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Chronic PPARγ Stimulation Shifts Amyloidosis to Higher Fibrillarity but Improves Cognition

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We undertook longitudinal β-amyloid positron emission tomography (Aβ-PET) imaging as a translational tool for monitoring of chronic treatment with the peroxisome proliferator-activated receptor gamma (PPARγ) agonist pioglitazone in Aβ model mice. We thus tested the hypothesis this treatment would rescue from increases of the Aβ-PET signal while promoting spatial learning and preservation of synaptic density. Here, we investigated longitudinally for 5 months PS2APP mice (N = 23; baseline age: 8 months) and App^{NL-G-F} mice (N = 37; baseline age: 5 months) using Aβ-PET. Groups of mice were treated with pioglitazone or vehicle during the follow-up interval. We tested spatial memory performance and confirmed terminal PET findings by immunohistochemical and biochemistry analyses. Surprisingly, Aβ-PET and immunohistochemistry revealed a shift toward higher fibrillary composition of Aβ-plaques during upon chronic pioglitazone treatment. Nonetheless, synaptic density and spatial learning were improved in transgenic mice with pioglitazone treatment, in association with the increased plaque fibrillarity. These translational data suggest that a shift toward higher plaque fibrillarity protects cognitive function and brain integrity. Increases in the Aβ-PET signal upon immunomodulatory treatments targeting Aβ aggregation can thus be protective.

Keywords: pioglitazone, Aβ-PET, App^{NL-G-F} mice, PS2APP mice, microglia, Aβ-plaque composition

INTRODUCTION

Alzheimer's disease (AD) has become the most common cause of dementia, and is imposing a significant burden on health care systems of societies with aging populations (Ziegler-Graham et al., 2008). During the past few decades, research on AD pathogenesis led to the formulation of a model that accumulation of amyloid beta (Aβ)-plaques and neurofibrillary tangles, the histologically characterizing hallmarks of AD (Braak and Braak, 1991), triggers a cascade of neurodegenerative events, leading to disease progression (Sasaguri et al., 2017). Additionally, novel emerging evidence indicates that neuroinflammation plays an important role in pathogenesis and progression of AD and many other neurodegenerative diseases (Zimmer et al., 2014; Heneka et al., 2015). In AD, activated microglial cells are able to bind and phagocytize soluble Aβ, and to some degree also the fibrillary Aβ aggregates, as part of the increased inflammatory response (Heneka et al., 2015). However, others report that Aβ-recognition receptors on microglia downregulate during the progression of AD, such that microglial cells eventually undergo senescence, characterized by reduced phagocytosis of Aβ-aggregates (Hickman et al., 2008). With time, the decreased microglial activity is permissive to expansion of fibrillar amyloidosis (Heppner et al., 2015; Blume et al., 2018) and a high proportion of dystrophic microglia were observed in human AD brain *post mortem* (Streit et al., 2014). These observations have led some to speculate that the microglial response is overwhelmed by the massive Aβ-deposition occurring in advanced AD, such that their chronic activation has a detrimental impact on disease progression (Hickman et al., 2008; Lee and Landreth, 2010).

It might follow that treatment with anti-inflammatory drugs should alleviate AD progression. Pioglitazone is an anti-inflammatory insulin sensitizer widely used to treat hyperglycemia in type 2 diabetes *via* activation of peroxisome proliferator-activated receptor gamma (PPAR-γ). Treatment with pioglitazone enables microglial cells to undergo a phenotypic conversion from a pro-inflammatory toward an anti-inflammatory and neuroprotective phenotype (Mandrekar-Colucci et al., 2012; Yamanaka et al., 2012). Furthermore, activation of PPAR-γ in the brains of AD mice initiate a coupled metabolic cycle with the Liver X Receptor to increase brain apolipoprotein E levels, which promotes the ability of microglial cells to phagocyte and degrade both soluble and fibrillary Aβ (Mandrekar-Colucci et al., 2012; Yamanaka et al., 2012). However, another study showed that only low-dose PPAR-γ agonist treatment, but not the conventional doses, promotes an Aβ-clearing effect by increasing (LDL Receptor Related Protein 1 (LRP1) in human brain microvascular endothelial cells (HBMECs) (Moon et al., 2012). Despite this compelling preclinical evidence, a meta-analysis encompassing nine clinical studies did not compelling support a beneficial effect of PPAR-γ agonist treatment on cognition and memory in in patients with mild-to-moderate AD (Cheng et al., 2016). Furthermore, a phase III trial of pioglitazone in patients with mild AD was discontinued due to lacking efficacy (Geldmacher et al., 2011). It remains a conundrum why the translation of PPARγ stimulation into human AD failed, which calls for further investigation to uncover the basis of the seemingly false lead. Conceivably, the efficacy of pioglitazone may be confined to a specific stage of AD, or in cases distinguished by a particular biomarker.

Given this background, we hypothesized that Aβ-load and composition would determine the individual efficacy of PPARγ stimulation effect in the progression of AD mouse models. Therefore, we undertook serial small animal positron emission tomography (μPET) with the Aβ-tracer [^{18}F]florbetaben (Manook et al., 2012; Rominger et al., 2013; Brendel et al., 2015a,b) in two AD mouse models with distinct Aβ-plaque composition. The transgenic PS2APP-line develops dense fibrillary Aβ-plaques with late debit whereas the knock-In mouse model App^{NL-G-F} develops more diffuse oligomeric Aβ-plaques with early debut. Both strains of mice were treated with pioglitazone or vehicle for 5 months during the phase of main Aβ accumulation. We conducted behavioral assessments of spatial learning and confirmed longitudinal PET findings by immunohistochemical analysis and biochemical analysis, thus aiming to test the hypothesis that response to pioglitazone would depend on the type of Aβ-plaques formed in transgenic mice.

MATERIALS AND METHODS

Study Design

Groups of PS2APP and App^{NL-G-F} mice were randomized to either treatment (PS2APP-PIO N = 13, all female; App^{NL-G-F}-PIO N = 14, N = 10 male, N = 4 female) or vehicle (PS2APP-VEH N = 10, all female; App^{NL-G-F}-VEH N = 23 N = 9 male, N = 14 female) groups at the age of 8 (PS2APP) and 5 (App^{NL-G-F}) months. In PS2APP mice, the baseline [^{18}F]florbetaben-PET scan (Aβ-PET) was performed at the age of 8 months, followed by initiation of pioglitazone treatment or vehicle for a period of 5 months and a follow-up Aβ-PET scan at 13 months. In App^{NL-G-F} mice, the baseline Aβ-PET scan was performed at the age of 5 month, followed by initiation of pioglitazone treatment or vehicle, for a period of 5 months. Follow-up Aβ-PET scans were acquired at 7.5 and 10 months of age, which was the study termination in App^{NL-G-F} mice. Mice were fed *ad libitum* with food pellets formulated with pioglitazone at a dose of 350 mg/kg or unaltered control pellets. The food was available to the mice without restriction.

For all mice, behavioral testing after the terminal PET scan was followed by immunohistochemical and biochemical analyses of randomized hemispheres. The TSPO-PET arm of the study and detailed analyses of neuroinflammation imaging are reported in a separate manuscript focusing on the predictive value of TSPO-PET for outcome of PPARγ-related immunomodulation (Biechele et al., 2022). The sample size estimation of the *in vivo* PET study was based on previous experience and calculated by G*power (V3.1.9.2, Kiel, Germany), assuming a type I error $\alpha = 0.05$ and a power of 0.8 for group comparisons, a 10% drop-out rate per time-point (including TSPO-PET), and a treatment effect of 5% change in the PET signal. Shared datapoints between the study arms are indicated.

Animals

PS2APP transgenic (Ozmen et al., 2008), App^{NL-G-F} APP knock-in (Saito et al., 2014) and wild-type C57Bl/6 mice were used in this investigation (for details see **Supplementary Material**). All experiments were performed in compliance with the National Guidelines for Animal Protection, Germany, with approval of the local animal care committee of the Government of Oberbayern (Regierung Oberbayern) and overseen by a veterinarian. The experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Animals were housed in a temperature and humidity-controlled environment with a 12-h light–dark cycle, with free access to food (Ssniff) and water.

Aβ-PET Acquisition and Reconstruction

[^{18}F]florbetaben radiosynthesis was performed as previously described (Rominger et al., 2013). This procedure yielded a radiochemical purity exceeding 98% and a specific activity of 80 ± 20 GBq/μmol at the end of synthesis. Mice were anesthetized with isoflurane (1.5%, delivered *via* a mask at 3.5 L/min in oxygen) and received a bolus injection [^{18}F]florbetaben 12 ± 2 MBq in 150 μL of saline to a tail vein. Following placement in the tomograph (Siemens Inveon DPET), a single frame emission recording for the interval 30–60 min p.i., which was preceded by a 15-min transmission scan obtained using a rotating [^{57}Co] point source. The image reconstruction procedure consisted of three-dimensional ordered subset expectation maximization (OSEM) with four iterations and twelve subsets followed by a maximum *a posteriori* (MAP) algorithm with 32 iterations. Scatter and attenuation correction were performed and a decay correction for [^{18}F] was applied. With a zoom factor of 1.0 and a 128 × 128 × 159 matrix, a final voxel dimension of 0.78 × 0.78 × 0.80 mm was obtained.

Small-Animal PET Data Analyses

Volumes of interest (VOIs) were defined on the MRI mouse atlas (Dorr et al., 2007). A forebrain target VOI (15 mm^3) was used for group comparisons and an additional hippocampal target VOI (8 mm^3) served for correlation analysis with spatial learning. We calculated [^{18}F]florbetaben standard-uptake-value ratios (SUVRs) using the established white matter (PS2APP; 67 mm^3; pons, midbrain, hindbrain and parts of the subcortical white matter) and periaqueductal gray (App^{NL-G-F}; 20 mm^3) reference regions (Brendel et al., 2016; Overhoff et al., 2016; Sacher et al., 2019).

Water Maze

Two different water maze tasks were applied due to changing facilities between the investigations of PS2APP and App^{NL-G-F} cohorts. We used a principal component analysis of the common read outs of each water maze task to generate a robust index for correlation analyses in individual mice (Biechele et al., 2020). The principal component of the water maze test was extracted from three spatial learning read-outs (PS2APP: escape latency, distance, platform choice; App^{NL-G-F}: escape latency, frequency to platform, time spent in platform quadrant). Thus, one quantitative index of water maze performance per mouse was generated for correlation with PET imaging readouts. The experimenter was blind to the phenotype of the animals.

Water Maze in PS2APP Mice

PS2APP and age-matched wild-type mice were subjected to a modified Morris water maze task as described previously (Sauvage et al., 2000; Busche et al., 2015; Keskin et al., 2017; Focke et al., 2019) yielding escape latency, distance to the correct platform and correct choice of the platform as read-outs.

Water Maze in App^{NL-G-F} Mice

App^{NL-G-F} mice (treated and vehicle) and 14 age- and sex-matched wild-type mice (vehicle) underwent a classical Morris water maze test, which was performed according to a standard protocol with small adjustments (Bromley-Brits et al., 2011) as previously described (Sacher et al., 2019). Details are provided in the **Supplementary Material**.

Immunohistochemistry

Immunohistochemistry in brain regions corresponding to PET analyses was performed for fibrillary as well as pre-fibrillary Aβ, microglia and synaptic density as previously published (Dorostkar et al., 2010; Brendel et al., 2017a,b). We obtained immunofluorescence labeling of pre-fibrillary Aβ using NAB228 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with a dilution of 1:500 (Monasor et al., 2020). For histological staining against fibrillar Aβ, we used methoxy-X04 (TOCRIS, Bristol, United Kingdom) at a dilution of 0.01 mg/ml in the same slice as for NAB228 staining. We obtained immunofluorescence labeling of microglia using an Iba-1 antibody (Wako, Richmond, United States) with a dilution of 1:200 co-stained with CD68 (BioRad, Hercules, CA, United States) with a dilution of 1:100. The synaptic density was measured using an anti-vesicular glutamate transporter 1 (VGLUT1) primary antibody (1:500, MerckMillipore, Billerica, Massachusetts, United States). Quantification was calculated as area-%. Details are provided in the **Supplementary Material**.

Biochemical Characterization of Brain Tissue

DEA (0.2% Diethylamine in 50 mM NaCl, pH 10) and RIPA lysates (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate) were prepared from brain hemispheres. The later was centrifuged at 14,000 g (60 min at 4°C) and the remaining pellet was homogenized in 70% formic acid (FA fraction). The FA fraction was neutralized with 20 × 1 M Tris-HCl buffer at pH 9.5 and used further diluted for Aβ analysis. Aβ contained in FA fractions was quantified by a sandwich immunoassay using the Meso Scale Aβ Triplex plates and Discovery SECTOR Imager 2400 as described previously (Page et al., 2008). Samples were measured in triplicates.

Statistics

The principal component of the water maze test was extracted using SPSS 26 statistics (IBM Deutschland GmbH, Ehningen,

Germany). Prior to the PCA, the linear relationship of the data was tested by a correlation matrix and items with a correlation coefficient < 0.3 were discarded. The Kaiser-Meyer-Olkin (KMO) measure and Bartlett's test of sphericity were used to test for sampling adequacy and suitability for data reduction. Components with an Eigenvalue >1.0 were extracted and a varimax rotation was selected. Water maze results were also used as an endpoint in the dedicated manuscript on serial TSPO-PET in both cohorts (Biechele et al., 2022). For immunohistochemistry quantifications GraphPad Prism (GraphPad Software, San Diego, California, United States) was used. All analyses were performed by an operator blinded to the experimental conditions. Data were normally distributed according to Shapiro−Wilk or D'Agostino-Pearson test. One-way analysis of variance (ANOVA) including Bonferroni *post-hoc* correction was used for group comparisons >2 subgroups. For assessment of inter-group differences at single time points, Student's *t*-test (unpaired, two-sided) was applied. All results are presented as mean ± SEM. *P*-values <0.05 are defined as statistically significant.

RESULTS

Long-Term Pioglitazone Treatment Provokes a Significant Increase of the Aβ-PET Signal in PS2APP Mice

First, we analyzed serial changes of fibrillar amyloidosis under chronic pioglitazone treatment by [^{18}F]florbetaben Aβ-PET in PS2APP mice and wild-type controls. Vehicle treated PS2APP mice showed an elevated Aβ-PET SUVR when compared to vehicle treated wild-type at 8 (+20.4%, $p < 0.0001$) and 13 months of age (+37.9%, $p < 0.0001$). As expected, the Aβ-PET SUVR of wild-type mice did not change between 8 and 13 months of age (0.831 ± 0.003 vs. 0.827 ± 0.008: $p = 0.645$). Surprisingly, pioglitazone treatment provoked a stronger longitudinal increase in the Aβ-PET signal of PS2APP mice (+21.4%) when compared to vehicle treated PS2APP mice (+14.1%, $p = 0.002$). At the follow-up time point, the Aβ-PET SUVR was significantly elevated when compared to untreated PS2APP mice (**Figure 1**; 1.140 ± 0.014 vs. 1.187 ± 0.011; $p = 0.0017$). Pioglitazone treatment in wild-type mice provoked no changes of Aβ-PET SUVR compared to vehicle-treated wild-type mice at the follow-up time-point (0.827 ± 0.008 vs. 0.823 ± 0.005: $p = 0.496$; for images of wild-type mice see **Supplementary Figure 1**). Taken together, we found a significant increase in the Aβ-PET signal, which implied an increase in fibrillary Aβ-levels under pioglitazone treatment in PS2APP mice.

Aβ-PET Detects a Strong Increase of the Fibrillar Aβ-Load in *App*$^{NL-G-F}$ Mice During Chronic PPARγ Stimulation

Next, we sought to validate our unexpected findings in PS2APP mice a mouse model with differing Aβ plaque composition, namely the *App*$^{NL-G-F}$ mouse, which has limited fibrillarity due to endogenous expression of APP with three FAD mutations (Saito et al., 2014). Strikingly, the effect of pioglitazone treatment on the Aβ-PET signal was even stronger in *App*$^{NL-G-F}$ mice than in PS2APP mice. There was a pronounced increase of the Aβ-PET signal during chronic pioglitazone treatment (+17.2%) compared to vehicle (+5.3%, $p < 0.0001$). *App*$^{NL-G-F}$ mice with pioglitazone treatment had a higher Aβ-PET SUVR at 7.5 (+4.6%, $p = 0.0071$) and 10 (+7.7%, $p < 0.0001$) months of age when compared to vehicle-treated *App*$^{NL-G-F}$ mice (**Figure 2**). The baseline level of Aβ-PET SUVR was non-significantly lower in treated compared to untreated *App*$^{NL-G-F}$ mice (0.878 ± 0.010 vs. 0.906 ± 0.006, $p = 0.1350$). In both mouse models, the Aβ-signal increase after pioglitazone-treatment compared to baseline scans was pronounced in the frontotemporal cortex and hippocampal area (**Figures 1A**, **2A**). In summary, the pioglitazone treatment augmented the Aβ-PET signal increase in both mouse models; this unexpected result was more pronounced in the *App*$^{NL-G-F}$ model, which expresses less fibrillary Aβ plaques.

Pioglitazone Triggers A Shift Toward Increased Aβ-Plaque Fibrillarity in Two Distinct Mouse Models of Amyloidosis

Given the unexpected *in vivo* findings, we set about to evaluate the molecular correlates of the potentiation of Aβ-PET signal during pioglitazone treatment in AD model mice. The (immuno)histochemical analysis showed that the observed increase of the Aβ-PET signal was predominantly explicable by a change in plaque composition rather than by a change in plaque density (**Figure 3**). In both mouse models, the proportion of fibrillary Aβ stained with methoxy-X04 increased significantly under pioglitazone treatment compared to vehicle treated animals (PS2APP: 29.6 ± 3.5% vs. 15.2 ± 0.7%, $p = 0.0056$, **Figure 3C**; *App*$^{NL-G-F}$: 9.1 ± 1.6% vs. 4.4 ± 0.4%, $p = 0.0001$, **Figure 3D**). Pioglitazone treatment had no significant effect on the proportion of pre-fibrillary Aβ stained with NAB228 in PS2APP mice (PS2APP: 65.4 ± 6.1% vs. 67.0 ± 6.9%, $p = 0.865$, **Figure 3C**). In *App*$^{NL-G-F}$ mice, however, the proportion of pre-fibrillary Aβ decreased significantly in treated animals (*App*$^{NL-G-F}$: 26.7 ± 1.7% vs. 34.5 ± 1.7%, $p = 0.0138$, **Figure 3E**). The effect size of pioglitazone treatment on plaque morphology was larger in *App*$^{NL-G-F}$ mice than in PS2APP mice, which was reflected by a significantly increased overlay of methoxy-X04 and NAB228 positive plaques proportions in relation to untreated mice (PS2APP: 40.4 ± 3.6% vs. 25.1 ± 2.1%, $p = 0.0075$, **Figure 3C**; *App*$^{NL-G-F}$: 35.0 ± 3.4% vs. 12.9 ± 1.3%, $p = 0.0005$, **Figure 3E**). We attribute this effect to the generally diffuse nature of the plaque composition of *App*$^{NL-G-F}$ mice, which predominantly contain high oligomeric and low fibrillary fractions of Aβ (Monasor et al., 2020) (compare **Figures 3A,B**).

The number of methoxy positive Aβ-plaques were similar between vehicle and pioglitazone treated groups for PS2APP (1,016 ± 107 vs. 1,118 ± 121, $p = 0.547$, **Figure 3D**) and *App*$^{NL-G-F}$ mice (242 ± 56 vs. 266 ± 33, $p = 0.722$, **Figure 3F**). Notably there was no significant effect of chronic pioglitazone treatment on the different insoluble Aβ species (Aβ40, Aβ42) as well as on the level of the soluble Aβ42-isoform observed in

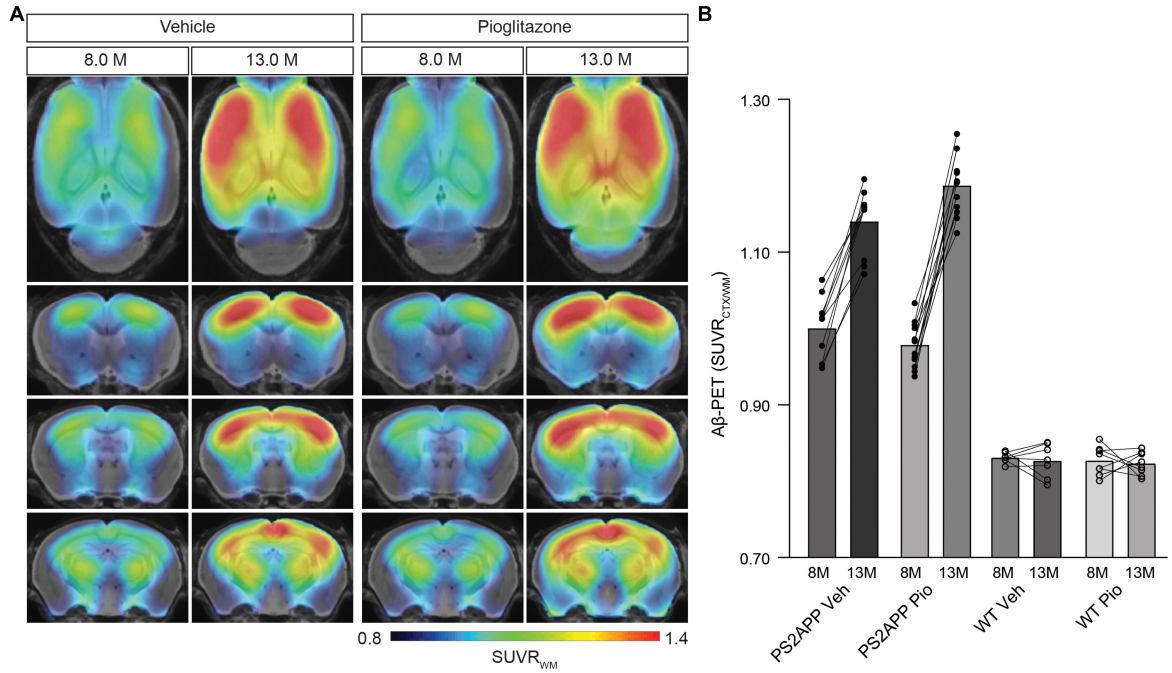

FIGURE 1 | PPARγ stimulation in PS2APP mice provokes an increase in the Aβ-PET signal. **(A)** Regional analysis of group-averaged standardized uptake value ratio (SUVR) images of the Aβ-PET radiotracer [^{18}F]florbetaben in untreated and in pioglitazone-treated PS2APP mice aged 8 and 13 months. Coronal and axial slices are projected upon a standard MRI template. **(B)** Plots show cortical SUVR values of [^{18}F]florbetaben in PS2APP and wild-type (WT) mice between 8 and 13 months of age under vehicle (Veh) or pioglitazone (Pio) treatment. The Aβ-PET signal increased in PS2APP mice during aging, but the increase was more pronounced in pioglitazone treated mice [$F_{(1,\ 12)}$ = 12.9; p = 0.0017]. In wild-type animals, no difference was observed between untreated and treated animals during aging [$F_{(1,\ 13)}$ = 0.490; p = 0.496]. Data are presented as mean ± SEM. P values of Bonferroni *post-hoc* test result from two-way ANOVA. N = 10–13 PS2APP; N = 7–8 WT. PET images of wild-type mice are provided in **Supplementary Figure 1**.

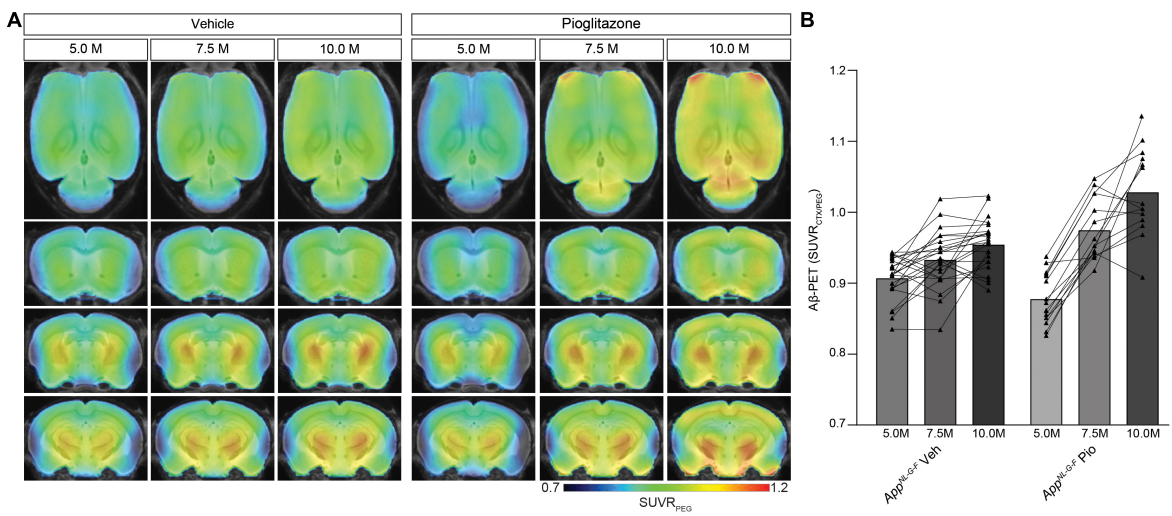

FIGURE 2 | Distinct Aβ-PET signal increase upon PPARγ stimulation in *App*$^{NL-G-F}$ mice with limited plaque fibrillarity and without overexpression of APP. **(A)** Regional analysis of group-averaged standardized uptake value ratios (SUVR) of the Aβ-PET radiotracer [^{18}F]florbetaben in untreated and in pioglitazone treated *App*$^{NL-G-F}$ animals at the age of 5, 7.5, and 10 months. Coronal and axial slices are projected upon a standard MRI template. **(B)** Plots show cortical SUVR of [^{18}F]florbetaben in *App*$^{NL-G-F}$ mice between the age of 5 and 10 months under vehicle or pioglitazone treatment. Aβ-PET signal increased in untreated mice during age but the increase was more pronounced in pioglitazone treated *App*$^{NL-G-F}$ mice [$F_{(2,\ 70)}$ = 20.12; p < 0.0001]. Data are presented as mean ± SEM. P-values of Bonferroni *post-hoc* test result from two-way ANOVA. N = 14–23.

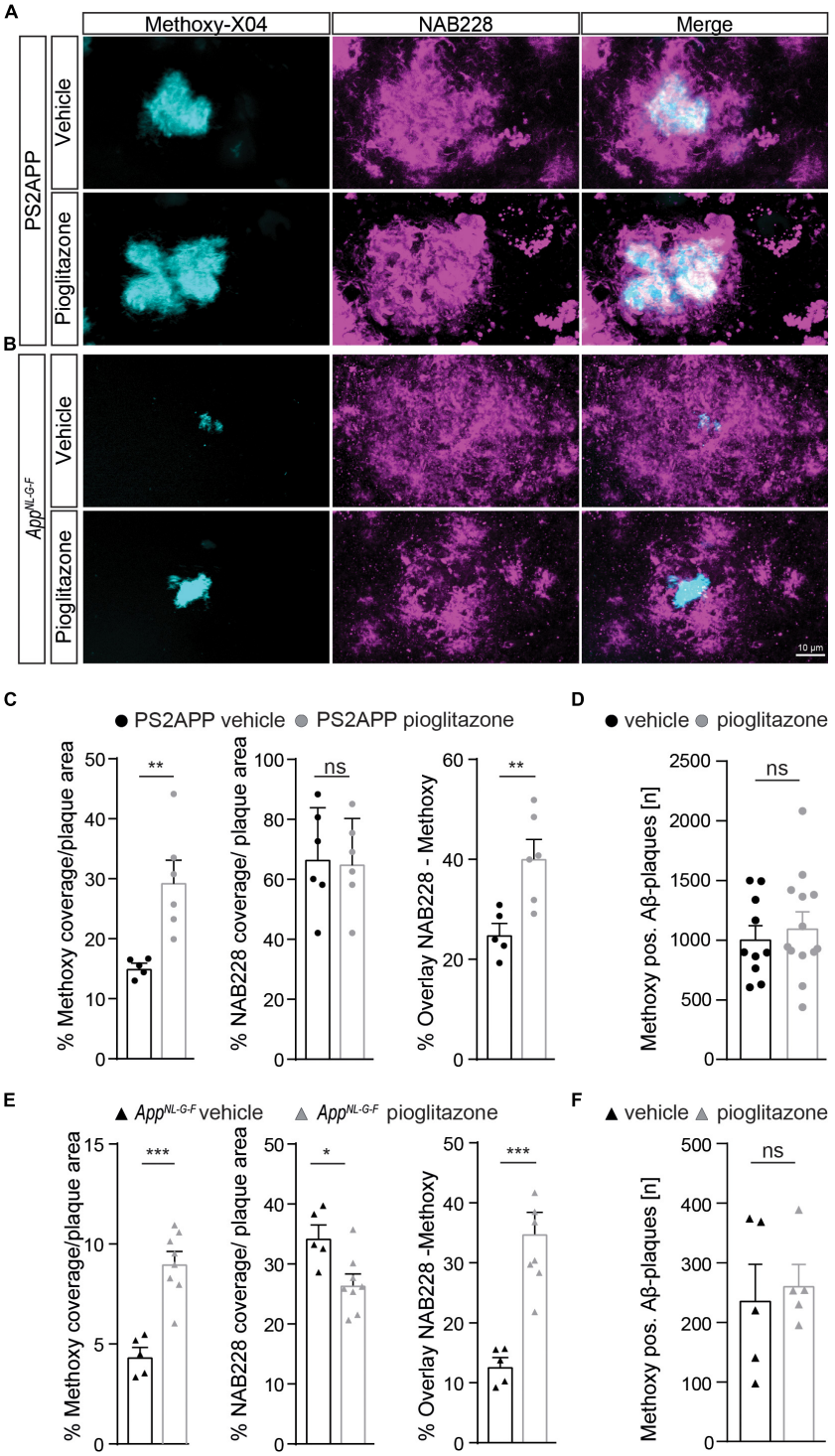

FIGURE 3 | Pioglitazone treatment triggers a change in plaque composition in two different mouse models of amyloidosis. Staining of fibrillary Aβ (methoxy-X04, cyan) and pre-fibrillary Aβ (NAB228, magenta) in vehicle and pioglitazone treated PS2APP mice **(A)** and App^{NL-G-F} mice **(B)**. **(C)** The plaque area covered by methoxy-X04 staining was significantly higher [$t_{(9)} = 3.612$; $p = 0.0056$], whereas the plaque area covered by NAB228 staining remained equal [$t_{(10)} = 0.175$; $p = 0.865$] in pioglitazone treated PS2APP mice. The overlay of NAB228 and methoxy staining increased under pioglitazone treatment [$t_{(9)} = 3.432$; $p = 0.0075$]. **(D)** The number of methoxy positive Aβ-plaques did not change under pioglitazone treatment in PS2APP-mice. **(E)** In App^{NL-G-F} mice, methoxy coverage [$t_{(11)} = 5.802$; $p = 0.0001$], NAB228 coverage [$t_{(11)} = 5.80$; $p = 0.0001$], as well as the overlay of both staining [$t_{(11)} = 2.93$; $p = 0.0138$], increased under pioglitazone treatment. **(F)** In App^{NL-G-F} mice, the number of methoxy positive Aβ-plaques did not change under pioglitazone. Data are presented as mean ± SEM; $n = 5$–13 mice. Two-sample student's *t*-test results: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

either mouse model (**Supplementary Figure 2**). Taken together, our results indicate that the potentiated increase of the Aβ-PET signal upon pioglitazone treatment reflected a change in plaque composition from less dense pre-fibrillar amyloid aggregates to fibrillary Aβ-fractions.

Microglial Activation Is Reduced Upon PPARγ Stimulation in Both Alzheimer's Disease Mouse Models

To confirm changes in the activation state of microglial cells, we performed Iba1 as well as CD68 immunohistochemical staining of activated microglia in both mouse models. We observed that pioglitazone treatment significantly decreased microglial activation in both mouse models (**Figure 4**). In PS2APP mice, PPARγ stimulation provoked a one-third reduction of area coverage of Iba1-positive microglial cells (area: 9.1 ± 0.6%) compared to untreated mice (14.0 ± 0.5%, p = 0.0003), and also a significant reduction of CD68-positive microglial cells area (7.6 ± 0.4% vs. 9.9 ± 0.3%, p = 0.0018). In pioglitazone treated App^{NL-G-F} mice, the area reduction was less pronounced, but still significant for Iba1-positive microglial cells (9.4 ± 0.2% vs. 10.6 ± 0.2%, p = 0.0015) and CD68-positive microglial cells (2.7 ± 0.1% vs. 3.0 ± 0.1%, p = 0.0141) compared to untreated mice. Thus, we observed a consistent net reduction of activated microglial coverage in both models; the lesser effect in App^{NL-G-F} mice might indicate partial compensation by triggering of microglial activation due to increased fibrillary Aβ levels (Sebastian et al., 2020).

Cognitive Function Is Improved by Chronic Pioglitazone Treatment in Association With an Increasing Aβ-PET Rate of Change

Finally, we aimed to elucidate whether the observed longitudinal changes in the composition of Aβ-plaques affected synaptic density and hippocampus related cognitive performance.

In PS2APP mice, treatment with pioglitazone resulted in a significant reduction of the water maze performance index compared to untreated mice during the probe trial (**Figure 5A**; p = 0.0155), whereas in wild-type animals there was no difference between treated and untreated animals ($p > 0.999$). The water maze performance index of pioglitazone treated PS2APP mice correlated strongly with the rate of increase in Aβ-PET signal (**Figure 5C**; R = 0.686; p = 0.0097). In App^{NL-G-F} mice, pioglitazone treatment did not result in a significant change of spatial learning performance (**Figure 5B**; $p > 0.999$). Accordingly, the water maze performance index and the rate of change in the Aβ-PET signal of pioglitazone treated App^{NL-G-F} mice did not correlate significantly (**Figure 5D**; R = 0.341; p = 0.254). There was no significant association between the water maze performance index and the Aβ-PET rate of change in vehicle treated PS2APP or App^{NL-G-F} mice.

To explore the basis of water maze results in PS2APP mice at the molecular level, we performed staining of synaptic density in the hippocampus. Aβ-oligomers are the primary neurotoxic forms of Aβ, while Aβ-fibrils have less neurotoxicity (Hardy and Selkoe, 2002; Haass and Selkoe, 2007; Zott et al., 2019). Thus, we hypothesized that pre-synaptic density in the hippocampal CA1-Area would be rescued upon pioglitazone-treatment. In wild-type mice we did not observe altered changed VGLUT1 density under pioglitazone treatment (**Figure 5E**, F; 0.519 ± 0.007 1/μm vs. 0.502 ± 0.008 1/μm, p = 0.810). In PS2APP mice, however, we found that pioglitazone treatment significantly rescued spine density in the CA1-region of the hippocampus compared to untreated animals (**Figures 5E,F**; 0.497 ± 0.006 1/μm vs. 0.459 ± 0.007 1/μm, p = 0.0012), supporting the hippocampal-dependent water maze results.

DISCUSSION

To our knowledge, this is the first large-scale longitudinal PET study of cerebral Aβ-deposition in two distinct AD mouse models treated with the PPARγ agonist pioglitazone. We combined *in vivo* PET monitoring with behavioral testing and detailed immunohistochemical analysis. Our main finding was an unexpected potentiation in both mouse models of the increasing Aβ-PET signal during 5 months of pioglitazone treatment. This increase occurred despite an improvement of spatial learning and prevention of synaptic loss in the PS2APP mice. Immunohistochemistry revealed a shift toward plaque composition of higher fibrillarity as the molecular correlate of the Aβ-PET signal in both mouse models. In PS2APP mice this increase was directly associated with improved cognitive performance, whereas in App^{NL-G-F} mice such an effect was not observed.

Aβ-PET enables longitudinal *in vivo* detection of Aβ-plaques, which plays an important role in AD diagnosis, monitoring disease progression, and as an endpoint for therapeutic treatment effects (Valotassiou et al., 2018). In our preceding observational and interventional studies, we validated in AD model mice the clinically established Aβ-PET tracer [^{18}F]florbetaben relative to histologically defined indices Aβ deposition (Brendel et al., 2015a,b). So far, an enhanced or increasing [^{18}F]florbetaben-PET signal has been interpreted as an indicator of disease progression or treatment failure (Laforce et al., 2018). Unexpectedly, we found that pioglitazone potentiated the increasing Aβ-PET signal in two mouse models compared to vehicle controls; in both cases, this increase was due to a shift of the plaque composition toward higher fibrillarity, and away from the more neurotoxic oligomeric form. However, ELISA measurements of plaque associated fibrillary Aβ extracted with formic acid did not indicate a change in the Aβ species composition in brain. This suggests that Aβ-PET imaging and immunohistochemical analysis detect treatment effects on Aβ-plaque composition that do not arise from a shift in the levels of Aβ species, and which may thus evade detection in studies of CSF or plasma content (Hansson et al., 2018).

Furthermore, our study provides evidence that rescued spatial learning deficits and prevented hippocampal synaptic loss can occur despite an increasing Aβ-PET signal upon immunomodulation. The combined results might sound contradictory, but according to the amyloid cascade hypothesis,

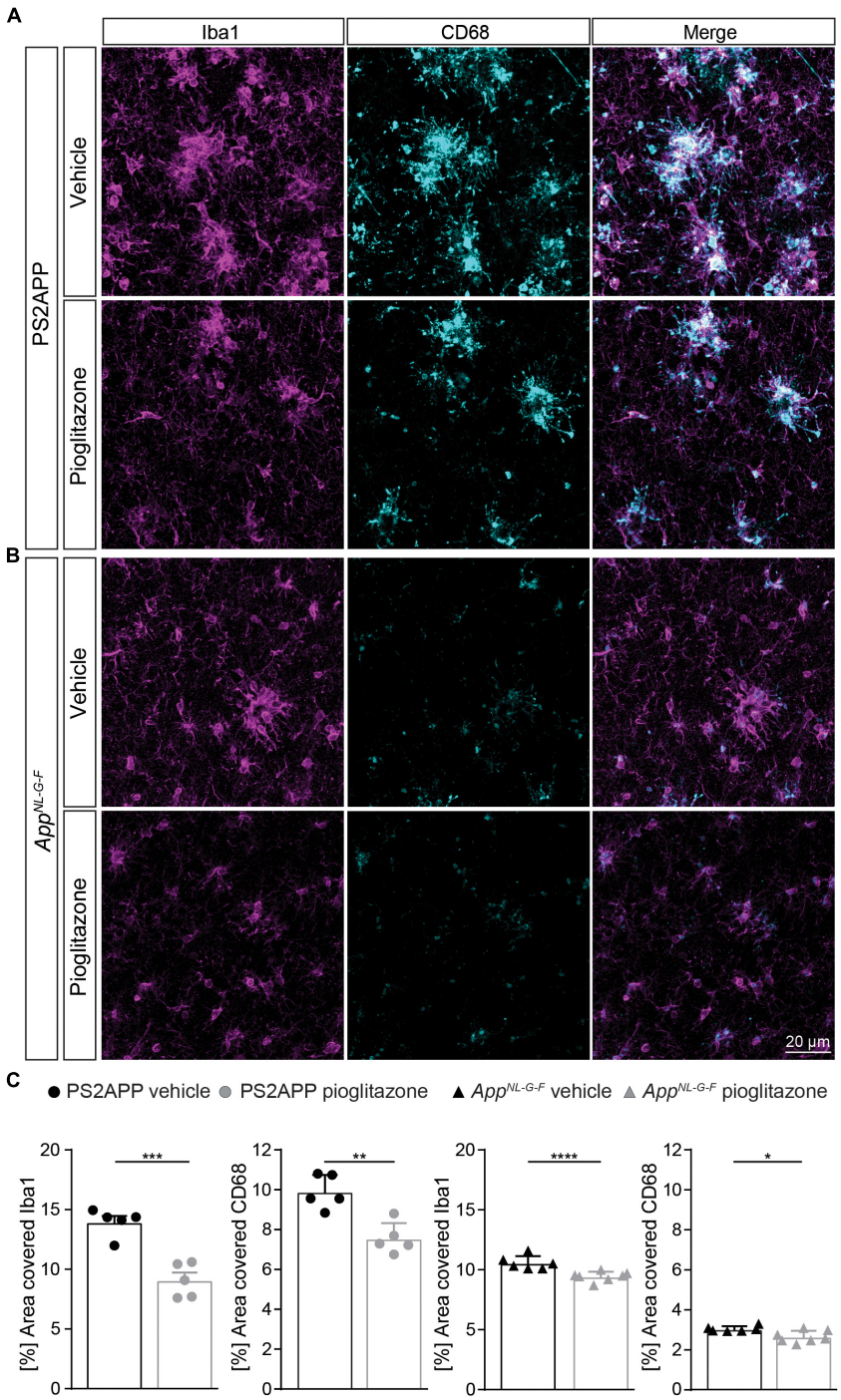

FIGURE 4 | Pioglitazone treatment reduces microglial activation in both AD mouse models. Iba1- (magenta) as well as CD68-(cyan) positive microglial cells in PS2APP **(A)** and App^{NL-G-F} mice **(B)**. **(C)** The area of Iba1 positive microglial cells [$t_{(8)} = 5.95$; $p = 0.0003$] as well as CD68 positive microglial cells [$t_{(8)} = 4.58$; $p = 0.0018$] decreased in treated PS2APP mice. The same effect was observed in App^{NL-G-F} mice were the area covered by Iba1 positive [$t_{(11)} = 4.21$; $p = 0.0015$] as well as CD68 positive microglial cells [$t_{(11)} = 2.91$; $p = 0.014$] were significantly reduced in treated compared to untreated mice. Data are presented as mean ± SEM; $n = 5$–7 mice. Two-sample student's t-test results: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

Aβ-oligomers rather than Aβ-fibrils are the neurotoxic Aβ-forms (Haass and Selkoe, 2007; Selkoe and Hardy, 2016). Indeed, high concentrations of Aβ-oligomers isolated from brain of AD patients correlated significantly with the degree of cognitive impairment prior to death (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). Furthermore, Aβ-oligomers have been shown

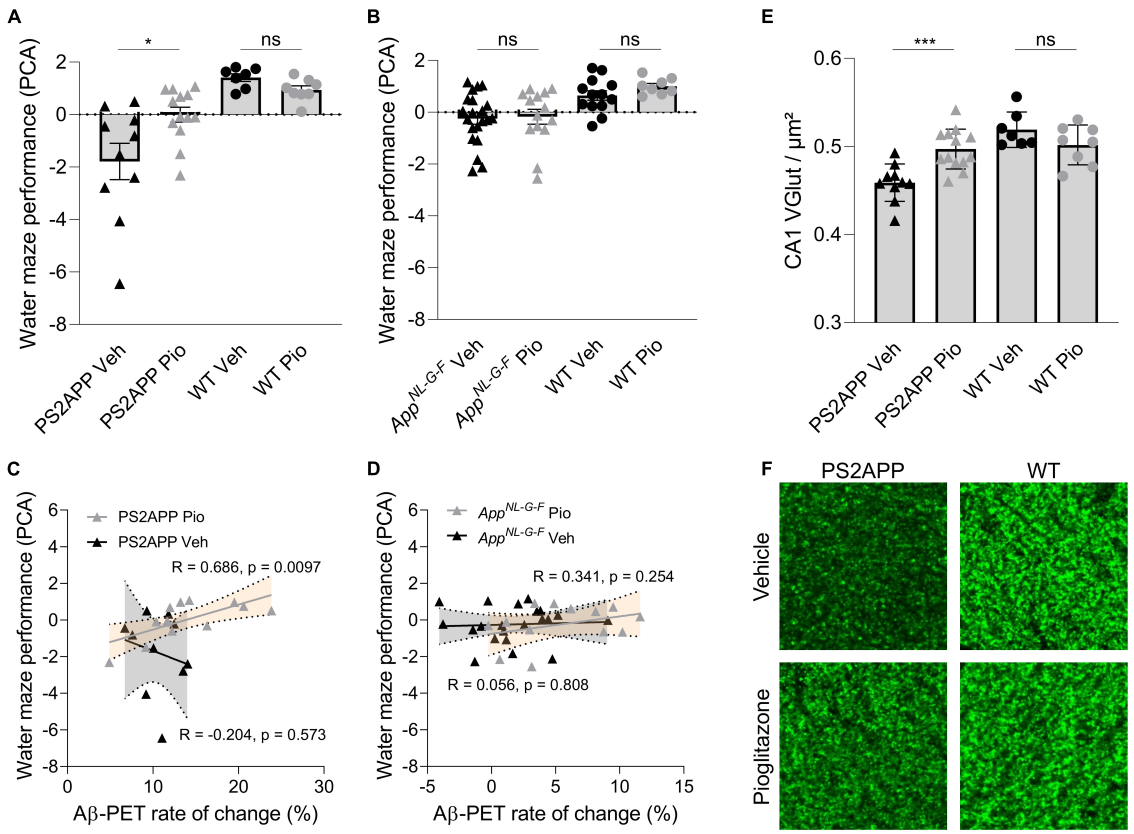

FIGURE 5 | Improved spatial learning correlates with an increased Aβ-PET rate of change in PS2APP mice. **(A)** One-way ANOVA revealed a significant difference of the water maze performance index between pioglitazone treated and untreated PS2APP and wild-type groups [$F_{(3,\ 34)} = 10.37$; $p < 0.0001$; $N = 7$–13]. Group-wise comparisons revealed that pioglitazone treated PS2APP mice achieved a higher performance index in the water maze test compared to untreated PS2APP mice ($p = 0.016$), whereas wild-type animals showed no significant difference between treatment groups ($p > 0.999$). **(B)** One-way ANOVA revealed a significant difference of the water maze performance index between pioglitazone treated and untreated App^{NL-G-F} and WT groups [$F_{(3,\ 34)} = 5.825$; $p = 0.0016$]. However, pioglitazone treated App^{NL-G-F} mice showed no difference in the water maze performance index when compared to untreated App^{NL-G-F} mice ($p > 0.999$) and wild-type animals again showed no significant difference between treatment groups ($p > 0.999$). Scatter plots show correlations between the Aβ-PET rate of change ([^{18}F]florbetaben; ΔSUVR) during the treatment period and individual cognitive testing scores in **(C)** PS2APP mice and in **(D)** App^{NL-G-F} mice (R indicates Pearson's coefficient of correlation) **(E)** The decrease in synaptic density in the hippocampal CA1-region as assessed by VGLUT1 staining was ameliorated in treated PS2APP mice when compared to untreated mice ($p = 0.0012$), whereas no such treatment effect was seen in wild-type animals [$p = 0.810$; group effect: $F_{(3,\ 34)} = 12.03$; $p < 0.0001$; $N = 7$–13]. **(F)** VGLUT1 staining in the hippocampal CA1-region of representative untreated and treated PS2APP mice (left column) as well as of representative untreated and treated wild-type (WT) mice (right column). Statistics of group wise comparisons derive from one-way ANOVA with Bonferroni *post-hoc* correction: $^{*}p < 0.05$; $^{***}p < 0.005$. Data are presented as mean ± SEM.

to disrupt long-term potentiation at synapses and provoke long-term depression (Cullen et al., 1997; Hu et al., 2008; Klyubin et al., 2014). Thus, improved spatial learning and rescued synaptic density could reflect a therapeutically induced shift of Aβ to hypercondensed plaques, in keeping with observations of greater neuritic damage in association with more diffuse plaques (Ulrich et al., 2014; Wang et al., 2016). Furthermore, strongly in line with our present data, a recent study argued that microglia promoted formation of dense-core plaques may play a protective role in AD (Huang et al., 2021).

The shift in plaque composition was more pronounced in App^{NL-G-F} mice than in the PS2APP model. Due to the expression of the Arctic mutation (Saito et al., 2014), the Aβ-deposits of the App^{NL-G-F} line consist predominantly of Aβ-oligomers (Sacher et al., 2019; Monasor et al., 2020). However, we observed no improvement in cognition in the APP knock-in mouse line after pioglitazone treatment. We attribute the lacking improvement of spatial learning to the minor deterioration of this model in water maze assessment at 10 months of age (Masuda et al., 2016; Sacher et al., 2019). Our present observation stand in contrast with previous studies showing that PPAR-γ agonists reduced Aβ-plaque formation by increasing Aβ-clearance (Camacho et al., 2004; Mandrekar-Colucci et al., 2012; Yamanaka et al., 2012). However, those studies only performed endpoint analyses, in part after short-term treatment of 9 days (Mandrekar-Colucci et al., 2012); the current work is the first to perform longitudinal *in vivo* monitoring of Aβ-deposition over a 5-month chronic PPAR-γ treatment period. We note that the divergent results could also reflect the different markers used for immunohistochemistry

compared to our present differentiated analysis of fibrillar and less dense pre-fibrillar Aβ components. As such, the decreased NAB228-positive plaque fraction in our treated App^{NL-G-F} mice fits to the earlier reported decrease of the 6E10-positive area in APPPS1 mice (Mandrekar-Colucci et al., 2012). We note that the biochemical source of the Aβ-PET signal is still a matter of controversy, since some studies found no impact of non-fibrillar plaque components (Catafau et al., 2016) whereas others postulated a significant contribution of non-fibrillar Aβ to the Aβ-PET signal (Ikonomovic et al., 2016, 2018, 2020). Recently, we were able to show that non-fibrillar components of Aβ plaques indeed contribute to the net Aβ-PET signal (Biechele et al., 2022). Therefore, increases in the [^{18}F]florbetaben-PET signal must be precisely differentiated and interpreted with caution. Development of new PET tracers that selectively target oligomeric Aβ may realize a more precise discrimination of neurotoxic Aβ plaque manifestation (Sehlin et al., 2016; Fang et al., 2019) and its impact on disease severity.

In line with previous pioglitazone studies (Mandrekar-Colucci et al., 2012; Yamanaka et al., 2012), we observed a decrease in microglial activity (Biechele et al., 2021), thus confirming the immunomodulatory effect of the drug. Since earlier studies have shown that fibrillary Aβ-deposits activate microglial cells (Sebastian et al., 2020) which then migrate toward the fibrillar deposits (Füger et al., 2017), resulting in an increased number of activated microglial cells surrounding Aβ-plaques (Blume et al., 2018), the inactivation and migration effects could cancel each other out. Based on our findings in both AD models, we conclude that, by increasing plaque fibrillarity, the immunomodulatory effect of pioglitazone overweighs the potential triggering of activated microglia. Modulating microglial phenotype to restore their salutogenic effects may prove crucial in new therapeutic trials (Lewcock et al., 2020). In several preclinical and clinical trials, pioglitazone proved to be a promising immunomodulatory approach for treatment of AD, especially in patients with comorbid diabetes (Liu et al., 2015; Cao et al., 2018). However, a large phase III trial of pioglitazone in patients with mild AD was discontinued due to lacking efficacy (Geldmacher et al., 2011). Our data calls for monitoring of the effects of PPARγ agonists by Aβ-PET, which may help to stratify treatment responders based on their individual rates of Aβ plaque accumulation. Based on our results, we submit that personalized PPARγ agonist treatment might be effective when the patient has capacity to successfully shift toxic pre-fibrillar Aβ toward fibrillar parts of the plaque.

We note as a limitation that PPARγ receptor agonists represent a rather unspecific class of drugs since PPARγ is involved in various pathways in addition to peroxisome activation, notably including glucose metabolism and insulin sensitization [48]. Future studies should address if the observed effects on Aβ plaque composition are also present for more selective immunomodulation strategies such as NLRP3 regulators [49]. Two different water maze examinations were performed in the present study due a switch of the laboratory. Hence, although we calculated a similar water maze performance index by a PCA of the main read-outs of each examination, the obtained results and the sensitivity to detect spatial learning deficits are not comparable between both Aβ mouse models.

CONCLUSION

In conclusion, chronic pioglitazone treatment provoked a longitudinal Aβ-PET signal increase in transgenic and knock-in mice due to a shift toward hypercondensed fibrillar Aβ plaques. The increasing rate of Aβ-PET signal increase with time was accompanied by ameliorated cognitive performance and attenuated synaptic loss after pioglitazone treatment. It follows that increasing Aβ-PET signal need not always indicate a treatment failure, since it is the composition of Aβ plaques that determines their neurotoxiticy. In summary, our preclinical data indicate that a shift toward increasing fibrillar amyloidosis can be beneficial for the preservation of cognitive function and synaptic integrity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Regierung von Oberbayern. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

KB, HA, AR, PC, MW, MMD, JH, and MB conceived the study and analyzed the results. TB, MD, and MB wrote the manuscript with further input from all co-authors. MD, GB, CSc, KW, FE, CSa, and CF performed the small animal, PET experiments, and small animal PET data analyses. TB, FP, YS, KO, GK, XX, MMD, and JH performed immunohistochemistry experiments, analyses, and interpretation. F-JG and SL performed PET tracer synthesis and analyses. NF analyzed and interpreted serial PET data and contributed to their analysis. GB, BZ, KW, and HA performed spatial learning tests and interpretation. BU-S, KB, and MW supplied the study with animal models and interpreted the dedicated results. All authors contributed with intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.854031/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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