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Leiden
The Netherlands

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Braakman, N.

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3 Longitudinal assessment of Alzheimer's β -amyloid plaque development in transgenic mice monitored by *in vivo* magnetic resonance microimaging*

3.1 Abstract

The development of β -amyloid plaques with age in the brains of the transgenic mouse model of Alzheimer's disease pathology was assessed by *in vivo* magnetic resonance microimaging. Towards this goal live transgenic mice (Tg2576) and non-transgenic littermates (control) were studied at regular intervals between the age of 12 and 18 months. Plaques were visualized using a T_2 weighted Rapid Acquisition with Relaxation Enhancement sequence. Changes in T_2 relaxation times were followed using a Multi-Slice Multi-Echo sequence. SCIL image software was used to calculate the plaque-load in the T_2 weighted MR images. A β plaques were clearly detected with the T_2 weighted RARE sequence in a scan time as short as 25 min in the hippocampal and cortical regions of the brain of Tg2576 mice but not in control mice. Following the plaque development in the same animals with age shows that the plaque-load and plaque size increased markedly, while T_2 relaxation times show a decreasing trend with age. These results demonstrate that μ MRI is a viable method for following the plaque developmental characteristics *in vivo* in the same animals and suggest that monitoring the effect of future therapeutic interventions over time in the same animals would ultimately be possible by μ MRI.

3.2 Introduction

Alzheimer's disease is the most common neurodegenerative disease and currently afflicts about 10% of the population over 60, with numbers still rising (1). The neuropathologic features of AD include the occurrence of senile plaques, neurofibrillary tangles, decreased synaptic density, and loss of neurons. The core of senile plaques consists mainly of aggregated amyloidogenic peptide A β which is derived from the amyloid precursor protein. The role of A β in AD may be substantial, as soluble A β polymers have

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been reported to be neurotoxic, both *in vitro* and *in vivo* (2). Although it is not yet clear whether senile plaques themselves are neurotoxic, A β plaque formation precedes disease onset by many years and is generally accepted as a biomarker for onset and progression of the disease (2,3). Early diagnosis of Alzheimer's disease is prevented by difficulties in visualizing A β plaques *in vivo* in the brain, and the only definite diagnosis for AD at present is post-mortem observation of A β plaques and neurofibrillary tangles in brain sections (4).

In order to study the pathogenesis of AD, its development over time, and to ultimately develop adequate therapeutic agents or preventive strategies, it is important to establish non-invasive *in vivo* imaging methods to visualize A β plaques and to validate if the μ MRI is feasible for quantitative monitoring of the plaque development with age in the same animals. Imaging methods such as single photon emission computed tomography or positron emission tomography use ionizing radiation and suffer from low resolution (5). MRI can provide much higher resolution than SPECT or PET without ionizing radiation and can theoretically resolve individual plaques non-invasively. The first effort to visualize A β plaques by MRI in fixed post-mortem human brain tissue was made by Benveniste *et al.* (6) using T_2^* -weighted MRI at 7T. Subsequently several different transgenic mouse models of AD pathology have been used to visualize A β plaques either *ex vivo* in fixed brain (5,7,8) or *in vivo* using targeted contrast agents (9,10) or amyloidophilic probes (2,11). However, the delivery of these agents requires a relatively invasive procedure. Very recently, initial efforts for *in vivo* detection of A β plaques using MRI without the aid of an exogenous plaque-specific contrast agent have also been reported in transgenic mouse models of AD (12,13) using T_2 -weighted spin echo and T_2^* -weighted gradient echo sequences. Vanhoutte *et al.* (13) used basic T_2^* -weighted MRI to visualize plaques associated with iron *in vivo* in the thalamus region of the brain, but failed to detect plaques in the cortex and hippocampus areas which are the main regions of A β deposit in the brain in human as well as in transgenic mouse models of AD pathology (14,15). Although imaging of A β plaques *in vivo* without the aid of an exogenous plaque-specific contrast agent still lacks sufficient sensitivity and requires further improvement, to date no longitudinal MR studies to follow the development of A β plaques with age in the same animals have been attempted. In recent longitudinal studies concerning the detection of A β plaque-load in mouse models of AD pathology, post-mortem biochemical and/or histological examinations were performed on different mice belonging to different age groups (16).

In the present study, high field μ MRI was used to detect A β plaques in a living transgenic mouse model of AD pathology, without contrast agent, and to track the development of plaques with age in the same animals. Our results demonstrate that μ MRI is a viable non-invasive method for longitudinal studies to assess A β plaque development in a quantitative manner and thus would be invaluable for evaluation of new anti-amyloid treatment strategies.

3.3 Methods

3.3.1 Transgenic mice

The transgenic mice used in this study contain as transgene the Swedish double mutation of the human amyloid precursor protein (APP₆₉₅), as developed and described previously by Hsiao et al (17). The transgene is expressed in C57B6 breeders. The N2 generation mice of both genders (n=5) were studied at ages between 12 and 18 months. Age-matched non-transgenic littermates served as controls. All animal experiments were approved by the Institutional Animal Care and Animal Use Committee in accordance with the NIH *Guide for the care and Use of Laboratory Animals*.

3.3.2 μ MRI

All μ MRI measurements were conducted on a vertical wide-bore 9.4T Bruker Avance 400WB spectrometer, with a 1000 mTm⁻¹ actively shielded imaging gradient insert (Bruker). The imaging coil used was a 25 mm volume coil (Bruker) which was best suited to acquire full brain images in both the coronal and the horizontal plane. The system was interfaced to a Linux pc running XWinNMR 3.2 and Paravision 3.02pl (Bruker Biospin).

For *in vivo* μ MRI measurements the mice were anesthetized using Isoflurane (Forene, Abott, UK) inhalation anesthesia, together with air and oxygen (1:1) at 0.3 l/minute. The anesthetic gas was administered via a special face mask, which also served as a fixation device for the mouse head by coupling it with a specially designed toothbar to hold the head in place (Bruker Biospin). While inside the probe, the respiration rate of the mouse was constantly monitored by means of a pressure transducer placed on the abdomen. The transducer was connected to a BioTrig acquisition module, which was interfaced to a BioTrig command module and laptop running BioTrig BT1 monitoring software (Bruker Biospin).

T_2 -weighted MR images were acquired using a RARE sequence (18) which employs a single excitation step followed by the collection of multiple phase-encoded echoes. This reduces the total scan time significantly compared to normal multi slice spin echo methods. Basic measurement parameters used for the RARE sequence were: TE = 10.567 ms (22.45 ms effective), TR = 5-6 s, flip angle = 90° , averages = 4+, RARE factor (echo train length) = 4. The field of view was $2.0 \times 2.0 \text{ cm}^2$, with an image matrix of 256×256 . This yields an effective in plane resolution of approximately $78 \text{ }\mu\text{m}$. Coronal (transverse) image slices (30-60) were acquired from the olfactory bulb to the cerebellum with a slice thickness of 0.2 mm spaced 0.2 mm between slices, or 0.5 mm spaced 0.5 mm between slices. The horizontal and coronal slices shown in Fig. 3.1 and those used for longitudinal studies (Fig. 3.4) were obtained with a slice thickness of 0.5 mm and a total scan time of approximately 25 minutes.

For T_2 relaxation measurements, an MSME sequence was used. Imaging parameters were: FOV $2.0 \times 2.0 \text{ cm}^2$, matrix size 256×256 , number of averages 2, number of slices 6 with slice thickness of 1 mm, number of echoes = 8 with TE of 8.5, 17.0, 25.5, 34.0, 42.5, 51.0, 59.5, and 68.0 msec, and a repetition time of 1.5 seconds. To calculate the T_2 relaxation time, regions of interest were drawn around the cortex and hippocampus in two adjacent mid-coronal slices. Another ROI in the muscle was used as an internal control according to Helpert *et al.* (7). The means and standard deviation of the T_2 relaxation times for each ROI were calculated. Mean values were compared using Student's t-test assuming equal variance, and significance was assigned at $P < 0.05$.

3.3.3 Brain preparation and histology

Following *in vivo* MR measurements, mice were deeply anesthetized and transcardially perfused with phosphate-buffered saline (pH 7.4) followed by 4% buffered paraformaldehyde (Zinc Formal-Fixx, ThermoShandon, UK) through the left cardiac ventricle. After perfusion fixation the brain was dissected out and placed in the same fixative for 48 h. Following fixation, the brain was dehydrated and embedded in paraffin. Subsequently coronal sections ($40 \text{ }\mu\text{m}$ thick) were carefully cut using a vibratome while maintaining as much as possible the same spatial orientation of mouse brain as in the MR imaging experiments. To detect the A β plaques, brain sections were subjected to immunohistochemistry using monoclonal anti-amyloid β (6E10) antibody at 1:1000 (Signet Laboratories, Inc). Immunolabeling was visualized by using the ABC kit (Vectastain) according to the manufacturer's instructions.

Detection of redox active iron associated with the A β plaques was done histochemically as described previously (19,20). Briefly, the brain sections were incubated for 15 h in 7% potassium ferrocyanide in aqueous hydrochloric acid (3%) and subsequently incubated in 0.75 mg/ml 3, 3'-diaminobenzidine (DAB) and 0.015% H₂O₂ for 5-10 min. This method involves the formation of mixed-valence iron (II/III), (Prussian blue) when iron (III) released from iron containing plaques by the hydrochloric acid reacts with potassium ferrocyanide. The mixed-valence complex then catalyzes the H₂O₂-dependent oxidation of DAB to give a brown color. Images of the histological sections were obtained using a Leica DM RE HC microscope, interfaced to a Leica DC500 3CCD digital camera. Quantitative histological analysis of A β plaque density and iron associated plaques was performed by SCIL image software (21).

For co-registration, immuno-histological images were matched with MR slices using common anatomical landmarks such as the ventricles, corpus callosum and hippocampal fissure using PhotoShop 7.0 (Adobe Systems, San Jose, CA). As a result of differences in the slice thickness in μ MRI (200 μ m) and histology (40 μ m), each MR image could be matched with at least 4 immuno-stained histological images.

3.3.4 Image analysis and quantification

For quantification of A β plaque-load and numerical density in MR images, image files with calibrated scale markers were imported in SCIL image software (21). The brightness of the images was altered so that the average optical density measurement for each imported image was similar. Regions of interest were manually drawn on the cortex and hippocampus, corresponding to the same anatomical markers used for histological A β quantification. Dark spots, with intensity below a preset threshold value (equal for all MR images), were considered as A β plaques. A β plaque-load (percentage hypointense area of the ROI) and numerical density of plaques (number of plaques per mm² of the ROI) were calculated for cortex and hippocampus.

3.4 Results

Figure 3.1 shows horizontal (axial) and transverse (coronal) slices through the brain of a living 18-month-old APP transgenic mouse (Tg2576) and its non-transgenic littermate (control) obtained by using a *T*₂-weighted RARE sequence. As can be seen in this figure, numerous dark spots were clearly evident in the cortex and hippocampus areas in both horizontal and transverse MR slices of the brain of Tg2576 transgenic mouse (Fig. 3.1, right panels).

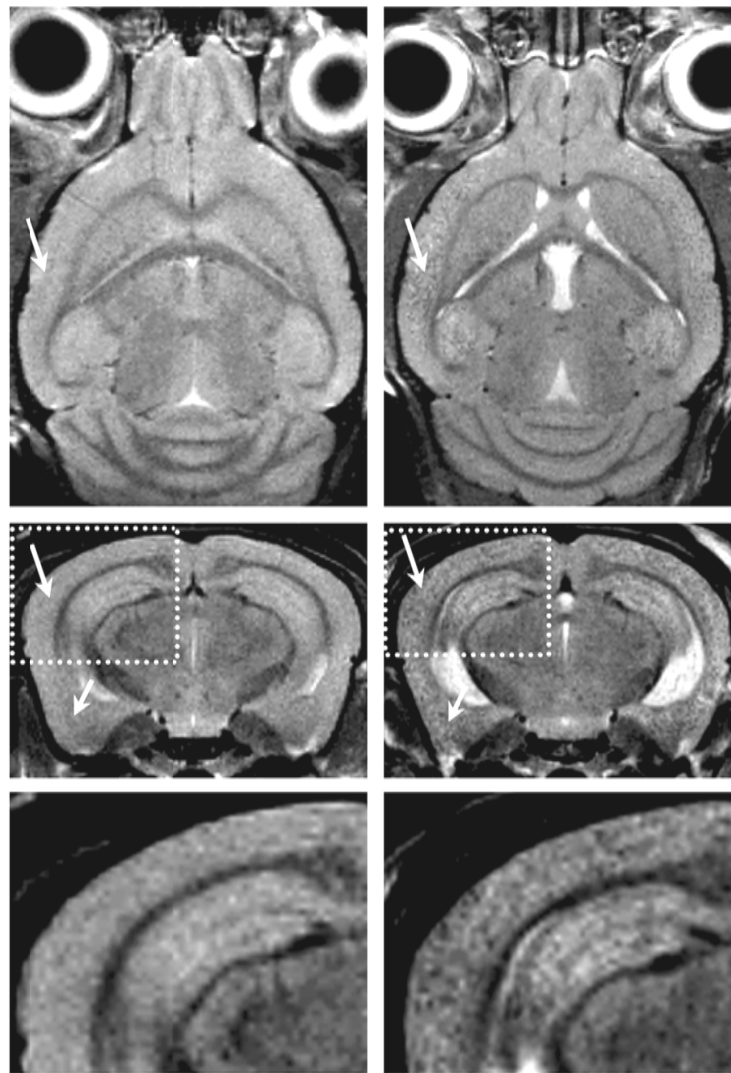


Figure 3.1: *In vivo* T_2 -weighted MR images of the brain of an 18-month-old control mouse (left panels) and AD transgenic Tg2576 mouse (right panels) obtained at 9.4T. The top row shows horizontal slices (Bregma: -6 mm), the middle row shows coronal slices (Bregma: -3.4 mm) and the bottom row shows magnified sub-sampled insets of the coronal slices, showing adequately resolved individual plaques in the bottom right panel. MR images were obtained with an in-plane resolution of $78\ \mu\text{m} \times 78\ \mu\text{m}$ ($TE = 10.567\ \text{ms}$, $TR = 6\text{s}$, echo train length = 4, averages = 4, total scan time 25 min). Arrows indicate areas with differences in the signal intensities between the Tg2576 mouse and its non-transgenic litter-mate. Numerous circular hypointensities can be clearly observed in the images of the cortical and hippocampal areas of the Tg2576 mouse right column), while no clear signal hypointensities are visible in the images collected from the control mouse shown in the left column.

These hypointense regions could be detected with scan times as short as 25 minutes. Such signal hypointensities were not observed in the brains of control mice (Fig. 3.1, left panels). MR hypointensities can be seen more clearly with higher magnification as displayed in the bottom row in Fig. 3.1.

To confirm that the hypointense signals seen in MR images correspond to the A β plaques, *in vivo* MR images of an 18-month-old transgenic mouse were compared with immunostained images obtained from the same mouse brain (Fig. 3.2). Immunolabeled sections show that dense-cored plaques are the predominant form of senile plaques in the cortical and hippocampal areas of the Tg2576 transgenic mouse brain (Fig. 3.2b). Many are giant plaques with core diameters above 100 μ m. In addition a few diffuse plaques were also observed. Co-registration of plaques between *in vivo* μ MRI and corresponding immunostained sections validates that many circular hypointense regions seen in μ MRI correspond to A β plaques (Fig. 3.2).

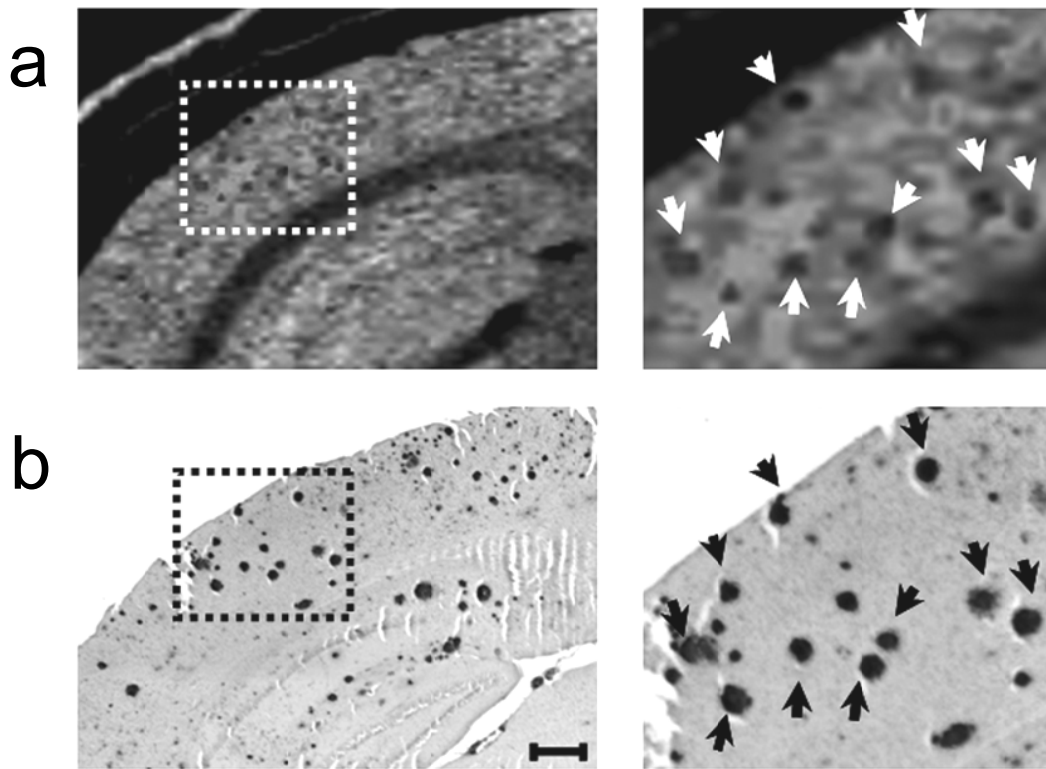


Figure 3.2: Co-registration of A β plaques seen in the brain of an 18-month-old AD mouse by (a) *in vivo* μ MRI and (b) histological section of the same mouse brain stained with A β antibodies. Many MR circular hypointense spots could be matched to immunostained A β plaques (arrows), seen more clearly in the higher magnification insets. Scale bar, 500 μ m.

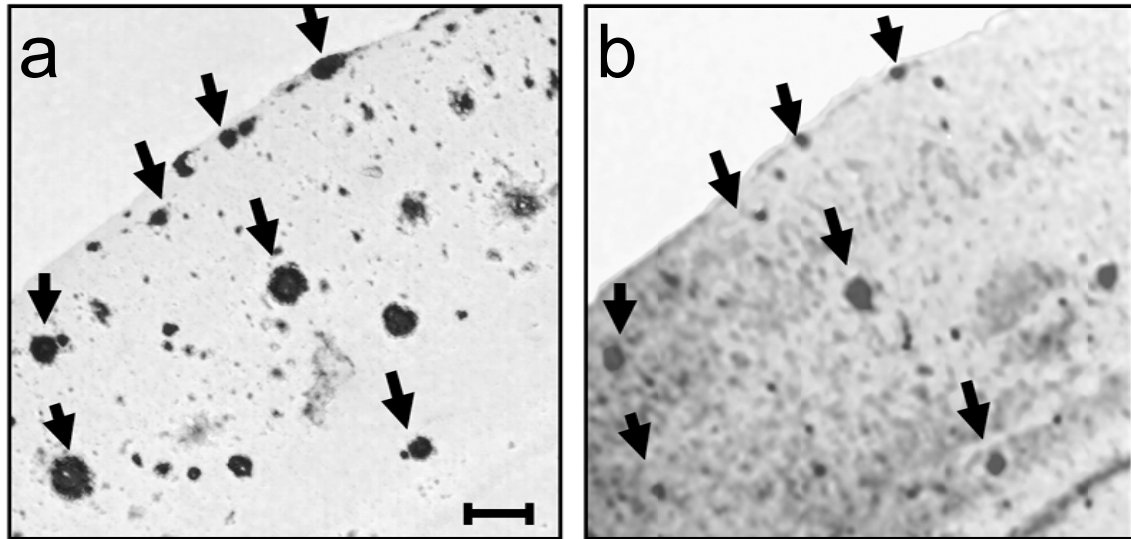


Figure 3.3: Co-registration of A β plaques and iron in the adjacent brain sections of an 18-month-old Tg2576 mouse. Brain sections (4 μ m thick) were stained with (a) an A β antibody (6E10) or (b) Perl's reaction following 3, 3-diaminobenzidine enhancement. Arrows indicate co-registered plaques. Scale bar, 250 μ m.

Although association of iron with plaques in human and in mouse models of AD pathology is known (19,22,23,24), it is yet to be established if it is the main reason for plaque specific T_2 contrast in MR images. As can be seen in Fig. 3.3, iron (III) was found to be associated with many dense-cored senile plaques, although not all A β plaques seen by immunohistology contained iron. Iron loaded plaques were restricted to the cortical and hippocampal regions of the brain (Fig. 3.3).

To examine the utility of μ MRI for detecting the development of A β plaques in longitudinal studies, the same mice ($n=5$ for each group) were imaged at regular intervals of approximately one month starting at the age of 12 months. An example of MR images obtained from the brain of a Tg2576 transgenic mouse at the age of 12, 14, 16 and 18 months is displayed in Fig. 3.4. A consistent increase in the MR hypointensities was observed with age in the cerebral cortical and hippocampal regions. In addition to the overall rise in MR hypointensities, the size of the circular hypointense spots also increased, suggesting an increase in the size of A β plaques with age (Fig. 3.4). Some of the A β plaques observed in the brain at the age of 12 months were detected consistently at all ages, while the sizes of these plaques showed an increasing trend with age (Fig. 3.4).

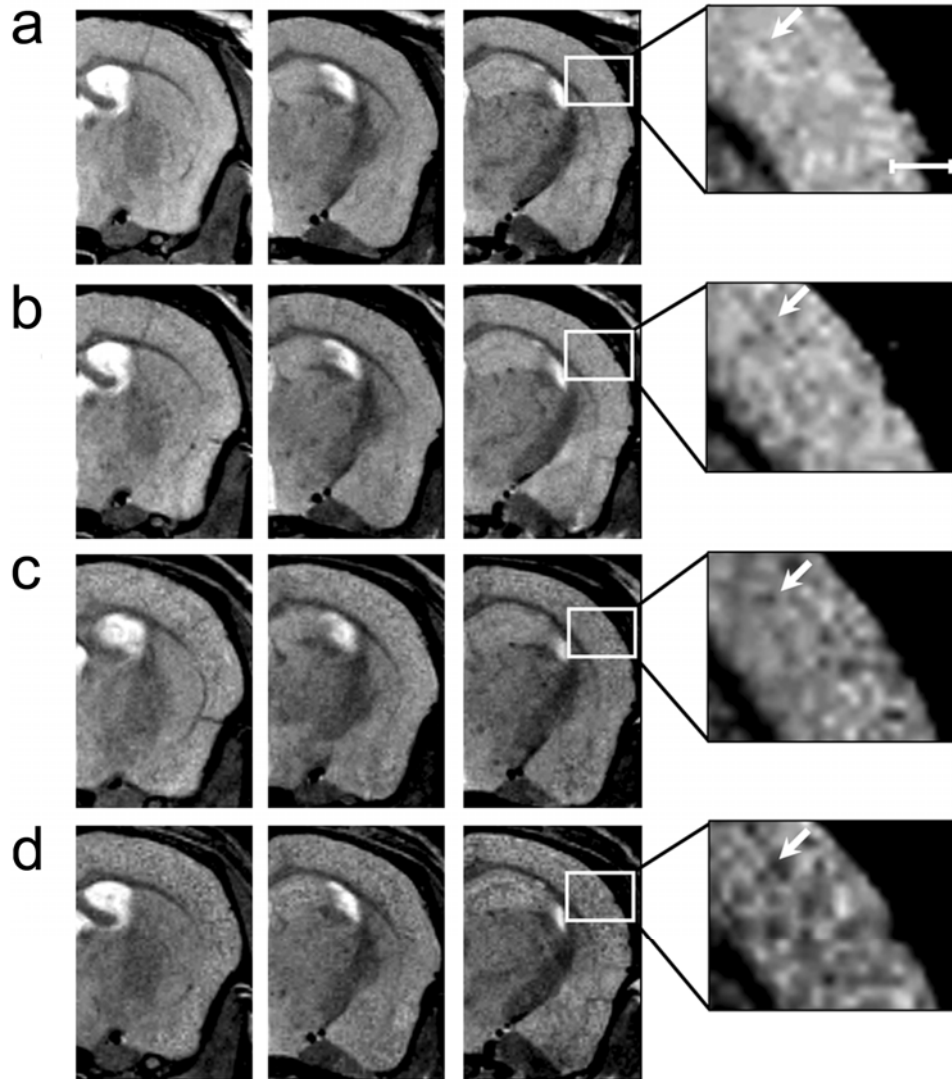


Figure 3.4: Development of A β plaques with age in the brain of AD transgenic mouse monitored by *in vivo* μ MRI. MR images showing successive coronal slices (left to right) of the brain of the same Tg2576 mouse at the ages of 12 months (a), 14 months (b), 16 months (c), and 18 months (d). Magnified subsampled areas on the right show an increase in plaque-load with age. Arrows indicate the same plaque seen at 12, 14, 16 and 18 months of age. Note the increase in size with age. Scale bar, 400 μ m.

Quantification of A β plaque-load in Tg2576 mice with age is shown in Fig. 3.5a. A marked age-related increase in both plaque-load and numerical density of the A β plaques is evident in this figure. Although the increase in numerical density of A β plaque shows an almost linear trend from 12 to 18 months of age, the overall plaque-load shows a much more rapid increase from 16 to 18 months of age (Fig. 3.5a).

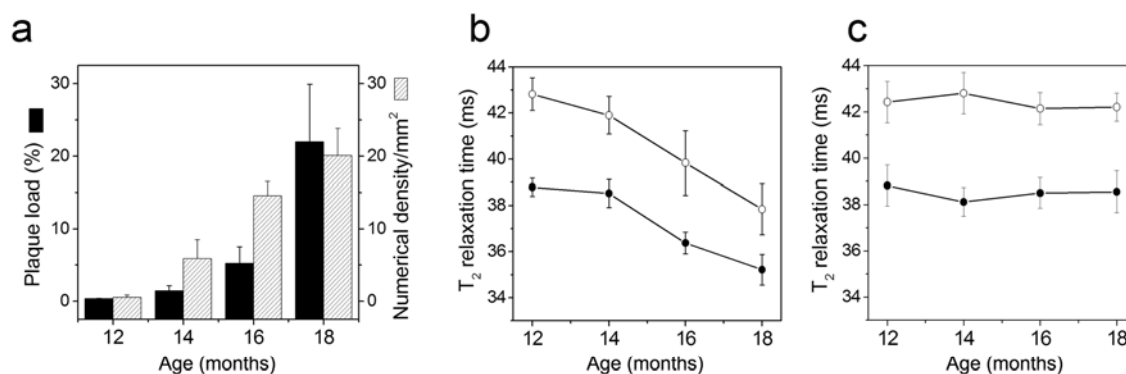


Figure 3.5: Age dependent changes in: (a) A β plaque-load and numerical density of A β lesions detected by μ MRI and T₂ relaxation time in the hippocampus (o) and cortex (●) regions of AD transgenic mice (b) and control mice (c). Data represents a mean of n=5 ($\pm$ SD).

An increase in the plaque-load with age was associated with a significant reduction in the mean transverse relaxation time T_2 in the hippocampus and cortex of Tg2576 mice (Fig. 3.5b). A region in muscle, used as the internal control, did not show any significant change in T_2 relaxation time with age. The T_2 relaxation time in muscle was 27.6 ± 0.95 ms at the age of 12 months, and 27.9 ± 0.82 ms at the age of 18 months. A similar study using the age-matched nontransgenic control mice did not show any significant age-dependent decline in T_2 relaxation time in the cortex or hippocampus (Fig. 3.5c).

3.5 Discussion

This study demonstrates the application of μ MRI to resolve A β plaque in the brain of living transgenic mouse model of AD pathology and to follow the development of the plaques in the same animals over time. Tg2576 mice overexpressing human APP₆₉₅ with the “Swedish” mutation develop A β plaques and memory deficit with age, (17,25,26,27) making them suitable for longitudinal MR studies.

The T_2 -weighted RARE sequence used in this study allowed clear identification of hypointense lesions corresponding to A β plaques identified by immunohistochemistry (Fig. 3.2). Previously, a similar fast spin echo sequence has been used to visualize plaques very clearly in *ex vivo* brain of AD mice at 7T (5). Our results suggest that a similar sequence with careful adjustment of MR parameters can be applied for detection of plaques in the brain *in vivo* at a moderately higher magnetic field of 9.4T. The characteristic size of A β plaques in Tg2576 mice varies from 20 to 150 μ m. Fig. 3.2 shows many giant plaques with sizes above 75 μ m in diameter, which is within the spatial resolution (78 μ m) of our MR experiments. Vanhoutte et al (13) have recently reported *in*

vivo visualization of A β plaques at 7T using intrinsic MRI contrast arising from the iron associated with plaques using a T_2^* -weighted 2D gradient echo sequence. One of the major limitations of their study was that the plaques were seen only in the thalamus but not in the cortex or hippocampus, which are the main areas for the A β plaque deposition in humans as well as in all known AD transgenic mouse models. In addition, the size of the plaques in T_2^* -weighted images is often overestimated (12). Jack *et al.* (12) recently demonstrated MRI visualization of plaques *in vivo* at 9.4T in another transgenic mouse model without the use of a contrast agent and using a trigger desensitizing modification of a T_2 -weighted spin echo sequence. With this method they resolved the plaques in an acquisition time as short as 1h 7 min. However, without trigger desensitizing modification or cardiorespiratory triggering, plaques could not be resolved (12). In our present study we use the multi-slice RARE method employing a single excitation step followed by the collection of multiple phase encoded echoes. With this method we have reduced the acquisition time significantly to 25 minutes and clearly identified plaques even with an in-plane resolution of 78 μ m (Figs. 3.1 and 3.4). In addition, plaques can be resolved without any cardiorespiratory triggering or trigger desensitizing modification. It is difficult to directly compare the results of this study to those of Jack *et al.* (12) due to the differences in the employed pulse sequence, as well as the different mouse models used.

The reason for plaque specific T_2 contrast is not known. It has been speculated that the presence of iron in the A β plaques may be responsible for MR contrast (5,12,13,22). Defective iron homeostasis, resulting in an increased iron level in AD brain, has been reported (9,28). In AD brain, iron is apparently mainly concentrated in amyloid plaques and may catalyze the formation of free radicals (29). The source of iron is unknown, but evidence suggests that induction of heme oxygenase, which occurs in AD, converts heme into tetrapyrrole and free iron (30). In AD such iron binds with the abnormal protein constituents of the lesions e.g. A β . It has been shown earlier that plaque-associated iron is redox active iron and is not bound to normal iron binding proteins but to the abnormal protein constituents of the A β lesions (19,20,24). To understand whether the presence of iron is the main reason for plaque specific T_2 contrast in MR images, we examined the distribution of iron in the adjacent histological sections of the Tg2576 mouse brain. The results show that iron is associated with many dense-cored A β plaques seen in the cortex as well as in the hippocampus (Fig. 3.3). Iron seems to be associated with the central region of the β amyloid deposits (Fig. 3.3). This observation is in line with earlier histochemical studies (19), which show that redox active iron is specifically localized to

the lesions of AD and not the glial cells surrounding senile plaques, which contain abundant iron binding proteins. Our results suggest that iron may be the source of the intrinsic MR contrast from A β plaques as has been recently proposed for this mouse model (22). However, signal hypointensities arising from the reduced water content in A β plaques compared to the surrounding tissue and from other unknown factors cannot be ruled out.

In addition to the presence of A β plaques, the lateral ventricles are visible as hyperintense regions in the coronal MR slices of the brain of AD mouse at Bregma -2.4. At the same location in the brain of wild-type mice, only very small portion of lateral ventricles is seen (Fig. 3.1, middle row). This can be explained by the fact that AD brains show immense enlargement of the lateral ventricles due to significant loss of surrounding tissue in comparison to control brains. The ventricular enlargement in AD brains was previously shown in humans (31) and another AD mouse model (32).

As illustrated in Fig. 3.4, Tg2576 mice show a marked age-related increase in amyloid deposition in the hippocampal and cortical regions of the brain (Fig. 3.4). The plaques increase rapidly in number, in size and in the degree of compactness. Only a few circular hypointense regions corresponding to A β plaques were observed in 12 month old Tg2576 mice, while the density of A β deposits, seen as dark circular hypointense regions in the cortical and hippocampal region, considerably increased at 18 months (Fig. 3.4).

A quantitative estimation of A β plaque-load and numerical density with age in the MR images shows that plaque burden increased markedly with age (Fig. 3.5a). The increase of plaque-load was more significant after 16 months than between 12-16 months. In comparison to A β plaque-load, the numerical density of the A β plaques shows a linear increase between 12 and 18 months. These results can be explained by a significant increase in the size of the plaques after 16 months contributing to an increase in the plaque area in the cortex and hippocampus. These results are well in line with immunohistochemical observations (16). Since the trend toward an increase in plaque burden seen by μ MRI is clearly significant within 12 and 18 months of age, it is suggested that monitoring the effect of anti-amyloid drugs in Tg2576 mice during that time window is feasible using *in vivo* μ MRI

A β plaques in mice and humans are quite similar in size (up to 200 μ m). In principle, the detection of A β plaques by MRI can be extended to human subjects. However, this would require improvement in instrumentation and MR sequences to permit imaging of human

brain with a similar contrast-to-noise ratio at a slightly lower spatial resolution, in a much shorter imaging time. With the growing awareness of the feasibility of human imaging at ultrahigh fields (≥ 7 T) and improvements in RF coil technology, it may be possible to apply this approach to humans in the future (12).

The spin-spin relaxation time T_2 is a specific attribute of spins that depends on their surroundings. Interaction between spins destroys the phase coherence and therefore, the T_2 relaxation time can be a sensitive indicator of impaired cell physiology. A lower T_2 relaxation time was previously observed in cortex and hippocampus of Tg2576 transgenic mice compared to non transgenic control (7). We followed the changes in T_2 relaxation time with age in Tg2576 transgenic mice. A good correlation has been observed between increase in the plaque-load and decrease in mean transverse relaxation time T_2 with age in both the hippocampus and cortex of Tg2576 mice (Fig. 3.5a and b). A similar study with age-matched non-transgenic control mice did not show any significant age dependent decline in T_2 relaxation time (Fig. 3.5c). These observations suggest an influence of plaque-load on T_2 reduction. Although the reason for reduction in T_2 time is not yet clear, earlier studies have speculated the involvement of iron associated plaques in reducing the T_2 time in AD brain (7,22).

In conclusion, we have applied μ MRI to resolve A β plaques in the brains of living transgenic AD mice without the aid of exogenous contrast agents in a reasonably short scanning time, and followed the development of the plaques in the same animals with age. Our results show that the developmental characteristics of A β plaques, such as number, size and compactness can be followed with age in the same animals using *in vivo* μ MRI. Such MR longitudinal studies may be a valuable tool for evaluating the efficiency of novel anti-amyloid treatment strategies for arresting the growth or preventing the development of new plaques using AD mouse models.

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