Positron emission tomography and functional characterization of a complete PBR/TSCO knockout

Richard B. Banati1,2,3,4, Ryan J. Middleton1, Ronald Chan2,3, Claire R. Hatty2,3, Winnie Wai-Ying Kam1,2,3, Candice Quin2,3, Manuel B. Graeber2,3,5, Arvind Parmar1, David Zahra1, Paul Callaghan1, Sandra Fok2, Nicholas R. Howell1, Marie Gregoire1, Alexander Szabo1,6, Tien Pham1, Emma Davis1 & Guo-Jun Liu1,2,3

The evolutionarily conserved peripheral benzodiazepine receptor (PBR), or 18-kDa translocator protein (TSPO), is thought to be essential for cholesterol transport and steroidogenesis, and thus life. TSPO has been proposed as a biomarker of neuroinflammation and a new drug target in neurological diseases ranging from Alzheimer’s disease to anxiety. Here we show that global C57BL/6-Tspo1m(GuwiyangWurra)-knockout mice are viable with normal growth, lifespan, cholesterol transport, blood pregnenolone concentration, protoporphyrin IX metabolism, fertility and behaviour. However, while the activation of microglia after neuronal injury appears to be unimpaired, microglia from GuwiyangWurra TSPO knockouts produce significantly less ATP, suggesting reduced metabolic activity. Using the isoquinoline PK11195, the ligand originally used for the pharmacological and structural characterization of the PBR/TSPO, and the imidazopyridines CLINDE and PBR111, we demonstrate the utility of GuwiyangWurra TSPO knockouts to provide robust data on drug specificity and selectivity, both in vitro and in vivo, as well as the mechanism of action of putative TSPO-targeting drugs.
According to current concepts, the most important function of the mitochondrial membrane protein peripheral benzodiazepine receptor (PBR)/18-kDa translocator protein (TSPO) is the regulation of steroid hormone production by helping to translocate cholesterol, the precursor to pregnenolone, across the aqueous mitochondrial intermembrane space, a view emphasized by the recent renaming of the PBR as translocator protein (TSPO)\(^1\). A large body of literature\(^2\) on the regulatory influence of the PBR/TSPO on steroid-mediated homeostasis, including the reported embryonic lethal effects of a Tspo knockout\(^5\), has established the PBR/TSPO as essential for life. Its critical role as an endocrine regulator is the prevailing explanation for the observed actions of PBR/TSPO binding drugs across an exceptionally broad therapeutic spectrum from the anti-inflammatory treatment of Alzheimer’s disease\(^6\) to anxiolysis without direct effects on the central GABA\(_\text{A}\) receptor protein complex\(^4\). However, recent observations that a conditional knockout in testicular Leydig cells appeared not to affect hormone production\(^8\) have controversially been interpreted as evidence that the PBR/TSPO, unlike the steroidogenic acute regulatory protein (StAR)\(^9\), is not an essential requirement for steroid hormone biosynthesis\(^10\). No further data indicating other potential impairments have been reported.

An important observation that has underpinned the growing interest related to the PBR/TSPO is the regularly seen increase of the PBR/TSPO in areas of brain injury and during ‘neuroinflammation’, most prominently in activated microglia\(^11\).

Our study provides a first extensive reference description of the constitutive phenotype of a global Tspo knockout animal model \textit{in vitro} and \textit{in vivo}. It addresses some of the fundamental questions, in regard to the long-term viability of animals without any potentially compensating PBR/TSPO-expressing cells, no further data indicating other potential impairments have been reported.

**Figure 1 | Generation and confirmation of global Tspo \(-/-\) mice.** (a) The Tspo gene was knocked out using a targeting construct with loxP sites flanking exons 2 and 3 (also see Supplementary Fig. 1). (b,c) Southern blot analysis (b) and PCR (BV2 mouse microglia were used as the positive control) (c) demonstrated the correct targeting of the Tspo gene. (d,e) Tspo mRNA expression across 13 tissues (triplicates, mean and standard deviation; normalized to Gapdh and Actb) (d) and measurement of TSPO protein by western blot of lysates from the kidney, spleen and testis (e) confirmed the complete absence of any Tspo gene product in Tspo \(-/-\) mice.

---

**Figure 1 | Generation and confirmation of global Tspo \(-/-\) mice.** (a) The Tspo gene was knocked out using a targeting construct with loxP sites flanking exons 2 and 3 (also see Supplementary Fig. 1). (b,c) Southern blot analysis (b) and PCR (BV2 mouse microglia were used as the positive control) (c) demonstrated the correct targeting of the Tspo gene. (d,e) Tspo mRNA expression across 13 tissues (triplicates, mean and standard deviation; normalized to Gapdh and Actb) (d) and measurement of TSPO protein by western blot of lysates from the kidney, spleen and testis (e) confirmed the complete absence of any Tspo gene product in Tspo \(-/-\) mice.

---

**Figure 1 | Generation and confirmation of global Tspo \(-/-\) mice.** (a) The Tspo gene was knocked out using a targeting construct with loxP sites flanking exons 2 and 3 (also see Supplementary Fig. 1). (b,c) Southern blot analysis (b) and PCR (BV2 mouse microglia were used as the positive control) (c) demonstrated the correct targeting of the Tspo gene. (d,e) Tspo mRNA expression across 13 tissues (triplicates, mean and standard deviation; normalized to Gapdh and Actb) (d) and measurement of TSPO protein by western blot of lysates from the kidney, spleen and testis (e) confirmed the complete absence of any Tspo gene product in Tspo \(-/-\) mice.
whether they have altered steroid levels or any other loss of function and whether specificity and selectivity can be assumed for some of the most widely used PBR/TSPO-ligands.

Results
Confirmation of global Tspo\(^{+/−}\) and Tspo\(^{−/−}\) knockout. Removal of exons 2 and 3 of the Tspo gene resulted in viable Tspo\(^{+/−}\) and Tspo\(^{−/−}\) animals. Following the removal of exons 2 and 3 only exons 1 and 4 remain, both of which do not contain any start codons in the TSPO reading frame. Therefore, no TSPO protein, or truncated TSPO protein can be produced (Fig. 1a).

A more detailed illustration of how the loss of exons 2 and 3 and subsequent merger of exon 1 and exon 4 cannot give rise to any functional fragment of the PBR/TSPO but possibly only an unrelated protein with no sequence similarity is shown in Supplementary Fig. 1.

The targeted deletion of Tspo and complete loss of TSPO protein was confirmed by Southern blot, PCR, RT-PCR, RT-qPCR, Western blot, (Fig. 1b–e and Supplementary Fig. 1), specific antibody staining against amino acids 156–169 at the C-terminus of the PBR/TSPO in tissues and macrophages from Tspo\(^{+/+}\), Tspo\(^{+/−}\) and Tspo\(^{−/−}\) mice (Fig. 2), in vivo tracer kinetic PET/CT studies using the PBR/TSPO ligand \([^{18}F]PBR111\)
Figure 3 | No constitutive TSPO ligand binding in Tspo−/− mice. (a) Tspo+/+, Tspo+/− and Tspo−/− mice are identical in external appearance and general behaviour. However, in vivo imaging (8 males of the same age for each genotype) with PET/CT using the radioligand [18F]PBR111, the 18F-labelled analogue to [125I]CLINDE, strikingly illustrates that (apart from occasional signals originating from the excretory pathways, such as gut and urinary bladder) Tspo−/− mice show no ligand binding (thus also demonstrating the selectivity of the used ligand), while both Tspo+/+ and Tspo+/− mice have the characteristic distribution of ligand binding, that is, in organs with known high TSPO expression, notably kidney and adrenal gland (green circle). The images are displayed with the colour scaling and are directly comparable (highest values are white). The time-point frame of the PET images is 15–20 min after injection and scaling is 3.8–17.9% ID per cm3.

(b) The kinetics of [18F]PBR111, and the displacement of the radioligand after injection (indicated by arrows) with cold PBR111 (1 mM) to establish non-specific binding, demonstrates that Tspo−/− mice do not have specific binding of [18F]PBR111 in any organs, while the ligand kinetic in Tspo+/+ and Tspo+/− mice indicates specific binding (ID = injected dose; n = 4 for each genotype; error bars denote standard deviation).

(Fig. 3), receptor-autoradiography and membrane receptor binding (Figs 4 and 5) using [3H]PK11195 (Fig. 6a) and [125I]CLINDE (Fig. 6b).

In addition, PBR/TSPO receptor membrane-binding data as well as extensive receptor autoradiographic validation for all major organs and the whole body of neonatal mice in all three genotypes confirm the absence of the PBR/TSPO protein in the Tspo−/− mice and the high selectivity of [3H]PK11195 in tissues where the PBR/TSPO is present (Fig. 4a,c,e,g,h and Supplementary Fig. 2). Further, we demonstrate in vivo and in vitro the high selectivity of [18F]PBR111 and [125I]CLINDE (Figs 3,4b,d,f and 5), which are thus the first new compounds for the PBR/TSPO validated in animals with a null background of any constitutive, or lesion-induced, specific TSPO binding.

Importantly, we show that in Tspo−/− animals, unlike in the normal wild-type, the microglial cell response in the facial nucleus after peripheral facial nerve lesion is not associated with an increase in the binding of the PBR/TSPO ligands, [3H]PK11195 and [125I]CLINDE. This demonstrates that in pathologic tissue changes the selectivity of [3H]PK11195 and [125I]CLINDE holds true and no additional non-selective binding emerges (Fig. 6a–e). Our data also indicate that the early stage of perineuronal microglial activation, with its typical change in microglial morphology, is not noticeably influenced by the loss of the PBR/TSPO and that the neuro-glial signaling mechanism remains intact (Fig. 6f, g).

We further demonstrate the background-free detection of syngeneic PBR/TSPO-expressing glioma cells growing in the brains of Tspo−/− animals in vitro and in vivo using the selective PBR/TSPO ligands, [3H]PK11195 and [18F]PBR111, as well as antibody staining against the PBR/TSPO. This approach tests simultaneously for the absence (respectively presence) of several recognition or binding domains that make up the full PBR/TSPO, simultaneously for the absence (respectively presence) of several recognition or binding domains that make up the full PBR/TSPO, whereas the Tspo+/+ tumours express the protein as expected (Fig. 7 and Supplementary Fig. 3).

General health and behavioural phenotyping. The observation of over 600 animals did not reveal any overt clinical impairment under standard diet (Supplementary Table 1) and normal housing conditions, nor increase in incidental pathologies in either heterozygous Tspo+/+ or homozygous Tspo−/− animals, both...
**Figure 4 | Comparative receptor autoradiography and membrane binding.** (a–f) The receptor autoradiographs (16 μm sections) show total binding of [³H]PK11195 (1 nM) and [¹²⁵I]CLINDE (3 nM) as well as competitive binding with 10 μM unlabelled CLINDE (CB(CL)), PK11195 (CB(PK)) and PBR111 (CB(PBR)) in the spleens (a,b), kidneys (c,d) and testes (e,f) of Tspo⁺/+ , Tspo⁺/- and Tspo⁻/- mice. Specific binding of 3 nM [¹²⁵I]CLINDE and 1 nM [³H]PK11195 is clearly visible in tissue sections from Tspo⁺/+ and Tspo⁺/- mice and is displaceable by the unlabelled ligands. There is no specific binding in Tspo⁻/- tissue. (g,h) Specific binding using [³H]PK11195 in testicular tissue (g) and kidney tissue (h) (n = 3 for each genotype Tspo⁺/+ , Tspo⁺/- and Tspo⁻/-). Tspo⁻/- mice (kidney (Bmax and Kd): 45,361.0 fmol per mg and 5.94 nM; testes: 3,696.0 fmol per mg and 7.32 nM) have approximately half the binding of Tspo⁺/+ mice (kidney: 108,934.0 fmol per mg and 12.90 nM; testes: 4,920.0 fmol per mg and 2.66 nM) while Tspo⁻/- mice have no appreciable binding. Curves represent non-linear regression of experimentally obtained data points and data are expressed as percentage relative to Tspo⁺/+ specific binding. Error bars denote standard deviation.
phenotype in both sexes remained largely unaffected by the heterozygous or homozygous loss of the Tspo gene, the only exception being a statistically significant trend increase in natural killer (NK) cells in female Tspo−/−− mice compared to female Tspo+/+− mice (Supplementary Table 3). Thus, the Tspo−/−− mice did not reveal the changes in cellular blood composition seen in zebrafish tspo gene silencing experiments15.

The concentrations of protoporphyrin IX (PPIX), thought to be an endogenous PBR/Tspo ligand, in the blood of Tspo+/+, Tspo−/−− and Tspo−−− mice were indistinguishable and increased similarly after treatment with 5-aminolevulinic acid (ALA), the first intermediate in haem biosynthesis and a precursor of PPIX (Fig. 8d), thus indicating normal haem metabolism.

Absence of compensatory transcriptomic regulation. Likewise, quantitative RT-PCR showed that the gene expression profiles for StAR, P450scc and Tspo2 across the major organs known to express the Tspo were similar across genotypes, indicating that the global loss of Tspo had not led to any compensatory transcriptomic regulation of those genes thought to be most closely linked to PBR/Tspo as a regulator of steroidogenesis (Fig. 8e).

Mitochondrial respiration and ATP production in microglia. Measurements of mitochondrial respiration and ATP production in primary cultures of mitochondria-rich microglia from Tspo+/+− and Tspo−−− mice using the Seahorse XF96 Cell Analyzer and selective inhibitors of the electron transport chain revealed that Tspo−−− mitochondria, compared to Tspo+/+−, have a consistently and significantly reduced ATP production as determined by inhibition of complex V (ATP synthase) with oligomycin (Fig. 9).

Discussion

Orthologues of the PBR, or TSP0, are found widely among eubacteria, archaea and eukaryotes2,3. Most members of the PBR/TSP0 protein family contain a specific binding site for the isoquinoline carboxamide PK11195, which has been used to pharmacologically define the PBR/TSP0 and distinguish it from other benzodiazepine-binding receptors, such as the GABA_A receptor protein complex13,16. An evolutionarily younger C-terminal cholesterol recognition amino-acid consensus (CRAC) domain is primarily found in the animal phylum. A parologue of the PBR/TSP0, the TSP02, exists in birds and mammals as the result of a gene duplication, which has retained the CRAC domain but lost the isoquinoline-binding site17.

In light of the evolutionary pressure to conserve the gene, its documented influence on steroidogenesis and the previously observed embryonic lethality caused by the knockout of either the Tspo5 or the endogenous PBR/TSP0 ligand, Acyl-CoA binding protein18, the survival and overtly normal phenotype of a global Tspo−−− knockout would be predicted to be discreet.

Our observation that microglia isolated from Tspo knockout mice have altered oxygen consumption and ATP synthesis rates points...
to a potential latent phenotype that may be elicited under those disease conditions where a significant regulation of the PBR/TSPO is known to occur. This raises the intriguing possibility that the disease course notably in inflammatory pathology of the brain, may be different in the absence of the normally observed, concomitant upregulation of microglial PBR/TSPO. Immune-modulatory functions of the PBR/TSPO have previously been suspected based on pharmacological studies using PBR/TSPO binding compounds that appeared to abrogate inflammatory responses.

Earlier work in bacteria suggests other functions than cholesterol transport for the PBR/TSPO, such as oxygen sensing and thus co-regulation of mitochondrial ATP production, which may become relevant under a range of disease conditions. Possible functional links of the TSPO to the Fo sub-unit of the ATP synthase through the VDAC on the outer and ANT on the inner mitochondrial membrane have been described.

Perceivably, PBR/TSPO-mediated changes in ATP production might explain indirect regulatory effects on the energy-dependent steroid biogenesis, particularly under stress challenges.

Figure 6 | No inducible TSPO ligand binding in Tspo−/− mice. (a,b) TSPO-binding ligands PK11195 and CLINDE/PBR111. (c) Axotomy of the facial nerve induces a retrograde neuronal reaction and highly reproducible microglial activation in the injured facial nucleus. (d) Autoradiography with [3H]PK11195 and [125I]CLINDE and immunohistochemical staining of the microglial activation marker CD11b on consecutive brain sections confirmed the previously reported localized induction of TSPO ligand binding in the injured facial nucleus contemporaneous to the activation of microglia of Tspo+/+ animals. (e) In contrast, no binding of [3H]PK11195 and [125I]CLINDE could be induced in Tspo−/− mice despite the undiminished presence of activated microglia in the injured facial nucleus, thus providing evidence that the high selectivity of [3H]PK11195 and [125I]CLINDE for their respective binding sites on the TSPO is retained in pathologically changed tissue. (f) Immunofluorescent anti-CD11b staining of activated microglia in the injured facial nucleus of Tspo−/− mice revealed no obvious differences in microglial activation, with its characteristic localization of activated, perineuronal microglia, here shown as higher magnification of the above section. Scale bars in (d,e) and the left image of (f) denote 1 mm, and that in the right image of (f) and in (g) 100 μm. The asterixes in (g) and (f) indicates the soma of neurons.
However, this alternative explanation of a functional association of the PBR/TSPO with steroid biogenesis necessitates re-examination of a substantial body of published research to separate direct from indirect or from non-selective, off-target effects. Many studies use compounds with purported selectivity for the PBR/TSPO for diagnostic or therapeutic purposes, such as those modulating complex behaviour in the apparent absence of binding to neural receptor systems\(^7\). Our study paradigmatically demonstrated for a long-established as well as a recently developed PBR/TSPO compound, that is, PK11195 and CLINDE/PBR111, respectively, that the Guwiyang Wurra Tspo\(^{-/-}\) strain is a powerful tool to discern selective from non-selective off-target binding and thus validate the purported drug actions in live animals under naturalistic conditions. We also anticipate innovative lipid membrane studies into the biophysical mechanisms by which ligands for the PBR/TSPO may either modify or introduce additional, potentially non-receptor, actions in the presence or absence of the protein\(^{1,27,28}\).

Lastly, the apparent irrelevance of the PBR/TSPO for the normal activation of microglia after neuronal injury indicates that neuro-glial interactions and inflammatory tissue responses are separate processes, though in vivo neuroimaging studies on both may regularly show high levels of PBR/TSPO expression\(^{13,14}\). The current concepts of ‘neuroinflammation’ do not adequately distinguish these and, therefore, too, merit revisiting.

The extensively characterized Guwiyang Wurra Tspo\(^{-/-}\) strain provides the broader research community with a means of dissecting the network of pathways through which the PBR/TSPO exerts its regulatory influence at the intersection of cell metabolism, innate immune response and endocrine regulation of behaviour in health and disease.

### Methods

#### Generation of Tspo\(^{-/-}\) mice

Tspo knockout mice were generated using a targeting construct that contained loxP sites flanking exons 2 and 3, and a neomycin cassette inserted between exons 3 and 4. The targeting construct was electroporated into C57BL/6 Bruce4 embryonic stem (ES) cells and cells correctly targeted by homologous recombination were injected into albino C57BL/6 blastocysts. Male chimeras were mated to female albino C57BL/6 mice and the resulting offspring with a black coat were screened for the presence of a targeted Tspo allele.

Mice positive for the presence of the targeted allele were crossed with C57BL/6 Cre deleter mice to remove the neomycin cassette and exons 2 and 3 to create heterozygous global Tspo deficient mice. To remove the Cre transgene, animals were bred to wild-type C57BL/6 mice. To produce animals for experiments, heterozygous mice were crossed to generate wild-type littermate controls. All animal procedures were approved by the University of Sydney Animal Ethics Committee and the ANSTO Animal Care and Ethics Committee. The Tspo\(^{-/-}\) mouse strain has been given the additional designation Guwiyang Wurra (‘fire mouse’ in the local Dharawal language). Future naming will thus be C57BL/6-Tspo\(^{+/+}\)Guwmu(Guwiyang Wurra).

Mice were genotyped by Southern blot analysis using genomic DNA isolated from tail biopsies. For routine genotyping, genomic DNA was isolated from tail biopsies, ear biopsies or stool samples and amplified by PCR to generate a 489-bp product for the wild-type allele and a 246-bp product for the knockout allele. See Supplementary Table 4 for primer sequences. The PCR consisted of an initial incubation at 95 °C for 2 min, followed by 4 cycles at 95 °C for 30 s, 68 °C for 30 s and 72 °C for 2 min, then 4 cycles at 95 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min, then 30 cycles at 95 °C for 30 s, 62 °C for 30 s and 72 °C for 2 min, and a final step at 72 °C for 5 min.

#### Immunoblotting

Lysates (20 μg protein) were mixed with 2× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), heated to 70 °C for 10 min and resolved on a 4–20% Mini-PROTEAN TGX gel (Bio-Rad). MagicMark XP (Life Technologies, Carlsbad, CA, USA) was used as the molecular weight marker. Proteins were transferred to a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad). The membrane was incubated with anti-TSPO antibody (#109497, Abcam, Cambridge, UK) diluted 1:10,000 or anti-GAPDH (#37168, Abcam) diluted 1:1,000 and peroxidase-conjugated anti-rabbit antibody (#A0545, Sigma-Aldrich, St Louis, MO, USA) diluted 1:10,000. Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA) was used and the membrane visualized using an ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

#### RT-PCR and quantitative real-time PCR

Tissue samples were homogenised in TRizol (Life Technologies) and total RNA isolated using the PureLink RNA mini kit (Life Technologies) with on-column DNase treatment following the manufacturer’s instructions. Purified RNA (1 μg) was reverse-transcribed to cDNA using the SuperScript III First-Strand Synthesis kit (Life Technologies). For RT-PCR, cDNA from the kidneys of Tspo\(^{+/+}\) and Tspo\(^{-/-}\) mice was amplified using

---

**Figure 7 | PBR/TSPO-expressing brain tumour in global Tspo\(^{-/-}\) mouse brain.** (a) [\(^{18}\)F]PBR111 PET/CT demonstrates a high-contrast signal confined exclusively to the Tspo\(^{+/+}\) brain tumour, while Tspo\(^{-/-}\) surrounding brain shows no ligand binding. (b,c) For the same animal shown in (a), in vitro autoradiography with [\(^{3}\)H]PK11195 (b) and subsequent immunohistochemical staining with anti-TSPO antibody on the same brain sections (c) confirm that PBR/TSPO ligand binding is strictly limited to the brain tumour region and no PBR/TSPO binding or recognition sites are present in Tspo\(^{-/-}\) tissue. Scale bars, 1 mm. The PET images show the time frame from 5 to 10 min after injection and scaling is 0–5.3% ID per cm\(^3\) for (a).
primes (Supplementary Table 4) located in exons 1 and 4. The PCR consisted of an initial incubation at 95 °C for 2 min, followed by 34 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final step at 72 °C for 5 min. The PCR products were confirmed by sequencing. For quantitative real-time PCR, cDNA was added to 2.5 µL of SsoFast EvaGreen Supermix (Bio-Rad) and 0.5 µL of each primer (Supplementary Table 4) in a final reaction volume of 5 µL. Reactions were performed on a CFX 384 Real-Time PCR detection System (Bio-Rad) by cycling at 98 °C for 30 s, followed by 45 cycles at 98 °C for 5 s and 63 °C (61 °C for the Tspo2 assay) for 10 s. A melt curve analysis was performed to confirm the specificity of each reaction. Each sample was run in duplicate. The Tspo2 primers were designed using Primer-BLAST and the specificity confirmed by DNA sequencing.

**Protoporphyrin IX.** Blood from euthanized mice was placed into heparin tubes on ice, plasma removed, cells washed twice in ice-cold phosphate-buffered saline (PBS) and then resuspended in PBS to the original volume. Samples were evenly split (300 µL each), one receiving 1 mM 5-ALA (Bio-Rad) and the other sterile H2O, and incubated in a shaking incubator at 37 °C for 4 h. Ethyl acetate/acetic acid (4:1) was added to each sample, then mixed and centrifuged before transferring the supernatant (1 ml) to a new tube with 1 ml of 1.5 M HCl. An aliquot of the lower HCl phase, after mixing and centrifuging the sample, was measured in a spectrophotometer (excitation wavelength 407 nm, slit width 10 nm, emission spectrum 450–800 nm in 1 nm increments). Glass tubes and containers were used throughout and samples were protected from light.

**Facial nerve anatomy.** Facial nerve anatomy25,30 was performed on Tspo+/+ and Tspo−/− mice (n > 4 per genotype) anaesthetized with 5% (v/v) isoflurane. A small incision (0.5–0.7 cm) in the skin 0.5 cm dorsal and lateral to the ear was made and the facial nerve transected under an operating microscope. The incision was closed with Vetbond Tissue Adhesive (3M, St Paul, MN, USA). The success of the operation was confirmed by the loss of ipsilateral whisker movement. Mice were monitored carefully and euthanized with CO2 3 days after the operation. Brains were dissected, placed in OCT, transferred to liquid nitrogen and stored at −80 °C until cryo-sectioning.

**Stereotactic implantation of GL261 mouse glioblastoma cells.** The Tspo+/+ (n = 6) and Tspo−/− (n = 4) mice were operated under isofluorane anaesthesia and aseptic conditions using a Kopf stereotactic apparatus and precision dental drill (Kopf Instruments, Tujunga, CA, USA). A small burr hole was made 3 mm anterior of the bregma and 2 mm lateral over the right frontal lobe (3 mm ventral). GL261 glioblastoma cells (NCI, Bethesda, MD, USA) at 3 × 10^3 µL in a volume of 10 µL were slowly injected over 2 min using a 10-µL Hamilton syringe connected to a pump by means of a 32-gauge needle. The animals were closely monitored daily for good recovery, including body weight and absence of neurological signs. Three and four weeks after implantation of the GL261 cells, the animals were imaged by means of microPET/CT and brains were dissected for histological studies and autoradiography.

**PET and CT imaging.** Mice, anaesthetized (5% (v/v) with isofluorane and maintained at 1–2%, were scanned using a small-animal Inveon PET/CT scanner (Siemens, Knoxville, TN, USA) according to the methods described previously31,32. Body temperature was maintained with a feedback regulated heating pad and...
Further extensive hematological and behavioural data are presented in Supplementary Tables 2 and 3.

After 40 min of imaging, PBR111 (1 mg per kg in 2% acetic acid–saline) was injected. The activity of HRP was detected with the peroxidase 3,3′-diaminobenzidine tetrahydrochloride liquid substrate system (Sigma-Aldrich). Sections were dehydrated with ethanol, incubated with xylene, and slides were mounted in DPX mounting media with a coverslip. Sections were visualized under an inverted Olympus BX51 microscope (Olympus, Tokyo, Japan), and captured with a Q-imaging camera and ImagePro 5.1 program.

A Zeiss LSM 710 confocal microscope with a 5 × EC Plan-Neofluar NA 0.16 objective was used for the fluorescence microscopy of the facial nucleus. Alexa Fluor 568 was excited with a 561-nm laser, using a 488/561/633 dichroic mirror, and emission captured from 565 to 640 nm.

For immunocytochemistry, peritoneal macrophages were isolated according to Zhang et al.35. After 2 days in complete medium the purity of macrophages was > 97% as confirmed with the macrophage-specific marker IB4 conjugated with FITC (Sigma-Aldrich). Cells on coverslips were fixed and incubated using the same method as for tissue sections, with the addition of a mouse anti-human/mouse mitochondrial electron transport chain complex IV antibody (#AB14705, Abcam), then incubated with the secondary antibodies Alexa Fluor (AF) 594-conjugated goat anti-rabbit antibody (Life Technologies) and AF488-conjugated goat anti-mouse antibody (Life Technologies). Cells on coverslips were mounted on microscope slides with ProLong Gold antifade reagent containing DAPI (Life Technologies) and viewed under a BX61WI Olympus microscope. Images were acquired with a digital camera (CoolSNAP, Photometrics, Tucson, AZ, USA) and the Image InVivo program (Photometrics). Deconvolution of images was performed with the AutoDeblur program (Photometrics) and further processed with ImageJ (NIH, Baltimore, MD, USA).

**Figure 9** Decreased metabolic activity in Tspo−/− microglia. (a–c) The basal, that is, mitochondrial and non-mitochondrial, oxygen consumption rate (OCR) is consistently and significantly lower in Tspo−/− microglia compared to Tspo+/+ microglia (Tspo−/−: 49.0 ± 16.3 pmol per min; n = 95; Tspo+/+: 81.0 ± 21.8 pmol per min, n = 154; Student’s t-test), while OCR after application of the ATPase inhibitor oligomycin (3 μM) is reduced to similar levels in both genotypes (n = 11–14 per group) (a). Thus, the oligomycin-inhibitable mitochondrial ATP production in Tspo−/− microglia is significantly lower than in Tspo+/+ microglia (P < 0.01; Student’s t-test) (c). Basal mitochondrial OCRs in Tspo−/− and Tspo+/+ microglia after inhibition of the electron transport chain complexes I and III with 10 μM rotenone + 10 μM antimycin A are reduced by similar amounts (c) and to similar levels (b) (n = 11–14 per group). (d) Metabolic activity is decreased in Tspo−/− microglia. Basal OCR vs ECAR (extracellular acidification rate) shows that both OCR and ECAR in Tspo−/− microglia (n = 95) are lower than those in Tspo+/+ microglia (n = 154). Error bars denote standard deviation for (a–d). Further extensive hematological and behavioural data are presented in Supplementary Tables 2 and 3.

**Autoradiography.** Tissue sections from Tspo+/+ and Tspo−/− mice were incubated at room temperature for 20 min in 170 mM Tris-HCl pH 7.4 containing 1 nM [3H]PK11195 (specific activity 84 Ci per mmol; Perkin Elmer, Waltham, MA, USA), washed twice for 5 min in 170 mM Tris-HCl, rinsed with 3 dips in ice-cold MiliQ H2O, and dried. Additional sections were incubated on ice for 1 h in 50 mM Tris-HCl pH 7.4 containing 3 nM [125I]CLINDE (specific activity 100 Ci per mmol; synthesized by ANSTO Life Sciences), washed twice for 2 min in ice-cold 50 mM Tris-HCl, for 1 min in ice-cold MiliQ H2O, and dried. Displacement binding was carried out in the presence of PK11195 (10 μM), CLINDE (10 μM) and PBR111 (10 μM). Single-emulsion films were exposed to sections and standards (14C or 3H) for 3 h ([3H]PK11195) or 10 weeks ([3H]PK11195).

**Immunohisto- and cytochemistry.** Immunohistochemistry was performed as previously described14,36. Tissue sections (used for autoradiography) were incubated with Tspo monoclonal antibody (#109497, Abcam) at 4 °C overnight and secondary HRP-conjugated goat anti-rabbit antibody at RT for 1 h (#A0545, Sigma-Aldrich). The activity of HRP was detected with the peroxidase 3,3′-diaminobenzidine tetrahydrochloride liquid substrate system (Sigma-Aldrich). Sections were dehydrated with ethanol, incubated with xylene, and slides were mounted in DPX mounting media with a coverslip. Sections were visualized under an inverted Olympus BX51 microscope (Olympus, Tokyo, Japan), and captured with a Q-imaging camera and ImagePro 5.1 program.

**Primary microglial cell culture.** Microglia were cultured from 0–2-day-old Tspo+/+ and Tspo−/− mice according to methods described previously35. Briefly, whole brains were dissected, cut into fine pieces and treated with 0.025% trypsin (Sigma-Aldrich). They were cultured in Dulbecco’s modified Eagle’s medium (DMEM/F12; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 1% penicillin-streptomycin-glutamine (Sigma-Aldrich) and 0.5 ng per ml GM-CSF (Abcam).
Microglia were purified by shaking at 350 revolutions per minute (r.p.m.) for 30 min at 37°C, pelleted by centrifugation, resuspended in supplemented DMEM/ F12 and placed (4 × 10^4 cells per well) into a 96 well Seahorse XF cell culture plate (Seahorse Bioscience, North Billerica, MA, USA) pre-coated with FBS for 15 min at room temperature. After 15 min the wells were washed twice to remove unattached cells. The purity of microglial cultures was >98% as confirmed by staining with the microglial marker Alexa Fluor-conjugated isoclitin GS-IB4 (Life Technologies). The 5 × 10^4 microglia from wild-type and knockout mice were plated into each well of the XF96 plate and were grown for 2–3 days before evaluating mitochondrial functions with the Seahorse XF 96 Cell Analyzer (Seahorse Bioscience). To rule out systematic differences in cell density and viability between Typo−/− and Typo+/+, cell density was monitored and assured throughout the experimental measurements, while viability, including apoptosis, was confirmed using caspase 3/7 (ref. 36).

**Measurements of mitochondrial respiration.** The oxygen consumption rate (OCR) and extracellular acidification rate in primary microglial cultures were determined using the Seahorse XF 96 Cell Analyzer. OCR is an indicator of mitochondrial respiration37,38. The day before the experiment 200 μl of the Seahorse XF Calibrant (pH 7.4) was added to each well of the XF 96-well utility plates. The sensor cartridge was placed on top of the plate and hydrated for 16 h at 37°C in a non-CO2 incubator. The protocol for basal measurements of the cells at 37°C (start, calibrate probes, equilibrate, mix 2 min, 1 min delay, measure 3 min) was repeated twice. Mitochondrial stress compounds (oligomycin, rotenone combined with antimycin A) were then injected, followed by 2 min mixing, 1 min delay, measure 3 min repeated (3 times).

On the day of the experiment, microglia (plating density 5 × 10^4 per well, purity >98% and viability >92% for both wild-type and knockout microglia) were washed 3 times. Mitochondrial stress compounds (oligomycin, rotenone combined with antimycin A) were used to determine mitochondrial ATP production and basal mitochondrial OCR36,37,38. Oligomycin and the combination of rotenone and antimycin A were injected at concentrations of 0.3, 1 and 3 μM (optimal for oligomycin) and 0.1 μM (optimal for rotenone/antimycin A).

The data for each reported parameter reflect 11–14 independent Seahorse XF measurement series from a total of seven independently repeated microglial cell culture preparations.

**Radioligand binding.** Organs were homogenized using a T25 digital Ultra-Turrax homogenizer (Ika, Wilmington, NC, USA) at speed setting 5 and 20,000 r.p.m. in ~45 ml of ice-cold TRIS buffer (pH 7.4) and washed twice in ice-cold 50 mM TRIS-HCl (pH 7.4) by centrifugation at 48,000 g. In 50 volumes of ice-cold 50 mM TRIS-HCl, and protein concentration was determined using a BCA protein assay kit (Thermo Scientific). Bmax and Kd were determined with saturation binding by incubating membrane protein (60 μl) with [3H]PK11195 (0.56–20 nM) on ice for 90 min before harvesting by rapid filtration through Whatman GF/C filters (GE Healthcare) presoaked in a 0.5% polyethylenimine (0.56–20 nM) on ice for 90 min before harvesting by rapid filtration through Whatman GF/C filters (GE Healthcare) presoaked in a 0.5% polyethylenimine solution. Non-specific binding was determined with 5 μM PK11195. Filters were placed in scintillation cocktail (Perkin Elmer) for 12 h and radioactivity was determined using a Tri-Carb 2100 TR Liquid Scintillation Counter (Perkin Elmer). Bmax and Kd values were obtained by non-linear regression using GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA, USA) by fitting total and non-specific binding.

**Cholesterol transport and biosynthesis.** P450bct metabolism was measured in mouse testis according to Tuckey et al.39 and Slominski et al.40. Briefly, an enriched mitochondrial fraction was prepared by centrifugation in 250 mM sucrose, 50 mM Tris (pH 7.4), and the enzymatic conversion of cholesterol to pregnenolone was conducted under the following conditions out to a maximum 2-h time-point: 50 mM HEPES pH 7.4, 250 mM sucrose, 20 mM KC1, 5 mM MgSO4, 0.2 mM EDTA, 1 mg per ml BSA, 8 μM Trilostane, 0.05 μM 4–hydroxycholesterol, 500 mM isocitrate and 5 mM NaNaDPP. Reactions were terminated with the addition of 4°C dichloromethane. The organic phase was retained and the aqueous phase was re-extracted twice more into dichloromethane. Extracts were combined, dried under nitrogen and dissolved in 100 μl of ethyl acetate. Pregnenolone and cholesterol were separated by thin-layer chromatography, with hexane/diethyltheretonic acid (15:15:1) mobile phase, and the amount of radioactivity in each spot quantified by liquid scintillation counting.

**Detection of serum pregnenolone.** Serum pregnenolone of male and female Typo+/+ and Typo−/− mice by cutting each cauda with a scalpel and incubating in 150 μl of pre-warmed Biggers–Whitten–Whittington (BWW) medium for 5 min at 37°C. After the incubation, an additional 250 μl of BWW medium was added, the tissue removed and the serum assayed using a computer-assisted serum analysis system (Hamilton Thorne, Beverly, MA, USA). Parameters assayed were total motile, progressive motile, VAP, VSL and VCL.

**Assessment of anxiety-related behaviours.** Behavioural tests were performed on mice aged between 3–4 months, handled regularly to reduce the impact of handling stress during testing. Anxiety-related behaviours were examined with the following: open-field test, emergence test, light/dark preference test and elevated plus maze. In each test the mice were allowed free exploration in the apparatus for 15 min. The duration and frequency of entering each compartment, total distance travelled, time spent active and the number of stool droppings were assessed. In the elevated plus maze risk assessment was defined as when an animal’s head was facing toward an open arm, open arm with more than 15% of its body in an open arm while maintaining its centre of mass within the closed arm or central platform. The genotype of the animals was blind to the experimenter during testing. Tests were carried out at least 7 days apart to reduce the effects of training history41. All experiments were recorded with an overhead camera on to DVD and later analysed using Motman Tracker 4.3 software (Motion Mensura, Cooks Hill, NSW, Australia).

**Data analysis.** Statistical significance was calculated using an unpaired two-tailed Student’s t-test for two-group comparisons. For comparisons of more than two groups univariate analysis of variance (ANOVA) with a Bonferroni’s post-hoc test was performed and considered significant at P<0.05. Data are presented as mean ± standard deviation, except where indicated otherwise.

**References**

1. Liu, G. J. et al. The 18kDa translocator protein, microglia and neuroinflammation. *Brain Pathol* 24, 631–653 (2006).

2. Papadopoulos, V. et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol. Sci.* 27, 402–409 (2006).

3. Gavish, M. et al. Enigma of the peripheral benzodiazepine receptor. *Pharmacol. Rev.* 51, 629–650 (1999).

4. Répondrecht, R. et al. Translocator protein (18kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. *Nat. Rev. Drug Discov.* 9, 971–988 (2010).

5. Papadopoulos, V. et al. Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids* 62, 21–28 (1997).

6. Barron, A. M. et al. Lignand for translocator protein reverses pathology in a remnant model of Alzheimer’s disease. *J. Neurosci.* 33, 8891–8897 (2013).

7. Rupprecht, R. et al. Translocator protein (18kDa) as target for anxiolytics without benzodiazepine-like side effects. *Science* 325, 490–493 (2009).
8. Morohaku, K. et al. A mammalian mitochondrial drug receptor is not required for steroid hormone biosynthesis. *Endocrinology* **155**, 89–97 (2014).
9. Stocco, D. M., Wang, X., Jo, Y. & Manna, P. R. Multiple signalling pathways regulating steriodogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. *Mol. Endocrinol.* **19**, 2647–2659 (2005).
10. Miller, W. L. Steroid hormone synthesis in mitochondria. *Mol. Cell. Endocrinol.* **379**, 62–73 (2013).
11. Papadopoulos, V. On the role of the translocator protein (18-kDa) Tspo in steroid hormone biosynthesis. *Endocrinology* **155**, 15–20 (2014).
12. Stocco, D. M. The role of PBR/Tspo in steroid biosynthesis challenged. *Endocrinology* **155**, 6–9 (2014).
13. Banati, R. B. et al. The peripheral benzodiazepine binding site in the brain in multiple sclerosis: quantitative in vivo imaging of microglia as a measure of disease activity. *Brain* **132**, 2321–2337 (2000).
14. Cagnin, A. et al. In vivo measurement of activated microglia in dementia. *Lancet* **358**, 461–467 (2001).
15. Rampon, C. et al. Translocator protein (18 kDa) is involved in primitive erythropoiesis in zebrafish. *FASEB J.* **23**, 4181–4192 (2009).
16. Le Fur, G. et al. Peripheral benzodiazepine binding sites: effect of PK 11195, I-(2-chlorophenyl)-N-methyl-(1-methylpropyl)-3 isoquinolinecarboxamide. II. In vivo studies. *Life Sci.* **32**, 1849–1856 (1983).
17. Fan, J., Rone, M. B. & Papadopoulos, V. Translocator protein 2 is involved in cholesterol redistribution during erythropoiesis. *J. Biol. Chem.* **284**, 30484–30497 (2009).
18. Landrock, D. et al. Acyl-CoA binding protein gene ablation induces pre-implantation embryonic lethality in mice. *Lipids* **45**, 567–580 (2010).
19. Domazet-Loso, T. & Tautz, D. An ancient evolutionary origin of genes associated with human genetic diseases. *Mol. Biol. Evol.* **25**, 2699–2707 (2008).
20. Barabasi, A. L., Gulbahce, N. & Loscalzo, J. Network medicine: a network-based approach to human disease. *Nature Rev. Genet.* **12**, 56–68 (2011).
21. Choi, H. B. et al. Inhibition of lipopolysaccharide-induced cytokine/oxidase-2, tumor necrosis factor-alpha and [Ca2+] i responses in human microglia by the peripheral benzodiazepine receptor ligand PK11195. *J. Neurochem.* **83**, 546–555 (2002).
22. Daugherty, D. J. et al. A TSPO ligand is protective in a mouse model of multiple sclerosis. *EMBO Mol. Med.* **5**, 891–903 (2013).
23. Rufli, M. R. et al. Benzodiazepine receptor-mediated chemotaxis of human monocytes. *Science* **229**, 1281–1283 (1985).
24. Yeliseev, A. A., Krueger, K. E. & Kaplan, S. A mammalian mitochondrial drug receptor functions as a bacterial ‘oxygen’ sensor. *Proc. Natl Acad. Sci. USA* **94**, 5101–5106 (1997).
25. Veenman, L. Potential involvement of F0F1-ATP(synth)ase and reactive oxygen species in apoptosis induction by the antineoplastic agent etrycophosphomoholcholine in glioblastoma cell lines: a mechanism for induction of apoptosis via the 18 kDa mitochondrial translocator protein. *Aptosis* **15**, 753–768 (2010).
26. Krestinina, O. V. et al. Effect of peripheral benzodiazepine receptor (PBR/TSP0) ligands on opening of Ca2+ – induced pore and phosphorylation of 3.5-kDa polypeptide in rat brain mitochondria. *Biochemistry (Mosc.)* **74**, 421–429 (2009).
27. Hatty, C. R. et al. Investigating the interactions of the 18 kDa translocator protein and its ligand PK11195 in planar lipid bilayers. *Biochim. Biophys. Acta* **1838**, 1019–1030 (2014).
28. Parisio, G., Sperotto, M. M. & Ferrarini, A. Flip-flop of steroids in phospholipid bilayers: effects of the chemical structure on transbilayer diffusion. *J. Am. Chem. Soc.* **134**, 12198–12208 (2012).
29. Banati, R. B., Myers, R. & Kreutzberg, G. W. PK (‘peripheral benzodiazepine’)– binding sites in the CNS indicate early and discrete brain lesions: microautoradiographic detection of [3H]PK11195 binding to activated microglia. *J. Neurocytol.* **26**, 77–82 (1997).
30. Mori, J. B. & Graeber, M. B. The facial nerve axotomy model. *Brain Res. Brain Res. Rev.* **44**, 154–178 (2004).
31. Disselhorst, J. A. et al. Image-quality assessment for several positron emitters using the NEMA NU-4/2008 standards in the Siemens Inveon small-animal PET scanner. *J. Nucl. Med.* **51**, 610–617 (2010).
32. Mattner, F. et al. Central nervous system expression and PET imaging of the translocator protein for relapsing-remitting autoimmune encephalomyelitis. *J. Nucl. Med.* **54**, 291–298 (2013).
33. Liu, G. J., Nagarajah, R., Banati, R. B. & Bennett, M. R. Glutamate induces directed chemotaxis of microglia. *Eur. J. Neurosci.* **29**, 1108–1118 (2009).
34. Graebner, M. B., Streit, W. J. & Kreutzberg, G. W. Axotomy of the rat facial-nerve leads to increased Cr3+ complement receptor expression by activated microglial cells. *J. Neurosci.* **21**, 18–24 (1998).
35. Zhang, X., Goncalves, R. & Mosser, D. M. The isolation and characterization of murine macrophages. *Current protocols in immunology* Chapter 14 (ed. Coligan, J. E. et al.) Unit 14 11 (John Wiley & Sons, Inc, 2008).