RESEARCH

Lack of adrenal TSPO/PBR expression in hamsters reinforces correlation to triglyceride metabolism

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Abstract

Despite being a highly conserved protein, the precise role of the mitochondrial translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), remains elusive. The void created by studies that overturned a presumptive model that described TSPO/PBR as a mitochondrial cholesterol transporter for steroidogenesis has been filled with evidence that it can affect mitochondrial metabolic functions across different model systems. We previously reported that TSPO/PBR deficient steroidogenic cells upregulate mitochondrial fatty acid oxidation and presented a strong positive correlation between TSPO/PBR expression and tissues active in triglyceride metabolism or lipid storage. Nevertheless, the highlighting of inconsistencies in prior work has provoked reprisals that threaten to stifle progress. One frequent factoid presented as being supportive of a cholesterol import function is that there are no steroid-synthesizing cell types without high TSPO/PBR expression. In this study, we examine the hamster adrenal gland that is devoid of lipid droplets in the cortex and largely relies on *de novo* cholesterol biosynthesis and uptake for steroidogenesis. We find that *Tspo* expression in the hamster adrenal is imperceptible compared to the mouse. This observation is consistent with a substantially low expression of *Cpt1a* in the hamster adrenal, indicating minimal mitochondrial fatty acid oxidation capacity compared to the mouse. These findings provide further reinforcement that the much sought-after mechanism of TSPO/PBR function remains correlated with the extent of cellular triglyceride metabolism. Thus, TSPO/PBR could have a homeostatic function relevant only to steroidogenic systems that manage triglycerides associated with lipid droplets.

Key Words

- ▶ adrenal cortex
- ▶ steroidogenesis
- ▶ mitochondria
- ▶ cholesterol
- ▶ lipid droplets

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Introduction

The precise function of the mitochondrial translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), has been an investigative question for decades (Gavish *et al.* 1999, Selvaraj & Stocco 2015, Gavish & Veenman 2018). Most proposed functions to date have been correlative or based on pharmacology, with a staple interest in drug discovery (Selvaraj & Tu

2016). With no rigorous testing for off-target effects, these research outputs have created a kaleidoscopic collection of purported functions (Liu *et al.* 2014, Selvaraj *et al.* 2015, Gatliff & Campanella 2016, Stocco *et al.* 2017). On this basis, TSPO-binding drugs have been proposed as therapeutic targets for a variety of conditions as represented by the patent landscape (Kim & Pae 2016a,b).

As a functional correlation to its abundant expression in the outer mitochondrial membrane of steroidogenic cells (Anholt *et al.* 1986), early pharmacological experiments in testicular Leydig cell lines reported TSPO function as linked to mitochondrial cholesterol import, the first step in *de novo* steroidogenesis (Mukhin *et al.* 1989, Krueger & Papadopoulos 1990, Papadopoulos *et al.* 1990). These studies by Vassilios Papadopoulos came at a time when there was substantial interest in identifying the rate-limiting step to steroid hormone biosynthesis (Stocco & Clark 1996). Subsequent studies by the same group that disrupted the *Tspo* gene (Papadopoulos *et al.* 1997a) or knocked-down *Tspo* gene expression (Hauet *et al.* 2005), again in Leydig cell lines, reported dramatic reductions to steroidogenesis. These studies were performed in response to the identification of the STAR protein (Clark *et al.* 1994, Caron *et al.* 1997), which contested the involvement of TSPO in this process. In several subsequent manuscripts, interpretation was asserted in that TSPO was ‘indispensable’ for *de novo* steroidogenesis (Culty *et al.* 1999) and *in vivo* viability (Papadopoulos *et al.* 1997b). As such, pharmacological effects have been frequently explained by extrapolation to the broad functions of steroids (Rupprecht *et al.* 2010).

In stark contrast to these engendering reports that were all from the Papadopoulos group, recent studies disagreed. *In vivo*, both conditional and global TSPO knockout mice failed to demonstrate any overt problems including those associated with *de novo* steroidogenesis (Banati *et al.* 2014, Morohaku *et al.* 2014, Tu *et al.* 2014, Wang *et al.* 2016, Klee *et al.* 2019). One study suggested age-related subtle steroidogenic abnormalities that were secondary and independent of *de novo* steroidogenesis (Barron *et al.* 2018). *In vitro*, CRISPR/Cas9-mediated deletion of TSPO in Leydig cells, showed no association to steroid biosynthesis (Tu *et al.* 2015) and, in glia, *de novo* steroidogenesis was confirmed independent of TSPO (Milenkovic *et al.* 2019). In support, the use of knockout models in studying TSPO-binding drugs have reported that effects are independent of TSPO (Tu *et al.* 2015, Singh *et al.* 2020). It has also been made clear that abundant TSPO expression is not exclusive to steroid-synthesizing cells; it is detectable in most tissue types with high expression also observed in brown and white adipose tissues (Tu *et al.* 2016). These reveals have not only marked progress to understanding, but also aligned current work to seek the precise function of TSPO, expanding to test alternate assumptions (Šileikyte *et al.* 2014, Zhao *et al.* 2016). Regrettably, however, they also revealed the imprecise and irreproducible qualities of prior

work on TSPO (reviewed in Selvaraj *et al.* 2015, Selvaraj & Tu 2016). Studies have now correlated TSPO to affecting mitochondrial metabolism and autophagy (Gatliff *et al.* 2014, Tu *et al.* 2016, Milenkovic *et al.* 2019, Kim *et al.* 2020), a clear departure from any ‘translocation’ actions. Nevertheless, as none have pinpointed a mechanistic structure–function explanation for TSPO, it has been difficult for groups engaged in drug discovery to explain effects and putative links to TSPO.

Papadopoulos has also weighed in to maintain that TSPO is crucial for ‘steroidogenesis’ and continues to publish results that interpret such a function, albeit a direct link to mitochondrial cholesterol import in these studies are not supported (Selvaraj *et al.* 2016, Selvaraj & Stocco 2018). We disagree with these misleading representations that force controversy, but do acknowledge that a TSPO function distinct from *de novo* steroid biosynthesis, common to all TSPO expressing cell types, could impact cellular physiology. The importance of TSPO function to long-term species survival is obviously indicated by its sequence conservation from bacteria to humans (Selvaraj & Stocco 2015). However, it must be noted that conserved does not mean fundamental; fitness under specific constraints can also lead to conservation.

A frequent correlation presented in support of a function in steroidogenesis is that TSPO is abundant in steroid-producing mitochondria and that there are no steroid-synthesizing cell types without abundant TSPO expression (Chung *et al.* 2020). As we previously found a strong correlation between high TSPO expression and cell types active in triglyceride metabolism and/or contain abundant lipid droplets (Tu *et al.* 2016), we decided to examine tissue TSPO expression levels in the hamster that is almost completely devoid of lipid droplets in steroidogenic cells of the adrenal cortex (Alpert 1950, Lehoux & Lefebvre 1980).

Materials and methods

Animals

Female C57BL/6 mice (8–10 weeks of age) and Syrian hamsters (*Mesocricetus auratus*; 10 weeks old) were killed for tissue collection. Adrenal glands, gonadal white adipose tissue, harderian glands and ovaries were dissected and processed. The tissues were either snap-frozen in liquid nitrogen for total RNA isolation or processed immediately. Animals were maintained in accordance to the NIH Guidelines for the Care and Use of Laboratory Animals.

The Institutional Animal Care and Use Committee of Cornell University approved all experiments in this work.

Histology

Unfixed tissues from mice and hamsters were immediately embedded in optimal cutting temperature compound (Sakura Fine-Tek®) and maintained frozen in -80°C . Frozen sections (5 μm thickness) were cut using a cryostat (CM1950, Leica), collected on glass slides, and stained for neutral lipid deposits using Oil Red O (Matheson Coleman & Bell). For staining, slides were first fixed with 4% formaldehyde during thaw for 60 min at room temperature and then washed with deionized water to remove the mounting compound. Sections were then immersed in Oil Red O dissolved in 60% isopropyl alcohol and incubated for 30 min at room temperature. This was followed by washing with deionized water, counterstaining using Fast Green FCF (Millipore, Sigma) and mounting with a coverslip using glycerol. Slides were viewed using a light microscope (Leica DM750 LED) and images were acquired using a high definition color camera (Leica ICC50W).

RNA isolation and cDNA synthesis

Total RNA was isolated from adrenals, white adipose tissue, harderian glands and ovaries (three biological replicates each) from both mice and hamsters using TRIzol™ (Life Technologies). RT for cDNA synthesis was carried out for 2 μg of total RNA using oligo-dT primers with the High Capacity cDNA RT kit (Applied Biosystems).

Absolute gene expression

Quantitation of *Tspo* expression was performed by generating a standard curve using a mouse *Tspo* template plasmid (pLenti-CMV-TSPO; Tu et al. 2016) using intron-spanning primers that were common for both mouse and hamster *Tspo* sequences (Table 1). For this method, the plasmid was first linearized using XbaI restriction digestion, followed by column purification (Zymo Research) and quantification (Nanophotometer N60, Implen). Mass of one copy of the linearized plasmid was determined by using the formula: $\text{mass} = (\text{size in bp}) \times (1.096 \text{ e} - 21 \text{ g/bp})$. Total linearized plasmid DNA required for a specific *Tspo* copy number (highest concentration) was calculated by multiplying mass of plasmid by copy number desired ($3 \text{ e} 6$ copies/ μL). This concentration was diluted serially (ten-fold) to achieve copy numbers extending the range from $3 \text{ e} 6$ to $3 \text{ e} - 1$ and DNA concentrations were confirmed. Standard quantitative PCR (qPCR) reactions were setup for each dilution in duplicate using *Tspo* primers, and amplification was quantified using SYBR-green, followed by melt curve analysis. Resulting cycle threshold (Ct) values were plotted against the known *Tspo* copy number for fitting a regression line, and a linear equation was obtained. Goodness-of-fit for the regression model was assessed using the coefficient of determination (R^2). Copy numbers of *Tspo* in different tissue samples were determined from their respective Ct values from qPCR using this standard curve equation. Specificity and amplification efficiency of the common *Tspo* primers were confirmed. Statistical analysis for significant differences was performed for each tissue comparing values obtained for mice and hamsters using the Student's *t*-test (Prism,

Table 1 Primer sequences used for gene expression assays.

Gene	Hamster	Mouse
<i>Tspo</i> ^a	5'-CCCTTGGGTCTCTACTGG-3' 5'-ACCCCACTGACAAGCAGAAG-3'	5'-CCCTTGGGTCTCTACTGG-3' 5'-ACCCCACTGACAAGCAGAAG-3'
<i>Ldlr</i>	5'-TCCTTGGCCATCTATGAGGAC-3' 5'-TGGGGACAAGAGGTTTTCAG-3'	5'-TCCCTGGGAACAATTCACC-3' 5'-CACTCTGTGCAAGCAGTCAG-3'
<i>Hsl</i>	5'-AAGCCCCAAAAGACCTCAGT-3' 5'-GCTGCCTCAGACACTCCT-3'	5'-TGGCACACCATTTTGACCTG-3' 5'-TTGCGGTTAGAAGCCACATAG-3'
<i>Cpt1a</i>	5'-GTGCAGGGAACAGGAAGGA-3' 5'-CAAACGTGAGGGACTTGTC-3'	5'-CTCAAACCTATTCGTCTCTG-3' 5'-TTGGATGGTGTCTGTCTC-3'
<i>Hmgcr</i>	5'-ATGCAGCAAACATCGTCACT-3' 5'-CTTCATTCTGTGGGACCACTT-3'	5'-AGCTTGCCGAATTGTATGTG-3' 5'-TCTGTTGTGAACCATGTGACTTC-3'
<i>Cyp11a1</i>	5'-GCAGTTGGTCCCACTCCTAA-3' 5'-TCGAAGTACCAGGTCATCCAC-3'	5'-AGGTCCTTCAATGAGATCCCTT-3' 5'-TCCCTGTAAATGGGGCCATAC-3'
<i>Vdac1</i>	5'-TTGCAGTTGGCTACAAGACG-3' 5'-GCACATACCTGGCTTTAGGG-3'	5'-AAGTGAACAACCTCCAGCCTGA-3' 5'-CACCAGCATTGACGTTCTTG-3'
<i>Gapdh</i> ^a	5'-GCCTTCCGTGTTCTACC-3' 5'-GCCTGCTTACCACCTTC-3'	5'-GCCTTCCGTGTTCTACC-3' 5'-GCCTGCTTACCACCTTC-3'

^aPrimer sequences used were identical for both hamster and mouse.

GraphPad). Differences were considered significant at $P < 0.05$.

Relative gene expression

Expression differences for specific genes were analyzed using qPCR on cDNA prepared from different tissues. Assays were performed with SYBR-green quantitation using intron-spanning primers designed independently for mouse and hamster genes: low-density lipoprotein receptor (*Ldlr*), hormone-sensitive lipase (*Hsl*), carnitine palmitoyltransferase 1A (*Cpt1a*), 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*) and voltage-dependent anion-selective channel protein 1 (*Vdac1*). Results were analyzed after normalization to the expression of glyceraldehyde-3-phosphate (*Gapdh*) as an internal control. All primer sequences are provided in Table 1. Relative quantification of fold-change was performed comparing Ct values from individual mice by applying the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Statistical analysis for significant differences was performed comparing ovaries to adrenals within mice and hamsters using the Student's *t*-test (Prism, GraphPad). Differences were considered significant at $P < 0.05$.

Results

Hamster adrenal cortex is devoid of lipid droplets

In order to investigate the relationship between *Tspo* expression levels and lipid metabolism, we chose to examine the hamster, which is distinct in its adrenal cholesterol sourcing for steroidogenesis. Staining for neutral lipids in frozen sections of the adrenals, harderian glands, and ovaries from mice and hamsters confirmed that the hamster adrenal cortex is devoid of intracellular lipid stores/droplets (Fig. 1). This was a systems divergence specific only to the adrenals as harderian glands and ovaries from both mice and hamsters showed morphologically comparable neutral lipid deposits within the expected functional compartments (Fig. 1). Secretory epithelial cells of the tubuloacini of the harderian glands contained lipid droplets in both species. The ovarian glandular interstitia and cells of the theca and granulosa layers showed lipid droplet content in both species.

Figure 1

Hamster adrenocortical cells are devoid of lipid droplets. Comparing mouse and hamster tissues for neutral lipid staining shows that the hamster adrenal is devoid of large lipid stores as observed in the mouse. Harderian glands and ovaries show morphologically comparable organization of lipid stores between the two species.

Imperceptible *Tspo* expression in adrenals of the hamster

To directly compare *Tspo* gene expression between mouse and hamster tissues, we developed an absolute gene expression assay by generating a standard curve (regression line) with known *Tspo* copy numbers and using primers common for both species. We observed a strong coefficient of determination ($R^2 = 0.9956$) between *Tspo* template copy number and the cycle threshold (Ct) detected in qPCR (Fig. 2A). This allowed not only a precise evaluation of *Tspo* gene expression levels, but also a

Figure 2

Hamster adrenal *Tspo* gene expression is imperceptible, a contrast to expression in mice. Regression line or standard curve generated with known *Tspo* copy numbers of a template plasmid using primers common for both the mouse and hamster genes (A). Absolute copy numbers of *Tspo* expression compared between adrenals, ovaries, harderian glands, and white adipose tissues of the mouse and hamster (B). Bars represent mean $\pm$ S.E.M.; significant differences: * $P < 0.01$, *** $P < 0.0001$.

comparison of the levels between the mouse and hamster. On this basis, our results showed that *Tspo* expression is imperceptible in hamster adrenals (mean copy number: 9.84) compared to mouse adrenals (mean copy number: 456.6); this was a 46-fold lower *Tspo* expression in hamster adrenals ($P < 0.0001$; Fig. 2B). Such a difference in *Tspo* expression was not observed in comparing ovaries ($P = 0.5906$) between hamsters (mean copy number: 70.25) and mice (mean copy number: 85.40). Among other tissues known to express high levels of *Tspo*, expression in the hamster remained either equivalent or higher in hamsters compared to mice. Harderian gland *Tspo* expression was 2.5-fold higher ($P < 0.01$) in hamsters (mean copy number: 1051.60) than mice (mean copy number: 423.48). White adipose tissue *Tspo* expression was not different ($P = 0.3491$) between hamsters (mean copy number: 865.47) and mice (mean copy number: 498.52).

Distinct differences in cholesterol and triglyceride metabolism in hamster adrenals

To compare steroidogenesis and lipid metabolic status between the mouse and hamster adrenals, we evaluated the expression of a few core genes involved in the regulation of these processes. We found that *Cpt1a*, a transferase essential for mitochondrial fatty acid oxidation (FAO), was substantially lower only in hamster adrenals relative to ovaries, but not in the mouse (Fig. 3A). Albeit not significant, expression of *Ldlr*, involved in lipid uptake, was approximately four-fold higher in hamster adrenals relative to ovaries, but not in the mouse (Fig. 3B). Showing a similar trend but with statistical significance, *Hmgcr*, involved in cholesterol synthesis, was eight-fold higher in hamster adrenals relative to ovaries, but not in the mouse (Fig. 3C). Expression of *Hsl*, an ester hydrolase involved in the release of free cholesterol and fatty acids from lipid droplet stores, was two-fold higher in mouse adrenals relative to ovaries, but not in the hamster (Fig. 3D). Expression of *Star* was 5.7-fold higher in the hamster adrenals relative to ovaries and 4.7-fold higher in mouse adrenals relative to ovaries (Fig. 3E). Albeit not significant, expression of *Cyp11a1*, the enzyme that converts cholesterol to the first steroid pregnenolone, was 0.6-fold lower in the hamster adrenals relative to ovaries, but not in the mouse (Fig. 3F). Expression of *Vdac1*, an ion channel in the outer mitochondrial membrane, was not different in comparisons between adrenals and ovaries in both hamster and mice (Fig. 3G).

Figure 3

Expression of genes linked to cholesterol and triglyceride metabolism in the adrenals of hamsters and mice. Graphs show expression of (A) *Cpt1a*, (B) *Ldlr*, (C) *Hmgcr*, (D) *Hsl*, (E) *Star*, (F) *Cyp11a1* and (G) *Vdac1* in the adrenals (Ad) in comparison to levels detected in the ovary (Ov) of hamsters and mice. Disparate metabolic features are observed in that there are distinctly low levels of *Cpt1a* and high levels of *Ldlr* and *Hmgcr* in the hamster adrenals compared to expression in the mouse adrenals. Bars represent mean $\pm$ S.E.M.; significant differences: # $P < 0.05$; ** $P < 0.001$.

Discussion

Cholesterol esters stored in steroidogenic cell lipid droplets of the adrenal cortex can act as a reservoir for rapid release of free cholesterol (Zoller & Malamed 1975, Bisgaier *et al.* 1985). Adrenocorticotrophic hormone (ACTH) stimulation of adrenals results in ester hydrolysis and release of free cholesterol and triglyceride fatty acids (Vahouny *et al.* 1978). Esterase activity of Hsl, critical in this mobilization, acts on both cholesterol esters and triglycerides (Holm *et al.* 1988). As a result, cholesterol release from droplets is coupled to triglyceride release that needs to be concurrently processed by the cells (Freeman & Ontko 1992). Although the specific disposition of these fatty acids remains to be delineated, pathophysiological accumulations have been shown to affect the rate of steroid biosynthesis (Sarel & Widmaier 1995, Meikle *et al.* 1996). In contrast to mice, rats and humans, the hamster adrenal cortex is almost completely devoid of esterified cholesterol/lipid droplets (Alpert 1950, Lehoux & Lefebvre 1980); a characteristic also confirmed in this study. This evolutionary divergence presents a unique physiology in that the hamster adrenal has substantial *de novo* cholesterol biosynthetic capability from endogenous substrates to meet the high demands for steroidogenesis (Lehoux & Lefebvre 1980), at a rate almost 25-fold higher than that recorded for the rat adrenal (Spady & Dietschy 1985). Cholesterol accumulation from biosynthesis in hamster adrenals was also measured at a rate ten-fold greater than the kinetics of uptake via low-density lipoproteins (LDLs) (Spady & Dietschy 1985). Our findings that indicate significantly higher hamster adrenal *Hmgcr* support these early reports. Higher levels of *Ldlr* expression also indicate an increased capacity to acquire lipids from extracellular sources in the hamster adrenal compared to the mouse adrenal. Correspondingly, with null lipid stores, the hamster adrenal cortex does not mobilize or process the considerable levels of triglycerides as observed in the adrenals of most other species. This is supported by hamster adrenal gene expression data that indicates dramatically lower mitochondrial FAO as shown by low *Cpt1a*. Levels of *Star* and *Cyp11a1* were functionally comparable in the adrenals of the two species.

Before the link between TSPO/PBR and steroidogenesis was fostered (Mukhin *et al.* 1989, Krueger & Papadopoulos 1990), a study that examined whole-body sections of neonatal rats with autoradiographic localization of TSPO using the small molecule [3H]Ro5-4864 concluded that expression of TSPO was correlated to tissues that derive their metabolic energy primarily from oxidative phosphorylation (Anholt *et al.* 1985). Supporting

this early evidence, triglyceride metabolism research comparing inbred mouse strains identified *Tspo/Pbr* as one of the six candidate genes that could influence triglyceride metabolism in mice (Leduc *et al.* 2011). Experimentally, we provided the first evidence linking TSPO/PBR and fatty acid oxidation (FAO) by demonstrating that TSPO/PBR deletion induces a shift in substrate utilization from glucose to fatty acids for energy production in steroidogenic cells and the adrenals (Tu *et al.* 2016). Although the precise mechanism for this effect continues to be a topic of ongoing research, several other groups across different organisms/systems have recently reported similar findings regarding the shift to metabolism (Liu *et al.* 2017, Jurkiewicz *et al.* 2018, Kim *et al.* 2020). It must be noted that this shift is not homologous to evolution in the hamster because the inherent presence of lipid droplets in the adrenal cortex remained unchanged in *Tspo* gene deleted mice (Tu *et al.* 2014). It is not anticipated that a loss-/gain-of-function studies in model systems, particularly for quantitative traits as noted for TSPO (Leduc *et al.* 2011), would represent the gamut of adaptations during evolution that conserves systemic function.

Divergence from dependence on lipid droplet stores could be considered the hallmark of adrenal steroidogenesis evolution in the hamster. In this context, conservation of TSPO expression was apparently not critical, supporting the correlation between TSPO and triglyceride metabolism rather than mitochondrial cholesterol import. Considering in entirety from trophic signaling to steroid hormone release, systemic coevolution, not only pertaining to energy metabolism, but also mechanisms that determine sourcing and traffic of cholesterol to the mitochondria, upstream of import, remain to be dissected in the hamster adrenal. For mitochondrial cholesterol import, it is clear that the critical role of STAR (Caron *et al.* 1997) is not dependent on TSPO (Morohaku *et al.* 2014, Tu *et al.* 2015). Although the mechanism of STAR-mediated mitochondrial cholesterol import remains to be fully elucidated (Miller & Bose 2011), it has been demonstrated that STAR is active prior to entry into the mitochondria (Arakane *et al.* 1996, 1998). Updated investigations on STAR and associated mitochondrial entry apparatus (Rajapaksha *et al.* 2013, Prasad *et al.* 2015) do not indicate TSPO involvement as previously suggested (Hauet *et al.* 2005).

Expression of *Vdac1*, a purported binding partner (McEnery *et al.* 1992), that resulted in the inclusion of TSPO in the mitochondrial permeability transition pore, was not different between the hamster and mouse adrenals.

It must be noted that TSPO involvement in mitochondrial permeability transition was disproven by studies using *Tspo* knockout mice (Šileikyte *et al.* 2014). Notwithstanding, levels of *Vdac1* indicated that mitochondrial content was comparable in the adrenals of the two species.

High TSPO expression in harderian glands has been documented in early studies (Verma *et al.* 1987). Associated with the orbit and releasing secretions on the surface of the nictitating membrane, these glands are absent in higher primates (Payne 1994). Lipids are their major secretory product in rodents (including mouse and hamster), releasing 1-alkyl-2,3-diacylglycerol that might act as a lubricant (Lin & Nadakavukaren 1981, Tvřizká *et al.* 1988). The murine harderian gland is known to have two epithelial cell types with different secretory products (Woodhouse & Rhodin 1963). Interestingly, female Syrian hamsters possess a harderian gland with only one epithelial cell type with clear distinctions to lipid droplet sizes (Bucana & Nadakavukaren 1973). Such intracellular specializations make the gland in each species functionally relevant. Our findings that indicate higher levels of *Tspo* in the hamster harderian glands compared to the mouse could be a result of these finer functional distinctions in these two species that might demand different respective levels of lipid metabolism.

Despite the unknowns regarding the concurrent evolution of lipid droplet loss and *Tspo* expression in the hamster adrenal glands, our findings advance understanding with two core interpretations: (1) there exist steroidogenic systems in nature without TSPO, supporting that it is not directly involved in the cholesterol to steroid biosynthesis process and (2) TSPO expression is consistently correlated to the level of triglyceride metabolism, a realistic step toward uncovering its physiological function.

It was not possible to examine hamster TSPO protein expression as there were no available species-specific antibodies. We attempted this using the following antibodies: (1) MAB from rabbit (C-terminal epitope from human, aa156-169, #ab109497, Abcam) (Morohaku *et al.* 2013); (2) polyclonal antibody raised in rabbit (N-terminal epitope from rat, sequence proprietary, #ab154878, Abcam); (3) polyclonal antibody raised in rabbits (N-terminal epitope from human, aa1-50; #A15649, ABclonal), and we did not detect cross-reactivity with hamster TSPO. Nonetheless, we do not believe that this impacts our interpretations in any way, as regulation of TSPO is at the transcriptional level and there exists strong transcript-protein correlation in all tissues examined (Wang *et al.* 2012).

In summary, *Tspo* research has been plagued with an overwhelming array of effects and functional justifications

that fail to provide any directional clarity toward a physiological function. We disagree with reports that attempt to repudiate work that refuted TSPO involvement in mitochondrial cholesterol import for steroidogenesis. It is extremely important that pharmacologists use *Tspo* gene disrupted mice or cells for target validation in reporting TSPO-associated functions. It is becoming increasingly clear that TSPO function, albeit not acutely essential, acts to provide some core aspects of organismal fitness that becomes vital for long-term survival of species.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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