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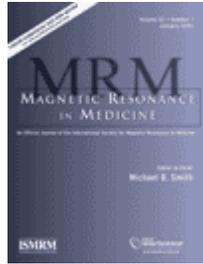
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Transversal relaxation time reflects brain amyloidosis in young APP/PS1 transgenic mice

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Running Head: : T2 reflects amyloidosis in young APP/PS1 mice

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Abstract

Amyloid deposits are one of the hallmarks of Alzheimer's disease, one of the most devastating neurodegenerative disorder. In transgenic mice modelling Alzheimer's pathology, magnetic resonance transverse relaxation time (T2) has been described to be modulated by amyloidosis. This modification has been attributed to the age-related iron deposition that occurs within the amyloid plaques of old animals. In the present study, young APP/PS1 transgenic mice without histochemically detectable iron in the brain were specifically studied. In vivo measurements of T2 in the hippocampus, at the level of the subiculum, were shown to reflect the density of amyloid plaques. This suggests that T2 variations can be induced solely by aggregated amyloid deposits in the absence of associated **histologically-detectable** iron. Thus T2 from regions with high amyloid load such as the subiculum is particularly well suited to follow plaque deposition in young animals, *id est* at the earliest stages of the pathological process.

Ref1, c1

Keywords: Alzheimer disease, amyloid- β peptide, iron, T2

1. Introduction

Alzheimer's disease (AD) is characterized by two main neuropathological lesions: senile plaques and neurofibrillary tangles. Plaques are extracellular deposits of amyloid- β ($A\beta$) peptides, while tangles are intraneuronal filaments of hyperphosphorylated tau proteins (1). Until now, the definitive diagnosis of AD requires post-mortem detection of these lesions. According to the amyloid cascade hypothesis, abnormal amyloid accumulation in the brain is a critical event in the disease process (2). Development of disease-modifiers targeting $A\beta$ pathology appears therefore to be of crucial importance.

Imaging strategies aimed at *in vivo* detection of cerebral $A\beta$ deposition in mice are actively investigated to monitor the progression of disease and to assess the effects of new therapies. Until now most of the protocols that allowed detection of brain amyloidosis in a non-invasive way in animals have relied on magnetic resonance imaging (MRI). Two strategies were developed. The first one is based on the direct detection of individual amyloid plaques by MR **microimaging**. This approach either exploits the natural contrast of the plaques (3) or uses specific contrast agents targeting $A\beta$ (4-6). This approach is hampered by the difficulty of imaging the smallest plaques because of the limited resolution of the MRI. The second strategy looks for "global" relaxation time changes associated with the presence of $A\beta$ instead of trying to detect individual plaques. For instance, it has been demonstrated that transversal relaxation times (T_2) of brain tissues from aged mouse models of AD are modified by the presence of $A\beta$ deposits associated with iron (7-9). The aim of the present study was to better understand and refine the origin of T_2 modifications observed in plaque-enriched brain tissue from living mice modelling AD's cerebral amyloidosis. These modifications might be caused by different/concurrent factors such as the presence of hydrophobic amyloid deposits or age-related accumulation of iron within

Ref 1, c8

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3 the plaques (3,9,10). To dissociate the impact of each variable the present study focused on
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5 young to middle-aged APP/PS1 transgenics (16-31 weeks of age).
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8 In several animal species, including humans cerebral iron increases with age and reaches a
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10 plateau in mature adults (11). In mice, age related increase of iron load seems to occur between
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12 the age of 2.8-3.4 months and 11 months (12,13)]. In a previous study, we further suggested that
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14 iron originating from surrounding tissue becomes progressively associated to the plaques in old
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16 double APP/PS1 transgenic mice (9). Interestingly, in this transgenic line, brain amyloidosis is
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18 very aggressive with an early onset at 2.5 months (14), presumably a few weeks before rising of
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20 cerebral iron load. Thus, one can expect that young APP/PS1 mice should start developing
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22 plaques without presenting amyloid-associated iron load, giving therefore the opportunity to
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24 isolate and refine the role of amyloid- β deposition per se on MR relaxation times. Indeed, in the
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26 present work, the youngest studied mice did not show histologically detectable iron deposition
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28 while still presenting variable amyloid loads. Analysis of regional T2 relaxation time was
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30 performed in the subiculum, one of the first brain areas to develop A β plaques in the studied
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32 transgenic model (14), and in the temporal association cortex in which no amyloid deposits were
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34 detected even in the oldest animals studied. We showed that even before the occurrence of
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36 histologically detectable iron in the plaques, the subicular T2 is negatively correlated to the
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38 amyloid load. This suggests that amyloid deposits or tissue modifications associated to amyloid
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40 deposits have a direct effect on T2 and that this parameter can be used as an early, iron- and age-
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42 independent, biomarker of the amyloid load.
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Ref 2, c1

Ref1, c1

Ref1, SC1

2. Materials and methods

2.1. Transgenic mice

Transgenic APP/PS1 mice (Thy1 APP751 SL (Swedish mutation KM670/671NL, London mutation V717I introduced in human sequence APP751) x HMG PS1 M146L), modelling early onset (at 9-14 weeks of age) and progressive cerebral amyloid deposition were used (n=11, 16-31 weeks of age) (14). Heterozygous “APP/PS1” mice were obtained by crossing heterozygous Tg APP_(+/-) mice with homozygous Tg PS1_(+/+) mice. The APP_(-/-)/PS1_(+/-) mouse littermates (“PS1”) resulting from crossings were used as controls (n=10, 16-31 weeks of age) as they display no amyloid plaques and therefore can allow to specifically evaluate the effects of the APP transgene on amyloid deposition in the brain.

Ref1, c 4

2.2. In vivo MRI experiments and data analysis

In vivo MR images were recorded on a 4.7 Tesla Bruker Biospec 47/30 system, equipped with a 12 cm diameter gradient system (200 mT/m). A surface coil (diameter = 30 mm), actively decoupled from the transmitting birdcage probe (Bruker GmbH) was used for signal acquisition. Animals were anesthetized with isoflurane (5% for induction, 1-1.5% for maintenance) in a mixture of N₂ (80%) and O₂ (20%) administered via a facemask. Respiration rate was monitored to insure animal stability until the end of the experiment. Body temperature of the mice was maintained by using a water filled heating blanket.

The MRI protocol was previously described (9). Briefly, a multislice multiecho sequence was used for T₂ measurements (echo times (TE)=12.2, 24.4, 36.6, 48.8, 61, 73.2 ms, repetition time (TR)=2000 ms, field of view (FOV)=1.5x1.5 cm², matrix 128x128, slice thickness=1 mm,

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3 acquisition time of 8min 49s). Parametric T2 maps were generated by fitting pixel intensity
4
5 values to single exponential curves using the Bruker fit package.
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8 Inversion recovery images (TE=10ms, TR=5000ms, TI=300ms, FOV=1.5x1.5cm², matrix
9
10 128x128, slice thickness=1mm) that had good contrast between cerebral structures were acquired
11
12 to localise regions of interest (ROIs) corresponding to the subiculum and to the temporal
13
14 association cortex. The subiculum is one of the first regions to show plaque deposition in
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16 APP/PS1 mice while the temporal cortex undergoes amyloidosis much later during the disease
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18 process. The ROI corresponding to the subiculum was placed underneath the cingulum on a slice
19
20 localized approximately between bregma -2.8 mm and bregma -3.8 mm according to the Paxinos
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22 atlas (15). ROIs corresponding to the temporal association cortex were drawn on this same slice.
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24 ROI outlines were then transferred to T2 maps. For all animals, T2 values were the mean of ROIs
25
26 drawn on the left and right side of the brain except for one APP/PS1 and one PS1 animals in
27
28 which T2 values were issued from only one side of the brain where the cingulum was accurately
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30 localized.
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38 **2.3. Histological analysis**

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40 Following completion of MRI studies, mice were sacrificed by decapitation. Brains were
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42 then extracted and fixed in 10% buffered formalin and kept in fixative at -4°C until being
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44 processed for histology (mean fixation time +/- SEM : 51 days +/- 7 days). Brains were then
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46 stored overnight in a solution of 20% glycerin and 2% dimethylsulfoxide in 0.1 M phosphate
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48 buffer for cryoprotection. Brains were subsequently sectioned into 10 series of 40 µm-thick
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50 coronal sections on a freezing microtome.
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54 Anti-A β immunohistochemistry was performed using the biotinylated 4G8 antibody
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56 (Biovalley, France). Endogenous peroxidase activity and non-specific antigenic sites were firstly
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Ref2, c2a

Ref 1, c7

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3 blocked using standard procedures before incubation with primary antibodies (1/10000; one night
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5 at room temperature). Following incubation in Avidin-Biotin-Horseradish peroxidase complex
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7 (Vector Laboratories, Burlingame, CA) final reaction made use of 3-3'-diaminobenzidine as
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9 chromogen (brown product). Amyloid deposits were concurrently labelled by standard Congo red
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11 staining on a second batch of slices (adapted from (16)). Each Congo-red stained slice was
12
13 digitized using a Super CoolScan 8000 ED high-resolution scanner (Nikon, Champigny sur
14
15 Marne, France). Regional amyloid loads (expressed as percent of tissue surface stained by the
16
17 Congo red dye) were quantified in the subiculum area that was previously investigated by means
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19 of MRI, using computer-based thresholding methods (17). Each stained section was binarized
20
21 after thresholding in Photoshop (Adobe Systems, Paris, France). Binarized images were then
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23 transferred to Image- Pro Plus software (Media Cybernetics, Silver Spring, USA) and submitted
24
25 to a particle-detection algorithm to measure the surface of plaques on each brain section (2-4
26
27 sections sampled per mice). The proportion of tissue area occupied by Congo red stained material
28
29 was then assessed. The ratio (surface stained/whole subiculum ROI surface) was taken as a
30
31 measure of the “amyloid load” in the subiculum..
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Ref 2, c2b

Ref 2, c2c

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39 Iron staining was performed on a third batch of serial sections using a protocol derived
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41 from the standard Perls-diaminobenzidine (Perls-DAB) method as previously described (9) (18).
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43 Although biochemical quantification of iron (eg atomic absorption spectrophotometry) is the
44
45 most valuable and precise method to assess iron concentrations, this approach was discarded as it
46
47 can not be used to measure iron loads in discrete brain areas. Quantification of Perls-stained
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49 material has been proven to reflect iron concentrations (19,20) as a linear relationship exists
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51 between the two measurements (20). In our experiments, final intensification with
52
53 diaminobenzidine of the Perls stain was carefully monitored under the microscope to get the best
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55 signal-to-noise ratio with a compromise between clear labelling and rising background staining.
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Ref1, c1

Ref 1, c2

Brain slices for all mice were simultaneously processed, and incubation in DAB was of the same duration for all animals.

3. Results

Neuropathological examination showed amyloid plaques in the subiculum of all studied APP/PS1 mice (Fig. 1). On the contrary, no plaques were observed in the temporal cortex, even in the oldest animals (Fig. 1C). Noticeably, intracytoplasmic positive 4G8 staining was found in the neurons of the subiculum, but not in other brain areas, of the three youngest mice (Fig. 1J). This staining was not observed (or was only weak) in the subiculum of older mice. As expected, PS1 mouse brains were plaque free. Quantitative analysis showed that, in the subiculum of APP/PS1 mice, the Congo red-assessed amyloid load was correlated with the age of the animals ($r=0.64$, $p<0.05$). In this region, iron was associated with the amyloid plaques of mice older than 23 weeks (Fig. 1K) but was not histologically detected in younger animals (compare G-H versus I in Fig. 1).

Ref 1, c7

Ref 2, c3a

MRI measurements performed on living mice before neuropathological examination indicated significantly lower T2 values in the subiculum of APP/PS1 mice as compared to age-matched PS1 animals (Fig. 2, $U=22.5$, $p<0.02$; $n_{APP/PS1}=11$ and $n_{PS1}=10$). On the contrary, no differences between T2 values in the temporal cortex from APP/PS1 and PS1 mice (Fig. 2, Mann Whitney's $U=51$; ns) were noticed.

Ref 1, c3

Correlative analysis revealed a significant negative linear relationship between T2 values and amyloid load in the subiculum of APP/PS1 mice ($r=-0.77$, $p<0.01$). This negative correlation was preserved even when analysis was restricted to the youngest APP/PS1 animals, for which amyloid was not associated with histochemically-detected iron (age<23 weeks; $n=8$, $r=-0.8$,

p<0.05, Fig. 3). Importantly, for PS1 and APP/PS1 animals of the same age group, T2 values were not significantly correlated with age whatever the brain region evaluated.

Population analysis in the youngest mice (age<23 weeks), with no histologically-detected iron in the subiculum, revealed that subicular T2 from PS1 mice (n=7) was in the same range as that of APP/PS1 mice with lower amyloid loads (<7%; U=13, ns) but was statistically longer than those of APP/PS1 mice with higher amyloid load (>7%, U=0, p<0.02). Finally, in agreement with correlative data (see above), the T2 of APP/PS1 mice with no histologically-detected iron but presenting high amyloid load was significantly shorter than that of iron-free APP/PS1 mice with low amyloid load (U=0, p<0.05).

Ref1, c1

Ref1, c1

4. Discussion

4.1. T2 is reduced in the subiculum of APP/PS1 mice

T2 values were assessed in the subiculum and temporal cortex of double transgenic APP/PS1 mice, modelling early onset and progressive amyloid deposition, and in single transgenic PS1 animals that were used as amyloid deposit free controls. The use of PS1 littermates as controls allowed us to specifically assess the effects of amyloid deposition on T2. We first showed that T2 values measured *in vivo* were lower in the subiculum of APP/PS1 than in PS1 mice. This result, obtained in a cohort of young adults (16-31 weeks), confirms previous data obtained in older animals (27-45 weeks of age) (9).

Ref1, c 4

In the present study, the lack of T2 changes in the temporal cortex of APP/PS1 mice as compared to PS1 animals might be explained by a lack of cortical plaques in this brain region in the APP/PS1 mice. However, in a previous work, we also showed that T2 was not modified in the cortex of older APP/PS1 animals that displayed plaques in the isocortex. For those aged animals,

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3 the lack of T2 differences between genotypes might be related to the relatively low amyloid load
4 in the isocortex (as compared to the subiculum) but, more likely, to the high and equal amount of
5 cortical iron deposition (associated or not to the amyloid plaques) in old APP/PS1 and PS1 mice,
6 which might mask the T2 effect caused by amyloid plaques *per se*.
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13 **4.2. Amyloid deposits lead to T2 decrease even in the absence of detectable iron**

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16 It is well known that iron accumulates in amyloid plaques (21,22). Thus several studies
17 suggested that the T2 decrease in the brain of transgenics with brain amyloidosis might be
18 associated with the effect of iron on relaxation times (7,10). In a previous study we showed
19 nevertheless that the overall brain iron load is not modified in APP/PS1 mice as compared to PS1
20 mice, and only its local distribution is altered between genotypes: while iron is "trapped" by
21 amyloid plaques in APP/PS1 mice, it is diffusely deposited in control PS1 animals. Iron trapping
22 within the plaques can explain the ability of detecting the plaques by MR microimaging.
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33 Regarding the global T2 decrease reported in previous studies, one may suspect that iron trapping
34 in focal aggregates leads to a T2 decrease via a "Magnetic relaxation switch" effect (9,23).
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Ref 2, c1

Ref1, c8

Ref 1, SC
Ref 2, c3cRef1, SC1
Ref2, c3c

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3 β proteins in the subiculum of the youngest studied APP/PS1 mice. This feature was previously
4 described in the same mouse model as the one used in our study (24). The presence of amyloid- β
5 peptides in the intracellular space modifies cell physiology and is considered to be a critical event
6 and primary physiopathological step in AD (see (25) for a review). T2 relaxation time, which can
7 be a sensitive indicator of impaired cell physiology (Brackmann,2006) can thus be affected. The
8 observed T2 reduction might also result from other pathological processes such as, for example
9 hypoperfusion (26) , associated to the amyloid load. It was shown that mouse models of AD have
10 a decreased cerebral blood flow and/or blood volume, which are associated with A β pathology
11 (27) and that T2 is sensitive to a reduction in the cerebral blood flow (26). Further studies are
12 needed to determine the relevant relaxation processes and the extend to which these factors affect
13 the T2 relaxation time.
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30 **4.3. T2 is an early in vivo marker of amyloid deposition**

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33 Previous methods to directly detect plaques by MR microimaging require a difference in
34 T2 between plaques and brain tissue, due for example to the accumulation of iron in the plaques,
35 which is an age-related phenomenon. Thus these protocols can only be performed in old animals
36 (typically after 9 months (3)). Furthermore, with these methods, small plaques can not be
37 detected because of the low resolution of MRI. On the contrary, evaluation of T2 relaxation times
38 in the subiculum allows quantitation of amyloid load at very early stages of its development in a
39 non-invasive way, and before iron has started to be detectable in the plaques by histological
40 methods. Also, as T2 measures reflect an "averaged" effect within each voxel, all the plaques,
41 even the small ones, are expected to participate to the detected T2 modifications. Such a "global"
42 effect is similar to that detected in humans with other markers such as PET agents (28). The only
43 requirement for using T2 measures to follow plaques deposition is to focus on brain regions with
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Ref 1, c9

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3 high amyloid content such as the subiculum, where the amyloid load was above 7% even in
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5 young animals.
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8 As a conclusion we show that T2 measures allow the detection of plaques in young
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10 animals at early stages of the pathological process. This makes of it a potential useful marker to
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12 evaluate the effects of drugs aimed at slowing down brain amyloidosis at a pre-clinical stage.
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For Peer Review

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7. Figure Legends

Figure 1. Neuropathology of APP/PS1 mice.

A. Brain slice (Nissl stain) illustrating the anatomical level where the subicular (SUB) and temporal cortical (Te) region were sampled for both MRI and histological measurements (dg : dentate gyrus of the hippocampus; wm: white matter). B. MR image (inversion recovery protocol (TR/TE/TI=5000/10/300ms)), illustrating the regions where T2 values were evaluated (labels as in A). C. Amyloid deposition in an old APP/PS1 mouse (31 weeks) showing plaques in the hippocampus, including the subiculum (white arrow heads) but paucity of brain amyloidosis in the cortical mantle, especially at the level of temporal regions (black arrow heads). D-F. Congo red stain to visualize amyloid deposits in the subiculum (area delimited by dotted lines) of APP/PS1 mice at different ages (D: 16 weeks; E: 22 weeks; F: 27 weeks); note the gradual increase of plaque density with progressive aging. G-I. Perls stain to detect iron in the brain of the same mice as shown in D-F; iron is virtually absent in the two youngest animals (G,H) but clearly detectable in the 27 week-old mouse (I) where it colocalized with amyloid plaques (white arrowhead). J. Intraneuronal accumulation of A β (4G8 immunostaining) in neurons of the subiculum (outlined area) of a 16-weeks old APPxPS1 mouse. Note that only cells from the subiculum do contain cytoplasmic A β . WM: white matter; Cx: overlying cortex. K. Double Congo red + Perls-DAB stain to show association of amyloid plaques (red) and iron (brown). Asterisk shows one example of amyloid plaque engulfed in iron deposits. All scale bars: 500 μ m (same scale bar for D-I).

Ref1, c7

Ref2, c3a

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Figure 2. T2 values in APP/PS1 and control PS1 mice.

In the plaque-enriched subicular region, T2 was significantly reduced in double APP/PS1 transgenics (n=11; mean age: 21.9 ± 1.4 weeks) as compared with plaque-free PS1 (n=10; mean age: 21.7 ± 1.9 weeks) mice. On the contrary, no genotype effect was noted when T2 was measured in the temporal cortex. Importantly, this isocortical brain region was amyloid-free in the studied APP/PS1 mice due to their young age (range: 16-31 weeks). *: p<0.05.

Ref1, c5

Figure 3. Association between *in vivo* T2 and amyloid load in the subiculum.

The correlation between amyloid load and subicular T2 value was studied in the youngest APP/PS1 mice (age <23 weeks; n=8) for which iron was not histochemically detected in plaques and therefore did not compete with amyloid-related effects on MR relaxation times. A negative correlation was observed between *in vivo* MRI measurements and plaque load (r=-0.8, p<0.05). Note that PS1 mice displayed subicular T2 values that are in the same range as those of APP/PS1 mice with the lowest (<7%) amyloid load.

Ref1, c1

Transversal relaxation time reflects brain amyloidosis in young APP/PS1 transgenic mice

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Running Head: : T2 reflects amyloidosis in young APP/PS1 mice

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Abstract

Amyloid deposits are one of the hallmarks of Alzheimer's disease, one of the most devastating neurodegenerative disorder. In transgenic mice modelling Alzheimer's pathology, magnetic resonance transverse relaxation time (T2) has been described to be modulated by amyloidosis. This modification has been attributed to the age-related iron deposition that occurs within the amyloid plaques of old animals. In the present study, young APP/PS1 transgenic mice without histochemically detectable iron in the brain were specifically studied. In vivo measurements of T2 in the hippocampus, at the level of the subiculum, were shown to reflect the density of amyloid plaques. This suggests that T2 variations can be induced solely by aggregated amyloid deposits in the absence of associated histologically-detectable iron. Thus T2 from regions with high amyloid load such as the subiculum is particularly well suited to follow plaque deposition in young animals, *id est* at the earliest stages of the pathological process.

Keywords: Alzheimer disease, amyloid- β peptide, iron, T2

1. Introduction

Alzheimer's disease (AD) is characterized by two main neuropathological lesions: senile plaques and neurofibrillary tangles. Plaques are extracellular deposits of amyloid- β ($A\beta$) peptides, while tangles are intraneuronal filaments of hyperphosphorylated tau proteins (1). Until now, the definitive diagnosis of AD requires post-mortem detection of these lesions. According to the amyloid cascade hypothesis, abnormal amyloid accumulation in the brain is a critical event in the disease process (2). Development of disease-modifiers targeting $A\beta$ pathology appears therefore to be of crucial importance.

Imaging strategies aimed at *in vivo* detection of cerebral $A\beta$ deposition in mice are actively investigated to monitor the progression of disease and to assess the effects of new therapies. Until now most of the protocols that allowed detection of brain amyloidosis in a non-invasive way in animals have relied on magnetic resonance imaging (MRI). Two strategies were developed. The first one is based on the direct detection of individual amyloid plaques by MR microimaging. This approach either exploits the natural contrast of the plaques (3) or uses specific contrast agents targeting $A\beta$ (4-6). This approach is hampered by the difficulty of imaging the smallest plaques because of the limited resolution of the MRI. The second strategy looks for "global" relaxation time changes associated with the presence of $A\beta$ instead of trying to detect individual plaques. For instance, it has been demonstrated that transversal relaxation times (T_2) of brain tissues from aged mouse models of AD are modified by the presence of $A\beta$ deposits associated with iron (7-9). The aim of the present study was to better understand and refine the origin of T_2 modifications observed in plaque-enriched brain tissue from living mice modelling AD's cerebral amyloidosis. These modifications might be caused by different/concurrent factors such as the presence of hydrophobic amyloid deposits or age-related accumulation of iron within

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3 the plaques (3,9,10). To dissociate the impact of each variable the present study focused on
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5 young to middle-aged APP/PS1 transgenics (16-31 weeks of age).
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8 In several animal species, including humans cerebral iron increases with age and reaches a
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10 plateau in mature adults (11). In mice, age related increase of iron load seems to occur between
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12 the age of 2.8-3.4 months and 11 months (12,13)]. In a previous study, we further suggested that
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14 iron originating from surrounding tissue becomes progressively associated to the plaques in old
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16 double APP/PS1 transgenic mice (9). Interestingly, in this transgenic line, brain amyloidosis is
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18 very aggressive with an early onset at 2.5 months (14), presumably a few weeks before rising of
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20 cerebral iron load. Thus, one can expect that young APP/PS1 mice should start developing
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22 plaques without presenting amyloid-associated iron load, giving therefore the opportunity to
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24 isolate and refine the role of amyloid- β deposition per se on MR relaxation times. Indeed, in the
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26 present work, the youngest studied mice did not show histologically detectable iron deposition
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28 while still presenting variable amyloid loads. Analysis of regional T2 relaxation time was
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30 performed in the subiculum, one of the first brain areas to develop A β plaques in the studied
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32 transgenic model (14), and in the temporal association cortex in which no amyloid deposits were
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34 detected even in the oldest animals studied. We showed that even before the occurrence of
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36 histologically detectable iron in the plaques, the subicular T2 is negatively correlated to the
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38 amyloid load. This suggests that amyloid deposits or tissue modifications associated to amyloid
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40 deposits have a direct effect on T2 and that this parameter can be used as an early, iron- and age-
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42 independent, biomarker of the amyloid load.
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2. Materials and methods

2.1. Transgenic mice

Transgenic APP/PS1 mice (Thy1 APP751 SL (Swedish mutation KM670/671NL, London mutation V717I introduced in human sequence APP751) x HMG PS1 M146L), modelling early onset (at 9-14 weeks of age) and progressive cerebral amyloid deposition were used (n=11, 16-31 weeks of age) (14). Heterozygous “APP/PS1” mice were obtained by crossing heterozygous Tg APP_(+/-) mice with homozygous Tg PS1_(+/+) mice. The APP_(-/-)/PS1_(+/-) mouse littermates (“PS1”) resulting from crossings were used as controls (n=10, 16-31 weeks of age) as they display no amyloid plaques and therefore can allow to specifically evaluate the effects of the APP transgene on amyloid deposition in the brain.

2.2. In vivo MRI experiments and data analysis

In vivo MR images were recorded on a 4.7 Tesla Bruker Biospec 47/30 system, equipped with a 12 cm diameter gradient system (200 mT/m). A surface coil (diameter = 30 mm), actively decoupled from the transmitting birdcage probe (Bruker GmbH) was used for signal acquisition. Animals were anesthetized with isoflurane (5% for induction, 1-1.5% for maintenance) in a mixture of N₂ (80%) and O₂ (20%) administered via a facemask. Respiration rate was monitored to insure animal stability until the end of the experiment. Body temperature of the mice was maintained by using a water filled heating blanket.

The MRI protocol was previously described (9). Briefly, a multislice multiecho sequence was used for T₂ measurements (echo times (TE)=12.2, 24.4, 36.6, 48.8, 61, 73.2 ms, repetition time (TR)=2000 ms, field of view (FOV)=1.5x1.5 cm², matrix 128x128, slice thickness=1 mm,

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3 acquisition time of 8min 49s). Parametric T2 maps were generated by fitting pixel intensity
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5 values to single exponential curves using the Bruker fit package.
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8 Inversion recovery images (TE=10ms, TR=5000ms, TI=300ms, FOV=1.5x1.5cm², matrix
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10 128x128, slice thickness=1mm) that had good contrast between cerebral structures were acquired
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12 to localise regions of interest (ROIs) corresponding to the subiculum and to the temporal
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14 association cortex. The subiculum is one of the first regions to show plaque deposition in
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16 APP/PS1 mice while the temporal cortex undergoes amyloidosis much later during the disease
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18 process. The ROI corresponding to the subiculum was placed underneath the cingulum on a slice
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20 localized approximately between bregma -2.8 mm and bregma -3.8 mm according to the Paxinos
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22 atlas (15). ROIs corresponding to the temporal association cortex were drawn on this same slice.
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24 ROI outlines were then transferred to T2 maps. For all animals, T2 values were the mean of ROIs
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26 drawn on the left and right side of the brain except for one APP/PS1 and one PS1 animals in
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28 which T2 values were issued from only one side of the brain where the cingulum was accurately
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30 localized.
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38 **2.3. Histological analysis**

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40 Following completion of MRI studies, mice were sacrificed by decapitation. Brains were
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42 then extracted and fixed in 10% buffered formalin and kept in fixative at -4°C until being
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44 processed for histology (mean fixation time +/- SEM : 51 days +/- 7 days). Brains were then
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46 stored overnight in a solution of 20% glycerin and 2% dimethylsulfoxide in 0.1 M phosphate
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48 buffer for cryoprotection. Brains were subsequently sectioned into 10 series of 40 µm-thick
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50 coronal sections on a freezing microtome.
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54 Anti-A β immunohistochemistry was performed using the biotinylated 4G8 antibody
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56 (Biovalley, France). Endogenous peroxidase activity and non-specific antigenic sites were firstly
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3 blocked using standard procedures before incubation with primary antibodies (1/10000; one night
4 at room temperature). Following incubation in Avidin-Biotin-Horseradish peroxidase complex
5 (Vector Laboratories, Burlingame, CA) final reaction made use of 3-3'-diaminobenzidine as
6 chromogen (brown product). Amyloid deposits were concurrently labelled by standard Congo red
7 staining on a second batch of slices (adapted from (16)). Each Congo-red stained slice was
8 digitized using a Super CoolScan 8000 ED high-resolution scanner (Nikon, Champigny sur
9 Marne, France). Regional amyloid loads (expressed as percent of tissue surface stained by the
10 Congo red dye) were quantified in the subiculum area that was previously investigated by means
11 of MRI, using computer-based thresholding methods (17). Each stained section was binarized
12 after thresholding in Photoshop (Adobe Systems, Paris, France). Binarized images were then
13 transferred to Image- Pro Plus software (Media Cybernetics, Silver Spring, USA) and submitted
14 to a particle-detection algorithm to measure the surface of plaques on each brain section (2-4
15 sections sampled per mice). The proportion of tissue area occupied by Congo red stained material
16 was then assessed. The ratio (surface stained/whole subiculum ROI surface) was taken as a
17 measure of the "amyloid load" in the subiculum..

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Iron staining was performed on a third batch of serial sections using a protocol derived from the standard Perls-diaminobenzidine (Perls-DAB) method as previously described (9) (18). Although biochemical quantification of iron (eg atomic absorption spectrophotometry) is the most valuable and precise method to assess iron concentrations, this approach was discarded as it can not be used to measure iron loads in discrete brain areas. Quantification of Perls-stained material has been proven to reflect iron concentrations (19,20) as a linear relationship exists between the two measurements (20). In our experiments, final intensification with diaminobenzidine of the Perls stain was carefully monitored under the microscope to get the best signal-to-noise ratio with a compromise between clear labelling and rising background staining.

Brain slices for all mice were simultaneously processed, and incubation in DAB was of the same duration for all animals.

3. Results

Neuropathological examination showed amyloid plaques in the subiculum of all studied APP/PS1 mice (Fig. 1). On the contrary, no plaques were observed in the temporal cortex, even in the oldest animals (Fig. 1C). Noticeably, intracytoplasmic positive 4G8 staining was found in the neurons of the subiculum, but not in other brain areas, of the three youngest mice (Fig. 1J). This staining was not observed (or was only weak) in the subiculum of older mice. As expected, PS1 mouse brains were plaque free. Quantitative analysis showed that, in the subiculum of APP/PS1 mice, the Congo red-assessed amyloid load was correlated with the age of the animals ($r=0.64$, $p<0.05$). In this region, iron was associated with the amyloid plaques of mice older than 23 weeks (Fig. 1K) but was not histologically detected in younger animals (compare G-H versus I in Fig. 1).

MRI measurements performed on living mice before neuropathological examination indicated significantly lower T2 values in the subiculum of APP/PS1 mice as compared to age-matched PS1 animals (Fig. 2, $U=22.5$, $p<0.02$; $n_{APP/PS1}=11$ and $n_{PS1}=10$). On the contrary, no differences between T2 values in the temporal cortex from APP/PS1 and PS1 mice (Fig. 2, Mann Whitney's $U=51$; ns) were noticed.

Correlative analysis revealed a significant negative linear relationship between T2 values and amyloid load in the subiculum of APP/PS1 mice ($r=-0.77$, $p<0.01$). This negative correlation was preserved even when analysis was restricted to the youngest APP/PS1 animals, for which amyloid was not associated with histochemically-detected iron (age<23 weeks; $n=8$, $r=-0.8$,

p<0.05, Fig. 3). Importantly, for PS1 and APP/PS1 animals of the same age group, T2 values were not significantly correlated with age whatever the brain region evaluated.

Population analysis in the youngest mice (age<23 weeks), with no histologically-detected iron in the subiculum, revealed that subicular T2 from PS1 mice (n=7) was in the same range as that of APP/PS1 mice with lower amyloid loads (<7%; U=13, ns) but was statistically longer than those of APP/PS1 mice with higher amyloid load (>7%, U=0, p<0.02). Finally, in agreement with correlative data (see above), the T2 of APP/PS1 mice with no histologically-detected iron but presenting high amyloid load was significantly shorter than that of iron-free APP/PS1 mice with low amyloid load (U=0, p<0.05).

4. Discussion

4.1. T2 is reduced in the subiculum of APP/PS1 mice

T2 values were assessed in the subiculum and temporal cortex of double transgenic APP/PS1 mice, modelling early onset and progressive amyloid deposition, and in single transgenic PS1 animals that were used as amyloid deposit free controls. The use of PS1 littermates as controls allowed us to specifically assess the effects of amyloid deposition on T2. We first showed that T2 values measured *in vivo* were lower in the subiculum of APP/PS1 than in PS1 mice. This result, obtained in a cohort of young adults (16-31 weeks), confirms previous data obtained in older animals (27-45 weeks of age) (9).

In the present study, the lack of T2 changes in the temporal cortex of APP/PS1 mice as compared to PS1 animals might be explained by a lack of cortical plaques in this brain region in the APP/PS1 mice. However, in a previous work, we also showed that T2 was not modified in the cortex of older APP/PS1 animals that displayed plaques in the isocortex. For those aged animals,

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3 the lack of T2 differences between genotypes might be related to the relatively low amyloid load
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5 in the isocortex (as compared to the subiculum) but, more likely, to the high and equal amount of
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7 cortical iron deposition (associated or not to the amyloid plaques) in old APP/PS1 and PS1 mice,
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9 which might mask the T2 effect caused by amyloid plaques *per se*.

13 14 **4.2. Amyloid deposits lead to T2 decrease even in the absence of detectable iron**

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17 It is well known that iron accumulates in amyloid plaques (21,22). Thus several studies
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19 suggested that the T2 decrease in the brain of transgenics with brain amyloidosis might be
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21 associated with the effect of iron on relaxation times (7,10). In a previous study we showed
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23 nevertheless that the overall brain iron load is not modified in APP/PS1 mice as compared to PS1
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25 mice, and only its local distribution is altered between genotypes: while iron is "trapped" by
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27 amyloid plaques in APP/PS1 mice, it is diffusely deposited in control PS1 animals. Iron trapping
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29 within the plaques can explain the ability of detecting the plaques by MR microimaging.
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31 Regarding the global T2 decrease reported in previous studies, one may suspect that iron trapping
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33 in focal aggregates leads to a T2 decrease via a "Magnetic relaxation switch" effect (9,23).
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35 However, another possible explanation is that the T2 decrease observed in APP/PS1 mice is
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37 related to the density of plaques *per se*. The present study showing a negative correlation between
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39 T2 values and amyloid load in a cohort of young mice that displayed subicular amyloid plaques
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41 without detectable iron aggregates indeed strongly supports this hypothesis. Our results indicate
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43 that mechanisms other than those associated to focal iron deposition within the plaques may be
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45 responsible for the reduced T2 relaxation in tissue containing amyloid deposits. Several
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47 mechanisms may account for this effect. First, the hydrophobic nature of amyloid deposits might
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49 be responsible for T2 shortening. Some other tissular alterations associated to the accumulation
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51 of amyloid plaques might however be involved. For instance, we detected intraneuronal amyloid
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3 β proteins in the subiculum of the youngest studied APP/PS1 mice. This feature was previously
4 described in the same mouse model as the one used in our study (24). The presence of amyloid- β
5 peptides in the intracellular space modifies cell physiology and is considered to be a critical event
6 and primary physiopathological step in AD (see (25) for a review). T2 relaxation time, which can
7 be a sensitive indicator of impaired cell physiology (Brackmann,2006) can thus be affected. The
8 observed T2 reduction might also result from other pathological processes such as, for example
9 hypoperfusion (26) , associated to the amyloid load. It was shown that mouse models of AD have
10 a decreased cerebral blood flow and/or blood volume, which are associated with A β pathology
11 (27) and that T2 is sensitive to a reduction in the cerebral blood flow (26). Further studies are
12 needed to determine the relevant relaxation processes and the extend to which these factors affect
13 the T2 relaxation time.
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30 **4.3. T2 is an early in vivo marker of amyloid deposition**

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33 Previous methods to directly detect plaques by MR microimaging require a difference in
34 T2 between plaques and brain tissue, due for example to the accumulation of iron in the plaques,
35 which is an age-related phenomenon. Thus these protocols can only be performed in old animals
36 (typically after 9 months (3)). Furthermore, with these methods, small plaques can not be
37 detected because of the low resolution of MRI. On the contrary, evaluation of T2 relaxation times
38 in the subiculum allows quantitation of amyloid load at very early stages of its development in a
39 non-invasive way, and before iron has started to be detectable in the plaques by histological
40 methods. Also, as T2 measures reflect an "averaged" effect within each voxel, all the plaques,
41 even the small ones, are expected to participate to the detected T2 modifications. Such a "global"
42 effect is similar to that detected in humans with other markers such as PET agents (28). The only
43 requirement for using T2 measures to follow plaques deposition is to focus on brain regions with
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3 high amyloid content such as the subiculum, where the amyloid load was above 7% even in
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5 young animals.
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8 As a conclusion we show that T2 measures allow the detection of plaques in young
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10 animals at early stages of the pathological process. This makes of it a potential useful marker to
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12 evaluate the effects of drugs aimed at slowing down brain amyloidosis at a pre-clinical stage.
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For Peer Review

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7. Figure Legends

Figure 1. Neuropathology of APP/PS1 mice.

A. Brain slice (Nissl stain) illustrating the anatomical level where the subicular (SUB) and temporal cortical (Te) region were sampled for both MRI and histological measurements (dg : dentate gyrus of the hippocampus; wm: white matter). B. MR image (inversion recovery protocol (TR/TE/TI=5000/10/300ms)), illustrating the regions where T2 values were evaluated (labels as in A). C. Amyloid deposition in an old APP/PS1 mouse (31 weeks) showing plaques in the hippocampus, including the subiculum (white arrow heads) but paucity of brain amyloidosis in the cortical mantle, especially at the level of temporal regions (black arrow heads). D-F. Congo red stain to visualize amyloid deposits in the subiculum (area delimited by dotted lines) of APP/PS1 mice at different ages (D: 16 weeks; E: 22 weeks; F: 27 weeks); note the gradual increase of plaque density with progressive aging. G-I. Perls stain to detect iron in the brain of the same mice as shown in D-F; iron is virtually absent in the two youngest animals (G,H) but clearly detectable in the 27 week-old mouse (I) where it colocalized with amyloid plaques (white arrowhead). J. Intraneuronal accumulation of A β (4G8 immunostaining) in neurons of the subiculum (outlined area) of a 16-weeks old APPxPS1 mouse. Note that only cells from the subiculum do contain cytoplasmic A β . WM: white matter; Cx: overlying cortex. K. Double Congo red + Perls-DAB stain to show association of amyloid plaques (red) and iron (brown). Asterisk shows one example of amyloid plaque engulfed in iron deposits. All scale bars: 500 μ m (same scale bar for D-I).

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Figure 2. T2 values in APP/PS1 and control PS1 mice.

In the plaque-enriched subicular region, T2 was significantly reduced in double APP/PS1 transgenics (n=11; mean age: 21.9 ± 1.4 weeks) as compared with plaque-free PS1 (n=10; mean age: 21.7 ± 1.9 weeks) mice. On the contrary, no genotype effect was noted when T2 was measured in the temporal cortex. Importantly, this isocortical brain region was amyloid-free in the studied APP/PS1 mice due to their young age (range: 16-31 weeks). *: $p < 0.05$.

Figure 3. Association between *in vivo* T2 and amyloid load in the subiculum.

The correlation between amyloid load and subicular T2 value was studied in the youngest APP/PS1 mice (age <23 weeks; n=8) for which iron was not histochemically detected in plaques and therefore did not compete with amyloid-related effects on MR relaxation times. A negative correlation was observed between *in vivo* MRI measurements and plaque load ($r = -0.8$, $p < 0.05$). Note that PS1 mice displayed subicular T2 values that are in the same range as those of APP/PS1 mice with the lowest (<7%) amyloid load.

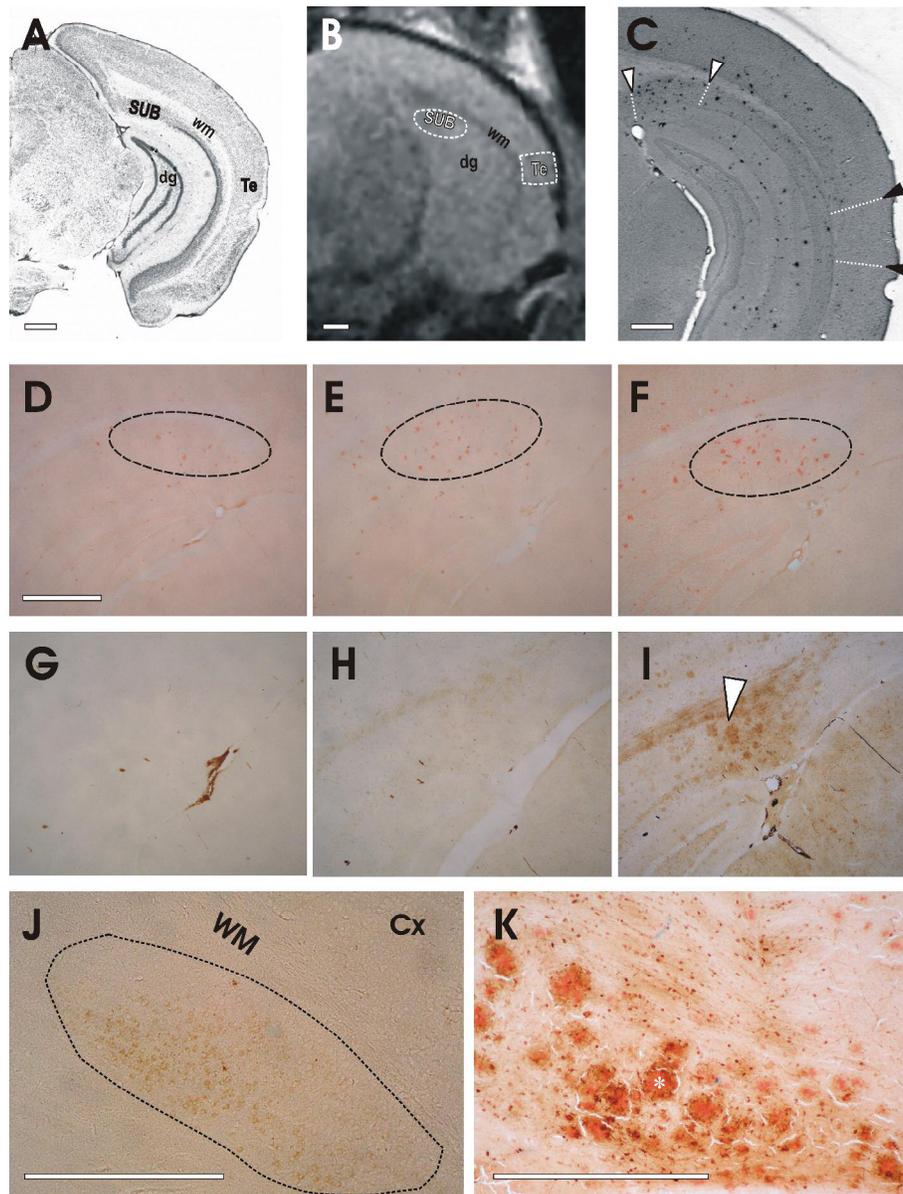
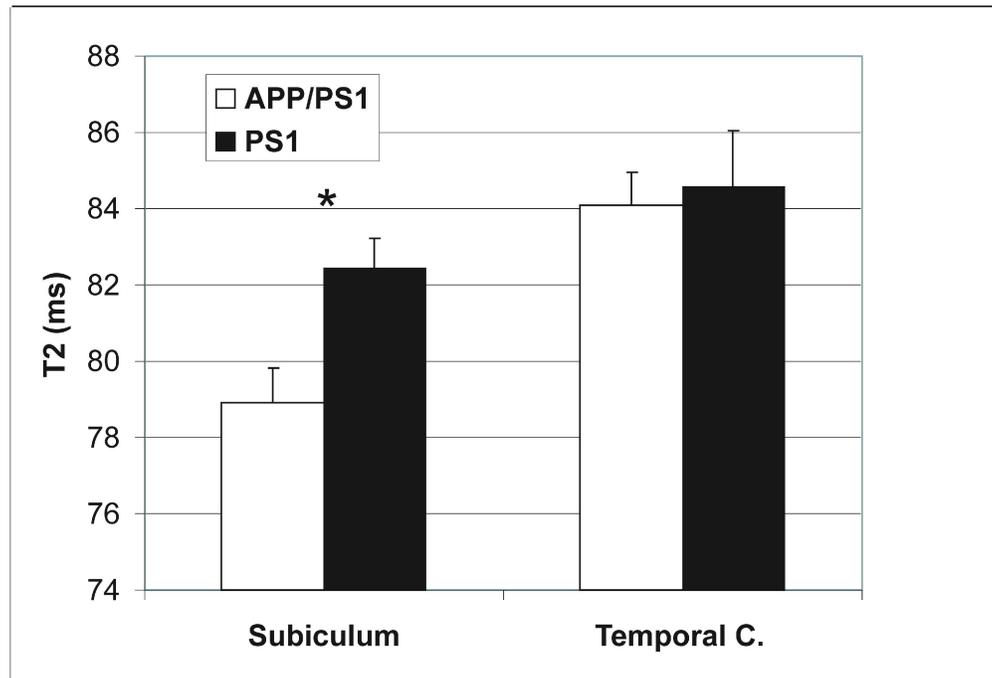


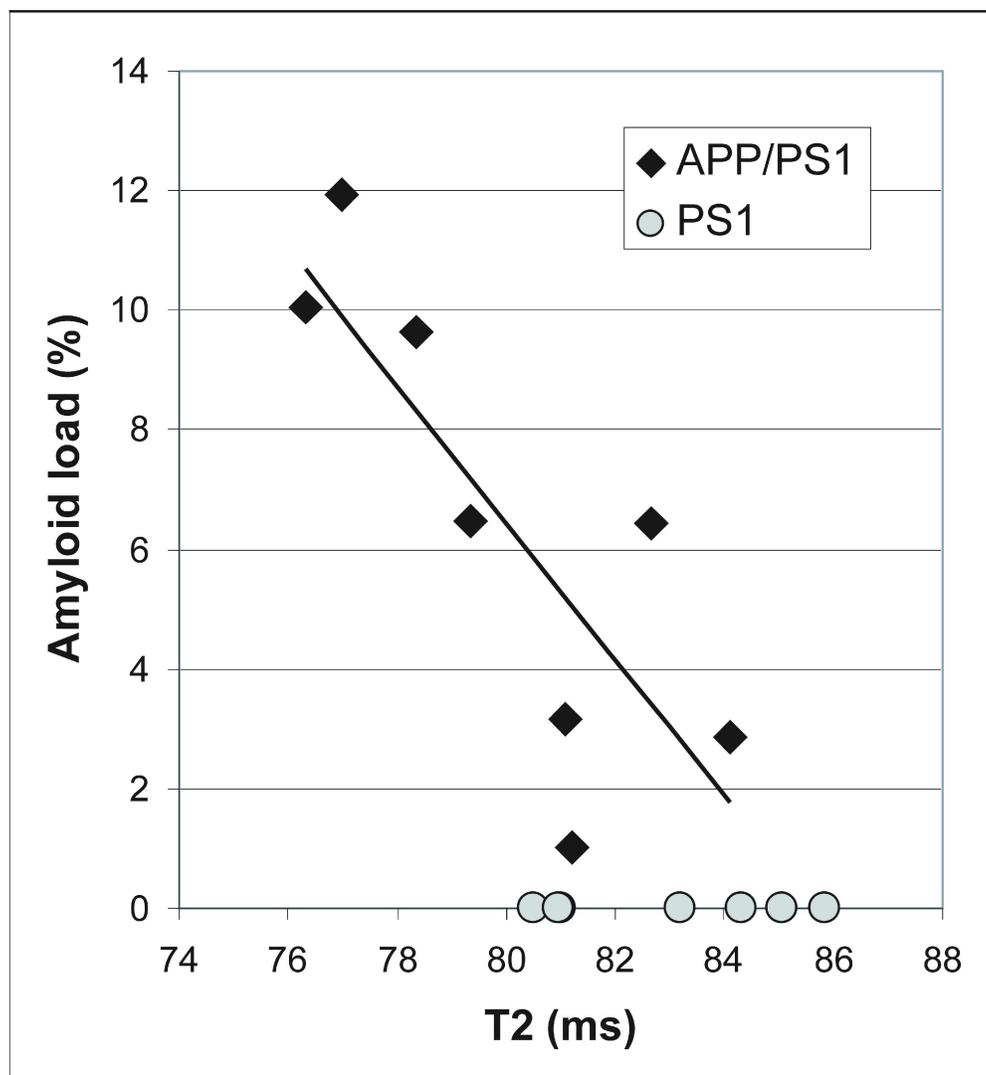
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Association between in vivo T2 and amyloid load in the subiculum. The correlation between amyloid load and subicular T2 value was studied in the youngest APP/PS1 mice (age <23 weeks; n=8) for which iron was not detected in plaques and therefore did not compete with amyloid-related effects on MR relaxation times. A negative correlation was observed between in vivo MRI measurements and plaque load ($r=-0.8$, $p<0.05$). Note that PS1 mice displayed subicular T2 values that are in the same range as those of APP/PS1 mice with the lowest (<7%) amyloid load.