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Received: 19 November 2018; Revised: 11 June 2019; Accepted: 13 June 2019
doi: 10.1002/acn3.50837

Abstract

Objective: Increased translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), in glial cells of the brain has been used as a neuroinflammation marker in the early and middle stages of neurodegenerative diseases, such as Alzheimer’s disease (AD) and Dementia with Lewy Bodies (DLB). In this study, we investigated the changes in TSPO density with respect to late stage AD and DLB. Methods: TSPO density was measured in multiple regions of postmortem human brains in 20 different cases: seven late stage AD cases (Braak amyloid average: C; Braak tangle average: VI; Aged 74–88, mean: 83 ± 5 years), five DLB cases (Braak amyloid average: C; Braak tangle average: V; Aged 79–91, mean: 84 ± 4 years), and eight age-matched normal control cases (3 males, 5 females: aged 77–92 years; mean: 87 ± 6 years). Measurements were taken by quantitative autoradiography using [3H]PK11195 and [3H]PBR28. Results: No significant changes were found in TSPO density of the frontal cortex, striatum, thalamus, or red nucleus of the AD and DLB brains. A significant reduction in TSPO density was found in the substantia nigra (SN) of the AD and DLB brains compared to that of age-matched healthy controls. Interpretation: This distinct pattern of TSPO density change in late stage AD and DLB cases may imply the occurrence of microglia dystrophy in late stage neurodegeneration. Furthermore, TSPO may not only be a microglia activation marker in early stage AD and DLB, but TSPO may also be used to monitor microglia dysfunction in the late stage of these diseases.

Introduction

Translocator protein 18 kDa (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), is located on the outer membrane of mitochondria and is a part of mitochondrial permeability transition pore. Outside of the central nervous system, it is ubiquitously expressed in the kidneys, heart, adrenal gland, and reproductive tract; in the brain it is expressed at a low level in the resting glial cells. TSPO is believed to be involved in the synthesis of steroids and the translocation of cholesterol from the outer to the inner mitochondrial membrane. TSPO is also involved in the regulation of cell death, cell proliferation, and inflammation. TSPO density has been found to be increased in many neurological diseases such as ischemic stroke, multiple sclerosis, amyotrophic lateral sclerosis, encephalitis, and frontotemporal dementia. The upregulation of TSPO is often accompanied by microglial changes from resting to activated morphology and the secretion of cytokines which can induce tissue inflammatory response; hence TSPO is currently accepted as a neuroinflammation marker.


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DAA1106.18 In these studies early stage patients with mild to moderate cognitive impairment (clinical dementia rate (CDR) 1 and 2) were usually recruited and increases in TSPO density were often found in multiple brain regions.7,10,15 Two studies showed no significant change in TSPO density between aged AD (CDR unknown) and the age-matched control brain.9,11 Because it is a marker of microglia activation, increases in TSPO density in early stage AD may reflect the involvement of microglia-mediated neuroinflammation in the pathogenesis of AD,19 although the enhanced phagocytosis by activated microglia has been reported to have a protective effect.20,21

Microglial dystrophy has been recently observed in the AD brain; this is thought to play a key role in the pathogenesis of AD.22–28 Both Tau pathology and amyloid beta pathology have been reported to promote microglial dystrophy,29,30 which may occur via telomere shortening.29,31,32 Although microglial dystrophy is closely related to AD stage, TSPO density in late stage AD and DLB has not been well documented until now. In this communication, we hypothesize that DLB and AD patients have more microglial dystrophy than healthy controls and TSPO PET may be useful for measuring late stage microglia dystrophy in DLB and AD patients. To explore the relationship between TSPO density change and microglial dystrophy, we systematically studied a group of late stage AD and DLB cases and measured TSPO density in multiple brain regions using quantitative autoradiography with two radioligands [3H]PK11195 and [3H]PBR28.

Postmortem human brain cases
Clinically and neuropathologically well-characterized human brain tissues were obtained from the Knight ADRC at Washington University School of Medicine including: seven AD (1 male, 6 females) aged 74–88 (mean: 83 ± 5) years at death, five DLB (2 males, 3 females) aged 79–91 (mean: 84 ± 4) years at death, and eight age-matched normal control cases (3 males, 5 females) aged 77–92 (mean: 87 ± 6) years at death. Dementia level was evaluated by CDR. Alzheimer disease pathological changes were assessed using Braak staging.33,34 Stages of amyloid beta deposition refer to initial deposits in the basal neocortex (A), deposits extended into the association areas of the neocortex (B), and heavy deposition throughout the entire cortex (C). Stages of neurofibrillar pathology correspond to transentorhinal (I–II), limbic (III–IV), and neocortical (V and VI). There is no significant difference between the average age and post mortem interval time among these three groups; whereas all the AD and DLB cases show a heavy burden of amyloid beta (Average: C) and neurofibrillary tangles (NFTs) (Average: VI for AD; V for DLB) and are significantly different from that of age-matched control cases (Average amyloid beta: A; NFTs: II). There exist significant differences in the brain weight and the CDR between the control cases and the AD or DLB cases. Detailed information on the clinical and pathological features is summarized in Table 1.

Materials and Methods

Ethics statement
After death, the written consent of the next of kin was obtained for brain removal per local Ethical Committee procedures (Washington University Institutional Review Board, Washington University School of Medicine). Use of this tissue for the postmortem receptor autoradiography study was approved by the Charles F. and Joanne Knight Alzheimer’s Disease Research Center (Knight ADRC) Leadership Committee.

Chemicals and radioligands
Chemical reagents and the standard compounds were purchased from Sigma-Aldrich (St. Louis, MO) and Tocris (Ellisville, MO). [3H]PK11195 (69.9 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA) and [3H]PBR28 (80 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO).
Quantitative autoradiography protocol

Sections on slides were incubated in an open staining jar with the respective radiotracer for 30 min as detailed below; slides were then rinsed five times at 1 min intervals with ice-cold buffer, then air dried. This protocol results in a free radioligand concentration loss < 5%, as described previously.35,36

TSPO binding

TSPO was labeled with [3H]PK11195 and [3H]PBR28. Briefly, adjacent brain sections were incubated for 30 min in buffer solution at room temperature with the addition of 2 nmol/L [3H]PK11195 and [3H]PBR28, respectively. Nonspecific binding was determined from incubating the slides in the presence of 1 µmol/L PBR28 for [3H]PK11195, and 1 µmol/L PK11195 for [3H]PBR28.

Quantification of total radioactivity

Slides were made conductive by coating the free side with a copper foil tape.Slides were then placed in the sample holder and loaded into a gas chamber containing a mixture of argon and triethylamine (Sigma-Aldrich) as part of a gaseous detector system, the Beta Imager 2000Z Digital Beta Imaging System (Biospace, France). After the gas was well mixed and a homogenous state was reached, exposure of the slides for 20 h yielded high-quality images. A [3H]Microscale (American Radiolabeled Chemicals, St Louis, Missouri) was counted simultaneously as a reference for total radioactivity quantitative analysis. Quantitative analysis was performed using the program Beta-Vision Plus (BioSpace, France) for anatomical regions of interest.

Statistical analysis

The receptor-bound radioligand binding apparent densities were calculated as described previously using the specific activity of each radioligand expressed as fmol/mg tissue.35,36 The experimenter was blinded to all conditions during the analysis. Comparison of receptor densities was analyzed using an unpaired Student’s t test. The correlation between receptor binding densities across different brain regions was assessed using Pearson product-moment correlation coefficient.

RESULTS

Quantitative analysis of TSPO binding

The sensitivity limit of the Beta Imager 2000Z Digital Beta Imaging System is 0.07 dpm/mm². A tritium

Table 1. Demographic details, clinical, and pathological features of cases.

<table>
<thead>
<tr>
<th>Case #</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Brain weight (g)</th>
<th>PMI (h)</th>
<th>Braak tangle</th>
<th>Braak amyloid</th>
<th>Exp CDR</th>
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<td>C</td>
<td>3</td>
<td>AD</td>
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</table>

AD, Alzheimer’s disease; Con, Control; D LB, Dementia with Lewy Bodies; PMI, Post-mortem interval; Exp CDR, Estimated clinical dementia rating at expiration.

standard was counted with each set of slides, the [3H] Microscale contains multiple strips with a known amount of radioactivity (ranging from 0 to 36.3 nCi/mg) and was used to create a standard curve. In each case the standard curve had a correlation coefficient (R) greater than 0.99. The binding density of TSPO was determined by quantitative autoradiography using 2 nmol/L [3H]PK11195 and [3H]PBR28 (Fig. 1). The nonspecific binding was determined by using the high affinity cold compound and was much higher with [3H]PK11195 than [3H]PBR28 (Figs. 2, 3, 4C and D). TSPO receptor density values in different brain regions are summarized in Table 2.

**TSPO densities in multiple brain regions of healthy control cases measured by [3H]PK11195 and [3H]PBR28**

In order to verify the consistency of [3H]PK11195 and [3H]PBR28 binding, we first compared TSPO densities measured by these two radioligands in different brain regions of control cases. Although the nonspecific binding of PK11195 is higher than [3H]PBR28, the TSPO densities measured by [3H]PK11195 and [3H]PBR28 showed no significant differences across all the brain regions measured (Fig. 1). The regional distribution of TSPO in control brains was similar, whether measured by [3H]PK11195 or [3H]PBR28: the lowest measured TSPO density level was in the RN, a modest level was found in the striatum (putamen and caudate), and the highest levels were found in the frontal cortex, thalamus, and SN (Fig. 1 and Table 2).

**TSPO density changes in multiple brain regions of AD, DLB cases compared to age-matched controls**

**Frontal cortex**

A similar binding pattern of [3H]PK11195 and [3H] PBR28 was observed in the frontal cortex; TSPO binding was mainly located in the grey matter, whereas TSPO binding in the white matter is relatively lower than in the grey matter (Fig. 2A and B). The nonspecific binding of [3H]PBR28 is lower than the binding of [3H]PK11195 (Fig. 2C and D). TSPO binding densities were similar for [3H]PK11195 and [3H]PBR28 in the frontal cortex of the control cases and the AD and DLB cases. Compared to the age-matched controls, no significant difference in cortical TSPO densities between control and AD or DLB cases was found by

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**Figure 1.** Comparison of translocator protein (TSPO) densities in the normal aged post mortem human brain measured by [3H]PK11195 and [3H] PBR28. Quantitative analysis of 2 nmol/L [3H]PK11195 and 2 nmol/L [3H]PBR28 binding in multiple brain regions of aged human healthy control cases (n = 8, aged 77–92 years). Non-specific binding was determined by the presence of 1 µmol/L unlabeled PBR28 for [3H]PK11195 and 1 µmol/L PK11195 for [3H]PBR28. Specific receptor binding densities (fmol/mg) were calculated by subtracting nonspecific binding from total binding. Data was calculated from the average of 2–4 adjacent sections of each case and presented as mean ± SEM. Th, Thalamus; SN, Substantia nigra; Fr, Frontal cortex; Cd, Caudate; Pu, Putamen; RN, Red nucleus.
either $[^3]H$PK11195 or $[^3]H$PBR28 autoradiography (Fig. 2F).

**Striatum**

TSPO was found to be homogeneously distributed in the striatum, including in the putamen and the caudate; whereas binding in the internal capsule was very low as measured by both $[^3]H$PK11195 and $[^3]H$PBR28 (Fig. 3A and B). The nonspecific binding was lower with $[^3]H$PBR28 than with $[^3]H$PK11195 (Fig. 3C and D). TSPO density as measured by $[^3]H$PK11195 was higher than that measured with $[^3]H$PBR28 both in the putamen and the caudate, but no significant differences in striatal TSPO densities were found between the control and the AD or DLB cases when measured using either $[^3]H$PK11195 or $[^3]H$PBR28 autoradiography (Fig. 3F and G).

**Thalamus**

TSPO was abundantly distributed in the thalamus and the density as measured by $[^3]H$PK11195 and $[^3]H$PBR28 gave similar results (Fig. 4A, B and H). However, nonspecific binding of $[^3]H$PK11195 was higher than that of $[^3]H$PBR28 in the thalamus (Fig. 4C and D). The decreased trend of thalamic TSPO binding in AD and DLB compared to the age-matched controls was found not to be significantly different when measured using $[^3]H$PK11195 and $[^3]H$PBR28 autoradiography (Fig. 4H).

**Red nucleus**

TSPO density was the lowest in the RN compared to the other regions (Fig. 4G; Table 2). There were no significant changes in the TSPO densities in the red nucleus between the control
and the AD or DLB cases when measured using either \[^{3}H\]PK11195 or \[^{3}H\]PBR28 autoradiography (Fig. 4G).

**TSPO density decreased in the substantia nigra of AD, DLB cases compared to age-matched control**

There was a high level of TSPO in the SN in control cases as measured by \[^{3}H\]PBR28 and \[^{3}H\]PK11195 (Figure 1; Fig. 4A and B; Table 2). Compared to the controls, the TSPO density was significantly decreased in AD as measured by both \[^{3}H\]PBR28 \((P = 0.01)\) and \[^{3}H\]PK11195 \((P = 0.02)\). A significant reduction in TSPO density was also found for DLB cases compared to that of the age-matched control measured by either \[^{3}H\]PBR28 \((P = 0.04)\) or \[^{3}H\]PK11195 \((P = 0.03)\) (Fig. 4F). TSPO density in the SN measured using either \[^{3}H\]PK11195 or \[^{3}H\]PBR28 was similar; a relatively higher nonspecific binding was found with \[^{3}H\]PK11195 than \[^{3}H\]PBR28 (Fig. 4C and D).

**Regional correlation of TSPO density in the frontal cortex, striatum and substantia nigra measured by \[^{3}H\]PBR28**

TSPO density in the frontal cortex significantly correlated with density in the striatal regions (both putamen and caudate); there was also a significant correlation between
TSPO density in the putamen and density in the caudate (Fig. 5A). No such correlations were found between the SN and the frontal cortex, putamen, or caudate (Fig. 5B); this is due to the significant correlation in TSPO density in the SN of AD and DLB cases.

**Discussion**

In this study, the TSPO density was measured in multiple brain regions of postmortem human brains in a group of late stage AD and DLB cases that had a heavy burden of...
amyloid beta and NFTs. TSPO density was measured by quantitative autoradiography using two different tritiated ligands, [3H]PK11195 and [3H]PBR28. Brain weight, Braak amyloid, and Braak NFTs stages of AD and DLB cases were significantly different from those of age-matched healthy controls. However, TSPO density appeared to be at a similar level in multiple brain regions including: frontal cortex, striatum, thalamus and red nucleus. Furthermore, we discovered that TSPO density was significantly decreased in the SN of AD and DLB cases.

Table 2. [3H]PBR28 and [3H]PK11195 binding densities in the late stage Alzheimer’s Disease (AD) (n = 7) and Dementia with Lewy Bodies (DLB) (n = 5) patients (fmol/mg) compared to age-matched control (n = 7).

<table>
<thead>
<tr>
<th></th>
<th>[3H]PBR28</th>
<th>[3H]PK11195</th>
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<tbody>
<tr>
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<td>DLB</td>
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<tr>
<td>Fr</td>
<td>418 ± 46</td>
<td>440 ± 48</td>
</tr>
<tr>
<td>Pu</td>
<td>297 ± 33</td>
<td>280 ± 30</td>
</tr>
<tr>
<td>Cd</td>
<td>302 ± 34</td>
<td>315 ± 48</td>
</tr>
<tr>
<td>SN</td>
<td>432 ± 63</td>
<td>291 ± 20*</td>
</tr>
<tr>
<td>Th</td>
<td>459 ± 100</td>
<td>298 ± 15</td>
</tr>
<tr>
<td>RN</td>
<td>180 ± 23</td>
<td>184 ± 10</td>
</tr>
</tbody>
</table>

Fr, Frontal cortex; Pu, Putamen; Cd, Caudate; SN, Substantia nigra; Th, Thalamus; RN, Red nucleus.

Data presented as mean value ± SEM. *P < 0.05 compared to control.

Figure 5. Regional correlation of translocator protein (TSPO) density measured by [3H]PBR28. Regional correlations in TSPO density measured using [3H]PBR28 were performed for all the cases. Significant correlation of TSPO densities was found between the frontal cortex with the putamen and the caudate and between the putamen and the caudate (A). Regional correlation of TSPO densities between substantia nigra with frontal cortex, the putamen, and the caudate are shown in B.
noise ratio using \([^{3}H]PK11195\). \([^{11}C]PR28\) is an improved TSPO radioligand with higher binding affinity and selectivity\(^{42-47}\) which has been successfully used to measure TSPO density in cerebral ischemia\(^{48}\) as well as in multiple sclerosis.\(^{49}\) TSPO densities measured by \([^{3}H]\)PK11195 and \([^{3}H]PR28\) in this study appeared to be at similar level, indicating a consistency of measurement for these two radioligands. Human subjects can have variable binding levels in PR28 PET and autoradiography human study due to polymorphism in the 18 kDa protein; codominance of the trait results in trimodal distribution.\(^{50-52}\) This is an important issue considering the reduction in TSPO density as measured by \([^{3}H]PR28\) in this study, however, it is unlikely that this reduction in TSPO density can be attributed to polymorphism. Two reasons indicate this: (1) In this study the reduction of TSPO density as measured by \([^{3}H]PR28\) was only slight (15–20% compared to that of age-matched control cases), but low affinity binders (a single nucleotide polymorphism (SNP) of PBR, Ala147Thr) have an affinity is 10-fold lower and while mixed affinity binders show ~50% reduction\(^{50}\) compared to the wild type; and (2) TSPO density measured by \([^{3}H]PK11195\) was at the same level as that of \([^{3}H]PR28\) and no low binder phenomenon has been reported for \([^{3}H]PK11195\). The cases used for our study can be safely assumed to consist of high affinity binders or mixed affinity binders as described previously.\(^{45,51,52}\)

No significant changes were found in TSPO density of the frontal cortex, striatum, thalamus, or red nucleus in late stage AD and DLB cases compared to that of age-matched control cases. This is different from most previous PET or autoradiography studies, in which an increase in TSPO density was usually found in the early and middle stage of AD. However, this is in line with a previous PET study using \([^{11}C]PK11195\) which showed no significant changes of TSPO density in AD compared to that of an age-matched control.\(^{9}\) It is also in line with another PET study using \([^{11}C]\)vinpocetine, which found a significant increase in TSPO density in the brain of aged healthy controls and aged AD patients compared to that found in young healthy control subjects, but no significant difference between aged AD patients and age-matched controls.\(^{11}\) Our findings, along with other results of previous studies, indicate that there may be a different pattern of changes in TSPO between early and late stage AD. In early stage AD, TSPO increases as a marker of microglial activation; whereas in late stage AD, this increase in TSPO density disappears. This may reflect the different states of microglia at different AD stages. This is interesting when considering recent findings showing that there were more dysfunctional microglia in the AD brain than in non-demented, amyloid-free control subjects\(^{29}\) and that Tau pathology was co-localized with dystrophic microglial cells.\(^{30}\) It is reasonable to deduce that with the progression of disease, the increase in TSPO density during early stage AD can become slower because of microglial dysfunction, which leads to no significant difference in TSPO density during late stage AD. This different temporal curve of TSPO density changes between the normal aging process and the AD brain may reflect a different microglial reaction pattern. In the AD brain, it seems that microglia are more predisposed to become dystrophic after initial activation than in the normally aging brain, in which a decrease in the up-regulation of MHCII expression on microglial cell surface, an indicator of microglia activation, was only observed at age > 80 years old in human post-mortem brain.\(^{53}\)

TSPO density was found to be significantly decreased in the SN, indicating a significant microglia dystrophy occurred in this region during the late stages of AD and DLB. This is different from previous reports indicating microglial activation in the SN during early stage DLB and AD cases.\(^{54}\) It is not surprising that decreased TSPO density, which may be a marker of microglia dystrophy, was also found in the SN of the AD brain. This is because a type of SN pathology which includes neuronal loss, Lewy bodies, and NFT has been proven to be present in AD.\(^{55-62}\) The significant reduction in TSPO density of the SN in the late stages AD and DLB is an interesting phenomenon which may imply an interaction between microglial dystrophy and neuronal loss in the same region. On one hand, dystrophic microglia can accelerate neuronal loss through the dysfunction of phagocytosis leading to more deposition of NFT and amyloid beta as well as through microglia-mediated neuroinflammation, which is well documented in dystrophic microglial cells. On the other hand, significant neuronal death in the SN may also prompt microglial dystrophy, although this possibility needs to be elucidated further.

It is more likely that the reduction in TSPO density of the SN in AD and DLB cases is mainly attributed to the loss of microglial cells, but it could also be due to down-regulation of TSPO on the mitochondrial membrane, or a decreased turnover rate of mitochondria in dystrophic microglia. The complete function of TSPO is not fully understood, but it has been reported that increased expression of TSPO can protect cells from apoptosis through the regulation of the mitochondrial permeability transition pore complex.\(^{2,63-65}\) In addition, TSPO can mediate protective effects against ROS damage.\(^{66}\) This is important because oxidative stress and mitochondrial dysfunction are thought to be closely related to neurodegenerative diseases.\(^{67-70}\) Taken together, the reduction in TSPO density in the late stages of AD and DLB found in this study may not only reflect the loss of microglial cells
but also may be a marker of dysfunction of mitochondria in microglial cells, which in turn contributes to microglia apoptosis and eventual neurodegeneration.71

In summary, this study provides quantitative information about TSPO changes in late stage AD and DLB brains, showing different patterns of change compared to that seen in early stage ADs. A presence of change or reduction in TSPO density may reflect dystrophy, senescence and death, or dysfunction of mitochondria in the microglia, in turn contributing to the neuronal loss in the late stage AD. Furthermore, the different pattern of change seen for TSPO density between early and late stage AD may indicate the importance of dynamic monitoring of TSPO through clinical PET imaging; not the initial increase, but rather the decrease in the upregulation of TSPO density may herald the progression of neurodegeneration.

Acknowledgments

The authors thank Ms. Erin E. Franklin and Mr. Michael Baxter of the Knight ADRC Neuropathology Core at Washington University School of Medicine, for coordination of the frozen tissue preparation and expert technical assistance. We also thank Ms. Lynne Jones and Mr. William Knight for editorial assistance. Research funded by National Institutes of Health (NIH) R01 NS092865, R01 AG052550, P01 AG026276, P01 AG03991, and P50 AG05681.

Author Contributions

JX, KRB, TSLB, and DMH involved in study concept and design. JX, JS, and RJP involved in collection of and qualitative analysis of data. JX, JS, RHM, and DMH involved in statistical analysis and interpretation. RJP, JX, and JS involved in preparation of tissue. JX, JCM, TSLB, and DMH obtained funding. JX, JS, and TSLB involved in drafting of the manuscript. JX and JS involved in autoradiography and preparation of figures/tables. JCM involved in critical revision of the manuscript for important intellectual content. JX and TSLB involved in study supervision.

Conflict of Interest

The authors have no conflicts of interest.

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