INTRODUCTION

The translocator protein 18 kDa (TSPO) is an ubiquitous mitochondrial protein enriched at the outer/inner mitochondrial membrane contact sites.\(^1\) The main function of TSPO is the transport of cholesterol within mitochondria for its conversion into pregnenolone (PREG), the precursor of steroids. TSPO is important in steroid-synthesising cells, including those in the central and peripheral nervous systems (CNS and PNS). TSPO ligands may play an important role.
in neuroprotection by modulating endogenous production of neurosteroids within the nervous system.2,3 The stimulation of neurosteroid production might be a beneficial strategy for Alzheimer’s disease (AD). Indeed, a drop of neurosteroid levels such as allopregnanolone (APα) was reported in the cerebral cortex of a triple transgenic mouse model of AD (3xTgAD), as well as post mortem in the brains of humans affected with AD.5,6 Neurosteroids regulate both repair and regeneration mechanisms in the brain because studies have shown the ability of APα to promote the regenerative processes in both the CNS and PNS.6,7

Abnormal Tau protein aggregation is a hallmark of AD brains and additional tauopathies including frontotemporal lobar degeneration. Tau impacts mitochondrial functions and dynamics by impairing mitochondrial complex I activity, mitochondrial respiration and mitochondrial-shaping proteins, leading to neurotoxicity.12,13 Of note, disturbed neurosteroid synthesis was also observed in a cellular model of tauopathy, suggesting that Tau-induced mitochondrial dysfunction might be involved in the impairment of steroidogenesis.14,15

To date, few investigations have examined the potential beneficial effects of TSPO ligands in experimental models of neurodegeneration within the CNS, especially in tauopathy.3 Ro5-4864 showed beneficial effects in functional outcomes and attenuated markers of inflammation in the rTg4510 transgenic model of tauopathy (tauTg), although in the absence of any benefit on Tau pathogenesis or neuronal loss.14 Because the TSPO ligands described in the literature suffer from a common problem of solubility,3 we developed new compounds based on an imidazo[1,2-c]quinazolinone scaffold and exhibiting nanomolar affinity to TSPO.17 We recently showed that both compounds, named 2a and 2b, improved the ATP production and PREG synthesis in human neuroblastoma cells overexpressing the human amyloid-beta protein precursor, a cellular model of AD.17,18 Compounds 2a and 2b are based on an imidazoquinazoline scaffold and acetamide moiety. Both compounds showed no affinity for central benzodiazepine receptor (up to 10 µmol L-1) but a promising affinity for TSPO vs both probes [3H]-PK11195 and [3H]-Ro5-4864 (400 nmol L-1 and 80 nmol L-1, respectively, for 2a)17 (see Supporting information, Figure S1). TSPO ligand 2b is closely related to 2a and presents an affinity of approximately 10 nmol L-1 vs both probes, in the same range as the compound of reference Ro5-4864.17

Because we previously demonstrated that our new TSPO ligands have beneficial effects against amyloid-beta-induced mitochondrial dysfunction, the present study aimed to evaluate their effects in a cellular model of AD-related tauopathy. We investigated whether our new TSPO ligands can attenuate the toxicity of abnormal Tau on mitochondria as well as their ability to promote steroidogenesis via the production of PREG in cells expressing either the wild-type form of Tau protein (wtTau) or the mutant form (P301L-Tau mutation, pathological form) compared to cells expressing the empty vector.13 The new ligands 2a and 2b were compared with compounds of reference (Ro5-4864, SSR180575 and XBD173) known from the literature for their high affinity and selectivity towards TSPO.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM), foetal calf serum (FCS) and penicillin/streptomycin were obtained from Sigma-Aldrich (St Louis, MO, USA). Glutamax was obtained from Gibco Invitrogen (Waltham, MA, USA). A ViaLight plus kit (ATP) was obtained from Lonza (Basel, Switzerland). TSPO ligands were synthesised by the laboratory CNRS, University of Strasbourg, UMR 7200, 170 Faculty of Pharmacology (Strasbourg, France). Especially, chemical compounds 2a and 2b were synthesised as described by Hallé et al17

2.2 Cell culture

Human neuroblastoma SH-SY5Y cells stably expressing the vector alone (pCEP4) were used and compared with cells stably transfected with human wild-type Tau (Htau40, wtTau cells) and P301L mutant Tau (P301L cells). Both wtTau and P301L cells showed similar Tau expression levels13,19; however, the P301L mutation is required for abnormal Tau hyperphosphorylation and filament formation.19 Of note, the three cell lines possess the same amount of mitochondria.13 Cells were regularly selected with G418 (300 µg mL-1) and screened on a regular basis by routine biochemical assays.20 Cells were cultured in DMEM with 1% penicillin/streptomycin/Glutamax 10% heat-inactivated FCS at 37°C in a humidified incubator chamber under a 7.5% CO2 atmosphere. The cells were kept in culture and in 10 cm2 dishes, split twice a week and plated when they reached around 80% confluence, 1 day prior treatment.

2.3 Assessment of steroidogenic capacity

To assess the ability of our new compounds to increase PREG synthesis, a standard protocol was used, as described in several previous analogous studies.21-26 The evaluation of the production of PREG was performed with a direct enzyme-linked immunosorbent assay (ELISA) test (DRG Diagnostics, Marburg, Germany), an enzyme immunoassay for the quantitative determination of PREG in cells. Cells were plated in four to eight replicates into a white 96-well cell culture plate at a density of 2 × 104 cells per well overnight. Cells were washed with a saline buffer (140 mmol L-1 NaCl, 5 mmol L-1 KCl, 1.8 mmol L-1 CaCl2, 1 mmol L-1 MgSO4 + 7H2O, 10 mmol L-1 glucose, 10 mmol L-1 Hepes/NaOH, 0.1% bovine serum albumin, pH 7.4) and treated for 2 hours with TSPO ligands (20 or 40 µmol L-1). To measure the production of PREG, the downstream conversion of PREG was blocked by the addition of trilostane (25 µmol L-1) and abiraterone (0.1 µmol L-1), which completely inhibits the conversion of PREG into progesterone or dehydroepiandrosterone. The cell supernatant was then collected and the ELISA test was performed in accordance with the manufacturer’s instructions. The plate was read at 450 nm using the plate reader Cytation 3 (BioTek Inc., Winooski, VT, USA).
2.4 | Determination of ATP levels and mitochondrial membrane potential (MMP)

For ATP and MMP measurements, cells were treated in DMEM + 10% FCS, 1 day after plating either with DMEM alone (untreated control condition), or with a final concentration of 10 nmol L⁻¹ of TSPO ligands made from a stock solution in dimethyl sulfoxide (DMSO), for 24 hours (final concentration of DMSO < 0.002%, no effect of the vehicle solution (DMSO) alone compared to the untreated condition).²⁷

Total ATP content was determined using a bioluminescence assay (VialightTM HT; Cambrex Bio Science, Walkersville, MD, USA) in accordance with the manufacturer’s instructions, as described previously.²⁷ Cells were plated in five replicates into a white 96-well cell culture plate at a density of 2 × 10⁴ cells per well. The bioluminescent method measures the formation of light from ATP and luciferin by luciferase. The emitted light was linearly related to the ATP concentration and was measured using the Cytation 3 Cell Imaging Multi-mode Reader (BioTek).

The mitochondria membrane potential was measured using the fluorescent dye tetramethylrhodamine, methyl ester and perchlorate. Cells were plated in six replicates into a black 96-well cell culture plate at a density of 2 × 10⁴ cells per well. Cells were loaded with the dye at a concentration of 0.4 μmol L⁻¹ for 15 minutes. After washing twice with Hank’s balanced salt solution, the fluorescence was detected using the Cytation 3 cell Imaging Multi-mode Reader (BioTek) at 531 nm (excitation)/595 nm (emission). Transmernbrane distribution of the dye was dependent on MMP.

For the experiments using inhibitors of steroidogenesis, cells were co-treated for 24 hours with TSPO ligands (10 nmol L⁻¹) and α-diaminoglutethimide (750 μmol L⁻¹; AMG, inhibitor of the cholesterol side-chain cleavage enzyme [P450scc]) or trilostane (25 μmol L⁻¹; T, inhibitors of the 3β-hydroxysteroid dehydrogenase) and abiraterone (0.1 μmol L⁻¹; A, inhibitor of the cytochrome CYP17A1). Next, ATP levels and MMP were measured. Each assay was repeated at least three times. Of note, a 24-hour treatment with AMG induced a reduction of 97% of PREG concentration in cell lysates (0.009 ng mL⁻¹ vs 0.21 ng mL⁻¹ in untreated cells, Student’s t test, $P = 0.0001$), whereas treatment with T/A induced a 200× accumulation of PREG after 24 hours (51.7 ng mL⁻¹ vs 0.21 ng mL⁻¹ in untreated cells, Student’s t test, $P < 0.0001$).

2.5 | Statistical analysis

Statistical analysis was performed with the Prism, version 5.02 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± SEM and normalised to the corresponding non-treated control group or to the vector non-treated cells (= 100%). For statistical comparisons of more than two groups, One-way ANOVA was used, followed by Dunnett’s multiple comparison tests vs the control. For statistical comparisons of two groups, Student’s unpaired t test was used. The experimental data are evaluated using Prism. $P < 0.05$ was considered statistically significant. Goodness of fit was estimated by the $r^2$ value using Pearson correlation and linear regression analysis.

3 | Results

3.1 | Mitochondrial impairments in Tau-overexpressing cells

Previous data obtained by our group showed that ATP and MMP levels were significantly reduced in P301L cells compared to wtTau cells.²⁷ In the present study, we evaluated PREG, MMP and ATP levels in Tau-overexpressing cells (wtTau and P301L cells) compared to empty vector transfected cells. PREG levels were significantly reduced in P301L cells compared to vector cells (Figure 1A). In line with our previous study, ATP levels were significantly decreased in P301L cells compared to vector cells (Figure 1C), which was paralleled by decreased MMP levels compared to vector and wtTau cells (Figure 1B).

Taken together, the results demonstrate that abnormal Tau exhibits a negative effect on the mitochondrial function leading to a...
down-regulation of the precursor of neurosteroid biosynthesis, PREG, a depolarisation of the mitochondrial membrane potential and a diminution of ATP production.

To evaluate the ability of TSPO ligands to increase steroidogenesis in vitro, we used an experimental design, adapted from Castellano et al., allowing the quantification of PREG levels after 2 hours of incubation in the presence of TSPO ligands at 20 or 40 µmol L⁻¹. All TSPO ligands increased PREG levels in vector cells (up to 42% increase with XDB173), with the concentration of 40 µmol L⁻¹ being the most effective (Figure 2A). At this concentration, our new TSPO ligands 2a and 2b increased PREG levels by 22.7% and 23.4%, respectively. Similarly, all of the tested ligands increased PREG synthesis in wtTau-transfected cells (Figure 2B) with the new compounds 2a and 2b increasing PREG levels at the 40 µmol L⁻¹ dose by up to 38.5% and 38.3%, respectively. In P301L-tau transfected cells, only Ro5-4864 showed no significant effect on PREG synthesis (Figure 2C). The other tested ligands were able to increase and normalise PREG levels (compared to the levels of vector cells), with compound 2a being the most effective (up to 33% increase at 20 µmol L⁻¹).

Thus, our new molecules 2a and 2b increased PREG synthesis in the three cell lines, with an effect similar to SSR-180,575 and Ro5-4864 and XBD173. Furthermore, the incubation of P301L cells with XBD173, SSR-180,575, 2a and 2b normalised PREG levels to those of the vector cells, especially at 20 µmol L⁻¹.

### 3.3 | TSPO ligands increase ATP levels in vector-, wtTau- and P301L-transfected cells

We recently showed that a 24-hour treatment with TSPO ligands, including our new compounds 2a and 2b, increased ATP levels in a cellular model of AD (SH-SY5Y overexpressing the amyloid precursor protein [APP]), with 10 nmol L⁻¹ being the most efficient concentration. Therefore, based on these previous data, we decided to assess the ability of TSPO ligands to increase ATP levels in our cellular models of tauopathy at 10, 100 and 500 nmol L⁻¹. In line with our previous data, Ro5-4864, XBD173, SSR-180,575, 2a and 2b significantly increased ATP levels in vector cells (Figure 3A). Again, the concentration of 10 nmol L⁻¹ was the most efficient dose for all the TSPO ligands. In wtTau and P301L cells, only treatments with Ro5-4864, 2a and 2b showed an improvement of ATP levels (up to 8.8% increase of ATP levels with the compound 2b at 10 nmol L⁻¹ in P301L cells) (Figure 3B,C). This increase partially rescued the deficit in ATP observed in the P301L cells compared to the vector cells (Figure 3C).

Thus, these data indicate that TSPO ligands (especially Ro5-4864, 2a and 2b) are able to increase ATP levels not only in the control cell line (vector), but also in the Tau-transfected cell lines (wtTau and P301L).

### 3.4 | TSPO ligands increase the mitochondrial membrane potential in vector-, wtTau- and P301L-transfected cells

Because the ability to synthesise ATP molecules depends on the MMP, we investigated whether the TSPO ligands were able to
modulate this parameter, especially in the Tau-transfected cells with a decreased MMP (Figure 1). We selected the concentration of 10 nmol L⁻¹, which was the most efficient for increasing ATP levels after 24 hours of treatment.

In the three cell lines, all the TSPO ligands were able to increase the MMP (Figure 4). In wtTau-overexpressing cells presenting a deficit in the MMP of approximately 25% compared to the vector cells, all of the ligands increased the MMP to a higher level than the untreated vector cells (up to 51% increase with SSR-180,575). The P301L-overexpressing cells presented a 60% decrease of MMP compared to the vector cells and a 25% decrease compared to wtTau-overexpressing cells. All of the ligands increased the MMP of P301L cells to a higher level compared to the untreated wtTau cells. Strikingly, the rise of MMP was more pronounced in the P301L cell
FIGURE 4  TSPO ligands increase the mitochondrial membrane potential (MMP). MMP was quantified after 24 hours of incubation with TSPO ligands (10 nmol L⁻¹) in (A) vector cells, (B) wtTau cells and (C) P301L cells. Values are the mean ± SEM and are normalised to 100% of the untreated group (Untreat.). In (B), the ATP levels of the vector cells (untreated cells) are indicated by a dashed grey line. In (C), the ATP levels of the vector and wtTau cells (untreated cells) are indicated by dashed grey lines. *P < 0.05; **P < 0.01; ***P < 0.001; one-way ANOVA and post-hoc Dunnett’s multiple comparison test vs untreated group

line (an increase after treatment of between 40% and 65%) compared to the vector and wtTau cells, suggesting a higher potency of the TSPO ligands with respect to increasing the MMP in the presence of the P301L Tau mutation.

3.5 | The increase of mitochondrial bioenergetics induced by our new TSPO ligands is not linked to the increased steriodogenesis

Next, we aimed to investigate the mechanisms underlying the effects of our new TSPO ligands, 2a and 2b, on the mitochondrial bioenergetics in vector, wtTau and P301L cells. We previously showed that neuroactive steroids are able to increase mitochondrial bioenergetics in neuronal cells. Therefore, we hypothesised that the increase of steriodogenesis induced by our new TSPO ligands is involved in the improvement of mitochondrial bioenergetic function.

To test this hypothesis, we inhibited the conversion of PREG into progesterone and dehydroepiandrosterone (DHEA) by incubating the cells with T/A (ie, inhibitors of the 3β-hydroxysteroid dehydrogenase and the cytochrome CYP17A1, respectively). The T/A treatment decreased ATP levels and MMP in the vector, wtTau and P301L cells, indicating that steriodogenesis is important for maintaining ATP synthesis (Figure 5A-C). Surprisingly, in cells treated with the compounds 2a or 2b, the absence of PREG synthesis did not affect the levels of ATP. Similar observations were made when measuring the MMP (Figure 5D-F).

These unexpected data indicated that the effects of our new TSPO ligands, 2a and 2b, on mitochondrial bioenergetic function are not mediated by their action on steriodogenesis but via mechanisms that remain to be determined.

4 | DISCUSSION

Abnormal Tau protein is known to have a negative impact on mitochondrial function. In the present study, we confirmed that ATP production and MMP are impaired in SH-SY5Y cells overexpressing the mutant form of Tau protein (P301L mutant Tau) (Figure 7) and we also showed that PREG synthesis is decreased compared to control cells (vector). Treatment with TSPO ligands, including our new compounds 2a and 2b, not only increased PREG levels in the vector and the wtTau-overexpressing cells, but also normalised the levels of PREG in P301L-overexpressing cells to those of the vector cells. Our new compounds also improve ATP synthesis in the three cell lines and increase the MMP with an efficacy similar to that of the TSPO ligands of reference (XBD173, SSR-180,575 and Ro5-4864) (Figure 7). Although steriodogenesis plays a role in the modulation of mitochondrial bioenergetic function, the action of compounds 2a and 2b on ATP synthesis and MMP does not appear to be link to their ability to increase steroid synthesis in our cellular models. Indeed, the effects of 2a and 2b on ATP production and MMP were still present in the presence of an inhibitor of PREG synthesis or inhibitors of PREG conversion in progesterone and DHEA (Figure 7), suggesting that other pathways are involved.

In the present study, we could reproduce previous data showing that abnormal Tau negatively impact mitochondrial function. In addition, we showed that neurosteroidogenesis (PREG synthesis)
is decreased in the presence of P301L-mutant Tau protein. On the one hand, abnormal Tau inhibits the mitochondrial complex I activity, which probably leads to the decrease in MMP and ATP production. On the other hand, studies showed that disruption of the MMP or ATP synthesis inhibited steroidogenesis. Thus, Tau may impair PREG synthesis by negatively impacting MMP and ATP production.

In a preliminary study, we showed that TSPO ligands are able to influence the mitochondria bioenergetic profile in SH-SY5Y cells. In the present study, we gained new insights about their effects in a cellular model of AD-related tauopathy by showing that TSPO ligands enhance the steroidogenesis in cell lines overexpressing Tau, by boosting PREG production and by increasing the MMP, with an efficacy comparable to the TSPO ligands described in the literature (Ro5-4864, XBD173 and SSR-180,575). Of note, along with Ro5-4864, the new TSPO ligands (2a and 2b) were the only compounds to increase ATP levels in wtTau and P301L cells. Interestingly, in the absence of pregnenolone (PREG) synthesis. (A-C) ATP levels and (D-F) MMP were still increase after 24 hours of treatment with the TSPO ligands 2a and 2b (10 nmol L⁻¹) in the presence of inhibitors of pregnenolone (PREG) metabolism in vector cells (A, D), wtTau cells (B, E) and P301L cells (C, F). *P < 0.05; **P < 0.01; ***P < 0.001; one-way ANOVA and post-hoc Dunnett’s multiple comparison test vs untreated group. **P < 0.05; **P < 0.01; ***P < 0.001; one-way ANOVA and post-hoc Dunnett’s multiple comparison test vs T/A group. T/A, trilostane/abiraterone, inhibitors of the 3β-hydroxysteroid dehydrogenase and the cytochrome CYP17A1, respectively.

**FIGURE 5** 2A and 2B increase ATP levels and mitochondrial membrane potential (MMP) in the absence of steroidogenesis. (A-C) ATP levels and (D-F) MMP were still increase after 24 hours of treatment with the TSPO ligands 2a and 2b (10 nmol L⁻¹) in the presence of inhibitors of pregnenolone (PREG) metabolism in vector cells (A, D), wtTau cells (B, E) and P301L cells (C, F). *P < 0.05; **P < 0.01; ***P < 0.001; one-way ANOVA and post-hoc Dunnett’s multiple comparison test vs untreated group. *P < 0.05; **P < 0.01; ***P < 0.001; one-way ANOVA and post-hoc Dunnett’s multiple comparison test vs untreated group. +P < 0.05; ++P < 0.01; +++P < 0.001; one-way ANOVA and post-hoc Dunnett’s multiple comparison test vs untreated group. T/A, trilostane/abiraterone, inhibitors of the 3β-hydroxysteroid dehydrogenase and the cytochrome CYP17A1, respectively.
FIGURE 7  Schematic representation of the effects of abnormal Tau protein and TSPO ligands on mitochondria. Abnormal Tau protein (TAU) inhibits mitochondrial complex I activity, leading to a decreased mitochondrial respiration, mitochondrial membrane potential (MMP) and ATP production. TSPO ligands increase pregnenolone (PREG) synthesis, MMP and ATP levels. Inhibition of PREG synthesis or PREG conversion to neurosteroids do not prevent the effects of TSPO ligands on ATP levels or MMP, suggesting that TSPO ligands act via other mechanisms, possibly involving the mPTP, 3β-HSD, 3β-hydroxysteroid dehydrogenase; AMG, α-aminogluthetimide; CYP17A1, cytochrome CYP17A1; ETC, electron transport chain; MMP, mitochondrial membrane potential; mPTP, mitochondria permeability transition pore; P450scx, cholesterol side-chain cleavage enzyme; PREG, pregnenolone; TSPO, translocator protein.

TSPO ligands were more efficient with respect to increasing MMP in P301L cells, which presented a stronger deficit in mitochondrial membrane polarisation compared to wtTau cells.

Although containing different chemical structures, all of the TSPO ligands improved the production of pregnenolone at 40 µmol L⁻¹ in vector and wtTau cells and at 20 µmol L⁻¹ in the P301L cells expressing abnormal Tau hyperphosphorylation. Of note, Castellano et al.²⁵ showed that the TSPO ligand PK11195 and its derivatives were able to induce pregnenolone production at 40 µmol L⁻¹ in C6 glioma cells. In line with several analogous studies,²¹⁻²⁵ a high (micromolar) concentration of TSPO ligands is used to detect an increase of steroidogenesis at the cellular level within 2 hours. The common use of TSPO ligand concentrations in the micromolar range following this standard protocol, aiming to confirm the steroidogenic capacity, highlights a recurrent issue concerning TSPO ligands: the lack of correlation between the binding affinity and the steroidogenic efficacy. This concern was specifically addressed by Costa et al.²⁵, who demonstrated that the residence time (ie, the time that a compound spends on its target) is a better parameter for estimating the steroidogenic effectiveness of a TSPO ligand than the binding affinity. Indeed, Costa et al.²⁵ observed a highly significant correlation between steroidogenic efficacy and residence time but not between the steroidogenic efficacy and binding affinity. Of note, when assessing the steroidogenic efficacy of the compound Ro5-4864, Costa et al.²⁵ found an EC₅₀ of 36.2 ± 2.5 µmol L⁻¹, which is in the range of concentration that we used in the present study for assessing pregnenolone synthesis (20 and 40 µmol L⁻¹).

In the same study, the Ro5-4864 binding affinity was estimated at 20.04 ± 2.36 nmol L⁻¹. Interestingly TSPO ligands appear to have specific effects in the nanomolar concentration range. For example, Ro5-4864 and PK 11 195 (another TSPO ligand) were shown to attenuate the tamoxifen-induced apoptosis and decrease of MMP in human breast cancer cell lines (MCF-7 and BT-20) at a concentration of 10 nmol L⁻¹.³⁰ Other TSPO ligands (PIGA) also increased the oxidative metabolism activity/proliferation index in U87MG human glioma cells in the nanomolar range.³¹ In line, we observed that TSPO ligands increased MMP and ATP levels at a low (10 nmol L⁻¹) concentration after 24 hours. We previously obtained similar data in a cellular model of AD (APP transfected SH-SY5Y cells) generating amyloid-β (Aβ) peptide.³²,³³ Indeed, we showed that our new TSPO ligands 2a and 2b boost PREG synthesis, increase mitochondrial respiration and ATP levels, and decrease Aβ levels. Furthermore, 2a and 2b were protective against hydrogen peroxide-induced oxidative stress because they reduced mitochondrial impairments, reactive oxygen species (ROS) levels and cell death.³³ Further experiments would be necessary to understand the discrepancy between the effects of TSPO ligands in the nanomolar concentration range and micromolar concentration range.

In a triple transgenic mouse model of AD (3xTgAD), the TSPO ligand Ro5-4864 reduced Aβ load, neuroinflammation and improved behavioural deficits.³² The neuroprotective effects of these molecules could be attributed to their ability to increase neuroactive steroid synthesis.

Neuroactive steroids, such as PREG, DHEA and allopregnanolone, as well as oestradiol, progesterone and testosterone, are involved in the regulation of brain-specific functions, including neurotransmission, learning and memory, and neuroprotection.¹⁵,³³,³⁴ Treatments with neuroactive steroids as allopregnanolone or progesterone have shown beneficial effects in animal models of AD.¹¹ TSPO ligands have been shown to promote the biosynthesis of allopregnanolone, which is currently undergoing clinical trials as a neuroprotector for treating AD, after showing efficacy in mouse models.¹¹,³⁵ We also showed that allopregnanolone and its analogue BR 297 exerted neuroprotective effects that counteract AD-related bioenergetic deficits.³⁶

Neuroactive steroids have been shown to increase cellular bioenergetics via the improvement of ATP production and mitochondrial respiration as well as the regulation of the redox homeostasis in neuronal cells.²⁷,²⁸ In addition, mitochondrial impairments induced by abnormal protein Tau were reduced after treatment with the neurosteroids progesterone, oestradiol and testosterone.²⁷ Based on these findings, we hypothesised that our new TSPO ligands exert their effects on mitochondrial function via their ability to increase the synthesis of neuroactive steroids. In the model that we used in the present study, we could not confirm this hypothesis because co-treatment with steroidogenesis inhibitors did not suppress the effects of our new TSPO ligands on ATP synthesis and MMP. Of note, suppressing steroid synthesis significantly reduced ATP levels and MMP in the cells, suggesting...
that this process is still important for mitochondrial bioenergetics. Because our new TSPO ligands were still efficient at increasing these parameters without steroidogenesis, other mechanisms may be involved. First, oxidative stress is a marker of tauopathies because higher levels of ROS were detected in the brain of aged Tau transgenic (pR5) mice.\textsuperscript{37-39} In our cellular model, we also measured higher cytosolic and mitochondrial ROS levels in the P301L-Tau transfected cell line compared to the wtTau cells (+7.3% and 10.7% respectively; data not shown). Given the induction of new TSPO ligands to decrease ROS levels under hydrogen peroxide-induced oxidative stress conditions in APP transfected SH-SYSY cells,\textsuperscript{18} the possibility cannot be excluded that TSPO ligands improve mitochondrial function (ie, ATP levels and MMP) via their antioxidant abilities. Second, the mitochondrial permeability transition pore (mPTP) opening is involved in cell death pathway in numerous diseases.\textsuperscript{40} Tau protein may play a role the mPTP opening because Tau ablation inhibited mPTP formation by reducing cyclophilin D protein, which improved mitochondrial bioenergetics in mouse hippocampal cells.\textsuperscript{41} Despite the role of TSPO in the modulation of the mPTP opening being questioned,\textsuperscript{42} there is some evidence indicating the blocking activity of new TSPO ligands against Aβ-induced mPTP opening, thus promoting cell survival.\textsuperscript{43} A recent study showed that novel benzimidazole derivatives, designed as modulators of mitochondrial activity, protect mouse immortalised hippocampal cells from Aβ-induced toxicity in vitro.\textsuperscript{44} One of these compounds was able to reduce Aβ-induced mitochondrial dysfunction in cells by decreasing ROS levels and increasing MMP, cellular viability and ATP production in vitro, and also ameliorated cognitive function in animal models of AD.\textsuperscript{44}

Interestingly, this novel benzimidazole derivative was also developed as an mPTP blocker.\textsuperscript{44} Thus, we can speculate that our new TSPO ligands are also able to modulate the mPTP, which may lead to the increase of MMP. Further investigations need to be conducted to determine the exact underlying mechanisms.

In sum, our strategy was designed to assess the neuroprotective effect of TSPO ligands via the investigation of neurosteroidogenesis and the modulation of the bioenergetic phenotype in cell models of tauopathy. Our findings convincingly demonstrate that our new imidazoquinazolinone TSPO ligands protect against mitochondrial dysfunction in Tau-related pathology, suggesting that these new compounds could be potential new therapeutic tools for the treatment of tauopathies.

**ACKNOWLEDGEMENTS**

The work was supported by a research grant from the Psychiatric University Clinics (UPK research Fonds to AE), the Swiss National Science Foundation (SNF#31003A_149728, to AE) and Synapsis Foundation to AG.

**CONFLICT OF INTERESTS**

The authors declare that they have no conflicts of interest.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**ORCID**

Amandine Grimm [https://orcid.org/0000-0003-3323-1756]
François Hallé [https://orcid.org/0000-0002-5324-8160]
Jürgen Götz [https://orcid.org/0000-0001-8501-7896]
Frederic Biehl [https://orcid.org/0000-0002-4122-0929]
Anne Eckert [https://orcid.org/0000-0002-9341-3669]

**REFERENCES**

1. Papadopoulos V, Lecanu L, Brown RC, Han Z, Yao Z-X. Peripheral-type benzodiazepine receptor in neurosteroid biosynthesis, neuropathology and neurological disorders. Neuroscience. 2006;138:749-756.
2. Rupprecht R, Rammes G, Eser D, et al. Translocator protein (18 kDa) as target for anxiolytics without benzodiazepine-like side effects. Science. 2009;325:490-493.
3. Rupprecht R, Papadopoulos V, Rammes G, et al. Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat Rev Drug Discov. 2010;9:971-988.
4. Porcu P, Barron AM, Frye CA, et al. Neurosteroidogenesis today: novel targets for neuroactive steroid synthesis and action and their relevance for translational research. J Neuroendocrinol. 2016;28:12351.
5. Wang JM, Singh C, Liu L, et al. Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of Alzheimer’s disease. Proc Natl Acad Sci USA. 2010;107:6498-6503.
6. Naylor JC, Kilts JD, Hulette CM, et al. Allopregnanolone levels are reduced in temporal cortex in patients with Alzheimer’s disease compared to cognitively intact control subjects. Biochim Biophys Acta. 2010;1801:951-959.
7. Wang JM, Johnston PB, Ball BG, Brinton RD. The neurosteroid allopregnanolone promotes proliferation of rodent and human neural progenitor cells and regulates cell-cycle gene and protein expression. J Neurosci. 2005;25:4706-4718.
8. Schumacher M, Hussain R, Gago N, Oudinet J-P, Mattern C, Ghomari AM. Progesterone synthesis in the nervous system: implications for myelination and myelin repair. Front Neurosci. 2012;6:10.
9. Chenyou S, Xiaoming OU, Jerry M, F, et al. Allopregnanolone increases the number of dopaminergic neurons in substantia nigra of a triple transgenic mouse model of Alzheimer’s disease. Curr Alzheimer Res. 2012;9:473-480.
10. Irwin RW, Wang JM, Chen S, Brinton RD. Neuroregenerative mechanisms of allopregnanolone in Alzheimer’s disease. Front Endocrinol. 2011;2:117.
11. Brinton RD. Neurosteroids as regenerative agents in the brain: therapeutic implications. Nat Rev Endocrinol. 2013;9:241-250.
12. Rhein V, Song X, Wiesner A, et al. Amyloid- and tau synergistically impair the oxidative phosphorylation system in triple transgenic Alzheimer’s disease mice. Proc Natl Acad Sci. 2009;106:20057-20062.
13. Schulz KL, Eckert A, Rhein V, et al. A new link to mitochondrial impairment in tauopathies. Mol Neurobiol. 2012;46:205-216.
14. Schaeffer V, Patte-Mensah C, Eckert A, Mensah-Nyagan AG. Modulation of neurosteroid production in human
10 of 10

neuroblastoma cells by Alzheimer’s disease key proteins. J Neurobiol. 2006;56:868-881.

15. Grimm A, Lim YA, Mensah-Nyagan AG, Götz J, Eckert A. Alzheimer’s disease, oestrogen and mitochondria: an ambiguous relationship. Mol Neurobiol. 2012;46:151-160.

16. Barron AM, Ji B, Sahara N, Zhang M-R, Suhara T, Higuchi M. Anti-inflammatory effect of tspo ligand in mouse model of tauopathy: Alzheimers Dement. 2017:13:P949.

17. Hallé F, Lejri I, Abarghaz M, et al. Discovery of imidazoquinazolone derivatives as TSPO ligands modulating neurosteroidogenesis and cellular bioenergetics in neuroblastoma cells expressing amyloid precursor protein. ChemistrySelect. 2017;2:6452-6457.

18. Lejri I, Grimm A, Hallé F, et al. TSPO ligands boost mitochondrial function and pregnenolone synthesis. J Alzheimers Dis. 2019:000:1-14. https://doi.org/10.3233/JAD-190127. [Epub ahead of print].

19. Ferrari A, Hoerndli F, Baechi T, Nitsch RM, Götz J. beta-Amyloid induces paired helical filament-like tau filaments in tissue culture. J Biol Chem. 2003;278:40162-40168.

20. Poirier Y, Grimm A, Schmitt K, Eckert A. Link between the unfolded protein response and dysregulation of mitochondrial bioenergetics in Alzheimer’s disease. Cell Mol Life Sci. 2019;76:1419-1431.

21. Da Settimo F, et al. Anxiolytic‐like effects of N, N‐Dialkyl‐2‐phenylindol‐3‐ylglyoxylamides by modulation of translocator protein promoting neurosteroid biosynthesis. J Med Chem. 2008;51:5798-5806.

22. Primofiore GN, et al. N-Dialkyl-2-phenylindol-3-ylglyoxylamides. A new class of potent and selective ligands at the peripheral benzodiazepine receptor. J Med Chem. 2004;47:1852-1855.

23. Campiani G, Nacci V, Fiorini I, et al. Synthesis, biological activity, and sars of pyrrolobenzoxazepine derivatives, a new class of specific “peripheral-type” benzodiazepine receptor ligands. J Med Chem. 1996;39:3435-3450.

24. Selvaraj V, Stocco DM. The changing landscape in translocator protein (TSPO) function. Trends Endocrinol Metab. 2015;26:341-348.

25. Brinton RD, Schneider LS, Law M. allopregnanolone, regenerative therapeutic for Alzheimer’s disease: phase 1b/2a update. Alzheimers Dement. 2017:13:P939-P940.

26. Lejri I, Grimm A, Miesch M, Geoffroy P, Eckert A, Mensah-Nyagan A-G. Allopregnanolone and its analog BR 297 rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement. Biochim Biophys Acta. 2017:1863:631-642.

27. Schmitt K, et al. Insights into mitochondrial dysfunction: aging, amyloid-beta, and tau-A deleterious trio. Antioxid Redox Signal. 2012;16:1456-1466.

28. Karpak S, Hauptmann S, Scherping I, et al. Proteomic and functional analyses reveal a mitochondrial dysfunction in P301L tau transgenic mice. J Biol Chem. 2005;280:23802-23814.

29. Alavi Naini SM, Soussy-Yanicostas N. Tau hyperphosphorylation and oxidative stress, a critical vicious circle in neurodegenerative tauopathies? Oxid Med Cell Longev. 2015:2015:151979.

30. Azarashvili T, Stricker R, Reiser G. The mitochondria permeability transition pore complex in the brain with interacting proteins – promising targets for protection in neurodegenerative diseases. Biol Chem. 2010:391:619-629.

31. Jara C, Aránguiz A, Cerpa W, Tapia-Rojas C, Quintanilla RA. Genetic ablation of tau improves mitochondrial function and cognitive abilities in the hippocampus. Redox Biol. 2018;18:279-294.

32. Barron AM, Garcia-Segura LM, Caruso D, et al. Ligand for translocator protein reverses pathology in a mouse model of Alzheimer’s disease. J Neurosci. 2013;33:8891-8897.

33. Giatti S, Garcia-Segura LM, Barreto GE, Melcangi RC. Neuroactive steroids, neurosteroidogenesis and sex. Prog Neurobiol. 2019;176:1-17.

34. Grimm A, Mensah-Nyagan AG, Eckert A. Alzheimer, mitochondria and gender. Neurosci Biobehav Rev. 2016;67:89-101.

35. Barron AM, Ji B, Sahara N, Zhang M-R, Suhara T, Higuchi M. Anti-inflammatory effect of tspo ligand in mouse model of tauopathy: Alzheimers Dement. 2017:13:P949.

36. Barron AM, Garcia-Segura LM, Caruso D, et al. TSPO PIGA ligands promote neurosteroidogenesis in human astrocyte well-being. Int J Mol Sci. 2016;17:1028.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Grimm A, Lejri I, Hallé F, et al. Mitochondria modulatory effects of new TSPO ligands in a cellular model of tauopathies. J Neuroendocrinol. 2020;32:e12796. https://doi.org/10.1111/jne.12796