropriate marker of neuroinflammation, the functional link between TSPO and neuroinflammation remains unknown. It is unclear whether increased TSPO expression is associated with pro-inflammatory and neurotoxic responses (often denoted as classically activated or M1), or with anti-inflammatory responses and tissue repair (denoted as alternatively activated or M2), or both. Studies that associate TSPO with specific inflammatory states are lacking, but are necessary to correctly interpret increased TSPO levels *in vivo* in clinical conditions and to adapt inflammatory therapies according to the need and disease stage of patients.

In this study, we aimed to better define in which neuroinflammatory conditions TSPO is induced. First, microglia were isolated from wild type brain and TSPO transcript expression was analyzed after polarization of the microglia into a pro- or anti-inflammatory state. In addition, we used a mouse model of peroxisomal β-oxidation deficiency, more specifically multifunctional protein-2 (MFP2) deficient mice, which develop robust and chronic neuroinflammation before the age of 8 weeks that progressively increases until death around the age of 17 weeks [14-16]. Different from most other neuropathological disease models in which neuroinflammation develops, *Mfp2*$^{-/-}$ mice do not show neuronal loss [16]. We previously characterized the proliferative *Mfp2*$^{-/-}$ microglia extensively and found that they are of local origin, are immunologically activated but display a mixed pro- (M1) and anti- (M2) inflammatory phenotype [16]. Of note, they do not express detrimental neuroinflammatory markers such as iNOS. We investigated whether TSPO is induced in this inflamed but non-neurodegenerative brain environment by immunohistological and gene expression studies and by *in vitro*, *ex vivo* and *in vivo* TSPO imaging using the TSPO specific radiotracer [^{18}F]DPA-714.

MATERIALS AND METHODS

Mouse breeding

Mfp2$^{-/-}$ mice were generated as previously described [17]. *Mfp2*$^{-/-}$ mice were bred on a Swiss/Webster background in specific pathogen free animal housing facility of the KU Leuven, had *ad libitum* access to water and standard rodent food, and were kept on a 12-hour light and dark cycle. All animal experiments were performed in accordance with the "Guidelines for Care and Use of Experimental Animals" and fully approved by the Research Ethical committee of the KU Leuven (#190/2012). Both wild type and heterozygous mice were used as controls as no differences were detected in our previous investigations [14-16].

Immunohistochemistry

Anesthesia of the mice and tissue processing for immunohistochemical (IHC) staining were performed as described [18]. Paraffin sections (7 µm) were used for immunofluorescent staining. The following primary antibodies were used: polyclonal rabbit anti-Iba1 (1:500; Wako D19-19741), rabbit anti-PBR (TSPO) (Abcam), rabbit anti-GFAP (Sigma). After overnight incubation with primary antibodies at room temperature (at 4°C for cryo sections) HRP-labeled secondary antibodies (1:200) were applied for 1 hour, followed by fluorescent labeling with a cyanine 2 (FITC) TSA kit (Perkin Elmer Life sciences, Boston, USA). When double immunolabeling was performed, sets of primary and secondary antibodies were applied sequentially. Cyanine 3 TSA kit (Perkin Elmer Life sciences) was used as second fluorescent label. Images were acquired with a motorized inverted IX-81 microscope connected to a CCD-FV2T digital camera (Olympus, Aartselaar, Belgium) and processed with LSM Image browser software (Zeiss, Germany).

Real time quantitative RT-PCR

RNA was extracted using PureLink RNA Mini Kit according to the manufacturer's protocol and converted to cDNA using Qiagen QuantiTect Reverse Transcription Kit.

Real time PCR was performed as previously described [19] using an ABI PRISM 7500 Real Time PCR instrument (Applied Biosystems, Lennik, Belgium). Validated primers and probe were ordered from Integrated DNA Technologies (IDT, Leuven, Belgium) as premade PrimeTime qPCR assay (TSPO,

NM_009775; TNFa, NM_013693; iNOS, NM_010927; Arg1, NM_007482). Assays were performed in triplicate in 10 µL TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Relative expression levels of the target genes were calculated taking into account the amplification efficiency as described [20]. The relative expression levels of the target genes were calculated as a ratio to the housekeeping gene β-actin. Statistics were carried out using Graphpad Prism v5.0 software (San Diego, CA).

Primary microglial cell culture

For the preparation of primary microglia cultures, control mice from C57BL6/J background were used. The cultures were derived from the cortices of mice at postnatal day 0-1 as previously described [21]. Briefly, mice where decapitated and cortices dissected. After removal of the meninges, cortices were mechanically dissociated and the cell suspension was diluted with low glucose DMEM supplemented with 10% fetal bovine serum and 0.01% penicilline-streptomycin and cultured in T75 flasks. At day 14 *in vitro* microglia cells were isolated by shaking and rinsing the flasks. The purity of obtained cultures was confirmed by means of Iba1 immunocytochemistry and Cd11b flow-cytometry and found to be higher than 95%.

For RNA isolation microglia cells were plated in a 12-well plate at density of 500 000 cells/well. One day after plating, cells were stimulated for 24h with vehicle (PBS 10µl/ml), lipopolysaccharides (LPS) (1µg/ml) or IL4 (20ng/ml) in order to shift them to the M0, M1 and M2 phenotype, respectively.

In vitro autoradiography

Mice (n=3/group) were sacrificed at an age of 19 weeks, brain was removed, rinsed with saline to remove blood, rapidly frozen in 2-methylbutane (-40 °C) and stored at -20 °C for 24 h. Brain sagittal sections were obtained using a cryotome (Shandon cryotome FSE; Thermo Fisher, Waltham, USA), mounted on adhesive microscope slides (Superfrost Plus; Thermo Fisher) and stored at -20 °C until autoradiography was performed. Brain slices were dried and preincubated in tris-HCl 50 mM buffer (pH 7.4) for 10 min at room temperature. Before incubation with [^{18}F]DPA-714, the brain sections were dried. The brain sections were incubated with 300 kBq (in 500 µl tris HCl 50 mM buffer) of tracer for 10 min. The brain sections were washed twice for 10 min in tris-HCl 50 mM (pH 7.4) + 0.3% BSA buffer at 4 °C. After a quick dip in water at 4 °C, the slides were dried. Autoradiograms were obtained by exposing the slides for 5 min to a high performance phosphor storage screen (super-resolution screen; Perkin Elmer,

Waltham, USA). The screens were read using a Cyclone Plus system (Perkin Elmer) and analyzed using Optiquant software (Perkin Elmer). The radioactivity concentration in the autoradiograms is expressed in digital light units (DLU)/mm² corrected for background. All statistical studies were performed with the unpaired two-tailed t-test. Calculations were carried out using GraphPad Prism v5.0 (San Diego, CA).

Ex vivo autoradiography

Mice (16 weeks old, n=6/group) were injected with the tracer (22 ± 0.6 MBq, mean ± SD) via a tail vein under anesthesia (2.5% isoflurane in O2 at 1 L/min flow rate) and were kept under anesthesia until they were sacrificed by decapitation at 30 min (n=3/group) and 60 min (n=3/group) post tracer injection. Brain was removed and rapidly frozen in 2-methylbutane (between -30 °C and -40 °C). 20 µm sagittal sections from brain were obtained using a cryotome (Shandon cryotome FSE, Thermo Fisher, Waltham, USA), mounted on adhesive microscope slides (Superfrost Plus, Thermo Fisher Scientific) and exposed to a phosphor storage screen film (super resolution screen; Perkin Elmer, Downers Grove, IL, USA) for 35 min. The screens were read using a Cyclone® Plus system (Perkin Elmer) and analyzed using Optiquant software (Perkin Elmer). The results were expressed as digital light units/mm^2 normalized for body weight of the animal and injected dose [(DLU/mm^2) * (body weight/injected dose)].

microPET

Imaging experiments were performed on a Focus 220 microPET scanner (Concorde Microsystems, Knoxville, TN, USA). Four *Mfp2*$^{-/-}$ (25.7 ± 2.7 g, mean ± SD) and five control mice (39.6 ± 5.9 g) were scanned at end stage of disease, between 14 and 19 weeks of age. Mice were injected with 22 ± 0.6 MBq (mean ± SD) of [^{18}F]DPA-714 via a tail vein between 14 and 19 weeks of age (volume injected <0.3 mL). During all PET sessions, animals were kept under gas anesthesia (2.5% isoflurane in O_2 at a flow rate of 1 L/min). List-mode 90-min microPET scans were acquired. Acquisition data were then Fourier rebinned in 27 time frames (4 x 15 seconds (s), 4 x 60 s, 5 x 180 s, 8 x 300 s, 6 x 600 s) and reconstructed using maximum a posteriori iterative reconstruction. The images were spatially aligned to a rat brain [^{18}F]FDG template in Paxinos coordinates [22] using an affine transformation, allowing the use of a predefined volumes of interest map. Time-activity curves (TAC) were generated for total brain for each individual scan using PMOD software (version 3.2; PMOD technologies, Zurich, Switzerland).

RESULTS

Induced TSPO expression is restricted to classically (M1) activated microglia *in vitro*

It is known that activated microglia exert dual functions, either pro-inflammatory (M1) functions that are rather harmful, or anti-inflammatory (M2) functions that are neuroprotective and regenerative [23]. While this M1-M2 distinction is an oversimplification and represents the extreme states, this subdivision is a nice strategy to associate certain disease phenomena with a specific activation state of microglia. Although TSPO is a well-known marker for general neuroinflammation, it is still unknown whether TSPO expression is induced during both pro- and anti-inflammatory activation states of microglia or during a specific activation state only. In this study, we aimed to associate induced TSPO expression with either a pro-inflammatory (M1) or anti-inflammatory (M2) activation state of microglia. Primary microglia isolated from wild-type mice were stimulated with either LPS or interleukin- (IL-) 4. We verified that LPS polarizes the microglia towards an M1 state by showing increased transcript expression of the pro-inflammatory markers *Tnfa* (Fig. 1A) and *iNOS* (Fig. 1B) compared to vehicle-activated (M0) microglia. Successful polarization to the M2 state with IL-4 was shown by increased arginase-1 *(Arg1)* expression (Fig. 1C) [24]. We found that *Tspo* transcript expression was induced in M1-activated microglia but did not change in M2-activated microglia (Fig. 1D). This demonstrates that *Tspo* expression is specifically increased in a pro-inflammatory microenvironment, but not in a healthy or solely anti-inflammatory environment.

TSPO transcript and protein levels are induced in *Mfp2*$^{-/-}$ brain

We previously demonstrated that in MFP2-deficient brain a robust and chronic neuroinflammation develops that is not accompanied by overt loss of neurons [16]. In order to analyse whether TSPO expression is increased in the CNS of *Mfp2*$^{-/-}$ mice, we first examined the transcript levels. Quantitative RT-PCR showed increased *Tspo* mRNA in brainstem of *Mfp2*$^{-/-}$ mice as compared to control mice at an age of 12 weeks (Fig. 2A). To ascertain that increased transcript levels are translated at the protein level, IHC was performed. Whereas TSPO immunoreactivity was hardly detectable in healthy control brain, TSPO was easily visualized throughout the whole *Mfp2*$^{-/-}$ brain (shown for brainstem and thalamus in Fig. 2B).

Upregulated TSPO levels colocalizes predominantly with reactive microglia in *Mfp2*$^{-/-}$ brain

Increased TSPO expression was generally considered to be dedicated to microglia, but recent literature suggests a potential contribution of reactive astrocytes [11, 25, 26]. In order to assess whether either microglia, astrocytes or both contribute to increased TSPO levels in *Mfp2*$^{-/-}$ brain, IHC staining of TSPO protein with cell type-specific markers (Iba1 for microglia and GFAP for astrocytes) was performed. Nearly all Iba1^{+} microglia express TSPO in *Mfp2*$^{-/-}$ brain (Fig. 3B) whereas only few GFAP^{+} astrocytes show TSPO expression (Fig. 3D), indicating that increased TSPO levels are mainly attributed to reactive microglia in the in *Mfp2*$^{-/-}$ brain.

Increased [^{18}F]DPA-714 uptake in *Mfp2*$^{-/-}$ brain: *In vitro* assessment of brain TSPO levels

In order to evaluate whether increased expression of TSPO in *Mfp2*$^{-/-}$ brain could also be detected by using radiolabeled ligands for this protein, *in vitro* autoradiography employing [^{18}F]DPA-714, an established PET tracer for TSPO, was used in a first approach. The results showed significantly higher tracer binding in the *Mfp2*$^{-/-}$ brain as compared to age-matched control brain ($P < 0.0001$; Fig. 3A,B). More specifically, high binding of [^{18}F]DPA-714 was observed in superior colliculus, brainstem, corpus callosum and hippocampus (Fig. 3A). We performed a blocking study using PK11195 (20 µM) as a blocking agent on brain slices of *Mfp2*$^{-/-}$ mice. PK11195 significantly reduced [^{18}F]DPA-714 binding ($P<0.0001$; Fig. 3C,D), demonstrating TSPO-specific binding of [^{18}F]DPA-714.

Increased [^{18}F]DPA-714 uptake in *Mfp2*$^{-/-}$ brain: *Ex vivo* assessment of brain TSPO levels

Ex-vivo autoradiography was used to verify whether the increased TSPO expression observed *in vitro* is sufficient to be detected after intravenous injection of [^{18}F]DPA-714. *Ex-vivo* autoradiography has a 10-fold higher resolution than microPET and is therefore more sensitive to reveal increased tracer concentration in small brain areas. Analysis of the *ex vivo* autoradiography data showed significantly higher tracer binding in the *Mfp2*$^{-/-}$ brain as compared to the age-matched control brain both at 30 min ($P = 0.0017$) and 60 min ($P < 0.0001$) after [^{18}F]DPA-714 injection (Figure 3E-H).

MicroPET: *In vivo* assessment of brain TSPO levels

Allthough the resolution of the microPET scanner (1.4 mm FWHM in the centre) is low relative to the brain size of mice so that partial volume effects may obscure increase of tracer binding in small brain

areas, we explored whether we would be able to detect increased [^{18}F]DPA-714 binding *in vivo* in *Mfp2*$^{-/-}$ mice compared to control mice. Figure 4 shows the time-activity curves (TACs) of the microPET scan of *Mfp2*$^{-/-}$ mice and age-matched control mice. The time activity curve expressed as nCi/cc of [^{18}F]DPA-714 shows slower wash out for *Mfp2*$^{-/-}$ mice compared with that of controls (Fig. 4A) in line with the *ex vivo* autoradiography findings. When the time activity curve is expressed as standard uptake (SUV) value, the initial brain uptake is clearly lower for *Mfp2*$^{-/-}$ mice compared to control mice whereas clearance rate from brain is slower for *Mfp2*$^{-/-}$ mice (Fig. 4B).

DISCUSSION

Microglia can perform a multitude of tasks in the CNS depending on physiological – pathological conditions and adapt their gene expression profile accordingly. Although TSPO has been widely used as a reactive microglia-specific neuroinflammation imaging target [27-29], it is unclear which microglial phenotypes are actually visualized [30]. By using *in vitro* and *in vivo* approaches, we demonstrate that TSPO is upregulated in pro-inflammatory microglia that however not necessarily reside in a destructive brain milieu.

In vitro, microglia can be manipulated and skewed into two extreme states, analogous to macrophages. We show in this study that transcript levels of TSPO only increase in pro-inflammatory (M1) and not in anti-inflammatory (M2) microglial cells. This suggests that TSPO rather marks a harmful inflammatory brain environment, and that TSPO is not induced in a purely neuroprotective or regenerative brain environment. The described M1 - M2 subdivision nicely reflects the Janus-like behavior of microglia regarding their promotion of either tissue injury (M1) or repair (M2), but it is an oversimplified concept and only represents extreme states [31]. Although microglia with either a pro- or anti-inflammatory signature can prevail in defined pathological conditions [32], it is now accepted that microglia *in vivo* mostly adopt mixed pro- and anti-inflammatory phenotypes in neurological disorders [16, 31, 33]. Despite its limitations, M1/ M2 polarization paradigms provide a useful tool to link molecular adaptations such as induced TSPO expression to specific disease phenomena. Future investigation needs to reveal whether the *in vitro* data obtained in this study, correlating increased TSPO levels to pro-inflammatory microglia only, can be extrapolated to *in vivo* conditions. Therefore, TSPO-specific radioligands should be applied to neurological conditions that are clearly associated with either pro- or anti-inflammatory microglia. For example, in a mouse model of ALS it was shown that M2 microglia are abundant in the early phase, whereas M1 microglia occur at end stage of disease [34].

This study also demonstrates that TSPO levels are clearly induced *in vivo* in a mouse model of peroxisomal MFP2 deficiency that develops robust neuroinflammation [16]. The results from IHC staining were confirmed by *in vitro* and *ex vivo* autoradiography with [^{18}F]DPA-714 demonstrating increased TSPO-specific binding in several brain areas of *Mfp2*$^{-/-}$ mice in comparison with control mice. The *in vivo* PET imaging data were more difficult to interpret probably due to the limited resolution of our micro PET scanner, although tracer clearance rate from the brain of *Mfp2*$^{-/-}$ mice was slower

compared to control mice as was clearly demonstrated by both the nCi/cc and SUV curves. This is indicative for a higher target expression in brain tissue of *Mfp2*$^{-/-}$ mice. but a higher tracer metabolism rate cannot be excluded. The difference between nCi/cc and SUV curves could be caused by *Mfp2*$^{-/-}$ mice being generally 25% smaller than control mice [35].

The difference between nCi/cc and SUV curves can be explained by a different brain-to-total-body mass ratio for *Mfp2*$^{-/-}$ vs control mice which influences the SUV values. *Mfp2*$^{-/-}$ mice are generally 25% smaller than control mice [35].

The cellular and molecular characteristics of the inflammatory response in *Mfp2*$^{-/-}$ mice were previously extensively characterized [16]. It is noteworthy that the massive increase in myeloid cells was not due to infiltration of peripheral monocytes. Furthermore, the microenvironment in *Mfp2*$^{-/-}$ brain deviates from the milieu in neurodegenerative disorders as there is neither neuronal loss nor oxidative stress during the entire lifespan of *Mfp2*$^{-/-}$ mice despite the robust neuroinflammatory response [16]. Although the expanded microglia population expressed mixed pro- and anti-inflammatory markers, it should be noted that *Mfp2*$^{-/-}$ microglia do not upregulate neurotoxic and oxidative stress markers such as Nos2, Il6 and Icam1 [15, 16]. Thus, our data reveal that TSPO labels pro-inflammatory microglia that do not have a neuro-destructive character.

In some but not all neuroinflammatory models, TSPO was shown to be upregulated in astrocytes in addition to microglia [6, 11, 36, 37]. In the *Mfp2*$^{-/-}$ brain, only a minority of astrocytes showed elevated TSPO levels, which is in contrast to microglia that virtually all induce TSPO. This supports the hypothesis that transition of resident microglia, and not astrocytes [38], from a resting to an activated phenotype principally underlies the increased TSPO expression.

Taken together, the *in vitro* data showing that TSPO expression is not increased in anti-inflammatory circumstances, supports its use as a marker for pro-inflammatory microglia imaging. However, TSPO upregulation does not distinguish whether microglia develop a neuro-destructive signature or not. An additional caveat regarding TSPO is that a human TSPO polymorphism with a trimodal distribution in binding affinity (high-affinity, low-affinity and mixed affinity binders) was uncovered for several TSPO

ligands [39]. This implicates that knowledge of binding status is needed to correctly quantify TSPO expression using these PET ligands in humans [39]. Also the fact that the particular function of TSPO in mounting the inflammatory response remains obscure, is a drawback for its use as a biomarker.

Therefore, it is highly desirable to identify new targets that allow specific visualization of neurotoxic microglia. Besides other candidates, P2X7 for which versatile radiotracers were already developed might be a good marker [40, 41]. In addition, in view of the therapeutic potential to convert pro-inflammatory into anti-inflammatory microglia [32], there is also a need to selectively detect the latter microglia by *in vivo* imaging in order to assess the therapeutic benefit. Given the ill-defined molecular phenotype of healing microglia [32], this will be a more difficult task.

FIGURE LEGENDS

Figure 1: *Tspo* transcript levels are increased in microglia during pro-inflammatory but not anti-inflammatory signaling.

(A,B) Analysis of transcripts levels by qRT-PCR confirms that LPS-treated (M1) microglia *in vitro* induce expression of typical pro-inflammatory genes such as *Tnfa* (A) and *iNOS* (B). (C) IL4-treated (M2) microglia induce expression of typical anti-inflammatory genes such as *Arg1*. (D) *Tspo* transcript levels are only significantly increased in M1-activated microglia but not in M2-activated microglia. Data are presented as mean ± SEM (n=3/group). *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2: Widespread increased TSPO transcript and protein levels in *Mfp2*$^{-/-}$ versus control brain predominantly localized in *Mfp2*$^{-/-}$ microglia.

(A) qRT-PCR analysis shows induced *Tspo* transcript levels in brain of *Mfp2*$^{-/-}$ mice as compared to age-matched controls at 12 weeks of age. Data are presented as mean ± SEM (n=3/group), **P < 0.01. (B-E) IHC staining shows virtually no immunoreactivity for TSPO (green) in CNS of wild type mice (B,D) but strong upregulation in *Mfp2*$^{-/-}$ brain (C,E), shown for brainstem and thalamus.

(F,G) IHC of TSPO (green) and Iba1 (red) to mark microglial cells. In healthy brain (F) TSPO levels are minimal so co-localization with Iba1^{+} microglia is not visible. In *Mfp2*$^{-/-}$ mice (G) TSPO immunoreactivity co-localizes with nearly all Iba1^{+} microglia (orange). (H,I) IHC of TSPO (green) and GFAP (red) to mark astroglia. Astrocytes of control mice (H) minimally express GFAP and TSPO. In CNS of *Mfp2*$^{-/-}$ mice (I) only few GFAP^{+} reactive astrocytes colocalize with TSPO protein (orange). Representative pictures are shown from inferior colliculus and brainstem regions.

Figure 3: *In vitro* and *ex vivo* autoradiography of brain slices of *Mfp2*$^{-/-}$ and control mice using [^{18}F]DPA-714. (A, B) Sagittal brain sections made of *Mfp2*$^{-/-}$ (A) and age-matched control (B) mice were incubated with [^{18}F]DPA-714. Significantly higher uptake is observed in brain sections of *Mfp2*$^{-/-}$ mice. (C,D) [^{18}F]DPA-714 binding was blocked in the presence of 20 µM PK11195 (D). (E-H) Ex vivo autoradiography of sagittal brain sections of *Mfp2*$^{-/-}$ (E,G) and age-matched control (F,H) mice at 30 (E, F) and 60 (G, H) min after [^{18}F]DPA-714 injection. Significantly higher uptake is observed in brain

sections of *Mfp2*$^{-/-}$ mice as compared to control at both 30 and 60 min after tracer injection ($P < 0.01$; two-tailed, un-paired T-test).

Figure 4: MicroPET in *Mfp2*$^{-/-}$ and control mice using [^{18}F]DPA-714.

(A,B) Time activity curves (TACs) expressed as nCi/cc (A) and standard uptake value (SUV g/ml) (B) as a function of time post tracer injection. n =4-5/group.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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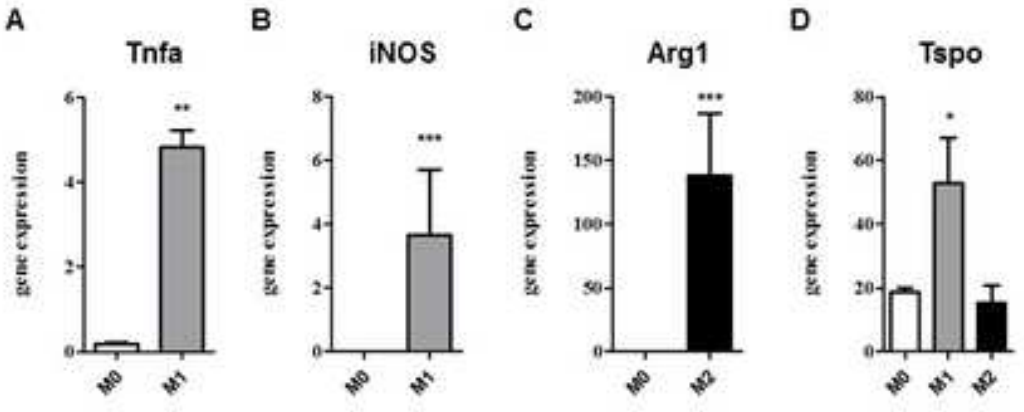

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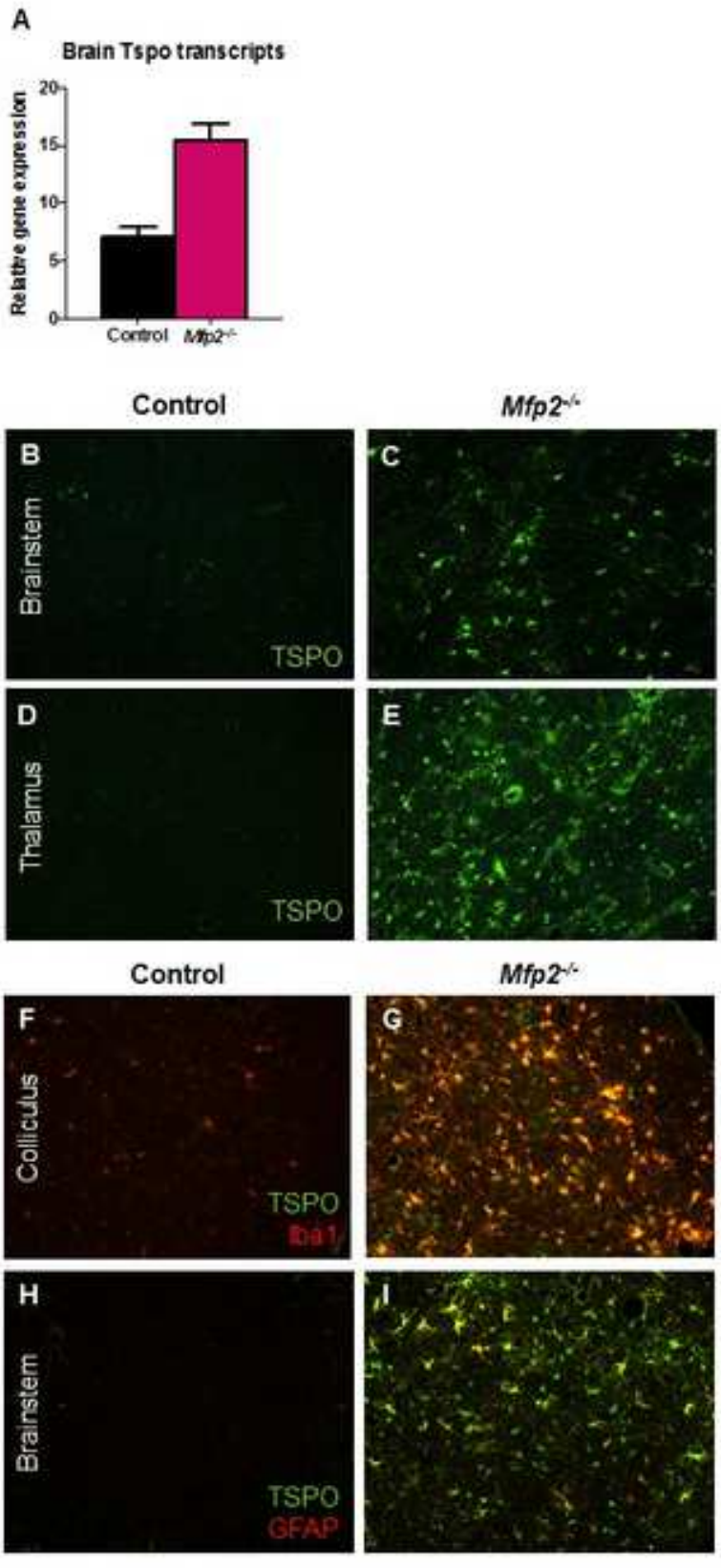

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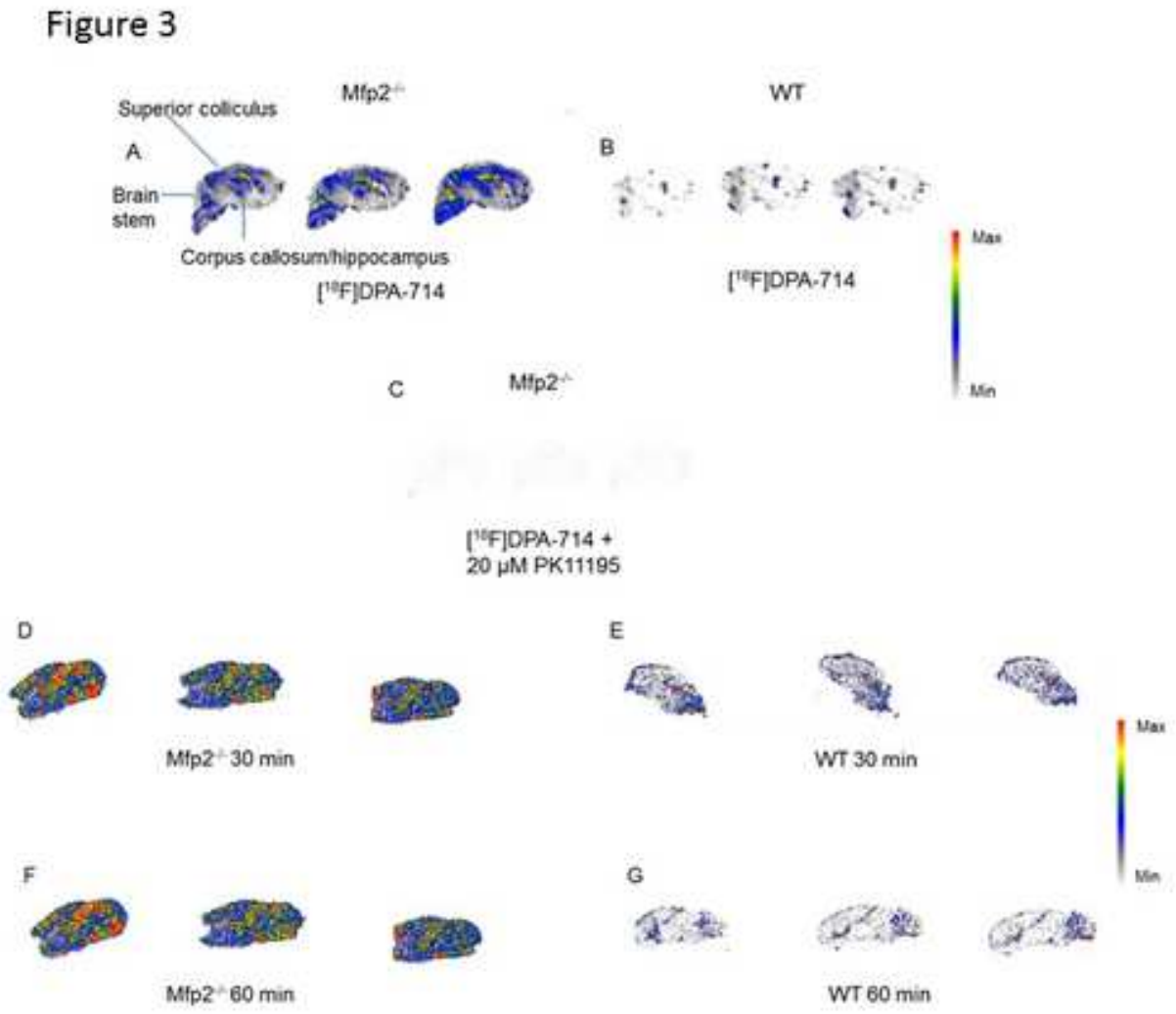

Figure 4

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