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TSPO ligand PK11195 improves Alzheimer-related outcomes in aged female 3xTg-AD mice

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Abstract

Alzheimer's disease (AD) pathogenesis is a multifactorial process that involves numerous pathways within the central nervous system. Thus, interventions that interact with several disease-related pathways may offer an increased opportunity for successful prevention and treatment of AD. Translocator protein 18 kD (TSPO) is a mitochondrial protein that is associated with regulation of many cellular processes including inflammation, steroid synthesis, apoptosis, and mitochondrial respiration. Although TSPO ligands have been shown to be protective in several neurodegenerative paradigms, little work has been done to assess their potential as treatments for AD. Female 3xTg-AD mice were administered the TSPO ligand PK11195 once weekly for 5 weeks beginning at an age 16 months, an age characterized by extensive β -amyloid pathology and behavioral impairments. Animals treated with PK11195 showed improvements in behavior and modest reductions of in both soluble and deposited β -amyloid. The finding that short-term PK11195 treatment was effective in improving both behavioral and pathological outcomes in a model of late-stage AD supports further investigation of TSPO ligands as potential therapeutics for the treatment of AD.

Keywords

Aging; Alzheimer's; β -Amyloid; Neuroinflammation; TSPO

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder with both genetic and environmental risk factors. These risk factors can perturb a variety of molecular pathways to lead to the hallmarks of AD neuropathology: β -amyloid ($A\beta$) deposition and neurofibrillary tangles. Aberrant protein trafficking and aggregation, altered inflammatory responses,

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Authors' contributions

AC and CJP designed the study. All experimental work and analyses were performed by AC. The manuscript was written and approved by AC and CJP.

Competing interests

The authors declare no competing interests with this study.

oxidative stress, mitochondrial dysfunction, metabolic disruption, and several other pathways have been implicated in contributing to the development and progression of AD (1, 2). The complexity of AD pathogenesis has likely contributed to the failure of highly specific interventions to effectively impede disease progression in clinical trials (3). An alternative therapeutic strategy that may overcome limitations of mono-therapy is the use of interventions with pleiotropic effects that are capable of targeting several AD-relevant pathways.

Translocator protein 18 kD (TSPO), previously known as the peripheral benzodiazepine receptor, is a protein located in the outer mitochondrial membrane that interacts with several other mitochondrial proteins. In addition to an apparent role in steroidogenesis, TSPO has been shown to have a number of other roles including regulation of mitochondrial function, apoptosis, and inflammation (4). In the brain, TSPO is expressed most highly in glial cells, particularly microglia (5). Because TSPO expression is upregulated in reactive glia, it has been used as an *in vivo* PET imaging marker of neuroinflammation (6). Its roles in inflammation, neurosteroidogenesis, and oxidative stress make it a prime candidate for therapies targeting neurodegenerative and neuroinflammatory diseases, including AD (7, 8).

Previous work in male mice has shown that TSPO ligands may be a potential therapeutic intervention to slow the progression or even reverse AD neuropathology (9). Barron et al. showed that the TSPO ligand, 4'-chlorodiazepam (Ro5-4864), could attenuate development of AD pathology in young castrated 3xTg-AD males as well as reduce AD-related pathology in aged male mice. Further, this work demonstrated that Ro5-4864 both increased levels of protective neurosteroids and reduced gliosis, results that suggest possible protective mechanisms. Like Ro5-4864, the TSPO ligand isoquinoline carboxamide (PK11195) previously has been shown to decrease microglial activation, inflammation, and cognitive deficits in response to several insults, including lipopolysaccharide (LPS) (10, 11). In male C57Bl/6 mice, PK11195 was associated with a decrease in soluble A β following chronic LPS treatment (11).

Because of numerous significant sex differences in AD including its disproportional prevalence in women, potential therapies must be evaluated in females as well as males. Recent work in aged female AD mouse models indicate that estradiol-based hormone treatments are ineffective in reducing AD-related neuropathology in aged female 3xTg-AD mice (12, 13), though such treatments are beneficial in young adult and middle-aged female AD mice (13–15). Unknown is whether aged female AD mice might benefit from TSPO ligands.

Here, the effects of short-term administration of the TSPO ligand PK11195 were investigated in aged female 3xTg-AD mice to model how this intervention might affect AD in later stages of the disease. The data show that both pathological and behavioral outcomes were improved in animals treated with PK11195. This evidence supports further investigation of different classes of TSPO ligands as therapeutics for AD.

2. Materials and Methods

2.1 Animals and treatments

3xTg-AD mice were maintained in a colony at University of Southern California. Sixteen month-old female mice were injected intraperitoneally once per week for 5 consecutive weeks with either vehicle (1% DMSO in safflower oil; N=6) or 3 mg/kg PK11195 (Tocris, Minneapolis, MN) (N=7), a dose demonstrated to induce neural effects (11). PK11195 was initially solubilized in pure DMSO then diluted in safflower oil to a final concentration of 1% DMSO. Animals were euthanized 24 hours after the fifth injection and brains were rapidly harvested. One hemibrain was immersion fixed in 4% paraformaldehyde for 48 hours, then stored at 4°C in 0.1M phosphate buffered saline with 0.03% sodium azide until immunohistochemical processing. The hippocampus was dissected from the second hemibrain, snap frozen, and stored at -80°C until use in PCR analyses. The rest of the brain was frozen and stored at -80°C until A β extractions. All procedures were conducted in accordance with the guidelines set forth by the USC Institution for Animal Care and Use Committee and under the supervision of USC veterinarians.

2.2 Behavior

Mice were assessed on spontaneous alternation behavior in the Y-maze, a test of attention towards novelty and spatial memory (16) that is dependent upon limbic system structures including the hippocampus (17), as previously described (13). In brief, mice were acclimated to the behavior room 30 minutes prior to the start of testing. Twenty-four hours after the fourth PK11195 injection, mice were placed in the long arm of a Y-maze and given 5 minutes to explore. Both number of total arm visits and number of correct alternations were scored for each animal. A correct alternation consisted of visits (2 paws in the arm) to three different arms in a row. Twenty-four hours later, animals were tested for anxiety-like behavior using the elevated plus maze, as previously described (18). Using standard approaches (19), mice were placed in the middle of the maze facing one of the closed arms and allowed to explore the maze for 5 minutes. Time spent in the open arms was measured.

2.3 Immunohistochemistry

Fixed hemibrains were completely sectioned in the horizontal plane at 40 μ m using a vibratome (Leica Biosystems, Buffalo Grove, IL). Sections were stored in PBS with 0.03% sodium azide at 4°C until immunohistochemistry was performed as previously described (13). Every eighth section (from a total of ~100) was immunostained for A β . In brief, tissue sections containing hippocampus were pretreated with 95% formic acid for 5 min, then washed 3 times for 5 min in TBS, followed by a 10 min rinse with an endogenous peroxidase blocking solution. Next, sections were rinsed in TBS/0.1% Triton-X before being blocked for 30 min in TBS with 2% BSA. Sections were incubated overnight at 4°C with anti-A β antibody (1:300; Life Technologies, Grand Island, NY; Cat #71-5800) diluted in the block solution. Sections incubated in primary antibody were washed and incubated with secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hour and processed for diaminobenzidine staining using Vectastain ABC Elite kit (Vector). Stained sections were air-dried overnight, dehydrated, then coverslipped with Krystalon (EMD Millipore, Billerica, MA). Our prior work has demonstrated that this A β immunostaining protocol

recognizes A β in 3xTg-AD mice but neither amyloid precursor protein nor its carboxyl-terminal fragments (15). Immunostaining for hyper-phosphorylated tau using phospho-tau antibody AT8 (1:750; Thermo, Waltham, MA) was performed as described for A β staining, but without the formic acid wash.

Digital images of A β staining in the hippocampus were taken using an Olympus BX-50 microscope fitted with an Olympus DP72 camera. Brightfield images were taken with a 20X objective. Images were imported to ImageJ 1.50b and converted to grayscale. These images were then measured for percent immunoreactive area, a measure of A β load. In brief, the area of the hippocampus being measured (subiculum, CA1 or CA2/3) was manually outlined. Images were adjusted via thresholding to remove background staining and create a binary image of positive versus negative immunoreactivity. The area of positive staining measured as A β load. Phosphorylated tau-immunopositive cells were counted from 4 sections per brain as previously described (13). Only cells that were darkly stained across the majority of the soma were scored.

2.4 Soluble β -amyloid quantification

A hemisphere, minus the hippocampus, was homogenized in 15 volumes of TBS with protease inhibitors (Calbiochem set I and Sigma sets II and III). The homogenate was centrifuged at 100,000 x g for 1 hr in an Optima XPN (Beckman Coulter). The supernatant was aliquoted and frozen at 80°C as the TBS-soluble fraction for later analysis. The pellet was resuspended in 2 mL of 0.01% Triton-X, mixed for 30 min at 4°C and centrifuged at 100,000 x g for 1 hr. The supernatant was aliquoted and frozen as the TX-soluble fraction for later analysis. Amounts of A β 42 were quantified in TBS- and TX-soluble fractions using human-specific ELISA (Meso Scale Discovery; Rockville, MD) according to the manufacturer's instructions.

2.5 Quantitative PCR

RNA extractions and real-time PCR were performed as previously described (20). Dissected hippocampi were lysed using TRIzol reagent (Life Technologies; Carlsbad, CA). Purified RNA (1 μ g) was used for reverse transcription using the iScript synthesis system (Bio-Rad; Hercules, CA). The resulting cDNA was used for real-time quantitative PCR using a Bio-Rad CFX96 Touch Real-Time PCR Detection System. The amplification efficiency was estimated from the standard curve for each gene. Relative quantification of mRNA levels from animal hippocampi was determined by the C_t method. The following primer pairs were used: CD11b: forward, 5'-CCAAGACGATCTCAGCATCA-3'; reverse, 5'-TTCTGGCTTGCTGAATCCTT-3'; F4/80: forward, 5'-TGCATCTAGCAATGGACAGC-3'; reverse, 5'-GCCTTCTGGATCCATTTGAA-3'; IL-1 β : forward, 5'-GGGCCTCAAAGGAAAGAATC-3'; reverse, 5'-TACCAGTTGGGGAAGTCTGC-3'; IL-6: forward, 5'-AGTTGCCTTCTTGGGACTGA-3'; reverse, 5'-TCCACGATTTCCAGAGAAC-3'. β -actin: forward, 5'-AGCCATGTACGTAGCCATCC-3'; reverse, 5'-CTCTCAGCTGTGGTGGTGAA-3'.

2.7 Statistics

All data are reported as the mean $\pm$ the standard error of the mean. Raw data were analyzed using GraphPad Prism version 5. T-tests were used to compare the effects of vehicle versus PK11195 treatments. Comparisons with $p < 0.05$ were considered significant.

3. Results

Vehicle- and PK11195-treated mice were behaviorally characterized on two measures. First, because TSPO ligands are known to reduce anxiety (21), we assessed performance in elevated plus maze, a measure of anxiety. Animals treated with PK11195 spent significantly more time in the open arm than control animals ($t = 2.06$, $p < 0.05$), consistent with established TSPO anxiolytic effects (Fig. 1A). Second, mice were evaluated on spontaneous alternation behavior in the Y maze, which involves aspects of attention and memory. PK11195 treatment was associated with a significant increase of $>25\%$ in alternations relative to vehicle treatment ($t = 2.48$, $p < 0.05$; Fig. 1B). There was no significant difference in total number of arm entries ($t = 0.83$, $p < 0.42$; Fig. 1C), indicating similar overall activity levels in the two groups.

To evaluate whether PK11195 treatment affected AD-related neuropathology, A β accumulation was quantified by two approaches. First, we measured the amount of deposited A β using quantitative immunohistochemistry. A β burden was significantly reduced in subiculum of PK11195-treated mice ($t = 1.91$, $p < 0.05$) and with statistically non-significant changes in the hippocampal regions CA1 ($t = 0.60$, $p = 0.28$) and CA2/3 ($t = 0.62$, $p = 0.28$; Fig. 2A-C). Second, we measured comparatively more soluble pools of A β using ELISA. PK11195 treatment was associated with a statistically non-significant trend of reduced A β_{42} in the TBS soluble fraction ($t = 1.29$, $p = 0.12$; Fig. 2D) and a significant decrease ($t = 3.51$, $p = 0.005$; Fig. 2E) in the detergent soluble fraction. We also counted numbers of pre-tangle-like cells immunoreactive for the phosphorylated tau marker AT8, which were not significantly affected by PK11195 treatment (vehicle: 179 ± 90 vs. PK11195: 164 ± 68 ; $t = 0.13$, $p = 0.45$).

Because TSPO ligands mediate some effects by regulating glial actions, we quantified several glia and inflammatory markers by qPCR. First, we assessed mRNA levels of two microglia markers that have been associated with activation, CD11b and F4/80. Expression levels of CD11b did not differ across groups ($t = 0.21$, $p = 0.42$; Fig. 3A) but F4/80 expression was significantly higher in PK11195- versus vehicle-treated mice ($t = 1.87$, $p < 0.05$; Fig. 3B). Next, we measured expression of two pro-inflammatory cytokines associated with gliosis, IL-6 and IL-1 β . Expression of IL-6 ($t = 1.98$, $p < 0.05$; Fig. 3C) but not IL-1 β ($t = 0.08$, $p = 0.47$; Fig. 3D) showed a significant increase in PK11195-treated animals.

4. Discussion

Short-term treatment with the TSPO ligand PK11195 was shown to significantly ameliorate cognitive deficits and modestly reduce A β in 16 month-old female 3xTg-AD mice. These findings are noteworthy for several reasons. First, efficacy was observed in aged 3xTg-AD mice when AD-related pathology is quite extensive and cognitive deficits are robust. Thus,

the observed benefits of TSPO ligand can be interpreted in the context of an AD treatment paradigm, which is significant given the limited efficacy of currently approved AD treatments. Second, benefits of TSPO ligand were apparent despite a limited administration protocol of five treatments, once per week. This finding suggests that the intervention quickly reversed established pathology and/or activated protective pathways to yield improved outcomes. Third, the results extend to PK11195 the previously reported ability of the TSPO ligand Ro5-4864 to yield benefits in aged 3xTg-AD mice (9). Because PK11195, Ro5-4864, and other TSPO ligands vary considerably in their abilities to confer protection across paradigms (6), that both PK11195 and Ro5-4864 are protective in aged 3xTg-AD mice affirms and generalizes the therapeutic potential of TSPO ligands for AD-related pathology. Fourth, the current results extend to females the utility of TSPO ligand protection against AD-related pathology. Prior studies of TSPO ligands in AD and neuroinflammation paradigms have primarily studied males (9–11). Because of the established sex differences of AD in both humans and rodent models (22), demonstrating efficacy of protective strategies across both sexes is essential.

TSPO ligands regulate numerous pathways that may contribute to the observed improvements in aged female 3xTg-AD mice. Because TSPO is expressed in several tissues (6), the observed effects of PK11195 may reflect actions at extra-neural sites. TSPO has long been implicated as an important mediator of steroidogenesis in tissues including gonads, adrenal gland and brain (23, 24), though recent observations suggest that it may not be essential to the process (25) or may be more important during aging (26). Because TSPO ligands are associated with increased expression of several steroids in brain that have been demonstrated to protect against A β pathology in female AD transgenic mice, including estradiol (14, 18), progesterone (18), and allopregnanolone (27), neurosteroidogenesis may be an important mechanism that contributes to the observed protection against AD-related endpoints. However, recent findings demonstrate that combined treatment with continuous estradiol and discontinuous progesterone improved behavior and reduced pathology in early but not late middle-aged female 3xTg-AD mice (13). These and other recent findings (12) demonstrate that at least some protective steroid hormones lose efficacy in aging females, suggesting that the observed PK11195 benefits may be mediated through additional mechanism(s).

TSPO ligands can also confer protection in neurodegenerative paradigms by regulation of glial activation. Because neural expression of TSPO is primarily within glia and its expression increases in response to neurodegenerative conditions, glia are presumed to be central to TSPO neuroprotective actions. TSPO ligands regulate glia in seemingly disparate ways, both increasing features of glial activation such as phagocytosis (28) and reducing indices of neuroinflammation including expression of pro-inflammatory cytokines (29). Our data are most consistent with activation of glia by PK11195. We observed that levels of the macrophage marker F4/80, which shows increased expression in activated microglia (30), were increased after PK11195 treatment suggesting that TSPO activation may be at least partially activating microglia. Activated microglia is a broad term and can refer to both harmful and beneficial actions of microglia with respect to A β plaques. Activated microglia in AD have been associated with increased A β phagocytosis (30), although they have also been shown to promote amyloid deposition (31). Depending on the type of activation state,

different cytokines can be released by glia. Here, PK11195 treatment increased expression of IL-6, a cytokine previously shown to increase A β phagocytosis and attenuate A β deposition (32). These observations suggest that regulation of glial activation may reasonably contribute to the mechanism(s) by which PK11195 improves outcomes in aged female 3xTg-AD mice, but more detailed studies will be required to confirm this possibility.

5. Conclusions

Despite the high prevalence of AD in the aged population, there remains a dearth of effective treatments. Findings in aged female AD transgenic mice provide new preclinical evidence that short-term treatment with the TSPO ligand PK11195 provides significant improvement in AD-related cognitive and pathological outcomes. Although determination of its protective mechanism(s) remains to be elucidated, TSPO has pleiotropic actions that are worthy of continued investigation in the context of AD treatment.

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Abbreviations:

TSPO	translocator protein 18 kD
AD	Alzheimer's disease

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Highlights

- Short-term treatment with the TSPO ligand PK11195 improved cognitive and behavioral performance in aged female 3xTg-AD mice
- PK11195 treatment modestly reduced both soluble and deposited β -amyloid in aged female 3xTg-AD mice
- Markers of glial activation were increased by PK11195, suggesting that TSPO modulation of glia may contribute to its observed benefits

Fig 1.

The TSPO ligand PK11195 improves behavioral outcomes. (A) Aged female 3xTg-AD mice spent significantly more time in the open arms of the elevated plus maze after treatment with PK11195. (B) In the spontaneous alternation task, PK11195 was associated with a significant increase in correct alternations, (C) but no significant difference in the number of total arm entries in the Y-maze; N=6–7. * indicates $p < 0.05$ relative to Vehicle group.

Fig 2.

Quantitative immunohistochemistry shows effects of PK11195 treatment on β -amyloid deposition in the hippocampus. Representative images show β -amyloid immunostaining in the subiculum of (A) vehicle and (B) PK11195-treated animals. Scale bar = 100 μm . (C) Quantification of β -amyloid load shows significant reduction associated with PK11195 treatment (filled bars) in the subiculum, but not in CA1 or CA2/3 regions of the hippocampus; N=6–7 (D) TBS soluble $A\beta_{42}$ was not significantly different between vehicle- and PK11195-treated mice. (E) Detergent soluble $A\beta_{42}$ was significantly decreased by PK11195 treatment; N=4–5. * indicates $p < 0.05$ relative to Vehicle group.

Fig 3.

Quantitative PCR data show effects of PK11195 treatment on mRNA expression of microglial and inflammatory markers. Data show mean (±SEM) relative expression levels in hippocampus of the macrophage/microglial markers (A) CD11b and (B) F4/80, and the pro-inflammatory cytokines (C) IL-6 and (D) IL-1β in Vehicle (open bars) and PK11195 treated (filled bars) mice; N=6–7. * indicates $p < 0.05$ relative to Vehicle group.