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Imaging microglial activation and glucose consumption in a mouse model of Alzheimer's disease

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Keywords: Alzheimer's disease; Inflammation; Microglia; Molecular Imaging; Positron emission tomography; PK11195; PPAR-gamma; Transgenic mice

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Abstract. In Alzheimer's disease (AD), persistent microglial activation as sign of chronic neuroinflammation contributes to disease progression. Our study aimed to *in vivo* visualize and quantify microglial activation in 13- to 15-month-old AD mice using [^{11}C]-(*R*)-PK11195 and positron emission tomography (PET). We attempted to modulate neuroinflammation by subjecting the animals to an anti-inflammatory treatment with pioglitazone (5-weeks' treatment, 5-week wash-out period). [^{11}C]-(*R*)-PK11195 distribution volume values in AD mice were significantly higher compared with control mice after the wash-out period at 15 months, which was supported by immunohistochemistry data. However, [^{11}C]-(*R*)-PK11195 μPET could not demonstrate genotype- or treatment-dependent differences in the 13- to 14 month-old animals, suggesting that microglial activation in AD mice at this age and disease stage is too mild to be detected by this imaging method.

Introduction. In AD, activated microglia are found in the direct vicinity of amyloid β ($\text{A}\beta$) plaques. Initially, they take on a neuroprotective role, but with disease progression, secretion of neurotoxic proinflammatory molecules results in a chronic neuroinflammatory stage and neurodegeneration (Perry et al., 2010). PK11195 (1-[2-chlorophenyl]-N-methyl-N-[1-methyl-propyl]-3-isoquinoline-carboxamide) binds to the translocator protein-18 kDa (TSPO) whose expression is low in the healthy brain and upregulated in activated microglia. [^{11}C]-(*R*)-PK11195 has been used in animal and human positron emission tomography (PET) studies to image the dynamics of activated microglia in neurological disorders including AD (Venneti et al., 2009). Here, we investigated the extent of microglial activation in a mouse model of AD using [^{11}C]-(*R*)-PK11195 and μPET . In addition, we evaluated the consumption of [^{18}F]2-fluoro-2-deoxy-D-glucose ([^{18}F]FDG) and the effect of pioglitazone, an agonist for the nuclear peroxisome proliferator-activated receptor γ (PPAR- γ), as compound with microglia-modulating properties (Kummer and Heneka, 2008).

Methods. Animals. *Hu/moAPP_{Swe}/huPSEN1 ΔE9* (APP/PS1) transgenic (Tg, $n = 7$) and wild-type (Wt, $n = 8$) mice (B6C3 background) were used in accordance with German Laws for Animal Protection after approval by the local animal care committee and governmental authorities. Mice were imaged at baseline (13 months), after daily treatment with 40 mg kg^{-1} of pioglitazone for 5 weeks (14 months) and after a 5 week wash-out period (15 months).

μPET and μMR imaging. Dynamic [^{11}C]-(*R*)-PK11195 and [^{18}F]FDG μPET imaging was performed (μPET Focus 220; Concorde Microsystems Inc., Knoxville, TN, USA) together with [^{18}F]fluoride μPET and magnetic resonance imaging (MRI; turboRARE T₂-weighted, echo time (TE) 32.5 ms, repetition time (TR) 5000 ms, 11.7 BioSpec animal scanner, Bruker BioSpin, Ettlingen, Germany). μPET and μMR images were co-registered (**Suppl. Fig. 1**) and volumes of interest (VOI) of the whole brain (WB), cortex (CX), hippocampus (HC), thalamus (TH) and cerebellum (CB) were generated. For each VOI, mouse and imaging period, percentage injected dose per cubic centimeter (% ID/cc), standardized uptake value (SUV), [^{11}C]-(*R*)-PK11195 distribution volume (DV) and [^{18}F]FDG Ki values were calculated.

Immunohistochemistry. At 12.5 and 15 months, brains of representative Tg and Wt mice were removed, cut, and stained with antibodies against Aβ (IC16, 1:400, and 2964, 1:200) and CD11b (MCA711, 1:200; marker for microglia).

Statistical analysis. Statistical analysis of quantified μPET data was performed using SPSS version 18.0 (SPSS, Chicago, IL, USA). [^{18}F]FDG Ki values were analyzed using a two-way mixed design ANOVA and [^{11}C]-(*R*)-PK11195 DV values with a two-tailed dependent and independent Student's *t* test. Posthoc Bonferroni correction was applied after every test, and statistical significance was set at a 5% level.

Results. No statistically significant differences for %ID/cc and SUV of [^{11}C]-(*R*)-PK11195 were found between different brain regions, genotypes, and imaging periods ($p > 0.05$, **Suppl. Table 1**). Representative parametric images of [^{11}C]-(*R*)-PK11195 DV and [^{18}F]FDG Ki values are shown in **Fig. 1A** and **Suppl. Fig. 3A**, respectively. Statistical analysis of [^{11}C]-(*R*)-PK11195 DV showed no significant differences between 13- to 14 month-old Tg and Wt mice ($p > 0.05$). At 15 months, a significantly higher DV could be observed in the WB, CX, HC and CB of Tg mice (off treatment, $*p < 0.05$, **Fig. 1B**), although this significance disappeared after posthoc Bonferroni correction for multiple comparisons. Treatment with pioglitazone failed to demonstrate statistically significant differences between the different imaging stages ($p > 0.05$, **Fig. 1B**). Analysis of [^{18}F]FDG Ki values showed no genotype-, brain region- or treatment-dependent differences ($p > 0.05$, **Suppl. Fig. 3B**).

Immunostaining revealed the presence of activated microglia clustered around amyloid plaques in the cortex of 12.5-month-old Tg mice as compared with Wt mice, which was more prominent after the wash-out period at the age of 15 months (**Fig. 1C**).

Discussion. In the attempt to non-invasively quantify A β -mediated microglial activation and its therapy-induced modulation in APP/PS1 mice by [^{11}C]-(*R*)-PK11195 and μPET , we did not observe any statistically significant genotype- or treatment-dependent differences in radiotracer DV at the age of 13-14 months, although immunohistochemistry verified substantial microglial activation in Tg mice at the age of 12.5 months. Venneti *et al.* (2009) found increased microglial activation in APP/PS1 mice as assessed by immunostaining (Iba-1) and [^{11}C]-(*R*)-PK11195 PET, only at older age (16-19 months) but not at younger age (13-16 months). Together with the current study, these data may indicate that at earlier Alzheimer's disease stages, the number of activated microglia is below the limit of detection by [^{11}C]-(*R*)-PK11195 μPET . Wiley *et al.* (2009) have come to similar conclusions in a recent multi-tracer human PET study in which they investigated the relationship between microglial activation and A β deposition in the brains of healthy subjects and of patients with early AD. They did not observe any differences in brain [^{11}C]-(*R*)-PK11195 retention between the different subject groups nor in relation to A β as indicated by [^{11}C] Pittsburgh compound B (PiB) retention.

After discontinuation of pioglitazone, we detected significantly higher radiotracer DV in 15-month-old Tg mice. Immunohistochemical analysis demonstrated characteristic microglial activation around A β depositions in the cortex of APP/PS1 mice at this stage, which was more pronounced than in the cortex of 12.5-month-old Tg mice. This finding may indicate natural disease progression or "rebound" activation of microglia after treatment discontinuation.

Taken together, our data suggest that [^{11}C]-(*R*)-PK11195 μPET is able to assess microglial activation in AD mice only when a certain threshold of activation is reached. Therefore, with the given limitations of the radiopharmaceutical, subtle differences in microglial activation may be missed by this imaging method. Newly developed TSPO-targeted radiotracers with an improved target-to-background ratio may be able to detect activated microglia at earlier disease stages.

Disclosure statement. All authors declare no conflict of interest related to this study. None of the authors' institutions have contracts relating to this research through which it or any other organization may stand to gain financially now or in the future. There are no other agreements of authors or their institutions that could be seen as involving a financial interest in this work. The data contained in the manuscript being submitted is original unpublished work and has not been submitted to any other journal for reviews. All animal procedures were carried out in accordance with the German Laws for Animal Protection and were approved by the local animal care committee and by the local governmental authorities. All authors have reviewed the contents of the manuscript being submitted, approve of its contents, and validate the accuracy of the data.

Acknowledgements. This work was supported in part by 6th FW DiMI-NoE LSHB-CT-2005-512146, IZKF-SchwJ3/001/11 and 7th FW INMiND GA N°278850. The studies were performed as part of the master thesis of SR during the European Master of Molecular Imaging (EMMI). We thank Drs. Sascha Weggen and Claus Pietrzik for providing the antibody IC16.

Appendix. Supplementary data. Supplementary data associated with this article can be found, in the online version, at doi.org/10.1016/j.neurobiolaging.2012.04.016.

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Figure Legend

Figure 1. (A) Representative coronal parametric images show no reproducible differences in [^{11}C]-(*R*)-PK11195 DV between Tg (*upper*) and Wt (*lower*) mouse brain at before, after and off treatment imaging. **(B)** Analysis of [^{11}C]-(*R*)-PK11195 DV in the whole brain of 13- to 14-month-old Tg and Wt mice shows no statistically significant genotype-dependent differences (mean $\pm$ SEM, 2-tailed independent Student's *t* test with posthoc Bonferroni correction, $p > 0.05$). After treatment wash-out, 15-month-old Tg mice have a statistically significant higher [^{11}C]-(*R*)-PK11195 DV compared with their age-matched controls (mean $\pm$ SEM, 2-tailed independent Student's *t* test, $*p < 0.05$). After posthoc Bonferroni correction, no statistically significant differences could be observed any longer between the two animal groups. Pioglitazone treatment did not lead to statistically significant differences between the different imaging stages (mean $\pm$ SEM, 2-tailed dependent Student's *t* test with posthoc Bonferroni correction, $p > 0.05$). **(C)** Immunostaining for microglia (CD11b, *red*) and A β (IC16 at 12.5 months and 2964 at 15 months, *green*) in the cortex of Tg mice (*upper*) shows increased staining for microglia in the vicinity of plaques compared with the plaque free cortex of Wt mice (*lower*) both at the baseline (12.5 months) and after the wash-out period (15 months; Scale bars, 100 μm).

1. Supplementary Introduction

Alzheimer's disease (AD) is the most common cause of dementia in elderly people. The disease is characterized by extracellular aggregation of amyloid β ($A\beta$) in the brain and intracellular aggregation of the tau protein resulting in $A\beta$ plaques and neurofibrillary tangles, respectively (Bettens et al., 2010; Bird; Castellani et al., 2010; Van Broeck et al., 2007). The exact mechanism of how these AD hallmarks cause damage to neuronal cells is still unclear, but one of the most compelling theories is the contribution of activated microglia to AD pathogenesis.

Microglial cells represent the native immune system of the brain and are the first line of defense against brain injury. Under physiological conditions, they reside in a resting state, continuously surveying their environment with motile processes. Upon neuronal injury microglial activation occurs, changing the behavior of the cells from patrolling to shielding. They migrate towards the injured site where they proliferate and secrete proinflammatory molecules, such as cytokines and reactive oxygen/nitrogen species, resulting in neuronal toxicity (Kofler and Wiley, 2011). In the AD brain, activated microglia are found in the direct vicinity of $A\beta$ plaques (D'Andrea et al., 2004; Itagaki et al., 1989). Initially, they take on a neuroprotective role by releasing amyloid-degrading enzymes for the clearance of amyloid fibrils in the early stages of the disease. As the disease progresses, however, these neuroprotective clearing mechanisms are insufficient or too slow to counteract the deposition of $A\beta$ in the AD brain, but the production of the neurotoxic pro-inflammatory molecules is preserved, maintaining chronic neuroinflammation and resulting in neurodegeneration (Cameron and Landreth, 2010; Combs, 2009; Perry et al., 2010; Rogers et al., 2002; Sastre et al., 2006).

The nuclear peroxisome proliferator-activated receptor γ (PPAR- γ) is involved in the inflammatory response of activated microglia. Through transcriptional deactivation, PPAR- γ

agonists are able to suppress this response by inhibiting gene expression of pro-inflammatory molecules originating from the activated microglia. Therefore, several PPAR- γ agonists have been studied as a possible therapeutic intervention for neuroinflammation in AD (Kummer and Heneka, 2008; Landreth et al., 2008).

The peripheral benzodiazepine receptor (PBR) derives its name from its function as a binding site for benzodiazepines and due to its high level of expression in peripheral organs. Additional research, however, found the receptor expressed all over the body, including in the brain and it was renamed translocator protein-18 kDa (TSPO) (Papadopoulos et al., 2006). The expression of TSPO is low in the healthy brain parenchyma but strongly upregulated in activated microglia as shown in both patient and animal studies. Therefore, the expression of TSPO is a marker of choice to study the extent and role of neuroinflammation in several neurological disorders with the use of radiolabeled TSPO-targeted compounds (Cagnin et al., 2007; Venneti et al., 2009b; Winkeler et al., 2010).

The pharmacological ligand PK11195 (1-[2-chlorophenyl]-N-methyl-N-[1-methyl-propyl]-3-isoquinolinecarboxamide) binds with high affinity and selectivity to TSPO, as shown in early *in vitro* and *in vivo* studies using tritiated PK11195 (Le Fur et al., 1983a; 1983b). Labeled with carbon-11, [^{11}C]-(*R*)-PK11195 has been used in several animal and human positron emission tomography (PET) studies to image the dynamics of activated microglia in various neurological disorders, including AD (Venneti et al., 2009a; Wiley et al., 2009). Although [^{11}C]-(*R*)-PK11195 is the first and most widely used TSPO ligand tracer, several limitations, such as short half-life, high level of non-specific binding, poor signal-to-noise ratio and low sensitivity, complicate quantification of the PET data and have led to a search for new and improved PET ligands for TSPO (Chauveau et al., 2009; Doorduyn et al., 2009).

In this study, we investigated neuroinflammation and cerebral glucose metabolism in APP/PS1 transgenic (Tg) mice, an animal model for AD. Through mutations, these mice

overexpress both the A β precursor protein (APP) and presenilin 1 (PS1) gene, causing rapid development of A β plaques clustered by activated microglia together with the exhibition of behavioral deficits (Garcia-Alloza et al., 2006; Gordon et al., 2002; Yan et al., 2009). Additionally, we evaluated anti-inflammatory treatment with the PPAR- γ agonist pioglitazone, a pharmaceutical drug used for the treatment of diabetes mellitus type 2. The effect of this treatment in both Tg and wild-type (Wt) mice as well as *in vivo* differences in microglial activation between the two groups were assessed with μ PET imaging using [^{11}C]-(*R*)-PK11195. Treatment- and genotype-dependent differences in neuronal function were evaluated using [^{18}F]fluoro-2-deoxy-2D-glucose ([^{18}F]FDG) and μ PET for the quantification of cerebral glucose metabolism.

2. Supplementary Methods

2.1 Animals and experimental procedures

13-month-old female *Hu/moAPP_{Swe}/huPSEN1 Δ E9* Tg ($n = 7$) and control Wt ($n = 8$) mice on a B6C3 background (Clinical Neurosciences Unit, University of Bonn, Germany) were used in this study. A drop-out of both Tg ($n = 4$) and Wt ($n = 3$) mice occurred during the study due to advanced age, and they were replaced by same aged additional animals. All animal procedures were carried out in accordance with the German Laws for Animal Protection and approved by the local animal care committee and governmental authorities.

Prior to imaging, mice were anesthetized with 3% isoflurane (Baxter GmbH, Unterschleissheim, Germany) in nitrous oxide/oxygen (60%/40%) in a small induction chamber and maintained with 1 to 2% isoflurane on a heated (37°C) pad with anesthesia system (Medres GmbH, Cologne, Germany). The amount of delivered anesthesia was monitored and maintained constant manually through observation of the respiratory rate. To facilitate tracer injection, a tail vein catheter was placed by inserting a 32-gauge needle (TSH Laboratory, Japan) connected to a polyethylene tube (Smiths Medical International Ltd, Kent, UK) with an inner diameter of 0.28 mm into the tail vein at a slight angle. Proper placement of the catheter was verified when blood backflow was observed in the tube. A small volume of a heparin-sodium solution (25000 I.E./5 ml, Ratiopharm GmbH, Ulm, Germany) was injected to flush the catheter in order to prevent the blood from clotting and the needle was fixed in the tail using a hypoallergenic adhesive (Leukosilk, BSN medical GmbH, Hamburg, Germany). A special multimodal animal carrier (Medres GmbH, Cologne, Germany) was used for μ PET imaging, allowing two mice to be imaged simultaneously for each scan, thereby reducing the number of experiments and increasing the efficacy of tracer usage, especially for tracers with a short half-life, like [^{11}C]-(*R*)-PK11195. The carrier was equipped with a nose cone to maintain anesthesia, a tooth bar to fix the animal and a rectal temperature

probe connected to an automatically controlled heating system (Medres GmbH, Cologne, Germany) to maintain the body temperature constant.

After baseline imaging (13 months), all animals were treated on a daily basis for 5 weeks by feeding them *ad libitum* standard chow complemented with 240 p.p.m pioglitazone (Actos™, Takeda Pharmaceuticals Co. Ltd., Osaka, Japan). During this period, animals were housed separately to allow observation of drug intake by weighing the animal and the residual food pellets daily. Based upon average daily food intake of 5 g of chow per mouse, the final dose of pioglitazone was calculated to 40 mg/kg/day. To assess the anti-inflammatory effect of the treatment, the imaging protocol was repeated after the treatment period (14 months) as well as after a subsequent 5-week wash-out period (15 months).

2.2 Radiochemistry

Radiosynthesis of [^{11}C]-(*R*)-PK11195 was accomplished by *N*-methylation using the method of Camsonne *et al.* (1984) with slight modifications. ^{11}C was produced by the $^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$ nuclear reaction using the MC16 cyclotron (Scanditronix, Uppsala, Sweden) at the MPI for Neurological Research. [^{11}C]CO₂ was converted to [^{11}C]methyl iodide using the automatic synthesis system FX_C Pro from General Electric Medical Systems (GEMS, Uppsala, Sweden). [^{11}C]methyl iodide was trapped in 0.3 ml of dimethylformamide (DMF), containing 1 mg NaH as a base and 1 mg (*R*)-*N*-desmethyl-PK11195 (ABX, Radeberg, Germany). The mixture was heated at 80°C for 3 min. After dilution with eluent (EtOH/H₂O 60/40 (v/v)), the product was isolated by preparative high-performance liquid chromatography (HPLC, Nucleosil 100 C18; Macherey-Nagel, Düren, Germany, 10x250 mm, flow: 3 ml/min). The product fraction was diluted with 50 ml of water, [^{11}C]-(*R*)-PK11195 fixed on a C18 SPE Cartridge (strata-X, Phenomenex, Torrance, CA, USA) and eluted with 0.7 ml of EtOH. The final formulation for injection consisted of 10% isotonic EtOH solution. Radiochemical purity

was > 99%. The specific activity was in the range of 0.5 to 2.2 Ci/ μ mol. The radiochemical yield amounted to 8 to 15% (decay corrected). [^{18}F]FDG was produced as described earlier by Hamacher *et al.* (1986).

2.3 μ PET and μ MRI imaging

Before tracer injection, the animal carrier with the anesthetized and fixed mice was positioned in a high-resolution (spatial resolution ~ 1.5 mm) μ PET Focus 220 scanner (Concorde Microsystems, Inc., Knoxville, TN, USA) with the mice brains placed close to the center of the field of view.

Dynamic μ PET imaging was performed on two different days of the same week at baseline, after a 5-week anti-inflammatory treatment and after a 5-week wash-out period using [^{11}C]-(*R*)-PK11195 and [^{18}F]FDG to compare the uptake and kinetics of the tracers between Tg and Wt mice. For co-registration purposes, anatomical information was obtained through bone imaging using [^{18}F]fluoride and high-resolution magnetic resonance imaging (MRI) of representative animals (turboRARE T₂-weighted, TE 32.5 ms, TR 5000 ms, 11.7 BioSpec animal scanner, Bruker BioSpin, Ettlingen, Germany).

Acquisition of emission data was started simultaneously with intravenous tracer injection in the first mouse for 30 min in the case of [^{11}C]-(*R*)-PK11195 (50 to 500 μ Ci per mouse) and 60 min for [^{18}F]FDG (400 μ Ci per mouse). After every [^{11}C]-(*R*)-PK11195 and [^{18}F]FDG data acquisition, an additional [^{18}F]fluoride (200 μ Ci per mouse) 10 min scan was performed, followed by a 10 min transmission scan using a Cobalt-57 source to correct for attenuation.

2.4 μ PET data analysis

2.4.1 Data reconstruction

Emission data were histogrammed in 21 time frames (12x30, 3x60, 3x120, 3x240 s) for [^{11}C]-(*R*)-PK11195 and in 29 time frames (12x30, 3x60, 3x120, 11x240 s) for [^{18}F]FDG,

reconstructed using 2D filtered back projection with a ramp filter and corrected for photon attenuation. For [^{18}F]fluoride, dynamic time framing and attenuation correction were not required.

2.4.2 Image analysis

The image analysis software VINCI (<http://www.nf.mpg.de/vinci3>, MPI for Neurological Research, Cologne, Germany) was used for image co-registration as well as for volume of interest (VOI) analysis. For every mouse, the [^{11}C]-(*R*)-PK11195- and [^{18}F]FDG μPET images were matched to each other with the help of the corresponding [^{18}F]fluoride μPET image. Additionally, the co-registered images were matched to a representative MR image, allowing correct placement of a three-dimensional mouse brain template generated from the Swanson mouse brain atlas (Swanson, 2001; **Suppl. Fig. 1**). The mouse brain atlas permitted anatomically correct localization of different brain regions, making the generation of VOIs of the whole brain (WB), cortex (CX), hippocampus (HC), thalamus (TH) and cerebellum (CB) possible. Using the co-registered images and the VOIs, [^{11}C]-(*R*)-PK11195 and [^{18}F]FDG kinetics were determined in Tg and Wt mice for each brain region at each imaging period.

2.4.3 Data normalization and quantification

% ID and SUV. The manually created brain VOIs were applied onto co-registered [^{11}C]-(*R*)-PK11195 μPET static images covering the entire acquisition time. For each VOI, each mouse and each imaging period, the mean activity in the VOI was divided by the injected radiotracer activity to calculate the percentage injected dose per cubic centimeter (% ID/cc) values which were in turn multiplied by the whole body weight of the mouse at the time of the experiment to determine standardized uptake values (SUV).

Kinetic modeling. Quantification of PET tracer kinetics requires the knowledge of the tracer blood input function (IF). Because of the difficulty to obtain blood samples from mice

during a μ PET scan, image-derived blood IFs were used. The use of these noninvasive IFs is highly desirable for quantification in longitudinal studies. From the co-registered [^{11}C]-(*R*)-PK11195 μ PET dynamic images, IFs were derived directly from the time activity curve (TAC) of the left ventricle of the heart and a Logan plot was applied voxel-wise (Herholz et al., 2004). From the slope of the plot, parametric images of the distribution volume of [^{11}C]-(*R*)-PK11195 were generated (slope = DV = $K_1/k_2 [1 + k_3/k_4]$). In the equation, the term $[k_3/k_4]$ describes the binding potential (BP) of the tracer. The DV of [^{11}C]-(*R*)-PK11195 is thus directly related to the BP of the tracer and therefore a measure for the local density of activated microglia in brain tissue.

From the co-registered [^{18}F]FDG μ PET dynamic images, IFs were derived from the liver TAC as described previously (Yu et al., 2009). To extract the liver TAC, a large VOI containing the liver was drawn manually onto the images. The TAC was calculated as the average TAC from the 15 voxels within the VOI that contained the highest activity during the first three minutes after tracer injection. The IF was then given by the liver TAC divided by the recovery coefficient (0.58) that accounts for the liver partial-volume effect (Green et al., 1998; Yu et al., 2009). Using this IF, [^{18}F]FDG kinetic rate constants (K_1 , k_2 , k_3 and k_4) were calculated for each brain voxel by applying a two-tissue-compartment model and parametric images were constructed. K_1 and k_2 represent [^{18}F]FDG forward and reverse transport between plasma and brain tissue, respectively. The constants k_3 and k_4 describe phosphorylation of [^{18}F]FDG and dephosphorylation of [^{18}F]FDG-6-phosphate, respectively. From these constants, the net influx K_i rate constant, which is related to glucose consumption, was calculated ($K_i [\text{mlcc}^{-1} \text{min}^{-1}] = K_1 \times k_3/[k_2+k_3]$) (Sokoloff, 1977).

2.5 Immunohistochemistry

Before the start of the treatment period (12.5 months) as well as after off treatment imaging (15 months), representative Tg and Wt mice were anesthetized and perfused

transcardially with phosphate buffered saline (PBS). After perfusion, the animals were killed and their brains were quickly removed and fixed in 4% paraformaldehyde (PFA). Brains were sagittally cut into free-floating 40 μ m thick sections using a vibratome (Leica, Wetzlar, Germany). For immunostaining, the selected sections were first fixed (15 min in 50% methanol), washed (3 times 5 min in PBS) and blocked (30 min in PBS, 0.1% Triton X100, 3% bovine serum albumin blocking buffer). Next, the sections were incubated overnight at 4°C with primary antibodies, washed (3 times 5 min in 0.1% Triton-X100 in PBS) and incubated for 90 min with secondary Alexa 488 or Alexa 594 conjugated antibodies (1:500, Invitrogen Molecular Probes, Eugene, OR, USA) after which they were washed a final time (3 times 5 min in 0.1% Triton-X100 in PBS). Following primary antibodies were used: against A β , mouse monoclonal IC16 at 12.5 months and rabbit polyclonal 2964 at 15 months (1:400, kindly provided by Dr. S. Weggen, University of Düsseldorf, Germany, and 1:200, University of Bonn, Germany, respectively) and against CD11b, rat monoclonal anti-mouse MCA711 (1:200, Serotec, Oxford, UK; marker for microglia). Finally, the stained sections were mounted onto glasses with 0.1% 1,4-diazobicyclo[2.2.2]octan and examined under a fluorescence microscope (Olympus BX61, Hamburg, Germany).

2.6 Statistical Analysis

All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). For [18 F]FDG, a two-way mixed design ANOVA was used with posthoc Bonferroni correction. In the case of [11 C]-(R)-PK11195, it was necessary to account for the drop-out of animals by analyzing mean values obtained within the complete animal groups at the different imaging stages (before, after and off treatment) using a two-tailed dependent Student's *t* test. Mean values obtained between the two groups were statistically analyzed using a two-tailed independent Student's *t* test. Posthoc Bonferroni correction was applied after every test to account for multiple testing. Statistical significance was set at a 5% level ($p < 0.05$).

3. Supplementary Results

3.1 Quantification of [^{11}C]-(*R*)-PK11195 and [^{18}F]FDG kinetic parameters using an image-derived IF

Typical blood IFs derived from the co-registered [^{11}C]-(*R*)-PK11195- and [^{18}F]FDG μPET images of one representative Tg and Wt mouse are shown in **Suppl. Fig. 2**. IFs demonstrate a rapid initial increase in tracer uptake and a relatively fast clearance from the blood during the remaining scan time (30 minutes for [^{11}C]-(*R*)-PK11195 and 60 minutes for [^{18}F]FDG).

3.2 [^{11}C]-(*R*)-PK11195 distribution volume and [^{18}F]FDG metabolism

μPET imaging using [^{11}C]-(*R*)-PK11195 and [^{18}F]FDG was evaluated in 13-, 14- and 15-month-old Tg ($n = 7$ to 5) and Wt ($n = 8$ to 5) mice at baseline level, after a 5-week treatment period and after a 5-week wash-out period, respectively.

For the semi-quantification of [^{11}C]-(*R*)-PK11195 uptake, % ID/cc and SUV values were calculated from the co-registered dynamic μPET images for all Tg and Wt mice at before, after and off treatment imaging stages. Summarized in **Suppl. Table 1** are the mean values with standard error in the WB, CX, HC, TH and CB. No statistically significant differences were found between different brain regions, genotypes and imaging periods ($p > 0.05$).

With the help of kinetic models, [^{11}C]-(*R*)-PK11195 DV and [^{18}F]FDG K_i values were calculated to assess microglial activation in the brain and changes in cerebral glucose metabolism, respectively. Examples of parametric images of representative Tg and Wt mice are shown in **Fig. 1A** ([^{11}C]-(*R*)-PK11195 DV) and **Suppl. Fig. 3A** ([^{18}F]FDG K_i values). Statistical analysis of the quantified data of [^{11}C]-(*R*)-PK11195 DV revealed a significant difference between Tg and Wt mice in the WB, CX, HC and CB at 15 months (off treatment,

* $p < 0.05$, **Fig. 1B**). However, after posthoc Bonferroni correction for multiple testing, no statistically significant differences in DV could be observed any longer between the two animal groups for any brain region. Pioglitazone treatment did not lead to statistically significant DV differences ($p > 0.05$, **Fig. 1B**). Analysis of [^{18}F]FDG Ki values and the possible anti-inflammatory effect of pioglitazone treatment on cerebral glucose metabolism revealed no statistically significant genotype-, brain region- or treatment-dependent differences ($p > 0.05$, **Suppl. Fig. 3B**).

3.3 Immunofluorescent staining

The extent of activated microglia and A β depositions was assessed with the use of antibodies against CD11b and A β , respectively, and determined in the cortex of 12.5- and 15-month-old Tg and Wt mice (**Fig. 1C**). In Tg mice, immunostaining revealed the presence of activated microglia in the vicinity of amyloid plaques at 12.5 months and, more prominently, at 15 months. On the contrary, in Wt mice no amyloid plaques were present at any time point, and hardly any CD11b-positive cells were detectable, demonstrating an increased microglial activation and plaque formation in Tg mice compared with Wt mice.

4. Supplementary Discussion

In the attempt to non-invasively quantify A β -mediated microglial activation and its therapy-induced modulation in an AD mouse model using [^{11}C]-(*R*)-PK11195 and μPET , we observed statistically significant higher radiotracer DV in 15-month-old Tg mice compared with control mice after discontinuation of the therapeutic modulator pioglitazone. Immunohistochemical analysis of the cortex of Tg mice demonstrated characteristic microglial activation around A β deposition at this stage. There was no evidence of microglial activation in Tg mice at the earlier age of 13-14 months as assessed by statistical analysis of [^{11}C]-(*R*)-PK11195 % ID/cc, SUV and DV values, although immunostaining verified the presence of activated microglia in the cortex of 12.5-month-old Tg mice as compared with Wt mice. No statistically significant differences between Tg and Wt mice were observed at any time point for glucose consumption using [^{18}F]FDG μPET . Taken together, our data may suggest that [^{11}C]-(*R*)-PK11195 μPET is able to assess microglial activation in AD mice when a certain threshold of activation is reached. Moreover, discontinuation of microglia-targeted anti-inflammatory therapy may cause a “rebound” activation.

AD is characterized by the deposition of insoluble A β plaques, which activate microglia to guide amyloid clearance. However, upon continuous activation, microglia secrete neurotoxic pro-inflammatory molecules that cause direct neuronal damage and induce a vicious cycle of chronic neuroinflammatory-mediated cell death (Glass et al., 2010). Noninvasive molecular imaging as well as the creation of Tg mouse models by introducing mutations in APP and/or PS1 genes play a relevant role in improving our understanding of the temporal disease dynamics of AD in humans (Waerzeggers et al., 2010). Several *in vitro* and *in vivo* staining agents (e.g. Thioflavine-S, Bussiere et al., 2004) and imaging tracers that bind to amyloid plaques (e.g. [^{11}C]PIB, Klunk et al., 2004), neurofibrillary tangles (e.g. [^{18}F]FDDNP, Shoghi-Jadid et al., 2002) and activated microglia (e.g. [^{11}C]-(*R*)-PK11195, Schroeter et al., 2009)

have already been used to provide new insights in disease pathogenesis and progression and to serve as biomarkers for early diagnosis and disease activity. Especially for immunotherapeutic strategies, imaging biomarkers have been used as therapy read-out in the experimental and clinical applications (Bacskai et al., 2001; Rinne et al., 2010).

In this study, we hypothesized that APP/PS1 mice have an increased level of activated microglia and thus neuroinflammation compared with control mice. Therefore, we investigated % ID/cc, SUV and DV values of [^{11}C]-(*R*)-PK11195 in brains of APP/PS1 Tg mice in comparison to control mice *in vivo* by μPET . We chose to assess microglial activation in animals between 13 and 15 months of age, having verified the presence of activated microglia in the cortex of 12.5-month-old Tg mice by the use of immunostaining. At the age of 13 months (baseline) however, the examined values showed no statistically significant differences between Tg and Wt mice for any of the brain regions. Moreover, no significant differences between the various brain regions were found within the animal groups, most likely due to the small size of the mouse brain, complicating discernment between brain regions of interest as a result of partial volume and spill-over effects. Further approaches to detect possible differences between the groups included the determination of binding potentials, which failed because we were not able to define a proper reference region with low (background) [^{11}C]-(*R*)-PK11195 uptake. These results are in line with previous publications. Although Ruan *et al.* (2009) validated the presence of activated microglia in the same AD mouse model using CD11b staining at an earlier stage of life, Venneti *et al.* (2009a) failed to confirm a significantly higher amount of activated microglia in the same AD mouse model using Iba-1 *in vitro* microglial staining and [^{11}C]-(*R*)-PK11195 *in vivo* μPET imaging compared with control mice in a 13 to 16 months age group. In the same study, Venneti *et al.* performed identical experiments in 16- to 19-month-old Tg and control mice. The results in this age group showed a significant correlation between both Iba-1 staining and [^{11}C]-(*R*)-PK11195 uptake, and the abundance of activated microglia in APP/PS1 mice compared with

control mice. Therefore, we speculate that the number of activated microglia is below the limit of detection by [^{11}C]-(*R*)-PK11195 μPET at earlier stages of AD. Wiley *et al.* (2009) have come to similar conclusions in a recent multi-tracer human PET study in which they investigated the relationship between microglial activation and A β depositions in the brains of healthy subjects and of patients with mild to moderate AD. They did not observe differences in brain [^{11}C]-(*R*)-PK11195 retention between the different subject groups nor in relation to A β as indicated by [^{11}C] Pittsburgh compound B (PiB) retention.

Follow-up measurements in our study were performed 5 and 10 weeks after the baseline study and were combined with the idea that microglial activation could be modulated by a PPAR- γ agonist. PPAR- γ is a nuclear receptor that plays an important role in inflammatory processes. Agonists that bind and activate PPAR- γ , suppress inflammation by inhibiting the expression of pro-inflammatory molecules at the transcriptional level. Therefore, we expected to detect a therapy-induced reduction in comparison to the baseline level of activated microglia in Tg mice after anti-inflammatory treatment with the PPAR- γ agonist pioglitazone. Lim *et al.* (2000) and Heneka *et al.* (2005) used in addition the PPAR- γ agonist ibuprofen in another Tg mouse model for AD, and immunohistochemistry showed a significant decrease of microglial activation in the ibuprofen-treated mice. We did not include an additional ibuprofen treated control group since the previous data from Heneka *et al.* (2005) had shown the most pronounced treatment effect in response to pioglitazone. To assess the efficacy of our treatment, we imaged Tg and Wt mice after a 5-week treatment period (14 months) as well as after a 5-week wash-out period (15 months). Quantification of [^{11}C]-(*R*)-PK11195 % ID/cc, SUV and DV values revealed no statistically significant differences between Tg and Wt mice at 14 months of age. At 15 months, however, DV values were found to be significantly higher in Tg mice compared with Wt mice. Posthoc Bonferroni correction for multiple comparisons, however, resulted in the deterioration of any significant genotype-dependent differences in the level of neuroinflammation.

Immunohistochemistry obtained from representative animals shortly after off-treatment imaging confirmed the presence of activated microglia clustered around A β plaques in the cortex of Tg mice, which was more pronounced than in the cortex of 12.5-month-old Tg mice. The presence of activated microglia has already been shown in the human AD brain (Itagaki et al., 1989) and in a mouse model of AD (Frautschy et al., 1998) and has been confirmed by other research groups in more recent studies (Bolmont et al., 2008; Meyer-Luehmann et al., 2008; Rodriguez et al., 2010). Our μ PET and immunohistochemistry results in the 15 months age group suggest the occurrence of neuroinflammation in the brains of APP/PS1 mice. The fact that we were not able to detect any statistical significant differences at earlier disease stages may as well be due to the fact that [^{11}C]-(*R*)-PK11195 has a high level of non-specific binding, a poor signal-to-noise ratio and relatively low sensitivity, all complicating PET quantification. Newly developed TSPO-targeted radiotracers with an improved target-to-background ratio may be able to detect activated microglia even at this early stage of disease progression (Chauveau et al., 2008).

In addition to non-invasive quantification of microglial activation, we aimed to investigate the correlation between neuroinflammation and neuronal function as determined by [^{18}F]FDG and μ PET. [^{18}F]FDG μ PET imaging was performed at every [^{11}C]-(*R*)-PK11195 imaging stage. We calculated the net influx K_i rate constant with the use of a two-tissue-compartment kinetic model to assess differences and changes in cerebral glucose metabolism in both Tg and Wt mice. As for [^{11}C]-(*R*)-PK11195, we compared [^{18}F]FDG metabolism between Tg and Wt mice in the same brain regions. K_i values between Tg and Wt mice at baseline, after treatment and after the wash-out period did not show any statistically significant differences and the PPAR- γ agonist pioglitazone presented no effect on [^{18}F]FDG metabolism either. Heneka *et al.* (2006) performed [^{18}F]FDG μ PET in APP23 mice, another mouse model for AD, and found no significant difference in cerebral glucose metabolism under basal conditions between AD and control mice, supporting our results. Only after inflicting

neuronal damage to the locus coeruleus, a significant difference between the two animal groups could be observed. A possible explanation for preserved cerebral glucose metabolism in Tg mice compared with Wt mice may be the lack of a global neuronal loss in the brains of APP and APP/PS1 mouse models, despite their extensive amyloid plaque pathology at the age of investigation (Irizarry et al., 1997; Takeuchi et al., 2000).

In conclusion, our multi-tracer μ PET study in APP_{Swe}/PS1_{dE9} mice and their littermate controls did not depict the hypothesized differences in [^{11}C]-(*R*)-PK11195 DV and [^{18}F]FDG Ki values as measured for altered microglial activity and glucose metabolism, respectively, at the ages of 13 and 14 months. At 15 months of age, after discontinuation of pioglitazone treatment, we observed increased [^{11}C]-(*R*)-PK11195 DV values in Tg mice which may indicate natural disease progression or “rebound” activation of microglia. With the given limitations of the radiopharmaceutical [^{11}C]-(*R*)-PK11195, subtle differences in microglial activation may be missed by this imaging method.

Disclosure statement

All authors declare no conflict of interest related to this study. None of the authors' institutions have contracts relating to this research through which it or any other organization may stand to gain financially now or in the future. There are no other agreements of authors or their institutions that could be seen as involving a financial interest in this work. The data contained in the manuscript being submitted is original unpublished work and has not been submitted to any other journal for reviews. All animal procedures were carried out in accordance with the German Laws for Animal Protection and were approved by the local animal care committee and by the local governmental authorities. All authors have reviewed the contents of the manuscript being submitted, approve of its contents, and validate the accuracy of the data.

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Supplementary Legends

Suppl. Table 1. [^{11}C]-(*R*)-PK11195 mean % ID/cc and SUV values with standard deviation were determined for each animal group in the WB, CX, HC, TH and CB at the different imaging stages. No genotype- or treatment dependent statistically significant differences were found for any of the brain regions (2-tailed dependent and independent Student's *t* test with posthoc Bonferroni correction, $p > 0.05$).

Suppl. Fig. 1. Example of co-registration of multi-tracer μPET images ([^{18}F]FDG, [^{11}C]-(*R*)-PK11195 and [^{18}F]fluoride) of a representative Tg mouse with its corresponding high-resolution MRI and the Swanson mouse brain atlas. The contour (in red) of the threshold of the [^{18}F]fluoride image is projected onto the other μPET images (here onto the [^{11}C]-(*R*)-PK11195 image) to guide the correct placement of the mouse brain atlas (projected here onto the [^{18}F]FDG image). From left to right a coronal, transaxial and sagittal view of the images is displayed.

Suppl. Fig. 2. Example of μPET image derived blood input functions from [^{11}C]PK11195, extracted from a heart ROI TAC in a representative Tg mouse (*open triangle*) and a Wt mouse (*open circle*), and from [^{18}F]FDG, derived from a liver ROI TAC in a Tg mouse (*closed triangle*) and Wt mouse (*closed circle*). All curves show a clear peak after bolus tracer injection, followed by a relatively fast clearance during the rest of the acquisition time (30 min for [^{11}C]-(*R*)-PK11195 and 60 min for [^{18}F]FDG).

Suppl. Fig. 3. (A) Representative coronal parametric images show no reproducible differences in [^{18}F]FDG K_i values between Tg (*upper*) and Wt (*lower*) mouse brain, nor visually significant differences between before, after and off treatment imaging. **(B)** Quantification of [^{18}F]FDG metabolism (presented as K_i values) in the whole brain of Tg and Wt mice shows no statistically significant genotype-, brain region- or treatment-dependent differences (mean $\pm$ SEM, two-way mixed design ANOVA with posthoc Bonferroni correction, $p > 0.05$).

List of abbreviations

% ID/cc	Percentage injected dose per cubic centimeter
A β	Amyloid beta
AD	Alzheimer's disease
APP	Amyloid precursor protein
BP	Binding potential
(μ)Ci	(micro)Curie
DMF	Dimethylformamide
DV	Distribution volume
FDG	2-fluoro-2-deoxy-D-glucose
HPLC	High-performance liquid chromatography
IF	Input function
MRI	Magnetic resonance imaging
p.p.m.	parts per million
PBR	Peripheral benzodiazepine receptor
PBS	Phosphate buffered saline
(μ)PET	(micro)Positron emission tomography
PFA	Paraformaldehyde
PPAR	Peroxisome proliferator-activated receptor
PS1	Presenilin-1
SUV	Standardized uptake value
TAC	Time activity curve
TE	Echo time
Tg	Transgenic
TR	Repetition time
TSPO	Translocator protein
VOI	Volume of interest
Wt	Wild-type

	Before Treatment (13m)		After Treatment (14m)		Off Treatment (15m)	
%ID/cc	Tg (<i>n</i> = 7)	Wt (<i>n</i> = 8)	Tg (<i>n</i> = 6)	Wt (<i>n</i> = 8)	Tg (<i>n</i> = 5)	Wt (<i>n</i> = 5)
WB	2.62 ± 0.76	2.25 ± 0.63	2.58 ± 0.46	2.64 ± 0.61	2.30 ± 0.33	2.70 ± 0.75
CX	2.56 ± 0.77	2.07 ± 0.58	2.43 ± 0.48	2.40 ± 0.54	2.20 ± 0.31	2.50 ± 0.63
HC	2.56 ± 0.83	2.27 ± 0.64	2.48 ± 0.48	2.49 ± 0.60	2.24 ± 0.32	2.62 ± 0.67
TH	2.77 ± 0.90	2.53 ± 0.79	2.57 ± 0.50	2.68 ± 0.65	2.36 ± 0.34	2.80 ± 0.74
CB	2.56 ± 0.62	2.13 ± 0.56	2.61 ± 0.50	2.72 ± 0.58	2.33 ± 0.27	2.72 ± 0.77
SUV	Tg (<i>n</i> = 7)	Wt (<i>n</i> = 8)	Tg (<i>n</i> = 6)	Wt (<i>n</i> = 8)	Tg (<i>n</i> = 5)	Wt (<i>n</i> = 5)
WB	79.8 ± 22.1	71.1 ± 24.9	84.4 ± 26.1	78.6 ± 17.9	82.3 ± 14.4	86.9 ± 31.3
CX	77.8 ± 22.9	65.6 ± 23.9	79.9 ± 27.0	71.6 ± 15.7	78.5 ± 14.4	80.5 ± 28.3
HC	77.7 ± 23.9	72.0 ± 26.7	81.6 ± 27.0	74.3 ± 18.0	80.0 ± 15.6	84.5 ± 30.6
TH	84.1 ± 25.6	79.6 ± 28.4	84.6 ± 28.3	79.9 ± 20.1	84.0 ± 14.9	90.3 ± 32.8
CB	78.2 ± 18.4	67.9 ± 24.5	85.5 ± 25.7	80.9 ± 16.2	84.1 ± 19.2	87.1 ± 30.5