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# Translocator protein (TSPO) ligands attenuate mitophagy deficits in the SH-SY5Y cellular model of Alzheimer's disease via the autophagy adaptor P62

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ABSTRACT

Mitochondrial dysfunction has been widely implicated in the pathogenesis of Alzheimer's disease (AD), with accumulation of damaged and dysfunctional mitochondria occurring early in the disease. Mitophagy, which governs mitochondrial turnover and quality control, is impaired in the AD brain, and strategies aimed at enhancing mitophagy have been identified as promising therapeutic targets. The translocator protein (TSPO) is an outer mitochondrial membrane protein that is upregulated in AD, and ligands targeting TSPO have been shown to exert neuroprotective effects in mouse models of AD. However, whether TSPO ligands modulate mitophagy in AD has not been explored. Here, we provide evidence that the TSPO-specific ligands Ro5-4864 and XBD173 attenuate mitophagy deficits and mitochondrial fragmentation in a cellular model of AD overexpressing the human amyloid precursor protein (APP). Ro5-4864 and XBD173 appear to enhance mitophagy via modulation of the autophagic cargo receptor P62/SQSTM1, in the absence of an effect on PARK2, PINK1, or LC3 level. Taken together, these findings indicate that TSPO ligands may be promising therapeutic agents for ameliorating mitophagy deficits in AD.



## 1. Introduction

Alzheimer's Disease (AD) is a devastating neurodegenerative disorder that is associated with a progressive and irreversible loss of cognitive and behavioral function. Pathologically, AD is characterised by the accumulation of two hallmark proteinopathies: extracellular amyloid-β (Aβ) plaques and intraneuronal tau neurofibrillary tangles (NFTs). In addition, mitochondrial dysfunction has been identified as an early feature of the neurodegenerative cascade, and is thought to play a causative role in the pathogenesis of AD [1]. Mitochondria are conserved organelles that supply energy in the form of adenosine triphosphate (ATP), which fuels various cellular processes required for neuronal homeostasis and survival, including synaptic transmission, calcium homeostasis, redox signaling, synaptic plasticity, and mitochondrial transport by motor proteins [2,3]. In addition, mitochondrial energy production has been linked to microglial inflammatory functions, such as phagocytosis, which play a critical role in removing Aβ in the AD brain [4]. However, mitochondrial quality is reducedwith aging and age-related diseases, such as AD. For instance, AD patients have been shown to exhibit an age-dependent decrease in ATP production [5,6], irregular mitochondrial morphology, and accumulation of damaged and dysfunctional mitochondria [7]. These damaged mitochondria are thought to trigger a range of neurotoxic effects in the brain, including elevated cytoplasmic calcium ($Ca^{2+}$) levels, increased reactive oxygen species (ROS) and the release of mitochondrial DNA (mtDNA), which are known to cause neuronal death [8,9].

Mitophagy is the major cellular mechanism responsible for maintaining mitochondrial quality and turnover, in which damaged mitochondria are sequestered into lysosomes where they undergo a process of degradation and recycling which is mediated via the PTEN-induced kinase 1 (PINK1)-Parkin pathway [10]. However,

*Abbreviations:* TSPO, Translocator Protein; AD, Alzheimer's Disease; Aβ, amyloid-β; APP, Amyloid Precursor Protein.

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impaired mitophagy has been identified as a hallmark feature of AD [7,11]. Both Aβ and tau have been shown to play a causative role in impairing mitophagy by inhibiting Parkin recruitment to mitochondria [12,13]. In addition, mitophagy enhancement through $NAD^+$ supplementation, urolithin A, and actinodin treatment decreased $A\beta_{1-42}$ levels, decreased tau hyperphosphorylation, and reversed memory impairments in pre-clinical AD models [11]. Thus, pharmacologic strategies aimed at enhancing mitophagy have been identified as promising therapeutic targets for the treatment of AD [11,14,15].

The translocator protein (TSPO) is an outer mitochondrial membrane (OMM) protein that is upregulated in AD [16], and TSPO has been shown to regulate a number of mitochondrial functions, including $Ca^{2+}$ signalling, ATP and ROS production, and inflammation [16–19]. Recently, ligands targeting TSPO have been shown to exert neuroprotective effects in AD by increasing phagocytic uptake of Aβ and decreasing Aβ accumulation [16,20–22], reducing brain atrophy [23], and reducing Aβ-induced mitochondrial dysfunction and oxidative stress [24]. Intriguingly, TSPO has been shown to form a complex with the voltage dependent anion channel (VDAC) [25,26], which is implicated in PINK1/Parkin-directed mitophagic clearance, via Parkin-mediated Lys 27 poly-ubiquitylation [27]. Moreover, studies using genetic knockdown or overexpression of TSPO have shown that TSPO modulates mitophagy in mouse fibroblasts, human mononuclear cells, and mouse models of depression [18,28,29]. However, whether TSPO ligands exert protective effects in modulating mitophagy in AD is unknown.

Therefore, the present study aimed to investigate the therapeutic potential of two TSPO ligands (Ro5-4864 and XBD173) on impaired mitophagy in AD. We found that SHSY5Y neuroblastoma cells overexpressing amyloid precursor protein (APP), which leads to a three-to four-fold increase in Aβ generation [30,31], exhibit decreased mitophagy compared to cells expressing the empty vector alone, following stimulation with the mitochondrial uncoupler FCCP. Both Ro5-4864 and XBD173 attenuated mitophagy deficits in APP-overexpressing cells, which appears to be modulated via P62/SQSTM1 protein.

## 2. Material and methods

### 2.1. Cell culture and Transfection

Human SH-SY5Y neuroblastoma cells were stably transfected with DNA constructs harboring the entire coding region of human wild-type APP (APP695, APP cells), or the vector alone (pCEP4, control cells: Mock) and used as described previously [32]. Stably transfected cell clones were selected with hygromycin (300 μg/ml). Cells were maintained in complete DMEM (10 % fetal calf serum (FCS), 5 % horse serum (HS), 1 % penicillin-streptomycin, and 1 % Glutamax) at 37 °C in 7.5 % $CO_2$. The cells were kept in culture in 10 $cm^2$ dishes, split twice a week and plated when they reached around 80 % confluence, 1 day prior treatment.

### 2.2. Treatment paradigm

Autophagy and mitophagy were assessed using cells transiently transfected with DsRed-LC3 for 24h. Cells were incubated with 10 nM of XBD173, Ro5-4864, or vehicle (DMSO, <0.002 %) for 24 h. This concentration has been widely used to yield neuroprotective effects in vitro [16–19]. Autophagic flux was assessed using SH-SY5Y cells stimulated with bafilomycin A1 (100 nM) or vehicle (DMSO) for 4h prior to fixation with 4 % paraformaldehyde (PFA). Mitophagy was assessed usingSH-SY5Y cells were treated overnight with carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP, 10 μM), followed by incubation with Mitotracker Deep Red FM (Thermo Fisher, M22426, 100 nM) for 40 min.

### 2.3. Microscopy and image analysis

Data were imaged in a single session using an inverted microscope (Leica Microsystems TCS SPE DMI4000) with the same imaging settings. Image analyses were performed using ImageJ software. Autophagy was quantified using z-stacks imaged at 40x resolution to capture multiple cells across the coverslip. Regions of interest (ROIs) were drawn around single cells in ImageJ, selected in the 568 nm channel containing the LC3 signal. The fluorescence intensity of DsRed-LC3 was measured above a set threshold to exclude background pixel values. Mitophagy was quantified by measuring both DsRed-LC3 and Mitotracker signals above a set threshold to exclude background pixel values, and the percentage of LC3 puncta that colocalize with mitochondria was calculated for each cell. To quantify the mitochondrial morphology, we utilized a computer-based analysis that calculated form factor (FF; mitochondrial shape) and aspect ratio (AR; mitochondrial length) using the Image J program.

### 2.4. Real-time quantitative PCR

Quantitative PCR (qPCR) was performed by isolating total cellular RNA using the RNeasy Mini kit (Qiagen, 74104). cDNA synthesis was performedusing the GoScript™ Reverse Transcription Kit (Promega, A2791). Only RNA samples with 260/280 nm of 2.0 ± 0.2 were used. Real-time PCR was performed using the GoTaq® qPCR kit (Promega, A6002). The sequence of primers is detailed in Table 1. Gene expression was measured as fold change and was evaluated by 2−ΔCT method. The data are represented as relative mRNA expression normalized to human GAPDH mRNA expression [33].

### 2.5. Statistical analysis

GraphPad Prism 8.2.1 (GraphPad Software) was used for statistical analyses and generation of plots. Levinès and Shapiro-Wilk tests were used to assess homoscedasticity and normality. For comparisons of more than two groups, One- or Two-Way ANOVA was used. Significant factors or interactions were further explored using Tukey's post-hoc comparisons.

## 3. Results

### 3.1. TSPO ligands attenuate mitophagy deficits in APP-overexpressing cells

Impaired mitophagy has been observed in both animal and cellular models of Alzheimer's disease [7,11]. We therefore sought to investigate the effect of TSPO ligands on AD-associated mitophagy dysfunction. To measure the levels of basal and FCCP-induced mitophagy, the colocalization of LC3 puncta with mitochondria was assessed in SH-SY5Y neuroblastoma cells transfected with LC3-DsRed and then stained with mitotracker. FCCP treatment markedly increased mitophagy levels in both Mock and APP-expressing cells (Two-way ANOVA; Main Effect (FCCP): $F_{(1, 394)} = 1690$, $p < 0.0001$), and a significant interaction effect of FCCP and TSPO treatment was observed (Interaction Effect (FCCP x TSPO Treatment): $F_{(3, 394)} = 17.43$, $p < 0.0001$). Specifically, in basal conditions, no significant effect of APP expression on cellular mitophagy was observed. However, following FCCP treatment, APP cells exhibited significantly reduced mitophagy compared to Mock cells (Pairwise Comparison (Mock + Veh + FCCP vs APP + Veh + FCCP): $p < 0.001$) (Fig. 1A and B). Intriguingly, both

TSPO ligands (Ro5-4864 and XBD173) significantly attenuated mitophagy deficits following FCCP treatment in APP cells compared to cells treated with vehicle alone (APP + Veh + FCCP vs APP + Ro5+FCCP): $p < 0.001$; (APP + Veh + FCCP vs APP + XBD + FCCP): $p < 0.001$; Fig. 1A and B).

### 3.2. No effect of APP or TSPO ligands on autophagic flux in cellular models of AD

To address the possibility that observed differences in mitophagy were due to a general effect on autophagy, we assessed autophagic flux. Cells were treated with TSPO ligands Ro5-4864 or XBD173, followed by the autophagosome-lysosome fusion inhibitor Bafilomycin A1 (BAF, 100 nM) or DMSO alone. A significant effect of BAFA1 treatment on LC3 puncta was observed (Two-way ANOVA; Main Effect (BAF): $F_{(1, 329)} = 301.3$, $p < 0.0001$). APP-overexpressing cells increased LC3 puncta levels to the same extent as Mock cells, and no effect of either RO5-4864 or XBD173 treatment on autophagic flux was observed (all $p > 0.3097$; Fig. 2A and B), suggesting that TSPO ligands specifically modulate selective autophagy of damaged mitochondria.

### 3.3. TSPO ligands increase P62/SQSTM1 expression in cellular models of AD

To corroborate these findings, gene expression levels of proteins involved in the mitophagy pathway were assessed by RT-qPCR. The mitophagy protein parkin (PARK2), was significantly decreased in APP-expressing cells compared to Mock cells following FCCP stimulation (One-way ANOVA: (Mock + Veh vs APP + Veh): $p = 0.035$; Fig. 3A). Similarly, a trend towards decreased PTEN Induced Kinase 1 (PINK1) and LC3 was observed in APP cells following FCCP stimulation, but this did not reach significance (all $p > 0.2894$, Fig. 3B and C). However, no effect of Ro5-4864 or XBD173 on PARK2, PINK1, or LC3 levels was observed (all $p > 0.2497$). P62/SQSTM1 levels were significantly reduced in APP cells compared to Mock cells following FCCP stimulation (One-way ANOVA: (Mock + Veh vs APP + Veh): $p = 0.0007$). However, TSPO ligands significantly increased P62/SQSTM1 in APP cells compared to cells treated with vehicle alone (APP + Veh vs APP + Ro5): $p = 0.0345$; APP + Veh vs APP + XBD): $p = 0.0015$; Fig. 3D).

**Table 1**
Primer sequences used for RTqPCR analysis.

| PRIMER | SEQUENCES |
|---|---|
| PARK2 | F-5′-GGA AGT CCA GCA GGT AGA-3′<br>R-5′-ATC CCA GCA AGA TGG ACC-3′ |
| PINK1 | F-5′-CCA TCA AGA TGA TGT GGA ACA-3′<br>R-5′-GAC CTC TCT TGG ATT TTC TGT AA-3′ |
| LC3 | F-5′-CTC AGA CCG GCC TTT CAA-3′<br>R-5′-CGA TGA TCA CCG GGA TTT TG-3′ |
| P62/SQSTM1 | F-5′-CGG CAG AAT CAG CCT CTG-3′<br>R-5′-GTC AGG CGG CTT CTT TTC-3′ |
| MFN1 | F-5′-CAG TCA CCA AGT AAA ACA ACA AA-3′<br>R-5′-GGG TAA TCT AGC AAT TTC TTC TTC-3′ |
| MFN2 | F-5′-CAG GAT TCA GGA AGC CCA G-3′<br>R-5′-GAT GCA CTC CTC AAA TCT CC-3′ |
| OPA1 | F-5′-CCA GGT GTG ATT AAT ACT GTG A-3′<br>R-5′-CCA TCT TGA ATA CAC AGT ATG ATG-3′ |
| FIS1 | F-5′-GCT CAA GGA ATA CGA GAA GG-3′<br>R-5′-AGT CCA TCT TTC TTC ATG GC-3′ |
| DRP1 | F-5′-TCA CGA GAC AAG TCT TCT AAA G-3′<br>R-5′-CCT CCA GAT GCA ACC TTG-3′ |
| GAPDH | F-5′-CAT GGT TTA CAT GTT CCA ATA TGA-3′<br>R-5′-GGA TCT CGC TCC TGG AAG-3′ |

*Abbreviations*: *PARK2*, Parkin; *PINK-1*, PTEN induced putative kinase 1; *LC3*, Microtubule-associated protein light chain 3; *P62/SQSTM1*, Sequestosome-1; *MFN1*, Mitofusin 1; *MFN2*, Mitofusin 2; OPA1, OPA1 Mitochondrial Dynamin Like GTPase; *FIS1*, Mitochondrial fission 1 protein; *DRP1*, Dynamin-1-like protein; *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase; *qRT-PCR*, quantitative real-time polymerase chain reaction.

### 3.4. TSPO ligands alter mitochondrial network connectivity but not pro-fission and -fusion genes

Mitochondrial dynamics, including the expression of fission and fusion-related proteins, have been shown to modulate mitophagy [34]. We thus investigated the effect of TSPO ligands on mitochondrial dynamics in APP-overexpressing cells. Mitochondrial morphology was quantified using a computer-based analysis that calculated Form Factor (mitochondrial shape) and length using Image J software. Consistent with previous studies [11,35–37]APP-overexpressing cells exhibited significantly more fragmented, punctate mitochondria compared to healthy controls, as evidenced by decreased Form Factor (One way ANOVA; $p < 0.0001$; Fig. 4A) and decreased Length (One way ANOVA; $p < 0.0001$; Fig. 4B). These parameters were significantly rescued following stimulation with TSPO ligands (APP + Veh vs APP + Ro5: $p = 0.0044$; (APP + Veh v APP + XBD): $p = 0.0181$). Given the alterations in mitochondrial morphology observed, we further corroborated the effect of APP overexpression on mitochondrial fission and fusion-related proteins (MFN1, MFN2, OPA1, DRP1). RT-qPCR analysis revealed a significant increase in OPA1 levels following XBD173 treatment in APP cells (One way ANOVA; F [3,27] = 3.247 $p = 0.0373$; Pairwise Comparison (APP + Veh vs APP + XBD): $p = 0.0174$), and a similar trend was observed in Ro5-treated cells, but this did not reach significance ($p = 0.055$; Fig. 4 D). DRP1 levels were significantly decreased in APP-overexpressing cells compared to Mock cells (One way ANOVA; F [3,20] = 4.136 $p = 0.0196$; Pairwise Comparison (Mock + Veh v APP + Veh): $p = 0.0071$), but no effect of TSPO ligands was observed (Fig. 4E). No effect of either APP overexpression or TSPO ligand treatment was observed on expression of MFN1, MFN2, and FIS1 (all $p >$ Fig. 4F–H).

## 4. Discussion

Decreased mitophagy capacity has been identified as an early feature of AD, leading to the accumulation of damaged or dysfunctional mitochondria [7,11]. Recent studies conducted in post-mortem tissues from AD patients, as well as cellular and animal models of AD, have revealed mitophagy defects linked to Aβ and tau [11,12]. Intriguingly, studies using genetic or pharmacologic enhancement of mitophagy in AD have reported decreased amyloid load and increased cognitive capacity, suggesting that mitophagy enhancement is a promising therapeutic strategy in AD [11,15,38,39]. Here, we provide evidence that the TSPO-specific ligands Ro5-4864 and XBD173 attenuate mitophagy deficits in cellular models of AD overexpressing APP.

In the current study, we observed a marked decrease in mitophagy levels in APP-overexpressing cells compared to Mock cells following FCCP stimulation, with mitophagy levels reduced by 35–40 %. The magnitude of this effect is comparable to that observed in animal models of AD, in which hippocampal mitophagy levels were 30–50 % lower than healthy controls [11]. We then tested the effect of TSPO ligand treatment on mitophagy levels in these cells using the ligands Ro5-4864 or XBD173 and found that both TSPO ligands significantly attenuated mitophagy deficits in APP overexpressing cells, returning mitophagy levels to normal. In line with this, previous studies using TSPO overexpression or deletion models have shown that TSPO modulates mitophagy via its interaction with VDAC [18,28]. However, this is the first study to date showing that TSPO ligands attenuate mitophagy deficits in the context of AD-related proteinopathy. This effect did not appear to be due to a general increase in autophagy, as APP overexpressing

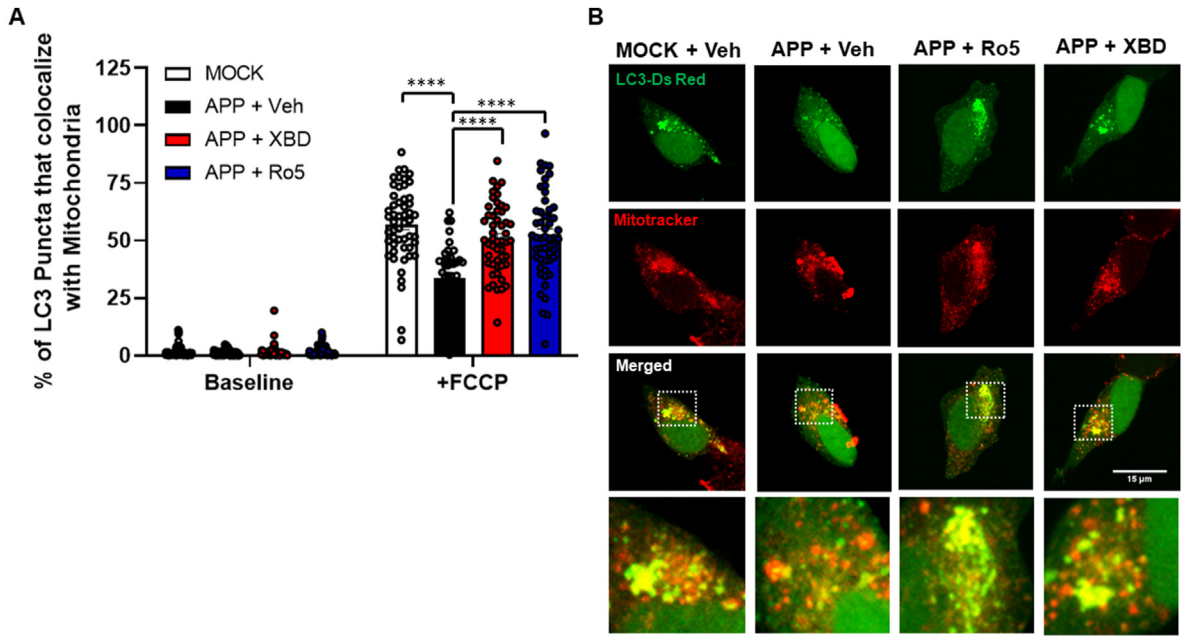


**Fig. 1. TSPO ligands attenuate mitophagy deficits in cellular models of AD.** APP or Mock cells expressing LC3-DsRed and stained with mitotracker were treated with TSPO ligands (10 nM, 24 h Ro5-4864 or XBD173) or vehicle (DMSO >0.002 %), followed by FCCP (10 μM, overnight). (**A**) Quantification of the percentage of LC3 puncta that colocalize with mitochondria. (**B**) Representative micrographs showing colocalization of LC3 (in green) and mitotracker (in red). Data were analysed by two-way ANOVA with Tukey's multiple comparisons test. Values are the mean ± SEM (n = 42–54 cells/group)****$p < 0.0001$.

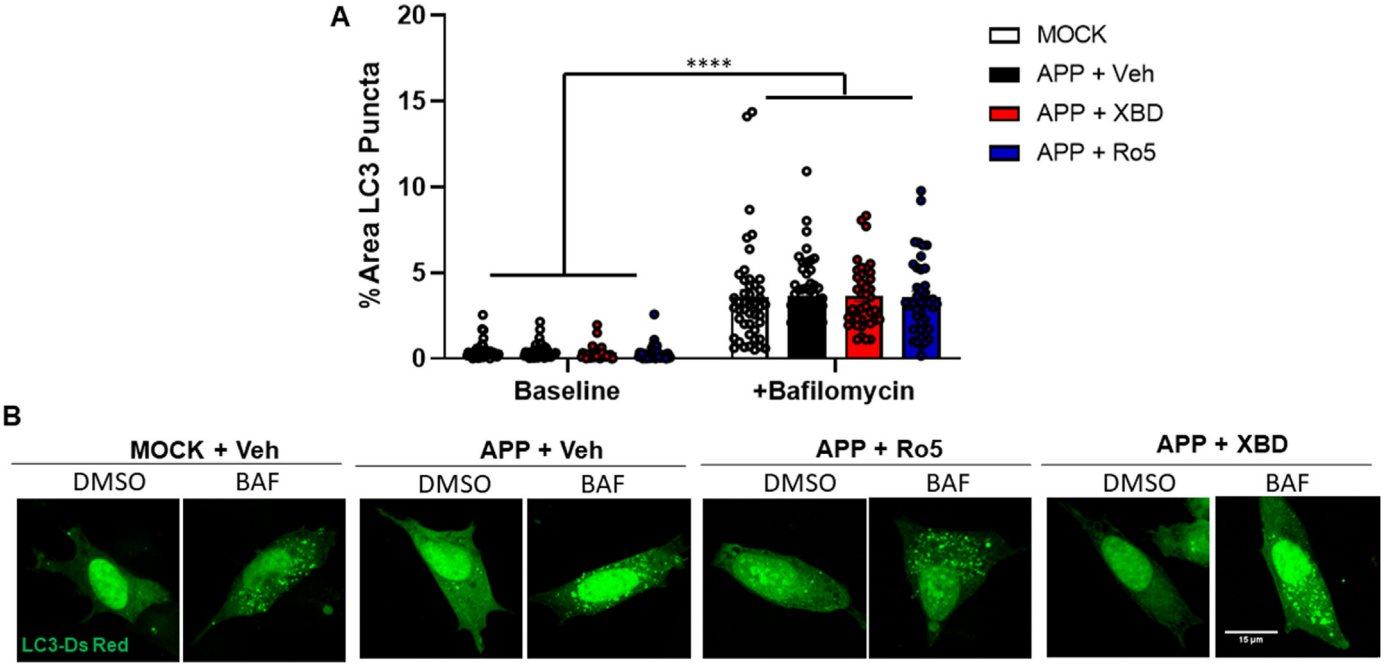


**Fig. 2. No effect of APP or TSPO ligands on autophagic flux.** APP or Mock cells expressing LC3-DsRed were treated with TSPO ligands (10 nM, 24 h Ro5-4864 or XBD173) or vehicle (DMSO >0.002 %), followed by stimulation with BAF (100 nM, 4h). **(A)** Quantification of the percentage area per cell of LC3-DsRed. **(B)** Representative micrographs of LC3 expression. Data were analysed by two-way ANOVA with Tukey's multiple comparisons test. Values are the mean ± SEM (n = 42–54 cells/group) ****$p < 0.0001$.

cells did not exhibit impairments in autophagic flux following bafilomycin A1 treatment, and no effect of TSPO ligands on autophagic flux was observed. Of note, in this study, mitophagy was assessed by measuring colocalization between LC3 and mitochondria, however future studies could further verify this finding using alternate measures of mitophagy such as LC3 lipidation or monitoring the degradation of mitochondrial proteins.

Previous studies have shown that Aβ interacts with the cytosolic protein PARK2 to disrupt mitophagy [40]. Consistent with this, using RT-qPCR we observed a marked decrease in gene expression levels of PARK2 in APP cells following FCCP stimulation. However, neither Ro5-4864 nor XBD173 modulated expression of PARK2, and neither APP nor TSPO ligands modulated the expression of other mitophagy-related genes PINK1 and LC3, suggesting that TSPO

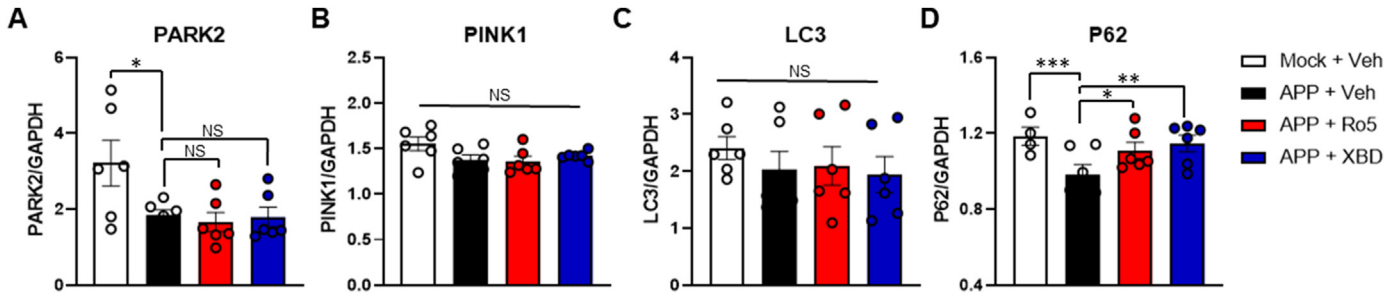


**Fig. 3. TSPO ligands increase P62/SQSTM1 expression in cellular models of AD.** RTqPCR analysis of fold-change in mRNA expression of genes encoding PARK2 **(A),** PINK1 **(B)**, LC3 **(C)**, and P62 **(D)** in APP and Mock cells stimulated with FCCP. Data were analysed by One-way ANOVA with Tukey's multiple comparisons test. Values are the mean ± SEM (n = 42–54 cells/group)*$p < 0.05$, **p < 0.01, ***$p < 0.001$.

modulates mitophagy via a different mechanism. Consequently, we measured gene expression levels of P62/SQSTM1, a protein that has previously been shown to promote ubiquitination of outer mitochondrial membrane (OMM) proteins independently of PARK2 and PINK1 during mitophagy [41], and is also upregulated following TSPO deletion [18]. Consistent with previous studies [42], we observed a marked reduction in P62 expression in APP cells. However, following treatment with either Ro5-4864 or XBD173, a significant increase in P62 expression was observed, suggesting a potential mechanism through which TSPO ligands increase mitophagy. In addition to binding LC3 on the phagophore, P62 is known to bind to diverse ubiquitylated proteins for autophagic clearance, including the COOH-terminal fragment of APP [43]. P62 is known to be sequestered in the cytosol by both Aβ and tau, which has been hypothesized to decrease the availability and physiological function of P62 in autophagic clearance in AD [44]. In line with this, previous studies have shown that increased p62 exerted neuroprotective effects and reduced the number of senile plaques [45,46] and was negatively associated with Aβ deposition in preclinical models of AD [44]. These findings suggest that TSPO-mediated increases in P62 levels could facilitate Aβ removal via autophagy activation, which may in turn alleviate Aβ-induced mitophagy deficits or freely available cytosolic P62 for the clearance of dysfunctional mitochondria. Future studies investigating the effects of TSPO ligands on P62 localization, protein expression, and autophagic removal of Aβ in APP-overexpressing cells could help elucidate this potential mechanism. However, we cannot exclude the possibility that the mitophagy-enhancing effect of TSPO ligand treatment may have been mediated through non-P62-related mechanisms since recent studies have proposed that a number of alternate receptors, including NBR1, NDP52, Tax1BP1, and OPTN play crucial roles in the recognition of autophagic cargo [47]. In addition, we did not investigate PINK1/Parkin-independent mitophagy pathways, which may also play a contributing role.

Mitochondrial dynamics refers to the process of fission and fusion that determines mitochondrial shape, quantity, and quality, and has also been shown to regulate mitophagy [34]. Because Aβ has been shown to disrupt mitochondrial fission and fusion proteins in animal and cellular models of AD [13,35], we hypothesized that TSPO ligands may indirectly enhance mitophagy via

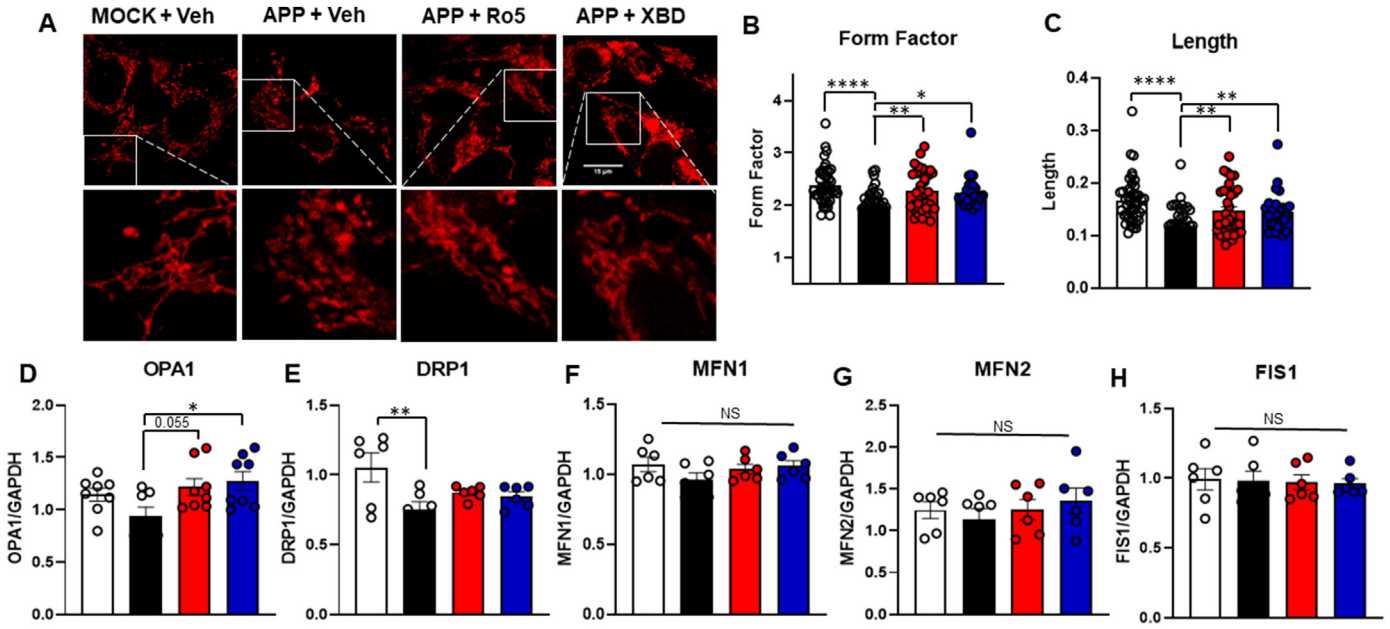


**Fig. 4.** TSPO ligands alter mitochondrial dynamics in APP-expressing cells. Representative micrographs of SH-SY5Y cells stably transfected with APP or an empty vector control (Mock), stained with mitotracker **(A).** Quantification of mitochondrial Form Factor **(B)** and Length in **(C)**. RTqPCR analysis of fold-change in mRNA expression of genes encoding OPA1 **(D)**, DRP1 **(E)**, MFN1 **(F)**, MFN2 **(G)**, and FIS1 **(H)**. Data were analysed by one-way ANOVA with Tukey's multiple comparisons test. Values are the mean ± SEM (n = 42–54 cells/group)*$p < 0.05$, **p < 0.01, ****$p < 0.0001$. Abbreviations: OPA1: Optic atrophy gene 1; DRP1: Dynamin-1-like protein; MFN1: Mitofusin-1; MFN2: Mitofusin-2; FIS1: Fission 1 homologue.

modulating mitochondrial dynamics. Consistent with previous studies [11,35–37], APP-overexpressing cells exhibited significantly more fragmented, punctate mitochondria compared to healthy controls, as evidenced by decreased Form Factor and Length. These parameters were significantly rescued following stimulation with TSPO ligands. This is in line with previous studies that showed another TSPO ligand PK11195 attenuated changes to mitochondrial ultrastructure following TNF-α exposure [48]. Moreover, genetic knock down of VDAC has also been shown to induce changes in mitochondrial shape and alters mitochondrial contact sites [49]. VDAC is a well-documented TSPO binding partner and Ro5-4864 binds to the TSPO/VDAC interphase, suggesting a potential mechanism through which TSPO ligands may alter mitochondrial dynamics. Intriguingly, despite observing an effect of TSPO ligands on Form Factor and Length in APP overexpressing cells, we did not observe a significant effect of TSPO ligands on pro-fission or -fusion gene expression. Other studies using TSPO deletion have reported similar effects on measurements of mitochondrial morphology, in which TSPO deletion altered mitochondrial aspect ratio and form factor but had no effect on the expression of pro-fission genes [18]. Future studies may shed light on the mechanism through which TSPO modulates mitochondrial morphology.

Aβ accumulates within the cytosol of neurons and microglia, where it binds to mitochondria, impairing mitochondrial bioenergetics and function [50–52]. Intriguingly, enhancement of mitophagy, including strategies that specifically target P62/SQSTM1 expression [53], have been shown to play a protective role in the degradation of Aβ proteins [11,39,54]. Similarly, previous studies have shown that treatment with TSPO ligands reduces Aβ accumulation [20–22], however Aβ levels were not directly assessed in the current study. Future studies aimed at assessing whether TSPO-mediated reductions in Aβ levels are modulated via mitophagy enhancement could provide further insight into the causal relationship between these phenomena. Furthermore, future studies could use TSPO knockout models to address the specific effect of TSPO ligands on Aβ and mitophagy, which may provide more insight into mechanism of neuroprotection.

In addition to their anti-amyloidogenic effects, TSPO ligands have been shown to exert protective effects on a number of mitochondrial functions in AD models, by reducing oxidative stress, decreasing ROS levels, and increasing ATP levels and mitochondrial membrane potential [22,24], however the mechanism underlying these effects is not known. Intriguingly, efficient removal of dysfunctional mitochondria via mitophagy has been shown to be crucial in modulating mitochondrial bioenergetics, ROS levels, and MMP, suggesting that mitophagy enhancement may underlie a wide range of mitochondria modulatory effects [54–56]. However, whether mitophagy enhancement plays a causal role in other measures of TSPO-mediated mitochondrial dysfunction remains to be tested.

## 5. Conclusion

Here we report the first evidence to date that TSPO ligands attenuate mitophagy deficits in cellular models of AD overexpressing APP. The increase in P62/SQSTM1 gene expression following TSPO treatment suggests a potential mechanism by which TSPO ligands reduce mitophagy impairments in AD. While mitophagy enhancement has previously been identified as a promising therapeutic strategy for the treatment of AD, our findings suggest that TSPO ligands are effective agents in modulating mitophagy deficits in response to Aβ.

## Author contributions

LHF, AG, and AE conceived the research; LHF and AG collected the data; LHF, AG, and SAH analysed the data; LHF, AG, and SAH drafted the manuscript. All authors edited and approve the manuscript.

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## Declaration of competing interest

None.

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