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High field magnetic resonance microscopy of the human hippocampus in Alzheimer's disease: quantitative imaging and correlation with iron

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Abstract

We report R_2 and R_2^* in human hippocampus from five unfixed post-mortem Alzheimer's disease (AD) and three age-matched control cases. Formalin-fixed tissues from opposing hemispheres in a matched AD and control were included for comparison. Imaging was performed in a 600 MHz (14T) vertical bore magnet at MR microscopy resolution to obtain R_2 and R_2^* ($62 \mu\text{m} \times 62 \mu\text{m}$ in-plane, $80 \mu\text{m}$ slice thickness), and R_1 at $250 \mu\text{m}$ isotropic resolution. R_1 , R_2 and R_2^* maps were computed for individual slices in each case, and used to compare subfields between AD and controls. The magnitudes of R_2 and R_2^* changed very little between AD and control, but their variances in the Cornu Ammonis and dentate gyrus were significantly higher in AD compared for controls ($p < 0.001$). To investigate the relationship between tissue iron and MRI parameters, each tissue block was cryosectioned at $30 \mu\text{m}$ in the imaging plane, and iron distribution was mapped using synchrotron microfocus X-ray fluorescence spectroscopy. A positive correlation of R_2 and R_2^* with iron was demonstrated. While studies with fixed tissues are more straightforward to conduct, fixation can alter iron status in tissues, making measurement of unfixed tissue relevant. To our knowledge, these data represent an advance in quantitative imaging of hippocampal subfields in unfixed tissue, and the methods facilitate direct analysis of the relationship between MRI parameters and iron. The significantly increased variance in AD compared for controls

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warrants investigation at lower fields and in-vivo, to determine if this parameter is clinically relevant.

Keywords

Human; Hippocampus; Alzheimer's disease; MRI; Iron; Variance

1. Introduction

The early detection and diagnosis of Alzheimer's disease (AD) remains a daunting task despite significant progress in establishing biomarkers for various stages of the disorder (Hsu et al., 2001; Jack et al., 2010). While clinical magnetic resonance imaging (MRI) enables structural tracking of atrophy in the mid- and late-states of AD, there is also the challenge of detecting the molecular changes that precede atrophy. Quantitative MRI utilizing parameters that are sensitive to such change (Ramani et al., 2006) is arguably underutilized in a clinical context.

A widely accepted observation in AD is the progressive atrophy of various regions in the brain. Increasingly this is being tracked with volumetric measurements in MRI, particularly in the hippocampus: a region of the brain intimately involved with learning, memory and cognition. In addition to volumetric changes stemming from atrophy, there are a variety of other tissue changes that may be tracked with MRI. These include the breakdown of myelin, which is understood to proceed in an age-dependant manner (Bartzokis et al., 2003; Bartzokis et al., 2004). White-matter pathology identified with clinical imaging has been attributed to myelin breakdown, and there is therefore potential to explore the relationship between AD and demyelination of white matter via MRI (Bartzokis et al., 2004; Ding et al., 2008).

Various processes and factors are implicated in the vulnerability of the hippocampus in AD, including myelin breakdown and disrupted metal ion homeostasis. It is therefore of particular interest to track molecular changes in this region that may precede quantifiable atrophy. Altered iron management and deposition has been linked to the generation of excess redox-active species (Brass et al., 2006) that can promote deleterious free-radical effects that may make the hippocampus more prone to the degenerative processes observed in Alzheimer's disease (Zhu et al., 2009), and changes in relaxation parameters in the hippocampus have in some clinical studies been attributed to alterations in iron concentration (Ding et al., 2009). Numerous studies have identified changes in iron storage and utilization as a contributing factor to the neurodegenerative process, with hallmarks of oxidative stress appearing significantly in advance of clinically detectable atrophy (Kell, 2010), and this, combined with the effect of iron in its various biogenic forms, particularly ferritin-bound (Harrison and Arosio, 1996), on MR parameters (Haacke et al., 2005; Jensen et al., 2010; Vymazal et al., 1996a; Vymazal et al., 1996b), has led to the consideration of iron as a potential biomarker for certain neurodegenerative disorders (Brar et al., 2009; Brass et al., 2006; Collingwood et al., 2008a; Haacke et al., 2005; House et al., 2007; Jara et al., 2006; Schenck, 2003, 2009).

The difficulty of isolating specific factors affecting MRI parameters means that the majority of findings are implicit, rather than direct. There are conflicting reports concerning the utility of employing quantitative MR properties to look at molecular changes, including utilising the influence of iron on MRI parameters (Fukunaga et al., 2010) to better understand AD pathogenesis. Two parameters that have been confirmed in-vitro and in-vivo to be affected by tissue iron are the proton transverse relaxation rate R_2 , and the related

parameter R_2^* which is primarily affected by differences in magnetic susceptibility between tissue compartments (Haacke et al., 2005; Yao et al., 2009). The susceptibility-related parameter R_2^* is being explored as a measure of brain iron in clinical studies (Yao et al., 2009), and some groups are working to directly quantify the susceptibility (Wharton and Bowtell, 2010), and phase (Haacke et al., 2005; Haacke et al., 2009; Jensen et al., 2010). Although correlations between R_2 , R_2^* and iron have been observed, their exact interpretation is non-trivial due to competing factors (particularly for R_2) and the challenges of interpreting the MR mechanisms at play. Even though significant shifts in iron concentration may be observed through mechanisms such as increasing rates of R_2 and R_2^* , it is necessary to have an overview of the primary chemical and structural changes taking place in the tissue that also affect the MRI measurement parameters in order to deduce the contribution of the iron. Factors such as oedema and inflammation, as well as demyelination and axonal loss, may confound signal changes due to iron (Ramani et al., 2006). House and co-workers recently concluded from a post-mortem study at 1.4 T that the main drivers of R_2 in human gray matter are water content and the concentration of iron (House et al., 2010), and utilized pre-existing data about tissue water content to determine the impact of iron on R_2 . Equivalent data are not yet available for very high field (> 7 T) studies. It has been suggested that in AD, increased iron concentrations increase de-phasing of nearby proton spins resulting in increasing R_2 , and that this effect may be offset by some effects of white matter demyelination, particularly the increased local concentration of water molecules (House et al., 2006). In addition to structural and chemical changes, there are well-documented temporal changes in brain iron concentration as a function of ageing, as well as indications of possible diurnal change (Unger et al., 2009).

In studies quantifying the transverse relaxation properties of AD and healthy brains (as opposed to comparing contrast), some workers have observed a prolongation of T_2 relaxation time (the inverse of the relaxation rate R_2), in the hippocampus of AD patients in low field MRI (0.04 T) as they progress from early stage ($T_2 \sim 115$ ms) to late stage ($T_2 \sim 150$ ms) (Kirsch et al., 1992), and in AD patients compared to healthy controls (Wang et al., 2004). Such reports are contradicted by those showing that no meaningful relationships can be derived between MRI relaxation times and AD, e.g. Campeau et al (Campeau et al., 1997). Differences in magnetic field strength, measurement sequence parameters, study size, age of the patients, and disease stage, may account for the conflicting findings. Other variables that also interfere with efficient assessment of MRI parameters in the clinical environment include variations in default pulse sequences from one platform to another and technical limitations associated with scanner hardware and software. Thus validation of the relaxation parameter trends seen in quantitative MRI is an area that is still actively developing, including work stemming from the Alzheimer's Disease Neuroimaging Initiative (Holland et al., 2009).

There has been extensive discussion concerning how MRI might be utilized to discover early indicators for the pathogenesis of Alzheimer's disease, including the possibility of detecting iron via clinical MRI platforms (Dobson, 2001; Haacke et al., 2005; House et al., 2008; Jara et al., 2006; Jensen et al., 2010; Schenck, 1995; Schenck and Zimmerman, 2004; Schenck et al., 2006; Zhu et al., 2009). If pathological changes in local concentrations or storage forms of iron precede atrophy (Kell, 2010), or accompany it on a clinically relevant timescale, then these changes hold potential as biomarkers that could be of particular value for differential diagnosis in neurodegenerative disorders, and for evaluating potential therapeutic interventions.

When assessing the influence of iron on MRI, the spatial resolution at which segmentation is performed, and parameters compared, may be very significant. Bulk analysis of specific regions of the brain may show differences in total iron between diseased and control, but

this does not necessarily correspond to a uniform elevation throughout in the tissue: some deposition may be highly localised. The form of the stored iron is also significant. Studies have indicated that the ferritin iron storage protein is the primary component in gray matter affecting R_2 (Vymazal et al., 1996a; Vymazal et al., 1996b), and efforts are also being made to differentiate between the particulate forms of biogenic iron, ferritin (soluble) and haemosiderin (insoluble) most commonly found in human tissue (Jensen et al., 2010). Recent electron microscopy, synchrotron microfocus X-ray fluorescence (μ XRF) and superconducting quantum interference device (SQUID) studies of AD tissue and plaque material have identified the presence of anomalous concentrations of biogenic magnetite (Collingwood et al., 2008a; Collingwood et al., 2005; Hautot et al., 2003; Pankhurst et al., 2008). Magnetite, an iron oxide with a magnetic moment more than 100 times higher than the normal iron storage protein ferritin, could also have an effect on MRI measurement (Gossuin et al., 2005) if present in higher concentrations in AD (Hautot et al., 2003; Quintana et al., 2004). It is of value to determine the distribution of such inclusions in-vivo, their chemical and mineral form, and their impact at various field strengths and imaging resolutions, if their presence is to be fully exploited.

Studies which allow characterization of iron in segmented tissue that has also been examined by MRI provide a means to support interpretation of clinical findings (Collingwood et al., 2008b; Fukunaga et al., 2010; House et al., 2007; House et al., 2008), and here we have applied this general approach to a high-resolution study of hippocampal subfields in AD and controls. Quantitative MRI was used to characterize magnetic relaxation properties of hippocampal subfields that we identified and imaged at near-cellular resolution. Such an approach has previously been explored at lower resolution for the Alzheimer's population with comparisons of magnetic relaxation parameters in various brain regions with established iron concentrations from autopsy analyses (House et al., 2007; House et al., 2008). There have been several recent studies of post mortem tissue where high resolution MRI data are successfully matched with pathologic features in the same stained sections; these measurements are typically achieved using fixed tissue (Meadowcroft et al., 2009; Nabuurs et al., 2010). As fixation has been shown to induce protein cross linking that affects R_2 (Dawe et al., 2009), to affect water diffusion properties (Shepherd et al., 2009b; Thelwall et al., 2006) and to leach various metal ions and change tissue magnetic properties over time (Dobson and Grassi, 1996; Gellein et al., 2008), it is of interest to study unfixed tissues in parallel with formalin fixed samples where the iron content is being assessed.

In this study, detailed R_2 and R_2^* maps are obtained for tissues that have not been subjected to chemical fixation. The use of a high magnetic field increases the spatial resolution that can be achieved in a limited time window, and enhances the impact of tissue iron on transverse relaxation and susceptibility. This approach, supported by the subsequent μ XRF characterization of iron distributions in the same tissue slices, has the potential to provide information that cannot otherwise be achieved with conventional staining and bulk tissue analyses.

2. Materials and Methods

All tissue was obtained under donor consent and provided by the Human Brain Tissue Bank (HBTB) at University of Florida. Appropriate ethical approval was obtained from the relevant authorities in the UK and USA prior to commencement of the studies. Diagnosis of Alzheimer's was confirmed at autopsy, and the age-matched controls were selected from cases with no vascular or other neurological complications. All fresh-frozen tissues had been stored at -80°C for 4–6 years at time of study.

2.1 Sample preparation

Fresh-frozen tissue ('unfixed'), and formalin-fixed tissue ('fixed') for comparison from opposing hemispheres for a matched AD and control pair (Table 1), were obtained from HBTB. For the frozen tissues, blocks of approximately 1 cm³ containing a cross-section of the hippocampal horn, including the dentate gyrus (DG) and surrounding Cornu Ammonis (CA), were carefully dissected from frozen (having been warmed overnight to just below freezing point) on a chilled cutting surface with an acid-washed ceramic blade and placed in 10 mm NMR tubes where they were suspended in Fluorinert (3M, Belgium NV) pre-cooled to -20 °C. The configuration is illustrated in detail in Figure 1. Minimal handling and careful temperature management were essential to preserve integrity of the small unfixed blocks. For the two cases fixed in 10% formalin, each dissected sample was washed thoroughly in several changes of double-distilled water prior to imaging, and then returned to 10% formalin post-imaging. For the unfixed samples, it was necessary to limit imaging time to 14 hours or less under refrigerated conditions to preserve the tissue structure for subsequent analysis. Samples were allowed to equilibrate from frozen to 3°C when inserted into the MRI probe. Shimming, and subsequent data collection, were performed with each sample at 3.0 ± 0.1 °C, and temperature was monitored throughout each experiment via a calibrated thermocouple in contact with the sample space. Samples were placed on dry ice immediately after measurement and stored at -80 °C prior to cryosectioning for μ XRF spectroscopy. Sample orientation was documented with photography prior to and post MRI, to enable correct positioning for the subsequent sectioning.

2.2 MRI measurements

Each sample was loaded into the vertical bore of a 600 MHz (14.1 T) Bruker Avance spectrometer equipped with a 10 mm TXI radiofrequency probe. The sample was tuned and matched to the ¹H channel and manually shimmed in XWin-NMR until a clean exponential decay profile was observed in the free induction decay, and the phase-corrected water peak was symmetrical and ~ 50 Hz full-width at half maximum. Image acquisition was performed using Paravision v3.0 (Bruker) interfaced to the spectrometer. Initially, a rapid gradient echo sequence FLASH (fast-low angle shot) was run to ensure positioning of the tissue within the centre of the magnet and radiofrequency coil. Quantitative imaging for the complete tissue block was initially performed with a set of low spatial resolution (250 m voxel) sequences from which R_2 , R_2^* , and the longitudinal relaxation rate R_1 could be computed. The contrast in these slices was used to select regions of interest for the high resolution imaging performed to obtain R_2 and R_2^* .

R_2 acquisition was performed using a multiple slice multiple echo routine (MSME). Multiple spin echoes were generated using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with slice-selective RF pulses. Excitation and refocusing of spins was performed with sinc3 RF pulses. The field of view (FOV) was 8 mm, with an in-plane resolution of 62 μ m, and a slice thickness of 80 μ m. Each image was generated with 15 different echo times. The effective T_E for each image was: 8.6, 17.2, 25.9, 34.5, 43.1, 51.7, 60.3, 69.0, 77.6, 86.2, 94.8, 103.4, 112.1, 120.7, and 129.3 ms, with repetition time T_R = 4000 ms, number of slices = 15, and number of averages N_A = 12. Twelve dummy scans were included prior to acquisition. Each echo in the Paravision MSME sequence is phase encoded and rewound, allowing signal to coherently build even with non-180° refocusing pulses. In the present study, the amplitude of the refocusing pulse was optimised prior to data acquisition so that R_2 measurement was not dependent on this feature.

R_2^* acquisition was performed using a multiple gradient echo (MGE) pulse sequence. The pulse sequence consists of slice selective excitation with a Gaussian pulse followed by a gradient echo train created by reversals of the readout gradient. All echoes have the same

phase encoding. The minimum first echo time was 3.7 ms, and a total of 18 echoes were obtained for each voxel with an echo spacing of 6.66 ms. The $T_R = 3500$ ms, and inclusion of dummy scans, N_A , and slice geometry were as for R_2 .

R_1 acquisition was achieved with a CPMG sequence using a fixed echo time ($T_E = 9.6$ ms) and variable T_R (4500, 3000, 1500, 1000, 750, 500, 350, and 250 ms) to determine recovery of magnetization. These scans were performed at lower resolution (250 μm voxels) with three dummy scans prior to each acquisition, and $N_A = 3$.

In order to obtain complete volumes for each block, offset slices were obtained in several sequential scans and interleaved post-imaging to recreate the complete region of interest. The MRI acquisition parameters used to obtain the data, including the duration of each individual sequence, are summarized in Table 2.

2.3 Data analysis

R_1 , R_2 and R_2^* were computed for regions of interest, accompanied by complete maps for display purposes, from a selection of slices in each measured tissue block. The R_2 and R_2^* sequences had been optimised to allow near-cellular resolution voxels with sufficient signal to noise to compute R_2 and R_2^* even in regions with fast decays. Fits were made assuming a mono-exponential decay, and for this study the approach was sufficient to achieve good fit statistics and to enable comparisons between regions.

Region of interest (ROIs) were drawn for up to nine subfields in each image of the hippocampus: the alveus (ALV), CA region (CA1-CA3, CA4), stratum radiatum (SR), stratum pyramidale (SP), regions of the dentate gyrus (DG) including the stratum granulosum (SG), polymorphic layer (PL), the stratum moleculare (SM), and where included - the subiculum (sub) (Figure 2). ROIs were drawn only for those regions that we could realistically identify in each MRI slice. Segmentation was based on information presented in: conventional staining methods for granular layers and stratum layers; an anatomic MRI atlas that combines histology, schematics, and high resolution MRI (Duvernoy and Bourgouin, 1998); and delineation of hippocampal subfields in a study based on diffeomorphic transformations of MRI data (Csernansky et al., 2005). With any MR segmentation protocol involving the hippocampus, there will remain subjectivity in classifying the border of one intra-ROI and the entry into another ROI region; this is especially true for the sub and CA regions (Konrad et al., 2009), and regions were grouped where appropriate. ROIs were drawn within each subfield, and the average R_1 , R_2 , and R_2^* value was obtained for each identifiable subfield from a set of slices in each tissue block. A standard Levenberg-Marquardt nonlinear least squares fitting algorithm was used to fit the R_2 and R_2^* decays (Figure 3) obtained via Equation (1) which takes finite background (A) into account:

$$y = A + Ce^{(-t.R_2)} \quad (\text{Equ. 1})$$

Computation of R_2 and R_2^* values for individual subfields was performed using the Levenberg-Marquardt nonlinear least squares fitting algorithm in Paravision v4.0 (Bruker), and the maps and histograms for display purposes (also computed with Equation (1)) were generated in the open source software ImageJ. Numerical outputs from each package were in good agreement. In ImageJ, it was necessary to add in the background removal step (corresponding to 'A' in Eqn. 1) in order to accurately fit the shortest decays, as background removal is not part of the standard open source tool.

Average R_2 and R_2^* parameters for each subfield were compared for AD and control samples using a one-tailed t-test assuming uneven variance (GraphPad, La Jolla, CA). When combining the findings for all the cases in a group, standard deviations were calculated as the average standard deviation (as opposed to simply the standard deviation of the mean values) in order to allow propagation of the standard deviations from each slice measurement.

In addition to detailed analysis of the subfields, R_2^* and R_2 values were obtained from the two primary regions in each sample, namely the CA4-DG and CA1-CA3 regions (as defined in Figure 2b). The outer layers (fimbria, alveus, subiculum) were excluded. The intensity data were exported from each region of interest in histogram form (range 0–500 s^{-1}), with 500 bins. The minimum T_E placed a practical constraint on the fitting of exponential decays corresponding to $R_2^* > 500 s^{-1}$, and this value was therefore treated as the upper threshold for R_2^* . (In practice, the very significant majority of R_2 and R_2^* values were 250 s^{-1} or less.) Tests for equal variances were performed in Minitab 16 using Levene's test, considering the distance of each observation from the sample median. The null hypothesis of equal variances was rejected at the 0.05 significance level.

2.4 Histology and μ XRF spectroscopy

The tissue blocks were cryosectioned post-MRI to allow confirmation of the tissue structure with staining, and direct visualisation of the metal-ion distribution in the tissue with synchrotron μ XRF, in order to enable correlation of these findings with the high-resolution MRI data. Each block was sectioned in the imaging plane with 30 μm slice thickness, with sections being mounted on quartz slides for synchrotron analysis, and on glass slides for histological evaluation. Each section was air-dried prior to measurement, and was protected by a Kapton film window that was sealed to the outer edge of the slide. Synchrotron μ XRF analyses were performed at the Diamond Light Source I18 beamline and the Advance Photon Source MRCAT beamline using methods similar to those described previously (Collingwood and Dobson, 2006; Collingwood et al., 2005; Mikhaylova et al., 2005). Mapping was performed using a 60 μm spot over the entire area of the section. The fluorescence spectrum obtained from each pixel enabled metal ion maps to be generated for each tissue section using the open-source software tool PyMCA (Solé et al., 2007). Adjacent tissue sections were stained with haematoxylin and Congo red to confirm the position of key features such as the SG cell layer, and to look for evidence of amyloid plaque deposition, respectively.

3. Results

Relaxation parameters for each subfield were sampled from eight slices in each high-resolution scan (from two scans obtained from each case), and averaged. The complete cross-sections were not compared because of slight differences in the relative areas of CA and DG sampled in each case. The R_2 and R_2^* parameters for the unfixed tissues are given by subfield for AD and control groups in Table 3.

While a mono-exponential decay cannot fully describe the relaxation process in post-mortem tissues, it can provide a good approximation in some systems (House et al., 2010; Jensen et al., 2010), and inspection of the fits (Figure 3) confirmed that the mono-exponential approach provided a reasonable basis for making comparisons between decay characteristics in the samples studied here.

It is noted that in the unfixed samples, the average R_2^* values are slightly decreased in the AD compared for the control group in those subfields where the differences reach statistical significance (ALV and SP, $p = 0.01$ and 0.05 respectively, when the values computed

initially as T_2^* ($= 1 / R_2^*$) values were compared using propagation of the average standard deviations). The R_2 mean values appear consistently increased in the AD compared for the controls in all subfields (Table 3); however, the differences in R_2 values do not reach significance at the 0.05 level.

The R_1 maps obtained at 250 μm resolution did not permit detailed segmentation; instead, values were obtained for the principal regions CA1-3 and CA4-DG, given in Table 4. Here, the R_1 values are consistently decreased in the AD cases compared for the controls for the unfixed samples (significant at $p = 0.01$)

In the two cases of fixed tissue (one AD, one control), the average R_2 and R_2^* values are increased in the AD fixed sample compared for the control (e.g. average R_2^* is 84 s^{-1} in the AD case versus 35 s^{-1} in the control in the CA1-CA3 region, and 89 s^{-1} vs 38 s^{-1} in CA4-DG). The fixed tissue R_2 and R_2^* values give rise to good contrast in the tissue, but the pattern of R_2 and R_2^* dependence on subfield is not as clear as in the unfixed tissue. The average R_1 values in the fixed samples are decreased in the AD compared for the control (e.g. average R_1 is 1.43 s^{-1} in the AD case versus 1.89 s^{-1} in the control in the CA1-CA3 region, and 1.37 s^{-1} vs 1.75 s^{-1} in CA4-DG). The most striking difference between fixed and unfixed tissue is the substantially increased R_1 in the unfixed cases.

The R_2^* values calculated for the subfields are displayed in box-plot format in Figure 4, where it is possible to assess the degree of spread for values in each subfield. Box plots allow one to determine if the values obtained for each case (even with a small sample size) follow a normal or non-parametric distribution as well as to see if the data conform to any pattern of skewness (Henderson, 2006). The R_2^* values show a narrow distribution and small areas for inter-quartile ranges which envelope a very small interval of values for the AD group, and an even smaller range for the control.

Histogram analysis of the difference in scatter for the R_2 and R_2^* distributions is shown for pooled data and for age and gender matched pairs of AD and control cases in Figure 5, along with results from Levene's test for equality of variances showing statistically significant differences between AD and control at the $p < 0.001$ level. The frequency scale for the histograms shown in Figure 5e) is logarithmic to demonstrate more clearly the anomalies either side of the main frequency distribution.

Cryosectioning of the samples post-MRI enabled staining and synchrotron X-ray analysis. Staining with haematoxylin confirmed the position of prominent cell layers in the hippocampus, and Congo red positive material consistent with amyloid deposition was evident in the AD tissues. Case AD1 was also examined with polarizing microscopy, revealing intact spherulite inclusions that were subsequently associated with copper during synchrotron μXRF mapping. This is reported in detail elsewhere (Exley et al., 2010; House et al., 2009). Iron distribution maps were measured with high sensitivity and specificity for iron using synchrotron μXRF . To enable comparison of the iron distribution (represented with a linear intensity scale) and the measured MRI parameters, R_2 and R_2^* maps were computed from the virtual tissue slices (Figure 6), and matched with cryosections obtained from the tissue blocks after the MRI measurement was complete. An example of a sectioned tissue slice (unstained) is shown in Figure 7 with the synchrotron X-ray fluorescence iron map from the same section, and the R_2 and R_2^* maps from the corresponding region in the original block. It is evident in Figure 7 that regions of higher iron intensity correspond to regions of increased R_2^* and R_2 , and the positive correlation is shown in Figure 8 for sixteen regions sampled (as shown in Figure 7) throughout regions across the section.

4. Discussion

This study uses fields and resolutions that are not clinically applicable; however, the detailed study of the properties of hippocampus tissue may provide background information that can support clinical imaging studies, particularly where the relationship between tissue iron distribution and MR parameters is concerned.

The development of structural MRI techniques for early detection of AD is in many ways attractive, as the scanning technology does not involve exposure to ionising radiation, provides good spatial resolution, and has scope for quantitative measurement of a range of parameters including tissue volume and relaxation parameters. It is of particular clinical importance that MRI scanner availability, particularly of higher field strength scanners, is on the increase in many communities.

At present, much of the emphasis on quantitative MRI in AD is on volumetric tracking of atrophy (Jack et al., 2010). However, if iron status in the hippocampus alters ahead of myelin breakdown and general atrophy whether as a causative factor or a by-product - then our combined observations in the unfixed and fixed tissues support the possibility that this feature may be evident in the hippocampus as a shift in the absolute value of R_2 or R_2^* . Such evidence of molecular change in the tissue might be of value alongside volumetric and other anatomic changes.

Various methods to quantify iron in-vivo have been explored, and arguably the most robustly validated is the R_2 measurement of iron loading in the liver where the approach was developed in conjunction with direct evidence of liver iron content from biopsy samples (St Pierre et al., 2005). Clinical MRI of brain iron has relied on referencing iron concentration data obtained postmortem, and studies combining clinical measurements and comparison of post-mortem tissue properties with actual iron concentration have been used to test the validity of this approach (House et al., 2006; House et al., 2007); one method utilizes the field-dependent response of ferritin cores (FDRI method) and has been used to observe differences in groups as a function of age and/or disease status, including Alzheimer's disease (Bartzokis et al., 2000). The linear signal from the iron storage protein ferritin and magnetic field relationship between the R_2 strength has been used as a reference for corroboration of brain iron measurements (Bartzokis et al., 2000; Gossuin et al., 2004), and it was recently shown that a strong linear relationship exists between brain iron concentration and R_2^* at magnetic field strengths extending from 1.5 T to 7 T (Yao et al., 2009). It has yet to be confirmed at what field this linear relationship breaks down due to saturation of ferritin, and studies are underway to link our observations in tissue and iron phantoms at 14 T with those obtained at lower field strengths.

Concerning the relaxation parameters obtained in the present study, it is striking as evidenced in the box plot in Figure 4 - how consistent the measured relaxation parameters are from one subfield to another, with the same pattern of relative values for AD and control, and it suggests that any variations in fragility of the unfixed tissue are not dominating the results.

A feature of interest is the difference in distribution of values for each subfield, where the maxima and minima (indicated by bars) indicate a much wider distribution in the AD subfields than in the controls. This is the case despite very similar averages and standard deviations in many of the subfields for AD and control (Figure 4). The difference in scatter for the R_2 and R_2^* distributions was further investigated using histogram analysis for slices from each individual case (Figure 5), and it was confirmed that in the AD tissues there are hyper-intensity pixels (and in some cases hypointensity pixels) in tails extending beyond the main histogram peak, compared to a more tightly confined peak in the control cases. Tests

for equal variance in the R_2^* distributions for AD versus controls in the CA4-DG and CA1-CA3 (Figure 5) confirmed that the variance was significantly different at the $p < 0.001$ level. The trend is towards a higher frequency of increased R_2^* values in the AD cases compared for the controls, and this is confirmed both in the comparisons of pooled data, and for individual matched AD-control pairs.

This difference in the distribution of R_2 and R_2^* values, with a wider spread for AD, is of particular interest. The hypo- and hyper-intensity pixel 'tails' may be due to artefacts resulting from a level of cell damage that is greater in the AD tissue than in the control; however, it is also possible that the hypo-intensity pixels relate to highly localised susceptibility artefacts that may include pathologic hallmarks of AD such as iron-rich deposits associated with microbleeds and/or senile plaques (Collingwood et al., 2008a; Meadowcroft et al., 2009).

The μ XRF iron map provided in Figure 7 illustrates the total iron distribution in the tissue with a high degree of sensitivity which cannot be achieved via conventional staining techniques, although there are, as one would expect, areas of consistency between observations made with non-haem iron staining of the hippocampus (Morris et al., 1994), and the μ XRF maps. There was a distinct pattern to the regional iron distribution, with higher levels in the SP, SR and SM layers outside the SG, and significantly lower levels in the DG and CA4. It is worth noting that while the general pattern of iron distribution did not appear significantly altered between the fixed and fresh-frozen samples, some other metal ions were extensively washed out in the fixed samples compared for the controls, and this will be reported in detail elsewhere.

The evidence in Figures 7 and 8 showing that regions of higher iron intensity correspond to regions of increasing R_2^* and R_2 is consistent with the literature already presented, in particular with the recent observations of Yao et al (Yao et al., 2009) at clinical field strengths, and supports the hypothesis that iron concentration affects R_2^* contrast. It is also useful confirmation that the effect can be observed at very high field strengths in unfixed tissue samples that have been subjected to freezing.

If the total iron in the hippocampus is higher in AD, and accumulates disproportionately in the subfields compared to controls, then this is not reflected in the R_2^* values in the unfixed tissue. The pattern of values is very similar for AD and control (Figure 4). By contrast, the two fixed samples did show a substantial increase of the R_2 and R_2^* values in the AD cases compared for the controls, and the values were markedly decreased in the fixed tissues compared for the unfixed tissue. However, despite good contrast within the sections, there was not such a consistent pattern of R_2 and R_2^* parameters from one subfield to another, and μ XRF confirmed a degree of metal ion leaching from these tissues which was no doubt exacerbated by having washed out the fixative prior to MRI measurement. R_1 for the fixed tissues is noticeably increased compared for the unfixed tissues, and decreased in all instances in the AD tissues than the controls (significant at $p = 0.01$ for both subfields in the unfixed samples). This decrease in the R_1 is consistent with anticipated altered water diffusion properties due to degeneration of tissue structure in the AD cases. Our findings corroborate previous studies indicating the impact of fixation on metal ions (Dobson and Grassi, 1996; Gellein et al., 2008), and of sample preservation methods on cross-linking of proteins (Dawe et al., 2009) and tissue water diffusion properties (Thelwall et al., 2006). Studies of relaxation effects on rat nervous system tissue have shown that fixation in 4% formaldehyde results in a reduction of up to 80% in estimated T_2 values and a 20% decrease in T_1 (Shepherd et al., 2009b), corresponding to increases in R_2 and R_1 . R_2 values are dependent not only on immersion fixation effects but also on the post-mortem interval required for removal, refrigeration, and chemical fixation of human autopsy tissue. The

impact on R_2 has been shown to be exacerbated for post-mortem intervals of greater than 4 hours (Shepherd et al., 2009a). Our preliminary analysis of fixed tissue which included one AD and one control show a distinct difference in R_2^* , R_2 and R_1 between the two cases, but taking into account the factors discussed above, it cannot be concluded to what extent these observations are impacted by sample preparation methods, and while a comparison of the differences between fixed and unfixed tissue requires a larger sample group, it is clear from these initial findings that the preservation methods used in ex-vivo studies of tissue is of critical importance. Previous work by Huesgen and co-workers (Huesgen et al., 1993) showed that MR microscopy of post-mortem fixed hippocampus showed little regional variability (specimens having been imaged at 7 T with 40 μm in-plane resolution, T_2 being measured with a CPMG sequence, eight echoes with $T_E = 10 - 80$ ms, $T_R = 2500$ ms); this is consistent with the lack of variation between subfields observed in our fixed tissue examples, and justifies the inclusion of unfixed samples in our study, where the observation of relative differences between subfields is likely more reliable in the data from the unfixed cases.

While the low-resolution R_1 sequence provided a useful point of comparison between samples, the limited time window available to measure the unfixed samples made it impractical to collect R_1 at the higher level of resolution used for R_2 and R_2^* due to the extended imaging times required to acquire good signal to noise at microscopy level resolution. This was not considered a major omission, as the priority was to obtain R_2 and R_2^* values from the hippocampal subfields. High resolution in-vitro R_1 data acquired at 3°C in defrosted tissue are arguably of less clinical relevance than R_2 and R_2^* , given that the magnitude of R_1 is highly temperature dependent, and the impact on R_1 of freezing and defrosting the tissue may dominate the R_1 contrast inherent in-vivo.

The MRI measurement protocols presented here have enabled high resolution images, along with R_2 and R_2^* parameters, to be obtained from hippocampal subfields in post-mortem unfixed tissues. This study does not purport to provide quantitative information that has direct application in clinical imaging of early-stage AD: the 14 T field is double the strength that may currently be used for clinical imaging, the regions of interest were necessarily defined utilizing a combination of atlases, histology, and computation, and while the R_2 and R_2^* values in the study were measured with sufficient accuracy for us to report clear trends in subfield values and borderline statistically significant differences between a couple of regions, the samples are from postmortem tissues of aged persons as opposed to early-stage AD patients. However, when the findings presented here are taken in context, they lend strength to the idea that altered tissue iron status may be identified by quantitative MRI prior to significant atrophy, as our findings are consistent with the hypothesis that the effect of iron on R_2^* and R_2 will not be masked until the physical degeneration of the tissue is established. It is therefore of particular interest to determine the magnitude, nature, timescale, disease-specificity, and reliability of pre-atrophy changes in tissue iron status for those who go on to develop Alzheimer's disease.

The changes in R_2^* and R_2 correlate broadly with iron distribution in the tissue; however, it is evident at this detailed level of segmentation that the contrast cannot be attributed solely to varying iron concentrations. Other parameters affecting water diffusion and susceptibility, including the variation in compartmentalization due to cell dimensions and density of packing, and differences in hydration between gray and white matter, are also presumed relevant. This makes the measure of a disease-related parameter that is largely independent of regional background signal change, of particular interest. Accurate quantification of R_2 and R_2^* is a challenge on MRI patient platforms, particularly those that are configured for clinical, as opposed to research, applications. We do not suggest that average R_2 or R_2^* in the hippocampus has potential as a tool to differentiate AD patients from healthy patients.

Even in studies where statistically significant differences in R_2 have been reported in other brain regions for AD compared with controls (reviewed elsewhere, (Ramani et al., 2006; Tofts, 2004)), the magnitude of these reported shifts is typically only a few percent of the R_2 value, and with the substantial overlap between study groups, we suggest it may be impractical to adopt this parameter as a tool for categorizing individual patients in routine clinical scanning. However, it is to be expected that elevated iron concentrations in neurons and glia, and amyloid plaque formation (especially magnetite-rich plaques (Collingwood et al., 2008a)) may contribute to pixels exhibiting anomalously increased R_2 and R_2^* , and our preliminary findings in these post-mortem tissues support this assertion. In this context, the significant variance of the signal intensity from the dentate gyrus and Cornu Ammonis is a parameter that warrants further investigation.

In conclusion, we have imaged AD and control hippocampus at MR microscopy resolution, and segmented multiple regions for R_2^* and R_2 analysis using a protocol that enables work with fragile unfixed tissues. Consistent results have been obtained for all the unfixed samples measured for the purposes of this study. Our findings show that in unfixed tissue at 14 T, the absolute R_2^* differences between AD and control are almost negligible in this small cohort of cases; however, the variance in the distribution of R_2 and R_2^* values in regions of interest large enough to be segmented in clinical scans is significantly different between AD and controls at $p < 0.001$. Preserving the iron status in the tissue through avoidance of fixation has allowed calculation and comparison of R_2^* and R_2 with iron maps obtained with synchrotron μ XRF, supporting the hypothesis that tissue iron concentration has a demonstrable relationship with R_2^* and R_2 at the microscopy level and at very high magnetic field strengths.

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Abbreviations

ALV	Alveus
CA	Cornu Ammonis
DG	Dentate Gyrus
SR	Stratum Radiatum
SP	Stratum Pyramidale
SG	Stratum Granulosum
sub	subiculum

PL	Polymorphic Layer
SM	Stratum Moleculare
CPMG	Carr-Purcell-Meiboom-Gill
MGE	multiple gradient echo
T_R	repetition time
T_E	echo time
μXRF	microfocus X-ray fluorescence
SQuID	Superconducting Quantum Interference Device

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Highlights

- High field MRI to quantify R_2 and R_2^* in human hippocampus subfields.
- Variance of R_2 and R_2^* significantly increased for Alzheimer's versus controls ($p < 0.001$).
- Direct positive correlation between R_2 , R_2^* and total iron distribution mapped in tissue.

Figure 1.

Schematic of measurement configuration for the MRI measurement of unfixed tissue blocks. The tissue is suspended in approximately 2 ml Fluorinert™ (FC-43) and positioned from above with gauze to prevent sample movement during data acquisition. Care was taken to avoid introduction of air bubbles, especially at the gauze-tissue interface, and any small pockets of trapped air were released with a fine plastic rod.

Figure 2.

Guide to the segmentation process adopted for the cross sections through the blocks of hippocampal tissue. a) Where image resolution permitted, regions of interest were selected from within the boundaries shown for the following subfields: Cornu Ammonis regions (CA1-CA3); Cornu Ammonis region 4 and dentate gyrus (CA4-DG); alveus (ALV); polymorphic layer (PL); stratum granulosum (SG); stratum moleculare (SM); stratum pyramidale (SP); stratum radiatum (SR); subiculum (sub, not evident in this particular slice). Some layers could not be segmented reliably at this resolution (e.g. stratum lacunosum), the position of the divisions between CA1, CA2, and CA3 cannot be determined in these MRI sections, and the stratum moleculare for the CA and DG was also treated as one region. The numbering and labelling system is from Fig.7 in Duvernoy and Bourgouin, illustrating segmentation of the human hippocampus matched to a high resolution MRI image (Duvernoy and Bourgouin, 1998). For histogram and R_1 analysis, the primary subfields CA1-CA3 and CA4-DG were selected as shown in b).

Figure 3.

Decay curves from regions of interest in fixed and unfixed tissues, including examples from the sequences used to obtain R_2^* and R_2 . A mono-exponential decay is fitted in Paravisionv 4.0 as outlined in Section 2.3.

Figure 4.

Box plots of R_2^* values for AD ($n=5$) and control ($n=3$) hippocampus subfields from unfixed tissue. The middle line represents the median, the bottom and top rectangles represent the 25th and 75th percentile of the R_2^* distribution respectively. Bars represent the maximum and minimum R_2^* values seen from the data. Diamonds represent average values calculated from adjacent slices in each tissue block in regions where the principal hippocampus structures could be clearly identified and segmented according to the schematic in Figure 2.

Figure 5.

Analysis of histograms for the R_2 and R_2^* distributions, using Levene's test for equality of variances. Pooled data for the AD and control cases are shown for R_2^* and R_2 in the two primary subfields, CA1-CA3, and CA4-DG. The variance is significantly different between AD and control for each condition illustrated in a) d), with more scatter in the AD cases than in the controls. In each condition, the Levene test output shows the 95% Bonferroni confidence intervals for standard deviations, with the outlier scatter (individual data points as black asterisks) either side. The pattern is also evident at the level of individual cases, as shown with two age- and gender-matched pairs in e), where the complete histograms (with frequency on a logarithmic scale) are accompanied by the Levene's test results showing the detail of the tails above and below the 95% confidence intervals. The variances are not equal in all instances, at significance level $p < 0.001$.

Figure 6. R_2^* (s^{-1}) and R_2 maps (s^{-1}) of representative MRI datasets from an AD case and a control.

Figure 7.

Qualitative comparison of the a) R_2^* map, b) R_2 map, c) unstained 30 μm thick tissue section cut from the corresponding region within the block of unfixed control case C3, and d) the iron fluorescence intensity map of the same section as measured with μXRF (60 μm in-plane resolution, linear intensity scale with black to white indicating low to high fluorescence intensity). Sixteen sites used for quantitative analysis (where the central 9 pixels were selected at each site) are outlined on the paired images.

Figure 8.

Plot of R_2 and R_2^* versus iron fluorescence intensity, using data sampled from regions throughout the gray and white matter of the hippocampus cross section as illustrated in Figure 7. There is evidence of a positive correlation in both examples, although it is clear (and consistent with expectations) that not all R_2 and R_2^* contrast in the MR microscopy maps can be directly attributed to iron concentration.

Table 1

Summary of hippocampus cases analyzed by MR microscopy (confirmed Alzheimer's disease: AD; control with no significant neuropathy: C). All tissues were obtained with IRB approval from the Human Brain Tissue Bank (HBTB) at University of Florida, and had been stored for six years or less at time of study. Unfixed (frozen) tissue had been stored at -80°C ; fixed tissue from the opposing hemisphere in two cases had been stored in 10% formalin.

Case	Age	Gender	Tissue Status
AD1	90	F	Frozen; Fixed
AD2	82	F	Frozen
AD3	86	M	Frozen
AD4	73	M	Frozen
AD5	90	F	Frozen
C1	90	F	Frozen; Fixed
C2	82	F	Frozen
C3	90	F	Frozen
Summary	Mean age (std dev)		
5 AD, 3 control	85.4 (6.1)	6 F, 2 M	

Table 2

Summary of MRI acquisition parameters for microscopy measurement of R_2^* (multi-gradient-echo), R_2 (multi-spin multi-echo CPMG sequence) and for lower resolution measurement of R_1 (CPMG sequence with variable repetition time T_R).

Parameters	R_2^*	R_2	R_1
Pulse Sequence	Multiple gradient echo (MGE)	Multi-spin multi-echo (MSME)	MSME Variable T_R (MSME-VTR)
Pulse shape	Gaussian	sinc3	sinc3
FOV (mm)	8×8	8×8	10×10
Matrix Size	128×128	128×128	40×40
Spatial Resolution	$62 \mu\text{m}$	$62 \mu\text{m}$	$250 \mu\text{m}$
Slice Thickness	$80 \mu\text{m}$	$80 \mu\text{m}$	$250 \mu\text{m}$
T_E (ms)	3.70 (minimum)	8.62 (minimum)	9.62
ΔT_E (ms)	6.66	8.62	-
# T_E	18	15	1
T_R (ms)	4500	4000	250 to 4500
# T_R	1	1	8
# Averages	12	12	3
Sequence duration	90 minutes	100 minutes	20 minutes

Calculated R_2 and R_2^* parameters (in seconds⁻¹) [mean (average standard deviation)] for AD and control tissue by hippocampus sub-field for all unfixed cases, using data from the high resolution scans, where the means and standard deviations are calculated from individually-computed pixel values obtained within each ROI and combined for the cases within each group. (Subfield abbreviations are defined in Figure 2.)

Table 3

REGION	R2* (s ⁻¹)		R2 (s ⁻¹)		Control	(n = 3)	AD	(n=5)	Control	(n=3)
	AD	(n=5)	AD	(n=3)						
ALV	56.8	(2.6)	64.9	(4.2)	37.2	(9.4)	32.7	(7.3)		
CA1-CA3	35.6	(2.4)	34.7	(2.0)	30.9	(5.3)	25.8	(4.0)		
CA4-DG	44.8	(2.6)	42.7	(4.7)	35.1	(3.8)	28.2	(2.8)		
PL	36.1	(2.0)	33.7	(4.2)	31.8	(4.1)	25.6	(3.4)		
SG	39.8	(2.1)	40.3	(2.8)	32.4	(3.8)	27.2	(2.7)		
SM	33.6	(2.0)	35.3	(2.6)	30.1	(4.4)	24.1	(3.6)		
SR	42.7	(1.6)	44.6	(5.0)	35.7	(5.4)	28.2	(5.3)		
Sub	32.3	(1.8)	33.8	(2.3)	29.2	(5.2)	25.0	(5.1)		
SP	34.8	(1.8)	38.8	(2.9)	30.4	(4.8)	25.1	(4.7)		

Table 4

Computed R_1 value (in seconds⁻¹) given as [mean (average standard deviation)] for CA1-CA3 and CA4-DG regions in unfixed tissue using the MRI data obtained at 250 μ m isotropic resolution. The R_1 values are consistently decreased in the AD cases compared for the controls (significant at $p = 0.01$).

	Unfixed tissue	(R_1 , s ⁻¹)
CASE	CA4-DG	CA1-CA3
AD 1	0.74 (0.07)	0.70 (0.02)
AD 2	0.64 (0.02)	0.69 (0.03)
AD 3	0.71 (0.04)	0.72 (0.07)
AD 4	0.62 (0.02)	0.61 (0.03)
AD 5	0.61 (0.02)	0.63 (0.03)
C1	0.76 (0.02)	0.76 (0.03)
C2	0.77 (0.02)	0.83 (0.12)
C3	0.76 (0.02)	0.74 (0.04)