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Automated detection of β -amyloid-related cortical and subcortical signal changes in a transgenic model of Alzheimer's disease using high-field MRI

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Abstract

In vivo imaging of β -amyloid load as a biomarker of Alzheimer's disease (AD) would be of considerable clinical relevance for the early diagnosis and monitoring of treatment effects. Here, we investigated automated quantification of *in vivo* T2 relaxation time as a surrogate measure of plaque load in the brains of ten APP/PS1 transgenic mice (age 20 weeks) using *in vivo* MRI acquisitions on a 7T Bruker ClinScan magnet. APP/PS1 mice present with rapid-onset cerebral β -amyloidosis, and were compared with eight age-matched, wild-type control mice (C57Bl/6J) that do not develop A β -deposition in brain. Data were analyzed with a novel automated voxel-based analysis that allowed mapping the entire brain for significant signal changes. In APP/PS1 mice, we found a significant decrease in T2 relaxation times in the deeper neocortical layers, caudate-putamen, thalamus, hippocampus and cerebellum compared to wildtype controls. These changes were in line with the histological distribution of cerebral A β plaques and activated microglia. Grey matter density did not differ between wild-type mice and APP/PS1 mice, consistent with a lack of neuronal loss in histological investigations. High-field MRI with automated mapping of T2 time changes may be a useful tool for the detection of plaque load in living transgenic animals, which may become relevant for the evaluation of amyloid lowering intervention effects in future studies.

Keywords

A β ; amyloid; transgenic mouse models; iron deposition; T2 relaxation time; high-field MRI; Alzheimer's disease

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Introduction

In vivo imaging of the characteristic neuropathological hallmarks of Alzheimer's disease (AD) would greatly enhance our ability to improve the early diagnosis of the disease, offering pathways for the evaluation and initiation of preventive treatments. A prominent target of *in vivo* imaging efforts in AD has been senile plaques, brain lesions that contain large amounts of aggregated β -amyloid ($A\beta$) peptides. Positron emission tomography (PET) technology using specific ligands such as ^{11}C -PIB (Pittsburgh compound B, [*N*-Methyl- ^{11}C]₂-(4'-methylaminophenyl)-6-hydroxybenzothiazole) can give a semiquantitative estimate of $A\beta$ load in the human brain [1]. However, magnetic resonance imaging (MRI) would offer several advantages over PET if MRI could be employed to detect $A\beta$ load *in vivo*: MRI provides better spatial resolution, is more widely available, requires lower costs and lacks radiation exposure as compared to PET. In addition, there is some uncertainty about the consistency with which PIB imaging can detect high $A\beta$ -load in humans [2,3], and the ligand does not bind avidly to $A\beta$ deposits in experimental animals [4,5].

There are three ^{18}F markers presently being evaluated in phase III studies (see www.clinicaltrials.gov), namely as Florbetaben ([^{18}F]-BAY94-9172) [6], Florbetapir ([^{18}F]-AV-45) [7], and Flutemetamol (GE-067) [8]. These tracers have shown similar properties to ^{11}C -PIB, however, with higher unspecific binding in the cerebral white matter and a lower rate of amyloid positive cognitively healthy control subjects [9]. More recent approaches aim at the measurement of fibrillar amyloid load *in vivo* using PET markers such as [^{18}F]AZD4694. This and similar markers are presently undergoing Phase II studies. ^{18}F -labeled PET tracers may help to make amyloid PET imaging wider available compared to ^{11}C tracers, but uncertainties remain in respect to the specificity of the amyloid binding in cognitively healthy subjects.

In contrast to PET, MRI has not yet achieved the detection of senile plaques in the living human brain, although several strategies to enhance MR contrast have successfully been applied to β -amyloid precursor protein (APP) transgenic mouse brains both *in vitro* and *in vivo* [10,11]. One important source of intrinsic contrast/paramagnetic susceptibility for MRI is iron, which is also associated with β -amyloid plaques [12–15]. The widely held view that iron found in and around senile plaques is the dominant cause of the hypointensities seen in MR images has recently been challenged, but still a close association between T2 time-reductions and plaque load has been confirmed [16]. This intrinsic contrast mechanism allows the detection of single, large senile plaques of more than 100 μm diameter at very high field strengths of more than 9T in living APP-transgenic mice without the aid of exogenous contrast agents [17,18]. Measuring the decline of T2 relaxation times is another approach to exploiting the intrinsic paramagnetic properties of plaque-bound iron [19]. Although this method does not allow visualization of individual plaques, it is applicable at lower field strengths and can be easily quantified. As it is not yet clear to what extent the individual imaging of large senile plaques at very high field strengths serves as a reliable estimate of overall cerebral lesion load in transgenic mice, the use of accessible T2 relaxation time mapping is an easier and faster alternative for characterizing plaque load *in vivo*. This approach is of further interest, because it may be more easily transferable to *in vivo* studies in humans than the imaging of individual senile plaques. This will become even more relevant with the broader availability of human MR imaging at very high field strengths, such as 7T. The size of dense-core plaques in the human brain is typically smaller than the detection threshold for single plaques of more than 100 μm diameter [20,21] in living transgenic mice [17]. Indeed, the large majority of senile plaques were not visible in T₂*-weighted MRI scans of post-mortem human brain specimens, even at 11 Tesla [22].

To date, measurement of T2 times in living transgenic mice has been mainly restricted to selected regions of interest in areas prominently affected by A β pathology, such as neocortex and hippocampus [17,23,24]. One study used automated image registration to facilitate ROI drawings, but did not apply voxel-based statistics to the co-registered T2 relaxation time maps [25]. The transgenic models investigated thus far develop considerable A β plaque load only after more than 1 year of age, extending the time required for larger scale longitudinal studies.

In the present study, we employed T2 time mapping, as originally proposed by Helpert et al. [19], in an APP/PS1 transgenic mouse model [26] that starts to develop cerebral β -amyloidosis by six weeks of age, and that shows considerable accumulation of A β at 10 weeks, even before the onset of neuronal loss at age 8 months and beyond. This rapid onset of cerebral β -amyloidosis enables the assessment of lesion load with *in vivo* imaging at a much earlier age than in previous studies. To extend previous studies of T2 time changes in transgenic models of AD, we complemented the ROI-based analysis of selected subregions of the brain with an automated voxel-based analysis in which the entire brain is mapped for changes in T2 relaxation times. This approach allows a rapid assessment of brain changes without the need for *a priori* selection of brain areas. We propose that the use of automated, voxel-based analysis of T2 time changes in a rapidly progressing transgenic model of cerebral β -amyloidosis is an easily accessible approach to investigating the potential utility of high-field MRI for the detection of senile plaque load in the living human brain.

Methods

Animals

We used 10 APP/PS1 heterozygote transgene-positive mice harboring a mutant human presenilin 1 (L166P variant) and a mutant human β -amyloid precursor protein (APPswe) in a C57BL/6J background driven by two separate Thy1 promoters [26], mean age 19.47 (SD 0.56) weeks, and 8 non-transgenic control mice (C57BL/6J), mean age 20.18 (SD 1.44) weeks. Both groups were not different in age ($T = 1.44$, 16 df, $p = 0.17$). Mice were housed under 12h/12h light/dark cycles and had access to food and water *ad libitum*. For imaging, the mice were anaesthetized using 0.5–2% isoflurane in pure oxygen (flow 0.5–1L/min). The anaesthesia was controlled by continued mechanical measurement of diaphragm movement (30 to 50 movements per minute) and isoflurane concentration was adjusted accordingly. The experiments were conducted as recommended by the NIH and compliant with minimal standards as defined by the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experiments were approved by the local institutional animal care and use committee (LALLF M-V/TSD/7221.3-2.4-020/08).

MRI acquisition

MRI measurements were performed on a 7T Bruker ClinScan magnet with a 20cm inner bore, capable of 290mT/m in 250 μ s (Bruker BioSpin MRI, Ettlingen, Germany). Images were received by a 2 $\times$ 2 phased-array RF coil, designed specifically for mouse brain studies, which was placed directly on the skull. For T2 relaxation time measurements, a multislice spin echo sequence was applied, with a field-of-view (FOV) of 2.0 $\times$ 2.0cm², matrix size 256 $\times$ 256 pixels, two averages, a repetition time (TR) of 4000ms and an echo time (TE) ranging from 15.1ms to 211.4ms in steps of 15.1ms. Six horizontal slices of 0.5mm slice thickness were acquired for each of the 14 echoes.

For morphological analysis, we acquired a T2-weighted turbo spin echo (TSE) sequence with the same FOV and orientation as the SE sequence. Ten slices of 0.6 mm thickness each,

with no gap between them, were acquired with TR = 5500 ms, TE = 33 ms, matrix size 640 × 640, 6 averages and echo train length = 7.

Histopathology

One day after the MRI scans, mice were killed by cervical dislocation and perfused with ice-cold, freshly prepared 4% paraformaldehyde, and then post-fixed for 48 hours. The brains were subsequently dehydrated, defatted and embedded in paraffin wax using a Histokinette (Leica Microsystems, Wetzlar, Germany). The paraffin blocks were cut into 4µm-thick sections that were baked for 60 minutes onto glass slides. Selected slides were stained with H&E, and others were immunostained as described previously [27,28] using a BondMaxx™ Immunostainer (Menarini, Berlin, Germany) with antibodies against β-amyloid (clone 4G8, host: mouse, Millipore, Schwalbach, Germany), glial fibrillary acidic protein (GFAP) (host: mouse, Dako, Hamburg, Germany), and Iba-1 (host: rabbit, Wako Chemicals GmbH, Neuss, Germany). Initially, slides were dewaxed, pretreated with formic acid for 1 hour (only for the Aβ stain) and incubated with the primary antibody (dilutions: 4G8-Aβ 1:400, GFAP 1:1000, Iba-1 1:1000). Nonspecific binding sites on the tissue sections were blocked and the immunoreaction product then developed using a mixed-DAB-Refine Kit (Menarini). The sections then were counterstained with hematoxylin.

Sections were double-stained with Aβ and Iba-1 using a BondMaxx™ (Menarini) automated immunostaining system. Sections were first pretreated with 98% formic acid for 5min and immunostained for Aβ using the anti-human Aβ clone 6F3D (1:200, Dako) and the Bond™ Polymer Refine Detection kit (Menarini). Microglia were then immunostained on the same sections using anti-Iba-1 (1:1000, Wako) and the Bond™ Polymer AP-Red Detection kit (Menarini). For iron-labeling, slides were incubated with sodium hexacyanoferrate and H₂O₂ (Prussian Blue method) and enhanced using DAB according to [29]. The Campbell-Switzer (CS) and LuxolFastBlue-Eosin (LFB) stains were performed as previously described [30,31]. Finally, the slides were scanned at 230nm resolution using a MIRAX Midi slide scanner (Zeiss MicroImaging GmbH, Jena, Germany) and analyzed using the AxioVision software package (Zeiss).

MR data processing

Prior to further processing of the MR images, the brain was manually edited from the skull using MRICro 1.40. We determined T2 time maps for each animal from the multiecho sequence by fitting a single exponential curve across the different TEs at each single voxel using a customized script in Matlab 7.0.1 (MathWorks, Natwick, USA) (Figure 2A).

T2 time measurements—Measurement of T2 relaxation was performed in two ways: (i) using manually placed regions of interest (ROI) on the T2 relaxation time maps in native space; and (ii) using automated voxel-based analysis across the entire grey matter in a group-specific common space. In a post-hoc analysis, after we had found strong effects in the cerebellum (see results section below), we used an approach to correct the T2 relaxation time maps for the amount of grey matter in each voxel based on the TSE scans in an attempt to control partial volume effects from CSF spaces.

ROI-based analysis: Manual measurement was implemented in Medical Image Processing, Analysis and Visualization, Version 4.1.3 (2008-10-15), (MIPAV, Center for Information Technology (CIT), National Institutes of Health (NIH); available at <http://mipav.cit.nih.gov>) [32]. ROIs were placed in the ME sequence at TE 15.1 ms, because this sequence provided good contrast between cortical and subcortical grey matter and white matter. The locations of the ROIs were then transferred to the T2 relaxation time maps that had been derived from the ME sequence (see above). We placed circular ROIs across the neocortical ribbon

including the cingulate cortex, as well as the hippocampus, and the caudate-putamen (CPu). ROIs were placed according to anatomical criteria with reference to a published mouse brain atlas (Figure 1) [33].

Each anatomical region was sampled in two consecutive slices, with the size of the ROIs adapted to the size of the anatomical area to be sampled. The following anatomical areas were defined:

- **Neocortical ribbon:** Twelve ROIs, with an area of 0.2502mm^2 each, were evenly distributed across the entire lateral neocortical band of each hemisphere in two consecutive slices. These slices were selected from the six available slices according to the clear visibility of the cortex. They corresponded to inter-aural distances (IAD) of 2.52 mm and 3.24 mm, respectively [33]. The three most anterior ROIs were grouped in the ventral insula across the two consecutive slices at IAD 2.52mm and 3.24mm. With the slice at IAD 2.52mm, we grouped the three ROIs of the middle anterior cortical portion, representing posterior aspects of the ventral insular cortex, the three ROIs of the middle posterior cortical portion, representing perirhinal and posterior insular cortex, and the most posterior three ROIs, representing entorhinal cortex. The latter three ROIs were averaged with the most posterior three ROIs in the more ventral slice at IAD 3.24mm, to represent entorhinal cortex. The middle-anterior three ROIs of slice IAD 3.24mm were averaged to represent primary and secondary somato-sensory cortex, and the following three ROIs (middle posterior) were grouped to represent auditory and temporal association cortex.
- **Hippocampal formation:** The hippocampal formation was sampled with a single ROI with an area of 1.1169mm^2 in each hemisphere in two consecutive slices. Of the six available slices, we selected the two in which the hippocampal formation could best be delineated, with IADs corresponding to 1.68 mm and 2.52 mm, respectively [33].
- **Caudate-Putamen:** The CPu was sampled in two consecutive slices corresponding to IADs of 2.52mm and 3.24mm [33]. One ROI was placed in the anterior CPu with an area of 0.9399mm^2 , and one ROI was placed in the posterior CPu with an area of 0.5737mm^2 .
- **Cingulate gyrus:** The cingulate gyrus (CG) was measured with a single ROI with an area of 0.2075mm^2 in one slice, which was selected from six available slices in which the CG could best be defined, with an IAD corresponding to 3.82mm [33].

T2 relaxation times were averaged within each ROI. The selection of the consecutive slices out of the six available slices was unambiguous in all animals. To control for background intensity, we used a control ROI placed in masseter muscle. For this measurement, we derived T2 relaxation time maps from the ME sequence without prior editing of the brain from the skull. Additionally, based on the findings from the voxel-based analysis, we sampled ventricular CSF space using a single ROI with an area of 0.3174mm^2 placed in the lateral ventricle in one slice. This slice was selected out of six slices in which the ventricular CSF space could best be outlined, with an IAD corresponding to 3.82mm [33].

Voxel-based analysis: Voxel-based analysis was implemented with Matlab 7.0.1 (MathWorks, USA) through Statistical Parametric Mapping [34,35] (SPM 5, Wellcome Department of Imaging Neuroscience, London; available at <http://www.fil.ion.ucl.ac.uk/spm>) and SPMouse (Wolfson Brain Imaging Centre, University of Cambridge, UK), a Matlab-based modification of SPM5, available at (<http://www.wbic.cam.ac.uk/~sjs80/spmmouse.html>) [36,37]. SPMouse implements grey

matter, white matter and CSF space probability maps from a high-resolution sequence that is in affine co-registration with a publicly-available digital C57BL/6J mouse atlas [38]. The processing of our data followed four subsequent steps that are illustrated (in a simplified fashion) in Figure 2.

In detail, in the first step, the ME sequence with a TE of 15.1ms was co-registered to the TSE sequence using affine transformation with a mutual information cost-function [39], implemented in SPM5 and adapted to murine data in SPMMouse. The affine transformation parameters were then applied to the T2 relaxation time maps that had originated from the ME sequence, resulting in T2 relaxation maps co-registered to the TSE sequence (Figure 2A).

In the second step, the TSE maps were segmented into grey matter and white matter maps. The SPM segmentation employs a mixture model cluster analysis (after correcting for non-uniformity in image intensity) to identify voxel intensities that match particular tissue types combined with *a priori* probabilistic knowledge of the spatial distribution of tissues derived from grey and white matter and CSF prior probability images (priors) [40]. Prior probability images were derived from the tissue maps implemented in SPMMouse that are in co-registration with a digital mouse atlas [36,37].

In the third step, the segmented grey matter maps derived from the TSE sequence were normalized to the standard grey matter map provided in SPMMouse using a set of nonlinear basis functions [41,42]. Normalized grey matter maps then were smoothed (0.5mm full width at half maximum isotropic Gaussian kernel) and averaged to obtain a group-specific grey matter template that was in co-registration with the digital C57 atlas [38] (Figure 2B and C).

In the fourth step, the segmented grey matter maps in native space were normalized to the group-specific grey matter template, the normalization parameters were applied to the grey matter maps themselves as well as to the T2 relaxation maps that were already co-registered with the TSE sequence (Figure 2C and D). This resulted in grey matter maps as well as T2 relaxation time maps that were spatially normalized into the group-specific standard space. The normalized maps were smoothed with a 0.5mm–full-width at half maximum isotropic Gaussian kernel before subsequent statistical analysis.

Post-hoc analysis of CSF partial volume effects: To our knowledge, there is no established method for partial volume effect (PVE) correction of T2 relaxation time data. However, to assess a potential effect of CSF partial volume effects on the cerebellum signal, we adapted an algorithm for PET atrophy correction based on the Müller-Gärtner algorithm [43]. The basic idea of the Müller-Gärtner algorithm in a more recent adaptation [44–46] includes the following processing steps:

- The PET scan is spatially aligned to the high resolution anatomical MRI scan.
- The anatomical MRI scan is automatically segmented into grey and white matter and CSF compartments.
- The segmented images are convoluted with the point spread function representing the spatial resolution of the PET camera.
- The white matter compartment is subtracted from the PET scan to obtain the grey matter PET scan, assuming that CSF radioactivity is negligible.
- The grey matter PET scan is divided by the convoluted grey matter MRI map, resulting in a PVE corrected PET scan.

Several factors are different in the case of PVE correction of the T2 relaxation time maps compared to the PET PVE correction:

- There is no equivalent for the point spread function of the PET camera in MRI. Therefore, we performed a sensitivity analysis where we used the T2-weighted turbo spin echo (TSE) sequence convoluted with the voxel resolution of the T2 relaxation time maps in a first analysis and the original voxel resolution of the TSE sequence in a second analysis. Since results were essentially unchanged between both analyses, in the following we only report the results of the unconvoluted TSE scans.
- In PET, grey matter voxel would have a higher, CSF voxel a lower signal. Here, signal was expected to decrease both in grey matter and CSF. Therefore, it would not be useful to divide voxel intensity of the T2 relaxation time maps by the grey matter probability maps. Therefore, binarized the segmented grey matter maps, where voxel with a grey matter probability of 0.5 and higher were assigned a value of 1, all other voxels were assigned a value of zero. We then multiplied the T2 relaxation time maps by the binarized grey matter maps. We used sensitivity analysis to determine the effect of the threshold used for binarization. Using a threshold of 0.33 probability for grey matter as well as a threshold of 0.66 probability did not essentially change the results so that here we report the results from the 0.5 threshold.
- The white matter compartment in the mouse brain is difficult to separate from grey matter and is of small spatial extent. Therefore, we considered no separate white matter compartment in our analysis.

In summary, we co-registered the T2 maps (via the multiecho sequence with a TE of 15.1ms) with the TSE sequence, as described above. The TSE sequence was segmented into grey and white matter maps as described above. The segmented grey matter maps then were binarized at a grey matter probability threshold of 0.5. Then the coregistered T2 maps were multiplied with the binarized grey matter maps to obtain PVE corrected maps which then were normalized into standard space, as described above.

Statistics

ROI data—According to the Shapiro-Wilk Test and visual inspection of scatterplots, the data from the transgenic group were not normally distributed. This was mainly due to one transgenic animal whose values lay more than 1.5 interquartile ranges above the third quartile for muscle, hippocampus and CPU measurements, corresponding to a mild outlier, and more than three interquartile ranges above the third quartile for all cortical measurements, corresponding to an extreme outlier. Upon checking the data quality and measurement accuracy, there was no indication of a problem with data acquisition or measurement in this animal. Therefore, we decided to leave the animal in the analysis and to use non-parametric tests for statistical inference to mitigate the effect of the outlier. This is a conservative decision, as the values of the outlier were in an *a priori* unexpected direction. However, effect-size calculations were repeated with the animal removed from the data set to assess the influence of the outlier on our findings.

ROI-based measurements were compared between groups using the Mann-Whitney U-test. The level of significance was set at $p < 0.05$.

Automated voxel-based analysis—For statistical analysis, we employed the general linear model on a voxel basis, where we assessed the effect of genotype on T2 relaxation times and grey matter density, respectively. Smoothed T2 relaxation times and grey matter

maps were masked for tissue outside of the grey matter using the segmented grey matter map from the group-specific brain template. Results were thresholded at a p level of < 0.05 , corrected for multiple comparisons using false discovery rate (FDR), and an extent threshold of 50 contiguous voxels was applied. FDR correction ensures that, on average, not more than 5% of the significant voxels are false positives [47]. We assessed effects in both directions, i.e. an increase as well as a decrease in T2 relaxation times and grey matter density in APP/PS1 transgenic mice relative to wildtype controls. The animal showing extreme values in the ROI-based analysis was kept in the voxel-based analysis as well for two reasons. First, the values were higher than expected, so that the risk of false positives would be decreased. Second, the average intensity of this animal after preprocessing was highest within the transgenic group, but not an outlier.

Post-hoc analysis of CSF partial volume effects—We employed a voxel-based linear model to determine group differences in T2 time measurements based on the smoothed normalized PVE corrected T2 maps. The p -value was set to a liberal level of 0.05, uncorrected, in order to determine whether T2 relaxation times would be different between groups in the cerebellum signal to the same extent than in cortical areas. Thus we controlled the type II error of erroneously not detecting an effect in the cerebellum after PVE correction.

Results

ROI analyses

In the APP/PS1 transgenic mice, Mann-Whitney U-tests revealed significant reductions in T2 relaxation times in all sampled brain regions except the dorsalmost cortical area representing entorhinal cortex ($p = 0.146$) (Table 1). The ventricular CSF also showed significantly reduced T2 relaxation time in the transgenic mice compared to wildtype controls. In contrast, the control region in the masseter muscle showed no significant difference between groups ($p = 0.146$, M-W $U = 23$). Effect size estimates are shown in Table 1, both with and without the values from the one animal presenting as an outlier.

Voxel based analyses

Data analysis using a linear model with automated VBM revealed extended reductions of T2 times in deeper cortical layers, hippocampus and CPU of transgenic mice at $p < 0.05$, FDR-corrected for multiple comparisons. Additionally, the APP/PS1 mice had significant clusters of diminished T2 times in the thalamus, septal nuclei and cerebellum (Figure 3). Effects also were found in ventricular CSF spaces. The opposite contrast, i.e., reduced T2 times in wildtype mice, showed no significant clusters anywhere in the brain at $p < 0.05$, FDR corrected.

The voxel-based analysis of the grey matter maps derived from the TSE sequences showed no significant increase or decline of grey matter volume in any brain region of the transgenic mice compared to wildtype controls at $p < 0.05$, FDR corrected.

Histological analyses

Mouse brains were analyzed using different conventional and immunohistochemical stains (Figures 4 and 5). The H&E stain revealed no obvious pathological or gross morphological changes (Figure 4A). Senile plaques were visualized using the Campbell-Switzer silver-staining method and A β -immunohistochemistry. The areal density of plaques was greater in deeper cortical layers compared to superficial cortical layers (Figures 4B and C). GFAP-positive astrocytes were located in close proximity to senile plaques and in central cerebellar regions. The density of astrocytes was higher in deeper cortical layers than in the superficial

layers (Figure 4D). Microglial staining labelled activated microglia only in proximity to A β deposits (Figure 4E). The Luxol-Fast Blue myelin stain showed no indication of myelin pathology or subcortical fibre tract reductions (Figure 4F). DAB-enhanced Prussian Blue staining showed that the senile plaques and adjacent microglia accumulate iron (Figure 5F).

Post-hoc analysis of CSF partial volume effects

Similar to the uncorrected data, in the voxel-based analysis using the PVE corrected data we found reductions in T2 relaxation times in cortical areas, however, the cerebellum was nearly devoid of any T2 relaxation time changes after PVE correction (Figure 6).

Discussion

We investigated 20-week old APP/PS1 transgenic mice developing early cerebral plaque-like β -amyloidosis using high-field MRI at 7T prior to the onset of neurodegeneration (after age 34–36 weeks). We found a significant reduction of T2 relaxation times in the transgenic animals compared to wildtype controls in deeper neocortical layers, hippocampus and caudate-putamen using both manual ROI measurements in native space as well as automated voxel-based analysis in a group-specific common space. Moreover, this effect was independent of local atrophy, as grey matter maps did not differ in wildtype and APP/PS1-transgenic animals at this timepoint. We focussed with our investigation on the detection of early changes at the beginning of the cerebral amyloidosis prior to the onset of marked neurodegeneration. The rapid onset of amyloidosis as compared to APP-only models (e.g. Tg2576) does not replicate the development of AD, but it helps to investigate *in vivo* changes due to cerebral amyloidosis within a shorter time frame. The localization of the T2 relaxation time effects mapped the spatial distribution of A β -plaques and activated microglia in histological sections of the brain (Figure 4B, C, E).

To our knowledge, this is the first description of the high-field MRI features of the Thy1-promoter-driven APP/PS1 double mutant mouse model *in vivo*. In this model, cerebral β -amyloidosis has been shown to start by 6 to 8 weeks of age, and to lead to significant plaque load in neocortex, hippocampus, thalamus and striatum by the age of 5 months [26]. At age 8 months, the entire forebrain is laden with A β -positive plaques and loss of neurons becomes detectable.

Consistent with the early onset and spatial distribution of plaques in the transgenic mice, our animals showed significantly reduced T2 relaxation times at age 4.4 to 5 months in neocortex, hippocampus, CPu, and thalamus. Several studies have demonstrated an association of iron with plaques in humans and in mouse models of AD pathology [12–15]. The iron has been localized to senile plaques and adjacent microglia, where it leads to paramagnetic susceptibility and consequent reductions of T2 relaxation times [17]. Consistently, we identified iron accumulation in the center of the amyloid plaques in our transgenic mice (Figure 5F).

In agreement with the report that significant neuron loss does not begin until 8 months of age in this double transgenic model [26], we did not find significant reductions of grey matter density in the transgenic mice when compared to the wildtype mice, and histological analysis with cell stains further indicates that neuronal loss is minimal or absent at age 5 months (Figure 4A). Moreover, there was no evidence of myelin changes or gross changes in the corpus callosum (Figure 4F) that would also indicate significant neuronal loss in the cortex. It should be noted, however, that other mechanisms could compensate volumetrically for minor neuronal loss, for example, the volume effect of β -amyloid plaques and accompanying astrogliosis (Figure 4D) or microgliosis (Figure 4E). Neuronal loss in this model has been reported at age 8 months and later [26]. For comparison, APP/PS1 models

resulting from intercrosses of two independent APP- and PS1-transgene strains exhibit atrophy at age 24 months and later that was detected by *in vivo* MRI and histological evaluation [48]. A PDAPP mouse model by [49] showed significant reductions of commissural fibers already at 3.6 months of age that became more pronounced at 17 months of age, as detected by MRI. It has been suggested that the very early changes of commissural connections in this PDAPP model represent a neurodevelopmental abnormality induced by the PDAPP transgene construct rather than an effect of neurodegeneration induced by amyloid pathology.

Unexpectedly, we found reduced T2 relaxation times in the ventricular CSF spaces of the transgenic animals. Though the explanation for this outcome remains uncertain, one study in transgenic mice overexpressing the London mutant form of APP at age 24 months found high levels of A β in CSF [50]. Therefore, high levels of A β , or possibly other substances related to cerebral A β deposition, in CSF may lead to reduced T2 relaxation times in CSF spaces, in agreement with the observation that higher viscosity of fluids decreases T2 relaxation times [51]. This issue clearly merits further study.

In addition, we found significantly reduced T2 relaxation times in the cerebellum in the voxel-based analysis. The spatial distribution of the effects matched the extension of the cerebellar folia. In the histological analysis, these areas were devoid of amyloid plaques and amyloid-related microgliosis (Figure 4H). Astrogliosis occurred in cerebellar structures, but was localized to the cerebellar peduncles, sparing the cerebellar folia (Figure 4G). The close association between CSF spaces and grey matter folia in the cerebellum suggests that the observed signal reductions in the cerebellum are most likely due to partial volume effects from CSF spaces that have decreased T2 relaxation times, as seen in the forebrain regions. This interpretation is supported by the finding that after correcting the T2 relaxation time maps for the content of grey matter within each voxel as assessed from the TSE data, cortical areas showed reduced T2 relaxation times, but the cerebellum was devoid of almost any signal reductions. Segmentation of TSE scans into different tissue compartments is less accurate than for human structural MRI data and therefore induces additional variability into the voxel-based analysis, which can also be seen by the reduced T-values compared to the uncorrected analysis. However, the lack of effects in the cerebellum is not only due to the reduced sensitivity of the analysis, since the effects in the cortex, although with reduced effect sizes, remained preserved.

In our histological analysis, we found iron accumulation in the center of the plaques (Figure 5F), supporting the association between T2 relaxation time reductions and plaque-related iron accumulation in forebrain regions. One study challenged the specificity of T2 relaxation time changes [24]. In this longitudinal study, T2 relaxation times declined significantly over a one-year follow-up in the hippocampus and cortex of an APP/PS1 model that shows slightly later onset of cerebral β -amyloidosis than does the model used in our study [26, 52]. However, PS1 single-transgenic mice, which do not develop A β plaques, showed a significant, age-associated decline of T2 relaxation times as well. At the same time, however, these mice were reported not to differ from wildtype controls at baseline, as well as at both follow-up time points in cross-sectional analyses. As the wildtype mice showed no significant longitudinal decline in T2 relaxation times, it remained unclear why the PS single-transgenic mice had progressive decline of T2 relaxation times but never differed from wildtype mice at any time point in the cross-sectional analyses. A likely reason is the severe attrition of animal numbers over time, since the study began with 23 PS1 single-transgenic mice at baseline and ended with only four animals at the second follow-up. This attrition renders the estimates of rates of change unreliable. In contrast, cross-sectional analyses were performed with reasonable group sizes after data from single time-point acquisitions had been added.

The transgenic mice employed in our study represent a valuable model to investigate early onset cerebral β -amyloidosis. However, the morphology and composition of amyloid plaques differs across different mouse models and between transgenic models and AD patients [53,54]. These differences may limit the translation of imaging findings in transgenic mouse models to human studies. The first attempts to undertake ultra-high-field MRI in human studies in AD at 7T suggest that plaque-related susceptibility alterations can be detected *in vivo* in cortical areas [55]. T2 relaxation time reductions were found to be associated with senile plaques, both in human brain tissue as well as in *post mortem* APP/PS1 transgenic mouse brains [16]. T2* contrast reductions were well-matched with iron deposits within and close to senile plaques in *post mortem* samples of AD patients' brains. In contrast, there were reductions of the T2* signal that corresponded to the location of plaques in APP/PS1 mice that develop cerebral β -amyloidosis after 1 year of age, but in the absence of significant iron deposits. The authors suggested that the detection of the murine plaques via the T2 effect was related to their higher fibrillar organization and density compared to human plaques. So different morphological changes may lead to similar signal changes in MRI, limiting the inference from MRI signal changes on the underlying plaque morphology. In our transgenic model, we consistently found a significant amount of iron within and in proximity to senile plaques, suggesting that iron is a significant contributor to T2 relaxation time reduction in this context.

The absolute T2 values we measured in our study were higher than those reported in other animal studies [17,19], but are comparable to those observed in a human investigation [56]. These differences may partly be due to the pulse sequence parameters used. Additionally, the processing of the data likely had an influence. In previous experiments, multiecho data were fitted to an exponential function, but no details were given as to whether the data were processed to exclude overall noise levels [17,19]. The human study did not employ noise level corrections [56]. We used the raw data without correcting for the effects of noise as well. However, we also estimated noise levels through the signal intensity in an ROI that was placed at the maximum distance from the measured object. Signal intensity in this ROI was nearly identical across the different echo times. Therefore, when considering noise levels, the signal was relatively more reduced with higher echo times, yielding a steeper decay function of the T2 signal. After correcting for noise levels, our T2 values were close to those in the previous animal studies [17,19]. However, since the between-group effects were not affected by the noise level correction of the data, we decided to use the T2 time measurements derived from the raw data.

There is a wide range of MRI based diagnostic markers in AD, from visual rating of hippocampus atrophy [57] through manual [58] and automated [59] hippocampus volumetry, and cortical thickness measurement [60] to automated detection of high resolution differences in brain morphology using deformation based morphology and multivariate analysis such as principal component analysis [61] or machine learning algorithms such as support vector machines [62]. These approaches yield an accuracy of about 80% in the prediction of AD in at risk subjects such as patients with the clinical syndrome of amnesic mild cognitive impairment [63]. *In vivo* amyloid imaging techniques will help in future to shift the diagnosis of AD into presymptomatic stages. A recent model on the dynamic use of biomarkers in AD [64] suggests that amyloid pathology precedes the loss of cortical neurons, as detected by structural MRI. However, the specificity of amyloid load findings in the brain for predicting AD in cognitively normal subjects remains to be shown. Additionally, amyloid imaging in combination with functional and structural markers of brain changes based on MRI will help us to better understand the dynamic sequence of molecular changes, such as amyloid accumulation, synaptic dysfunction and neuronal loss. In future, the combined use of these markers will help to improve our models on the development of AD pathology in the brain.

In summary, we found significant reductions of T2 relaxation times in cortical and subcortical brain areas that corresponded to the pattern of A β accumulation and reactive gliosis shown in subsequent histological analyses (Figure 4). The APP/PS1-related effects on T2 relaxation times occurred in the absence of obvious morphological brain changes (besides the occurrence of senile plaques), as shown both *in vivo* in the grey matter density maps and *post mortem* in histological sections. Our findings suggest that the APP/PS1 transgenic model can serve as a useful model for the high-field MRI analysis of brain changes related to cerebral β -amyloidosis. Due to the earlyonset and rapid progression of A β pathology, the APP/PS1 mouse also could be employed for the efficient testing of therapeutic interventions that would be expected to influence A β load and associated MR signal. Moreover, our data suggest that voxel-based analysis can readily be employed as an observer-independent and automated approach to detecting *in vivo* surrogate markers of β -amyloid load and related tissue changes throughout the brain.

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Figure 1. Regions of interest (ROIs) in native space

Regions of interest are shown on slices: A) corresponding to IAD 1.68 mm, B) corresponding to IAD 2.52 mm, C) corresponding to IAD 3.24, D) corresponding to IAD 3.82.

Abbreviations are as follows:

CPu(a) - anterior caudate-putamen

CPu(p) - posterior caudate-putamen

ai - anterior ventral insular cortex

pi - posterior ventral insular cortex

PRhpi - perirhinal and posterior insular cortex

Ent - entorhinal cortex

hi - hippocampus

vi - ventral insular cortex

s1s2 - primary and secondary somato-sensory cortex

AuTeA - auditory and temporal association cortex

cg - cingulate cortex

v - ventricle

Figure 2. Processing stream for voxel-based analysis of T2 time maps

A: A T2 time map is derived from the multiecho sequence

B: The T2 time map is linearly aligned to the high-resolution TSE sequence (via the multiecho sequence; not shown in detail due to space restrictions)

C: The high-resolution TSE sequence is non-linearly normalized into an *a priori* defined standard space of the group-specific template brain

D: The deformation field generated in Step C is applied to the linearly aligned T2 map to generate the T2 map in the *a priori* defined standard space.

For further details refer to text.

Figure 3. Voxel-based analysis of T2 time reductions in APP/PS1 mice as compared to wildtype mice

Significant reductions of T2 relaxation times in APP/PS1 transgenic mice compared to wildtype mice are indicated by the colored regions where the color intensity corresponds to the voxel-wise T_{16} -value (see color bar in the lower right). Cluster extension set at ≥ 50 contiguous voxels passing the significance threshold of $p < 0.05$, FDR corrected. Effects are projected on the group-specific, averaged TSE image in standard space representing the template brain. The right side of the image corresponds to the right side of the brain.

Figure 4. Histological analysis of an APP/PS1 mouse at the age of 20 weeks

A) H&E stain overview shows only minor changes, e.g., faint senile plaques. In particular, there is no obvious evidence of neuronal loss or apoptotic cells. B) Campbell-Switzer silver stain (CS) shows fibrillar and amorphous amyloid deposits with a predominant localization in the deeper cortical layers. C) Immunohistochemical stain for β -amyloid detects predominant deposits in the deeper cortical layers (#1, see also inset “I”) as compared to subpial cortical regions (#2). D) GFAP-positive astrocytes are visualized in deeper cortical layers (#1), CPu, and hippocampus corresponding to A β plaque load and T2 relaxation time measurements. E) Double-immunohistochemistry of β -amyloid deposits (6F3D) and corresponding microglia (Iba-1) shows activated microglia only co-localized with A β deposits. F) Myelin stain (LuxolFastBlue-Eosin) does not reveal changes in subcortical white matter tracts, nor of large fibre tracts, e.g. the corpus callosum. G) The GFAP stain shows marked astrogliosis only in the cerebellar peduncles adjacent to the brain stem, and not in the cerebellar folia (magnification from D). H) No A β plaques are detectable in the cerebellum. APP/PS1 mice do not express the transgene in cerebellum (Thy1 promoter) (magnification from C). I) A β plaques in cortical layers (#1 & #2) and in the caudate-putamen (CPu) (magnification from C). (Scale bars: A–F 1mm (left) 200 μ m (right), G–I 250 μ m).

Figure 5. Detailed histological presentation of senile plaques in deeper cortical layers

A) H&E stain shows the disruption of neuropil by dense-core senile plaques. B) Visualization of A β by immunohistochemistry shows the dense-cored character of a plaque surrounded by a diffuse corona of A β . C) Activated microglial cells cover large portions of the β -amyloid deposits. D) Plaque as seen in a LuxolFastBlue-Eosin stained section. E) Reactive astrocytes surround β -amyloid deposits and lead to intensive encapsulation. F) Central parts of the plaques accumulate iron as visualized by Prussian Blue / DAB-enhancing method. G) Fibrillar character of a senile plaque is visualized by the Campbell-Switzer staining method. (Scale bar: 20 μ m for all images).

Figure 6. Voxel-based analysis of T2 time reductions in APP/PS1 mice compared to wildtype mice after correction for partial volume effects

Significant reductions of T2 relaxation times after PVE correction in APP/PS1 transgenic mice compared to wildtype mice are indicated by the colored regions where the color intensity corresponds to the voxel-wise T_{16} -value (see color bar in the lower right). Cluster extension set at ≥ 50 contiguous voxels passing the significance threshold of $p < 0.05$, uncorrected. Effects are projected on the group-specific, averaged TSE image in standard space representing the template brain. The right side of the image corresponds to the right side of the brain. For details on PVE correction refer to the text.

Table 1

Mean values and effect sizes for regional T2 time measurements

Measure mean (SD)	APP/PS1-pos [‡]	Wildtype controls	Cohen's d ^a	Cohen's d ^b
Muscle	95.46 (16.56)	103.02 (9.85)	1.14	0.57
Hippocampus	65.58 (6.06) [*]	73.82 (6.9)	1.93	1.36
anterior caudate-putamen	63.81 (5.15) [*]	67.38 (2.76)	1.95	0.89
posterior caudate-putamen	63.42 (4.93) [*]	67.59 (3.06)	2.03	1.05
cingulate cortex	64.63 (8.67) [*]	66.31 (2.59)	1.71	0.27
anterior ventral insular cortex	64.05 (9.75) [*]	65.69 (2.68)	1.88	0.23
posterior ventral insular cortex	63.75 (10.59) [*]	65.87 (2.88)	2.30	0.27
posterior insula and perirhinal cortex	64.69 (10.97) ^{**}	67.7 (3.84)	2.34	0.37
somato-sensory cortex	61.53 (7.24) [*]	63.84 (2.63)	1.98	0.43
auditory and temporal association cortex	62.59 (7.19) [*]	65.55 (3.76)	1.99	0.53
entorhinal cortex	67.91 (12.76)	69.8 (4.69)	1.04	0.20
Ventricle	86.00 (10.1) ^{**}	104.99 (6.73)	2.25	2.29

Means and standard-deviations (SD) of the ROI-based measurements of T2 times in ms and effect sizes (Cohen's d) (

^a – excluding.^b including the one animal with outlying values).

Mann-Whitney U-test. Including all animals:

^{*} significantly reduced in transgenic animals compared to wildtype controls at $p < 0.05$ ^{**} significantly reduced in transgenic animals compared to wildtype controls at $p < 0.01$ [‡] APP/PS1 heterozygote animals