Peripheral Benzodiazepine Receptor/Translocator Protein Global Knock-out Mice Are Viable with No Effects on Steroid Hormone Biosynthesis**

Lan N. Tu 1, Kanako Morohaku 2, Pulak R. Manna 3, Susanne H. Pelton 4, W. Ronald Butler 4, Douglas M. Stocco 4, and Vimal Selvaraj 5

From the 6Department of Animal Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, New York 14853 and the 7Department of Cell Biology and Biochemistry, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Background: Translocator protein (TSPO) has been considered a mitochondrial cholesterol transporter critical for steroid hormone production. TSPO knock-out mice were reported to be embryonic lethal.

Results: TSPO knock-out mice are viable with no effects on steroidogenesis.

Conclusion: TSPO is not essential for steroidogenesis and is not necessary for sustaining life.

Significance: This study rectifies a serious inaccuracy in the current understanding that is critical for treating steroid hormone disorders.

Translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor, is a mitochondrial outer membrane protein implicated as essential for cholesterol import to the inner mitochondrial membrane, the rate-limiting step in steroid hormone biosynthesis. Previous research on TSPO was based entirely on in vitro experiments, and its critical role was reinforced by an early report that claimed TSPO knock-out mice were embryonic lethal. In a previous publication, we examined Leydig cell-specific TSPO conditional knock-out mice that suggested TSPO was not required for testosterone production in vivo. This raised controversy and several questions regarding TSPO function. To examine the definitive role of TSPO in steroidogenesis and embryonic development, we generated global TSPO null (Tspo−/−) mice. Contrary to the early report, Tspo−/− mice survived with no apparent phenotypic abnormalities and were fertile. Examination of adrenal and gonadal steroidogenesis showed no defects in Tspo−/− mice. Adrenal transcriptome comparison of gene expression profiles showed that genes involved in steroid hormone biosynthesis (Star, Cyp11a1, and Hsd3b1) were unchanged in Tspo−/− mice. Adrenocortical ultrastructure illustrated no morphological alterations in Tspo−/− mice. In an attempt to correlate our in vivo findings to previously used in vitro models, we also determined that siRNA knockdown or the absence of TSPO in different mouse and human steroidogenic cell lines had no effect on steroidogenesis. These findings directly refute the dogma that TSPO is indispensable for steroid hormone biosynthesis and viability. By amending the current model, this study advances our understanding of steroidogenesis with broad implications in biology and medicine.

The peripheral benzodiazepine receptor (PBR) was first identified as a protein with distinct pharmacology and high binding affinity to benzodiazepines in "peripheral" sites, as opposed to "central" sites found in brain tissue (1–4). The central benzodiazepine receptor is the γ-aminobutyric acid type A (GABA A ) receptor that has specific function in inhibitory neurotransmission (5, 6). However, the function for the PBR has remained a topic of debate for decades (7, 8), despite its prominence as a therapeutic target in a variety of diseases/disorders (9–12).

Genetic sequence comparisons show that the PBR gene is highly conserved from archaea to metazoans (13). In mammals, highly prominent PBR protein expression has been identified in steroidogenic tissues (14–16). Binding sites for PBR ligands have also been identified in other tissues including heart, brain, adrenal, kidney, salivary gland, platelets, brown adipose tissue, skin, and liver (7). In subcellular fractions, binding sites for the PBR were identified to be present in the outer mitochondrial membrane (OMM) (14, 17).

The transport of cholesterol from intracellular stores and the OMM to the inner mitochondrial membrane (IMM) forms the first and rate-limiting step in steroid hormone biosynthesis (18, 19). Cholesterol is then converted to the first steroid, pregnenolone, by CYP11A1 (cytochrome P450 side chain cleavage), an enzyme located on the matrix side of the IMM (20, 21). The initial link between PBR and steroid hormone biosynthesis emerged by studying the effect of a PBR-binding chemical PK11195 that triggered steroidogenesis in the Y1 mouse adrenal tumor cell line (22). In agreement, targeted disruption of *This work was supported by funds from Cornell College of Agriculture and Life Sciences (startup funds to V. S.), Robert A. Welch Foundation Grant B1-0028 (to D. M. S.), a fellowship from the Vietnam Education Foundation (to L. N. T.), and National Institutes of Health Grant HD-17481 (to D. M. S.).

1 To whom correspondence should be addressed: Dept. of Animal Science, 204 Morrison Hall, Cornell University, Ithaca, NY 14853. Tel.: 607-255-6138; Fax: 607-255-9829; E-mail: vs88@cornell.edu.

2 The abbreviations used are: PBR, peripheral benzodiazepine receptor; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; TSPO, translocator protein; Bt2cAMP, dibutyryl cyclic AMP.
PBR in the R2C rat Leydig tumor cell line inhibited steroidogenesis (23).

The structure of murine PBR showed a five α-helix fold, each forming a transmembrane segment (24), and the possibility of a channel-like structure with an interior surface for potential substrate translocation (25). Cholesterol binding to PBR was defined by the identification of a cholesterol recognition amino acid consensus sequence at the C-terminal region (26). These structural features combined with functional considerations led to the modeling of PBR as a membrane-associated cholesterol transport protein (9, 27). Based on these findings, to more accurately represent its subcellular role, PBR was renamed as translocator protein (TSPO) (28).

Independent of studies on PBR/TSPO, another mitochondrial protein that is expressed in rapid response to steroidogenic stimuli, the steroidogenic acute regulatory protein (STAR), was identified in the MA-10 Leydig tumor cell line (29). Induced expression of STAR resulted in increased steroid hormone production even in the absence of hormonal stimulation (29). Anchoring STAR in a mitochondrion-affixed TOM20-STAR fusion protein in MA-10 cells resulted in the constitutive production of steroid hormone (30). In humans, STAR mutations cause lipid congenital adrenal hyperplasia that ranges from an almost complete inability to synthesize steroids (31) to less severe forms that retain partial STAR protein activity (32). Also in support, Star gene-deleted mice showed an almost complete inability to synthesize steroid hormones underscoring its critical role in steroidogenesis (33).

Two key experiments appeared to cement a functional link between STAR and PBR/TSPO. First, inhibition of protein synthesis that blocks STAR production and steroidogenesis (34) did not affect the ability of PK11195 to trigger steroidogenesis (35). Second, knockdown of PBR/TSPO in mitochondrion-affixed TOM20-STAR fusion protein expressing MA-10 cells resulted in a reduction in their sterogenic capacity (36). Thus, the PBR function was linked to transport of cholesterol from the OMM to the IMM (37).

In a recent study, we questioned this model showing that PBR/TSPO expression is not required for in vivo testicular steroidogenesis (38). This result has indeed raised a lot of questions and controversies regarding the current understanding of the precise PBR/TSPO-mediated functions in steroid hormone production (39). Moving forward, there remained an absolute need to examine specific deficits in TSPO global knock-out mice to gain additional functional understanding of this protein (40).

In a review article published in 1997, it was claimed that efforts to generate a Pbr/Tspo gene-deleted model failed as a result of early embryonic mortality in Pbr/Tspo-deficient mice (41). This led to the conclusion that in addition to steroidogenesis, TSPO is involved in basic functions that are critically necessary for embryonic development. However, this review article (41) failed to explain experimental methods covering the recombination strategy used and the exact stage of embryonic mortality; these specific methods/results were never published. Given the current gap in understanding PBR/TSPO function in vivo, we decided to revisit this global knock-out question.

With the primary objective of studying the role of PBR/TSPO in steroidogenesis and other vital functions (if any) that may be critical for embryonic development, we used a germ cell specific knock-out approach to generate Pbr/Tspo global gene-deleted mice. Our results are unexpected and contrary to the prevailing view in that we find PBR/TSPO knock-out mice are viable and fertile with no effects on steroid hormone biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Generation of Tspo<sup>−/−</sup> Mice**—Mice with floxed alleles for Pbr/Tspo with loxP sites flanking exons 2 and 3 (Tspo<sup>β/β</sup>) were generated and validated as described previously (38). In this study, Tspo<sup>β/β</sup> female mice were crossed with Ddx4-cre+ (FVB-Tg(Ddx4-cre)1Ddxac/J) (42)) males to generate a germ cell-specific deletion of PBR/TSPO (TspocΔ+/+) in both male and female mice. TspocΔ+/+ Ddx4cre+ positive offspring were then back-crossed to generate TspocΔ/Δ mice of both sexes. These sperm- and oocyte-specific knock-outs were bred to generate global PBR/TSPO null (Tspo<sup>−/−</sup>) offspring in the C57BL/6 background. We also observed that oocyte-specific TspocΔ/Δ Ddx4-cre+ mice induced global recombination directly as described previously for the Ddx4-cre+ strain of mice (42). Cre was subsequently bred out from Tspo<sup>−/−</sup> mice (Fig. 1). Breeding colonies for Tspo<sup>−/−</sup> and Tspo<sup>β/β</sup> mice were maintained separately, and offspring were genotyped for all experiments (Fig. 1) using primers that have been previously validated (38). ROSA26-tdTomato (R26-tdTom) reporter females (B6.Cg-Gt(Rosa)26Sor<sup>tm14(CAG-tdTomato)Hze</sup> (43)) were used to confirm recombination and bred to generate Tspo<sup>β/β</sup>-R26-tdTom homozygous mice to directly mark PBR/TSPO deletion. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of Cornell University approved all experiments in this work.

**Phenotypic Analysis**—Growth rate of Tspo<sup>β/β</sup> and Tspo<sup>−/−</sup> mice were examined from postnatal day 0 to week 5. Litter sizes from homozygous crosses in Tspo<sup>β/β</sup> and Tspo<sup>−/−</sup> mouse colonies were examined in at least eight unique pairings. Recombination was examined in different tissues by both PCR of genomic DNA using specific primers, as validated previously (38), and tdTomato reporter expression indicating cre-mediated recombination, as described previously (38).

**Histology**—Tissues were processed for hematoxylin and eosin staining as described (38). Immunohistochemistry for PBR/TSPO was performed using a rabbit monoclonal anti-TSPO antibody (Abcam) that specifically recognizes amino acids 156–169 at the C terminus of exon 4 as described (16, 38). For examining neutral lipid deposits, 9-μm frozen sections were prepared, and lipid staining was performed using Oil Red O (Matheson Coleman & Bell) in 60% isopropyl alcohol for 30 min. Fluorescent tdTomato was also visualized in 9-μm frozen sections in multiple tissues. Images were acquired using either a DFC365FX or an ICC50HD camera (Leica) or a Meta 510 confocal scope (Zeiss). Density of Oil Red O staining density was quantified using ImageJ (44).

**Immunoblots**—Proteins were separated by SDS-PAGE and immunoblotted using rabbit monoclonal primary antibodies against TSPO (Abcam), isocitrate dehydrogenase 2 (Abcam),...
PBR/TSPO Is Not Essential for Steroidogenesis

and a rabbit polyclonal STAR antibody (45). Each primary antibody was multiplexed with the loading control ACTB (Li-Cor). Simultaneous detection was performed using IRDye 700 and 800 under a laser fluorescence scanner (Li-Cor) and quantified as described previously (38). For immunoblots performed in knockdown experiments, TSPO polyclonal (Cell Signaling) and ACTB (Invitrogen) primary antibodies were used. Specific band intensities in knock-down experiments were calculated by image analysis using Quantity One software (Bio-Rad).

Hormone Assays—Plasma was collected from 8- to 10-week-old Tspo^{fl/fl} and Tspo^{−/−} mice. Levels of different steroid hormones were measured using radioimmunoassay as follows: corticosterone, aldosterone, and testosterone (Siemens), estradiol and progesterone (Serono Maia), as described previously (38, 46). For adrenal/corticosterone stimulation test, 500 ng/g body weight of adrenocorticotropin (ACTH) fragment 1−24 (Sigma) was administered subcutaneously, and plasma was collected after 1 h. For the testis/testosterone stimulation test, 10 IU of human chorionic gonadotropin (EMD Biosciences) was administered intraperitoneally to male mice, and plasma was collected after 1 h.

Transmission Electron Microscopy—Adrenal glands were fixed in Karnovsky’s fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.08 M phosphate buffer) overnight. After fixation, tissues were processed for transmission electron microscopy as described previously (12). Stained sections were examined in a Tecnai™ T12 Spirit electron microscope (FEI), at 120 kV. High magnification images were acquired using a high resolution CCD camera Megaview III (Olympus Soft Imaging System) to examine cellular and mitochondrial morphology within regions in the adrenal cortex.

RT-PCR and Quantitative PCR—Total RNA was extracted from adrenal glands of 8–10-week-old Tspo^{fl/fl} and Tspo^{−/−} mice.

![FIGURE 1. Generation of TSPO knock-out mice. A, schematic showing genomic Tspo locus in wild type (Tspo^{+/+}), intact floxed (Tspo^{fl/fl}), and knock-out (Tspo^{−/−}) mice. Tspo^{fl/fl} mice contained exons 2 and 3 flanked with LoxP sites (open arrowheads). Tspo^{fl/fl} female mice were bred with Ddx4-cre male mice to generate germ cell-specific deletion of TSPO as heterozygotes. These sperm and oocyte-specific knock-out mice were bred to generate Tspo^{−/−} offspring. Ddx4-cre transgene was subsequently bred out from this colony. B, panel showing genotyping for floxed alleles and cre transgene in Ddx4-cre, Tspo^{fl/fl}, Tspo^{fl/fl}/Ddx4-cre mice, Tspo^{−/−}/Ddx4-cre mice, Tspo^{−/−}, and Tspo^{−/−}/Ddx4-cre mice. C, genomic DNA PCR for recombination at the Tspo locus detects deletion of exons 2 and 3 in Tspo^{−/−} mice (product sizes: Flox-2697 bp, Null-872 bp, and Con-161 bp).](image1)

![FIGURE 2. Validation of Tspo^{−/−} genotype. A, Tspo^{−/−}—ROSA26-tomato reporter mice were used to examine cre-mediated recombination induced in offspring from germ cell-specific deletions. Global recombination was clear in R26-tdTom-Tspo^{−/−} P3 pups. B, Tspo monoclonal antibody specific for peptide corresponding to TSPO exon 4 detected a protein of expected size (18 kDa) in Tspo^{fl/fl} tissues but not in Tspo^{−/−} tissues. ACTB and mitochondrial isocitrate dehydrogenase 2 were used as controls. Scale bar, 1 cm. C, representative images of different tissues from an R26-tdTom-Tspo^{−/−} mouse with no tdTomato fluorescence in contrast to tissues from an R26-tdTom-Tspo^{−/−} mouse showing tdTomato fluorescence (red) indicating recombination. Nuclei were counterstained with DAPI (blue). Scale bar, 50 μm.](image2)
TABLE 1
Litter sizes from Tspos/n and Tspos/~ male and female mice

| Mating pairs                  | n | Litter size* ± S.E. |
|-------------------------------|---|---------------------|
| Tspos/~ female × Tspos/~ male | 17 | 8.11 ± 0.44         |
| Tspos/~ female × Tspos/~ male | 8  | 8.00 ± 0.53         |

* No significant differences in litter size were detected between the two crosses.
Sex ratios in these litters were also different.

FIGURE 3. TSPO deletion does not affect gonadal steroidogenesis. A, testes sections showing TSPO localization in Leydig and Sertoli cells in Tspos/~ but not in Tspo/+ testis. Functional morphology of seminiferous tubules was not affected in Tspos/~ testis. B, ovary sections showing TSPO localization in interstitial cells and surface epithelium, weak in theca and granulosa cells in Tspos/~ ovary; no staining was observed in Tspos/~ ovary. Functional morphology was not affected in Tspos/~ ovary. C, base-line plasma testosterone levels were not different between Tspos/~ and Tspos/~ male mice (n = 24–25/group). D, increase in plasma testosterone levels after human chorionic gonadotropin stimulation was similar between Tspos/~ and Tspos/~ male mice (n = 6–7/group). E, base-line plasma estradiol levels were significantly higher in Tspos/~ compared with Tspos/~ (p < 0.05), but the difference was modest (n = 12–15/group). F, base-line plasma progesterone levels were not different between Tspos/~ and Tspos/~ mice (n = 12–15/group). Scale bar, 200 μm.

Adrenal Transcriptome Shotgun Sequencing—Adrenal gland total RNA from Tspos/~ and Tspos/~ mice were extracted, and poly(A) RNA were isolated using oligo(dT)25 Dynabeads (Invitrogen). Fragmentation and strand-specific RNA-seq library preparation was performed as described (49). Each sample with unique bar codes was pooled to one lane for obtaining short single-reads in a HiSeq 2000 sequencer (Illumina). RNA-seq reads were first aligned to the ribosomal RNA gene database (50) using Bowtie (51), and the mapped reads were discarded. The resulting filtered reads were aligned to the mouse genome (Ensembl) using TopHat (52). Subsequent to alignments, raw counts for each mouse gene were derived and normalized to reads per kb of exon model per million mapped reads. Differentially expressed genes were identified using the DESeq package (53). Raw p values of multiple tests were corrected using false discovery rate (54).

FIGURE 4. TSPO deletion has no effect on morphology and lipid deposits in the adrenal cortex. A, adrenal sections from Tspos/~ and Tspos/~ mice showing TSPO localization in adrenocortical cells with a higher density in the zona glomerulosa; no staining was observed in Tspos/~ adrenal. No difference in adrenocortical morphology was apparent between two genotypes. B, Oil Red O staining of adrenal glands showed no difference in neutral lipid deposits between Tspos/~ and Tspos/~ adrenals. C, quantification of Oil Red O (ORO) labeling density was similar in both Tspos/~ and Tspos/~ adrenal cortex (n = 5/group; mean ± S.E.). Scale bar, 200 μm.

TSPO Knockdown in Different Steroidogenic Cell Lines—Specific small interfering RNAs (siRNAs) were used to knock down endogenous TSPO expression in different steroidogenic cell lines as described previously for other genes (55). Tspos siRNAs and the Silencer® negative control (scrambled) were obtained as annealed oligonucleotides (Invitrogen). Sense and anti-sense strand sequences for TSPO siRNAs utilized were as follows: 1) 5’-GGAAAGAGCCCGGGAGGUGC-3’; 2) 5’-GCGUCUCAUGUCUGGAAUtt-3’; 3) 5’-UAGUCUGGAAUtt-3’; and 3) 5’-UGGCGCCUGCUAGCUGUGCATT-3’. Cell lines MA-10, Y-1, H295R, R2C, and MLTC cells (ATCC) were transfected with 100 nM Tspos siRNA–3 or control using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were examined for TSPO protein expression.
and used to estimate steroidogenic potential with 0.5 mM dibutyryl cyclic AMP (Bt2cAMP) as described previously (55).

RESULTS

Ts\(^{po-/-}\) Mice Are Viable and Fertile—The Ts\(^{po+/?}\) locus allows cre-mediated deletion of exons 2 and 3 of the Ts\(^{po}\) gene (Fig. 1A). Exon 1 does not contain any start codons. Removal of exons 2 and 3 eliminates the start codon, and exon 4 does not have any in-frame start codons. Therefore, deletion of exons 2 and 3 will completely eliminate TSPO protein expression (38). All germ cell-specific Dex4-cre mice were used to induce germ cell Ts\(^{po}\) deletion in F1 and global recombination in F2 generations. Litters from all crosses were genotyped, and the Ddx4-cre deletion in F1 and global recombination in F2 generations. Ts\(^{po}Tspo\) germ cell-specific and 3 will completely eliminate TSPO protein expression (38). Therefore, deletion of exons 2 and 3 eliminates the start codon, and exon 4 does not have any in-frame start codons. Therefore, deletion of exons 2 and 3 will completely eliminate TSPO protein expression (38). All germ cell-specific Dex4-cre mice were used to induce germ cell Ts\(^{po}\) deletion in F1 and global recombination in F2 generations. Litters from all crosses were genotyped, and the Ddx4-cre deletion in F1 and global recombination in F2 generations.

FIGURE 5. TSPO deletion has no effect on adrenal steroid hormone production. A, plasma aldosterone base-line levels were not significantly different between Ts\(^{po+/?}\) and Ts\(^{po-/-}\) mice (n = 7/group). B, plasma corticosterone base-line levels were not significantly different between Ts\(^{po+/?}\) and Ts\(^{po-/-}\) mice (n = 24–30/group). C, increase in plasma corticosterone in response to ACTH stimulation was similar between Ts\(^{po+/?}\) and Ts\(^{po-/-}\) mice (n = 10–14/group). D, representative Western blot showing no compensatory increase in STAR and TSPO protein expression after ACTH stimulation in both Ts\(^{po+/?}\) and Ts\(^{po-/-}\) mice. Quantification of STAR (E) and TSPO (F) protein levels also showed no significant increase at 1 h after ACTH stimulation (n = 3). A.U., arbitrary units. Data are represented as mean ± S.E.

TABLE 2

Genes differentially expressed between Ts\(^{po+/?}\) and Ts\(^{po-/-}\) adrenal glands

| Genes | Gene name/description | Ts\(^{po+/?}\) (mean) | Ts\(^{po-/-}\) (mean) | Ratio | \(p\) value |
|-------|-----------------------|-----------------------|-----------------------|-------|------------|
| BCO18473 | Unclassified noncoding RNA gene | 0.01 | 1.86 | 186.33 | 1.77E-13 |
| Trim12a | Tripartite motif-containing 12A | 1.97 | 0.01 | 0.01 | 1.19E-12 |
| Gmi12196 | Predicted gene 12196 | 0.26 | 40.59 | 156.12 | 5.94E-12 |
| Pydc4 | Pyrin domain containing 4 | 1.63 | 0.02 | 0.02 | 7.36E-10 |
| Gmi4955 | Predicted gene 4955 | 3.25 | 0.07 | 0.02 | 9.45E-09 |
| Gmi16510 | Predicted pseudogene 16510 | 1.19 | 23.5 | 19.81 | 5.13E-06 |
| Gmi5540 | Predicted pseudogene 5540 | 0.71 | 19.88 | 25.41 | 0.0018 |
| Prrc2a | Prolin-rich coiled-coil 2A | 1.16 | 20.69 | 17.78 | 0.0084 |
| Rap33-81C12.1 | Known lincRNA | 0.93 | 22.69 | 24.49 | 0.0084 |
| Atxn2l | Ataxin 2-like | 1.5 | 16.44 | 14.99 | 0.0147 |
| Abhd1 | Anhydrase domain containing 1 | 1.34 | 10.53 | 7.84 | 0.0157 |
| Ankr52d | Ankyrin repeat domain 52 | 0.35 | 5.62 | 15.91 | 0.0166 |
| Nse2p | Neurexophilin and PC-esterase domain family, member 2 | 0.8 | 0.12 | 0.15 | 0.0173 |
| Trm117B | Transmembrane protein 178 | 16.36 | 3.04 | 0.19 | 0.0188 |
| Eps1 | Epsin 1 | 0.8 | 9.97 | 12.47 | 0.0320 |
| Zbkb7 | Zinc finger and BTR domain containing 7B | 0.38 | 5.51 | 14.64 | 0.0346 |
| Osg1 | Oxidative stress induced growth inhibitor 1 | 2.46 | 19.15 | 7.79 | 0.0346 |
| Cyp21a2-ps | Cytochrome P450, family 21, subfamily a, polypeptide 2 pseudogene | 159.94 | 1352.39 | 8.5 | 0.0346 |

\(^{*}\) Data are presented in the order of significance based on \(p\) value.
Gonadal Steroid Hormone Production Is Not Affected in TsPo−/− Mice—The lack of TSPO did not affect the functional morphology of the testis (Fig. 3A) or the ovary (Fig. 3B) in TsPo−/− mice compared with TsPo+/+ controls. In male mice, examination of plasma testosterone concentration showed that base-line levels were not different between TsPo−/− and TsPo+/+ mice (Fig. 3C). The increase in plasma testosterone in response to human chorionic gonadotropin in vivo was not different between TsPo−/− and TsPo+/+ mice (Fig. 3D). Cauda epididymal sperm counts were not different between TsPo−/− and TsPo+/+ mice (data not shown). In female mice, examination of plasma estradiol and progesterone concentration showed that TSPO deletion did not decrease base-line values (Fig. 3, E and F). In actuality, TsPo−/− mice showed modest but significantly higher estradiol levels compared to TsPo+/+ cohorts. We did not observe any abnormalities in gonad size, reproductive behavior, and fecundity in TsPo−/− mice (data not shown).

Adrenal Steroid Hormone Production Is Not Affected in TsPo−/− Mice—The lack of TSPO expression did not affect the functional morphology of the adrenal cortex (Fig. 4A) in TsPo−/− mice. Quantitation of neutral lipid staining in the adrenal cortex did not indicate any major steroid transport defects in TsPo−/− mice (Fig. 4, B and C). Base-line levels of aldosterone (Fig. 5A) and corticosterone (Fig. 5B), two major hormones produced by the adrenal cortex, were not different between TsPo−/− and TsPo+/+ mice. An increase in plasma corticosterone in response to ACTH in vivo was not different between TsPo−/− and TsPo+/+ mice (Fig. 5C). There was also no evidence for compensatory increase in STAR expression in adrenals of TsPo−/− mice compared with TsPo+/+ mice after ACTH treatment (Fig. 5, D–F).

Loss of TSPO Does Not Affect Steroidogenic Gene Expression in Adrenal Glands—Comparison of gene expression profiles by transcriptome shotgun sequencing of TsPo−/− and TsPo+/+ adrenals revealed only a short list of differentially expressed genes (Table 2), none of which were directly associated with steroidogenesis. Mining reads representing TsPo mRNA confirmed deletion of exon 2 and exon 3. A majority of the differentially expressed genes was involved in immune activity. Validated expression of genes involved in the steroid hormone biosynthetic pathway, Star, Cyp11a1, and Hsd3b1 (Fig. 6, A–C), and genes encoding TSPO-interacting proteins as follows: Vdac1, Ant, Hsx2, Pap7, and Acpb, were not affected in TsPo−/− adrenals (Fig. 6, E–L). Complete dataset is available through NCBI-SRA SRP043599. To evaluate possible functional redundancy, we also examined expression levels for TsPo2, a TsPo paralog (56). We did not detect TsPo2 expression in both TsPo+/+ and TsPo−/− adrenal glands (Fig. 6D).

Ultrastructure of Adrenal Cortex Remains the Same in TsPo−/− Mice—Examination of steroidogenic cells of the adrenal cortex using transmission electron microscopy did not show any aberrations in cellular ultrastructure in TsPo−/− mice (Fig. 7). Despite its presence in the OMM, TSPO deficiency did not affect mitochondrial morphology. Lipid droplet distribu-
PBR/TSCO Is Not Essential for Steroidogenesis

FIGURE 7. Ultrastructure of cells in the adrenal cortex is not affected by TSCO deletion. Transmission electron micrographs showing cell morphology in the zona glomerulosa (A) and zona reticularis (B) of the adrenal cortex. Subcellular organelle morphology, including mitochondria and lipid droplets, is identical between both Tspo+/+ and Tspo−/− mice.

TSPO Knockdown in Steroidogenic Cell Lines Does Not Affect Steroidogenesis—Progesterone production after Bt2cAMP treatment by the different murine (MA-10, Y1, and MLTC) and human (H295R) steroidogenic cell lines was not adversely affected by knockdown of TSPO expression (Fig. 8, A–D). TSPO protein expression in murine cell lines decreased by approximately 85, 75, and 82%, respectively; surprisingly, base-line TSPO protein expression was not detectable in human H295R cells (confirmed by extremely low levels of Tspo mRNA (Fig. 8, F and G)). This observation showed that H295R cells are capable of making steroid hormones without TSPO. In all conditions, TSPO knockdown showed a modest but significant decrease in progesterone production in conditions with and without Bt2cAMP treatment compared with scrambled controls (Fig. 8E). Mono-allelic Tspo gene deletion in R2C cells was reported to abolish TSPO expression and inhibit steroidogenesis (23). We find this hard to reconcile, as we do not find any evidence for allelespecific TSPO expression in our in vivo studies (Fig. 9). Moreover, R2C cells are pathologically constitutive in steroid hormone production, the mechanism of regulation of which is not physiologically relevant. Data from TSPO knockdown experiments in three different murine cell lines together with the discovery that TSPO expression is below the detection levels in H295R cells confirm that TSPO is not essential for steroidogenesis in vitro.

DISCUSSION

According to existing literature, TSPO function is absolutely essential for steroid hormone biosynthesis (23), and global Tspo gene deletion results in early embryonic lethality in mice (41). In direct contrast to those observations, our experimental findings disprove these functional arguments and definitively demonstrate that TSPO is not essential for steroidogenesis and that Tspo−/− mice are viable and fertile. These concrete results form a key step toward rectifying the prevailing model of steroidogenesis and underscore that the precise role of TSPO in mammalian physiology has yet to be uncovered.

In a previous study, we demonstrated that conditional Tspo gene deletion in testicular Leydig cells did not affect testosterone production (38). At that time, the finding was met with skepticism that Amhr2 cre-mediated recombination may not have complete penetrance in Leydig cells and criticism that it may not be the case for all steroid-synthesizing cells (39). From observations in this study in which Tspo has been deleted globally, it is clear that TSPO is not involved in physiologically relevant steroid hormone biosynthesis. This calls into question the previously suggested functional cooperation between STAR and TSPO for mitochondrial cholesterol import (57).

It could be argued that Tspo−/− mice respond to the loss of TSPO with some unknown compensatory mechanism. If this is the case, it is surprising that redundant mechanisms were not evident in earlier studies that claimed an indispensable role for TSPO in the steroidogenic machinery (23). Tspo2, a paralog of TSPO (35% homology) and a likely candidate for functional compensation, is restricted to the adult bone marrow and is not expressed in steroidogenic tissues (56), negating such a possibility. Moreover, there is no experimental evidence that multiple mechanisms are involved in mitochondrial cholesterol import. Based on observations in STAR knock-out mice, it appears unmistakable that there is one rate-limiting mechanism of mitochondrial cholesterol import that is governed by...
STAR (33). Therefore, a model for TSPO in mitochondrial cholesterol transport is highly unlikely.

Given these results, it is unclear how the global TSPO knock-out reported in an earlier study by Papadopoulos et al. (41) could be embryonic lethal. As exact experimental details for that study were not published, we can only speculate that an error occurred in genome manipulation during this previous attempt. Similarly, a study from this same research group reported that more than 70% TSPO knockdown in cell lines resulted in cell death (58). This is certainly not the case in both our in vivo and in vitro experimental observations and indicates that experimental conditions used in these earlier studies may have not been optimal. This conclusion is supported by another recent study that independently generated conditional liver- and heart-specific Tspo/H11002/H11002 mice and did not report any incidence of cell death after abolishing TSPO expression; TSPO was found not to be involved in mitochondrial permeability transition and cell death (59).

At base-line physiology, gene expression differences between Tspofl/fl and Tspo/H11002/H11002 adrenals were surprisingly few. A majority of these genes are involved in immune modulation as follows: Tmem178 is a negative regulator of inflammatory cytokine production (60); Prrc2a is a gene within the major histocompatibility complex class III region and is involved in the inflammatory process (61); Osgin1 encodes an oxidative stress response protein that regulates inflammation (62); Zbtb7b is a regulator of T cell-mediated tumor-induced immunity (63); Pydc4 is part of a PYHIN domain family of proteins involved in innate immunity (64); and Trim12a is a putative antiviral gene (65). Two genes were involved in vesicular transport and lipid sequestration as follows: Epn1 is an endocytic adaptor involved in clathrin-mediated endocytosis (66), and Abca2 encodes an endoly-
PBR/TSPO Is Not Essential for Steroidogenesis

sosomal protein with a role in intracellular lipid trafficking (67). Three genes expressed were of unknown function: Ankrd52, Abhd1, and Atxsn2. The remaining were two noncoding RNA genes, two predicted genes, and three pseudogenes. Careful consideration of these differentially expressed genes and the phenotype observed in Tspo−/− mice indicates that TSPO is not essential for sustaining physiological function.

TSPO binding chemicals have been shown to increase steroid hormone production in isolated mitochondria from steroidogenic cells (35), MA-10 cells (37), and even live rats (68). Given our current findings, we can offer two possible explanations for these pharmacological effects. With ligand binding, TSPO may undergo a conformational change (as suggested previously (24, 69)), resulting in the release of bound cholesterol that subsequently becomes available to mitochondrial transport systems and steroidogenesis. Alternatively, the effect of pharmacological agents could be due to drug-membrane interactions resulting in TSPO-independent biological effects (70).

It has also been discovered that TSPO ligands like PK11195 can insert themselves into lipid bilayers affecting membrane properties (71), something that can modulate cholesterol availability to the steroidogenic machinery and also the nature of TSPO-ligand interactions. Nevertheless, as TSPO is considered a therapeutic target for numerous inflammatory conditions (9–12), these properties of TSPO ligands may be important to assess specificity and side effects.

The sexual disparity in body weight differences in that only female Tspo−/− mice showed subtle but higher growth weights than their Tspofl/fl cohorts suggests a hormone-dependent metabolic mechanism (72). But the overall lack of phenotype in Tspo−/− female mice indicates that TSPO is not essential for sustaining physiological function.

In summary, this study provides irrefutable evidence that PBR/TSPO is not essential for steroid hormone biosynthesis and that Tspo−/− mice are viable and fertile. These findings form a critical step toward understanding TSPO function and will help rebuild a defined model for steroid hormone biosynthesis. In addition, this understanding also forces reevaluation of interpretations made for TSPO function across multiple fields, including neuroscience, immunology, and oncology.

Acknowledgments—We thank Dr. Jim Giovannoni and Dr. Zhangjun Fei at the Boyce Thompson Institute for assistance in RNA-seq and data analysis; John Grazul at Cornell Center for Materials Research (supported by National Science Foundation Grant DMR-112-296); University of California at Davis Diagnostic and Research Electron Microscopy Laboratory for assistance in transmission electron microscopy; Dr. Johanna Dela Cruz at the Cornell University Biotechnology Resource Center for assistance in whole animal imaging and Dr. Mario Ascoli for MA-10 cells.

Note Added in Proof—In the original version of the manuscript that was published in JBC Papers in Press, the ACTB subpanels used to demonstrate equal protein loading in panels A, B, and E of Fig. 8 were incorrect. The correct ACTB images from the blots used to generate the original TSPO and STAR data in these panels are now provided in the revised Fig. 8. This correction does not change the interpretation of the results or the conclusions.

REFERENCES

1. Braestrup, C., Albrechtsen, R., and Squires, R. F. (1977) High densities of benzodiazepine receptors in human cortical areas. Nature 269, 702–704
2. Braestrup, C., and Squires, R. F. (1977) Specific benzodiazepine receptors in rat brain characterized by high-affinity [3H]diazepam binding. Proc. Natl. Acad. Sci. U.S.A. 74, 3805–3809
3. Regan, J. W., Yamamura, H. I., Yamada, S., and Roskes, W. R. (1981) High affinity [3H]diazepam binding: characterization, localization, and alteration in hypertension. Life Sci. 28, 991–998
4. Davies, L. P., and Huston, V. (1981) Peripheral benzodiazepine-binding sites in heart and their interaction with diprydamole. Eur. J. Pharmacol. 73, 209–211
5. Takahashi, H., Nagashima, A., and Koshino, C. (1958) Effect of γ-aminobutyric acid on the electrical activity of the cerebral cortex. Nature 182, 1443–1444
6. Macdonald, R., and Barker, J. L. (1978) Benzodiazepines specifically modulate GABA-mediated postsynaptic inhibition in cultured mammalian neurones. Nature 271, 563–564
7. Gavish, M., Bachman, I., Shoukrun, R., Katz, Y., Veenman, L., Weisinger, G., and Weizman, A. (1999) Enigma of the peripheral benzodiazepine receptor. Pharmacol. Rev. 51, 629–650
8. Gatiloff, J., and Campanella, M. (2012) The 18 kDa translocator protein (TSPO): a new perspective in mitochondrial biology. Curr. Mol. Med. 12, 356–368
9. Rupprecht, R., Papadopoulos, V., Rames, G., Baghai, T. C., Fan, J., Akula, N., Groyer, G., Adams, D., and Schumacher, M. (2010) Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat. Rev. Drug Discov. 9, 971–988
10. Qi, X., Xu, J., Wang, F., and Xiao, J. (2012) Translocator protein (18 kDa): a promising therapeutic target and diagnostic tool for cardiovascular diseases. Oxid. Med. Cell. Longev. 2012, 162934
11. Vlodavsky, E., Palzur, E., and Soustiel, J. F. (2014) 18 kDa translocator protein as a potential therapeutic target for traumatic brain injury. CNS Neurol. Disord. Drug Targets 12, 1–10
12. Daugherty, D. J., Selvaraj, V., Chechenova, O. V., Liu, X. B., Pleasure, D. E., and Deng, W. (2013) A TSPO ligand is protective in a mouse model of multiple sclerosis. EMBO Mol. Med. 5, 891–903
13. Yeliseev, A. A., and Kaplan, S. (1995) A sensory transducer homologous to the mammalian peripheral-type benzodiazepine receptor regulates photosynthetic membrane complex formation in Rhodobacter sphaeroides 2.4.1. J. Biol. Chem. 270, 21167–21175
14. Anholt, R. R., de Souza, E. B., Oster-Granite, M. L., and Snyder, S. H. (1986) Peripheral-type benzodiazepine receptors: autoradiographic localization in whole-body sections of neonatal rats. J. Pharmacol. Exp. Ther. 233, 517–526
15. Wang, H. J., Fan, J., and Papadopoulos, V. (2012) Translocator protein (TSPO) gene promoter-driven green fluorescent protein synthesis in transgenic mice: an in vivo model to study Tspo transcription. Cell Tissue Res. 350, 261–275
16. Morohaku, K., Phuong, N. S., and Selvaraj, V. (2013) Developmental expression of translocator protein/peripheral benzodiazepine receptor in reproductive tissues. PLoS One 8, e74509
17. Anholt, R. R., Pedersen, P. L., De Souza, E. B., and Snyder, S. H. (1986) The peripheral-type benzodiazepine receptor. Localization to the mitochondrial outer membrane. J. Biol. Chem. 261, 576–583
18. Simpson, E. R., and Boyd, G. S. (1967) The cholesterol side-chain cleavage system of bovine adrenal cortex. Eur. J. Biochem. 2, 275–285
19. Simpson, E. R., McCarthy, J. L., and Peterson, J. A. (1978) Evidence that the cycloheximide-sensitive site of adrenocorticotrophic hormone action is in...
the mitochondrion. Changes in pregnenolone formation, cholesterol content, and the electron paramagnetic resonance spectra of cytochrome P-450. J. Biol. Chem. 253, 3135–3139
20. Yago, N., and Ichii, S. (1969) Sub mitochondrial distribution of components of the steroid 11β-hydroxylase and cholesterol sidechain-cleaving enzyme systems in hog adrenal cortex. J. Biochem. 65, 215–224
21. Churchill, P. F., and Kimura, T. (1979) Topological studies of cytochromes P-450ccc and P-45011β in bovine adrenocortical inner mitochondrial membranes. Effects of controlled tryptic digestion. J. Biol. Chem. 254, 10443–10448
22. Mukhin, A. G., Papadopoulos, V., Costa, E., and Krueger, K. E. (1989) Mitochondrial benzodiazepine receptors regulate steroid biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 86, 9813–9816
23. Papadopoulos, V., Amri, H., Li, H., Boujrad, N., Vidic, B., and Garnier, M. (1997) Targeted disruption of the peripheral-type benzodiazepine receptor gene inhibits steroidogenesis in the R2C Leydig tumor cell line. J. Biol. Chem. 272, 32129–32135
24. Muriel, S., Robert, J. C., Coic, Y. M., Neumann, J. M., Ostuni, M. A., Yao, Z. X., Papadopoulos, V., Jamir, N., and Lacapère, J. J. (2008) Secondary and tertiary structures of the transmembrane domains of the translocator protein TSPO determined by NMR. Stabilization of the TSPO tertiary fold upon ligand binding. Biochim. Biophys. Acta 1778, 1375–1381
25. Korkhov, V. M., Sachsse, C., Short, J. M., and Tate, C. G. (2010) Three-dimensional structure of TsP0 by electron cryomicroscopy of helical crystals. Structure 18, 677–687
26. Li, H., and Papadopoulos, V. (1998) Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern. Endocrinology 139, 4991–4997
27. Lacapère, J. J., Delavoie, F., Li, H., Pérani, G., Maccario, J., Papadopoulos, V., and Vidic, B. (2001) Structural and functional study of reconstituted peripheral benzodiazepine receptor. Biochem. Biophys. Res. Commun. 284, 536–541
28. Papadopoulos, V., Baraldi, M., Guilarte, T. R., Knudsen, T. B., Lacapère, J. J., Lindemann, P., Norengen, M. D., Nett, D., Weizman, A., Zhang, M. R., and Gavish, M. (2006) Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol. Sci. 27, 402–409
29. Clark, B. J., Wells, J., King, S. R., and Stocco, D. M. (1994) The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (STAR). J. Biol. Chem. 269, 28314–28322
30. Bose, H. S., Lingappa, R. V., and Miller, W. L. (2002) Rapid regulation of steroidogenesis by mitochondrial protein import. Nature 417, 87–91
31. Lin, D., Sugawara, T., Strauss, J. F., 3rd, Clark, B. J., Stocco, D. M., Saenger, P., Rogol, A., and Miller, W. L. (1995) Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 267, 1828–1831
32. Baker, B. Y., Lin, L., Kim, C. J., Raza, J., Smith, C. P., Miller, W. L., and Achermann, J. C. (2006) Non classic congenital lipoid adrenal hyperplasia: a new disorder of the steroidogenic acute regulatory protein with very late presentation and normal male genitalia. J. Clin. Endocrinol. Metab. 91, 4781–4785
33. Caron, K. M., Soo, S. C., Wetsel, W. C., Stocco, D. M., Clark, B. J., and Parker, K. L. (1997) Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. Proc. Natl. Acad. Sci. U.S.A. 94, 11540–11545
34. Privat, C. T., Crivello, J. F., and Jefcoate, C. R. (1983) Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. Proc. Natl. Acad. Sci. U.S.A. 80, 702–706
35. Krueger, K. E., and Papadopoulos, V. (1990) Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. J. Biol. Chem. 265, 15015–15022
36. Hauet, T., Yao, Z. X., Bose, H. S., Wall, C. T., Han, Z., Li, W., Hales, D. B., Miller, W. L., Culty, M., and Papadopoulos, V. (2005) Peripheral-type benzodiazepine receptor-mediated action of steroidogenic acute regula-
tions of the 18-kDa translocator protein (TSPO): regulation of apoptosis and steroidogenesis as part of the host-defense response. *Curr. Pharm. Des.* **13**, 2385–2405

59. Sileikyte, J., Blachly-Dyson, E., Sewell, R., Carpi, A., Menabò, R., Di Lisa, F., Richelli, F., Bernardi, P., and Forte, M. (2014) Regulation of the mitochondrial permeability transition pore by the outer membrane does not involve the peripheral benzodiazepine receptor (TSPO). *J. Biol. Chem.* **289**, 13769–13781

60. Decker, C. (2012) The American Society for Bone and Mineral Research 2012 Annual Meeting, Minneapolis, MN, October 12–15, 2012, ASBMR, Washington, D. C.

61. Nieters, A., Conde, L., Slager, S. L., Brooks-Wilson, A., Morton, L., Skibola, D. R., Novak, A. J., Riba, J., Ansell, S. M., Halperin, E., Shanafelt, T. D., Agana, L., Wang, A. H., De Roos, A. J., Severson, R. K., Cozen, W., Spinelli, J., Butterbach, K., Becker, N., de Sanjose, S., Benavente, Y., Cocco, P., Staines, A., Maynadié, M., Foretova, L., Boffetta, P., Brennan, P., Lan, Q., Zhang, Y., Zheng, T., Purdie, M., Armstrong, B., Krickre, A., Vajdic, C. M., Grulich, A., Smith, M. T., Bracci, P. M., Hanock, S. J., Hartge, P., Cerhan, J. R., Wang, S. S., Rothman, N., and Skibola, C. F. (2012) PRRC2A and BCL2L11 gene variants influence risk of non-Hodgkin lymphoma: results from the InterLymph consortium. *Blood* **120**, 4645–4648

62. Li, R., Chen, W., Yanes, R., Lee, S., and Berliner, J. A. (2007) OKL38 is an oxidative stress response gene stimulated by oxidized phospholipids. *J. Lipid Res.* **48**, 709–715

63. Mariani, F., Sena, P., Pedroni, M., Benatti, P., Manni, P., Di Gregorio, C., Manenti, A., Palumbo, C., de Leon, M. P., and Roncucci, L. (2013) Th1-inducing POZ-Kruppel factor (ThPOK) is a key regulator of the immune response since the early steps of colorectal carcinogenesis. *PLoS One* **8**, e54488

64. Keating, S. E., Baran, M., and Bowie, A. G. (2011) Cytosolic DNA sensors regulating type I interferon induction. *Trends Immunol.* **32**, 574–581

65. Schaller, T., Hué, S., and Towers, G. J. (2007) An active TRIM5 protein in rabbits indicates a common antiviral ancestor for mammalian TRIM5 proteins. *J. Virol.* **81**, 11713–11721

66. Chen, H., Ko, G., Zatti, A., Di Giacomo, G., Liu, L., Raiteri, E., Perucco, E., Collesi, C., Min, W., Zeiss, C., De Camilli, P., and Cremona, O. (2009) Embryonic arrest at midgestation and disruption of Notch signaling produced by the absence of both epsin 1 and epsin 2 in mice. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13838–13843

67. Mack, J. T., Beljanski, V., Soulka, A. M., Townsend, D. M., Brown, C. B., Davis, W., and Tew, K. D. (2007) “Skittish” Abca2 knockout mice display tremor, hyperactivity, and abnormal myelin ultrastructure in the central nervous system. *Mol. Cell. Biol.* **27**, 44–53

68. Chung, J. Y., Chen, H., Midzak, A., Burnett, A. L., Papadopoulos, V., and Zirkin, B. R. (2013) Drug ligand-induced activation of translocator protein (TSPO) stimulates steroid production by aged brown Norway rat Leydig cells. *Endocrinology* **154**, 2156–2165

69. Lacapère, J. J., and Papadopoulos, V. (2003) Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. *Steroids* **68**, 569–585

70. Seneviratne, M. S., Faccenda, D., Di Biase, V., and Campanella, M. (2012) PK11195 inhibits mitophagy targeting the F1Fo-ATP synthase in Bcl-2 knock-down cells. *Curr. Mol. Med.* **12**, 476–482

71. Hatty, C. R., Le Brun, A. P., Lake, V., Clifton, L. A., Liu, G. J., James, M., and Banati, R. B. (2014) Investigating the interactions of the 18kDa translocator protein and its ligand PK11195 in planar lipid bilayers. *Biochim. Biophys. Acta* **1838**, 1019–1030

72. Anholt, R. R., De Souza, E. B., Kuhar, M. J., and Snyder, S. H. (1985) Depletion of peripheral-type benzodiazepine receptors after hypophysectomy in rat adrenal gland and testis. *Eur. J. Pharmacol.* **110**, 41–46