In-vivo mapping of hippocampal subfields in mesial temporal lobe epilepsy: relation to histopathology

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ABSTRACT

A particularly popular automated MRI hippocampal subfield mapping technique is the one described by Van Leemput et al. (2009) that is currently distributed with Freesurfer software. This method assesses the probabilistic locations of subfields based on *a priori* knowledge of subfield topology determined from high-field MRI. Many studies have applied this technique to conventionally acquired T1-weighted MRI data. In the present study, we investigated the relationship between this technique applied to conventional T1-weighted MRI data acquired at 3 Tesla and postsurgical hippocampal histology in patients with medically intractable mesial temporal lobe epilepsy (mTLE) and hippocampal sclerosis (HS). Patients with mTLE (n=82) exhibited significant volume loss of ipsilateral CA1, CA2-3, CA4-dentate gyrus (DG), subiculum and fimbria relative to controls (n=81). Histopathological analysis indicated that the most significant neuronal loss was observed in CA1, then CA4 and CA3, and more subtle neuronal loss in CA2, consistent with classical HS. Neuronal density of CA1 significantly correlated with MRI-determined volume of CA1, and increasingly so with CA2-3 and CA4-DG. Patients with increased HS based on histopathology had greater volume loss of the ipsilateral hippocampal regions on MRI. We conclude by suggesting that whilst time efficient and fully reproducible when applied to conventional single acquisition sequences, the use of the automated subfield technique described here may necessitate the application to multi-acquisition high-resolution MR sequences for accurate delineation of hippocampal subfields.

INTRODUCTION

The human hippocampus is composed of a complex and heterogeneous structure. However, the hippocampus is usually modelled as a single structure in neuroimaging research; this is particularly due to the insufficient resolution of conventional magnetic resonance imaging (MRI) to identify the boundaries of intra-hippocampal morphology. The estimation of global hippocampal volume on MRI is a sensitive marker of pathology in patients with mesial temporal lobe epilepsy (mTLE) and Alzheimer's disease in particular, as an extensive literature testifies. However, hippocampal pathology does not always occur globally within these disorders. For example, classical hippocampal sclerosis (HS) in patients with mTLE is associated with neuronal loss of CA1, CA3 and CA4 neurons, with relative preservation of area CA2 (Bratz, 1899; de Lanerolle, et al., 2003; Margerison and Corsellis, 1966; Wyler, et al., 1992). Other patterns of HS may be identified in mTLE, with differential clinicopathological characteristics (Blumcke, et al., 2007; Thom, et al., 2010). Furthermore, Alzheimer's disease is preferentially associated with neuronal loss of region CA1 (West, et al., 1994; West, et al., 2004). The early identification of hippocampal pathology is important for the diagnosis, clinical management and surgical consideration (in the case of refractory mTLE) for these disorders. Given that assessment of the entire hippocampus as one structure may obscure subtle or circumscribed hippocampal damage, it is important to develop and validate methods that permit analysis of hippocampal subfields in patients with neurological disorders. Early detection of circumscribed hippocampal subfield pathology may potentially provide important diagnostic information.

There have been several attempts to identify and quantify hippocampal subfields on MRI *in vivo*. At 7 Tesla, studies have identified individual CA regions, the dentate gyrus (DG), and subiculum (Prudent, et al., 2010), quantified the thickness of CA1 (Kerchner, et al., 2010), and manually segmented CA1, CA2, CA3, and subiculum separately, and CA4 and DG together (Wisse, et al., 2012). At 4.7 Tesla, the volume of CA1, CA2 and CA3 (together), DG, and subiculum has been estimated (Huang, et al., 2013; Malykhin, et al., 2010). At 4 Tesla, separate measurements of CA1, CA2, and subiculum, and CA3, CA4 and DG together have been made (Mueller, et al., 2011; Mueller, et al., 2009; Mueller, et al., 2007; Mueller and Weiner, 2009). Whilst many of the sequences used in these higher field MRI studies could be adapted for scanning within clinically acceptable time frames, routine scanning of patients in clinical settings presently almost invariably requires the use of 1.5 and 3 Tesla MRI systems, which offer lower resolution images at clinically-acceptable acquisition times, and as a consequence make it difficult to distinguish intra-

hippocampal anatomy. Moreover, the aforementioned high field studies that have quantified subfield volume or thickness have done so manually. Manual estimation is more reliable than automated human-independent methods, but they can be considerably time consuming, which may take two to four hours for a highly trained expert (Yushkevich, et al., 2010). Given the laborious and time consuming nature of manual measurements, some studies have measured subfields in only selected sections, and discounted the vast majority of hippocampal tissue (Mueller, et al., 2009). Manual assessment is permitted only by skilled anatomists, which makes assessment in a routine clinical context impractical, and raises issues with respect to intra- and inter-rater reliability of measurements.

In order to overcome the shortcomings of manual techniques applied to high-field acquired data, there have been some recent developments in the automated assessment of hippocampal subfields. One popular method is the one described by Van Leemput et al. (2009), which incorporates a statistical model with Markov random field priors to label subfields from high-resolution images obtained from a 3 Tesla MRI system with an acquisition time of >35 mins. This approach has been particularly popular given its distribution in context of the widely used Freesurfer software (http://surfer.nmr.mgh.harvard.edu/). Whilst this approach was developed for application to averaged multi-acquisition high-resolution T1-weighted images, many studies have applied this technique to single-acquisition conventionally acquired T1-weighted images (Hanseeuw, et al., 2011; Li, et al., 2013; Lim, et al., 2012; Lim, et al., 2013; Pereira, et al., 2013; Teicher, et al., 2012). It is unknown to what extent the automated subfield segmentation technique as applied to conventionally acquired T1-weighted data reflects the underlying pathology of the disorders investigated. It is important to investigate whether alterations in subfield volume reflect actual histopathological alterations underlying neurological disorders.

Medically intractable mTLE offers a unique opportunity to assess the performance of automated hippocampal subfield mapping techniques for two reasons. Firstly, the hippocampus ipsilateral to the side of seizure onset is typically structurally abnormal (i.e. atrophic), and permits assessment of the automated technique's ability to adapt to the abnormally shaped hippocampus. There is a well-established histopathology underlying volume atrophy of the hippocampus, manifest as a loss of pyramidal cells and gliosis within the *Cornu Ammonis* (Blumcke, et al., 2007; Bratz, 1899; de Lanerolle, et al., 2003;

Margerison and Corsellis, 1966; Wyler, et al., 1992). Secondly, in suitable refractory patients, the disorder is amenable to surgical resection of the hippocampus, which permits comparison of the presurgical MRI mapping of hippocampal subfields and the histological architecture and pathology of subfields on resected specimens. In the present study, we performed an unprecedented comparison of automatically acquired hippocampal subfield volumes determined using the technique described by Van Leemput et al. (2009) applied to presurgical MRI with histologically determined neuronal density measures of subfields in patients with mTLE who underwent amygdalohippocampectomy. Importantly, the automated analysis of MRI hippocampal subfields were based on conventionally acquired 3 Tesla MR acquisitions in context of clinical evaluation of patients, as most frequently done (Hanseeuw, et al., 2011; Li, et al., 2013; Lim, et al., 2012; Lim, et al., 2013; Pereira, et al., 2013; Teicher, et al., 2012). There were two primary objectives of the present study: (i) to compare the MRI-determined hippocampal subfield volumes between a large sample of patients with mTLE who had unilateral HS on routine clinical neuroimaging evaluation and age- and sex-matched healthy controls; and (ii) to compare the presurgical MRI-determined subfield volumes with subfield neuronal density measures in patients who underwent amygdalohippocampectomy.

METHODS

Participants

We recruited 82 consecutive patients (mean age 41.9 yrs, SD 13.9; 51 left HS, 31 right HS) with clinical evidence of unilateral mTLE and neuroradiological evidence of HS from quantitative MRI. All patients underwent comprehensive routine pre-surgical evaluation for medically intractable TLE, including detailed seizure semiology, interictal EEG, long-term video EEG monitoring, if clinically necessary also invasive electrophysiological investigations, clinical conventional MRI (T1-weighted, T2-weighted and FLAIR), and neuropsychological assessment (Kral, et al., 2002). All patients had complex partial seizures of presumed temporal lobe origin and no evidence of dual pathology. We also recruited 81 neurologically and psychiatrically healthy age-matched control participants (mean age 40.0 yrs, SD 13.8). All patients and controls provided written informed consent and the local ethics committee approved this study.

Pre-surgical MRI

All 163 participants underwent MRI at the Life & Brain Center in Bonn on a 3 Tesla scanner (Magnetom Trio, Siemens, Erlangen, Germany). An eight-channel head coil was used for signal reception. Clinical T2-weighted and FLAIR images were not quantitatively analysed. All quantitative MRI analyses were performed on T1-weighted magnetization-prepared rapid acquisition gradient echo (MPRAGE) images (160 slices, TR = 1300 ms, TI = 650 ms, TE = 3.97 ms, resolution 1.0 × 1.0 × 1.0 mm, flip angle 10°, acquisition time approx. 7 minutes). Images were analysed using Freesurfer software (v.5.1.0).

To obtain hippocampal subfield volume estimates, each MPRAGE image was first submitted to the routine automated Freesurfer processing stream that has been described in detail elsewhere (Dale, et al., 1999; Fischl, 2012; Fischl and Dale, 2000; Fischl, et al., 2002; Fischl, et al., 2004; Segonne, et al., 2004). Briefly, images were skull stripped and brain-only tissue extracted (Segonne, et al., 2004), spatially registered using an automated Talairach transformation, deep grey matter was segmented from subcortical white matter to provide automated volumetric labelling of subcortical structures (Fischl, et al., 2002), and the cortical surface was reconstructed (Dale, et al., 1999), permitting analysis of cortical thickness (Fischl and Dale, 2000). Data pertaining to cortical thickness was not used in this study. Information on the practical implementation of Freesurfer processing can be found online (http://ftp.nmr.mgh.harvard.edu/fswiki/recon-all). Routine Freesurfer processing previously performed excellent cortical reconstruction, brain segmentation and

structural labelling with MPRAGE data acquired on the same MR system as the present study in patients with mTLE (Keller, et al., 2012b).

The hippocampal subfield segmentation technique (Van Leemput, et al., 2009) was then performed on the Freesurfer-processed data. Similarly to the standard Freesurfer processing stream, information on the practical implementation of the hippocampal subfield approach can be found online (www.freesurfer.net/fswiki/HippocampalSubfieldSegmentation). The method uses a probabilistic atlas of hippocampal anatomy based on manual delineations of hippocampal subfields from multiple high resolution T1-weighted MRI scans. In particular, the technique uses a statistical model with Markov random field priors to delineate subfield boundaries based on T1-weighted MPRAGE MR sequences acquired from a 3 Tesla Siemens Trio MRI system, with a slice thickness of 0.8 mm, 208 coronal sections, and an acquisition time of 7.34 mins. For the development of this method, five separate acquisitions were collected and motion-corrected to obtain a single resampled high contrast volume (Van Leemput, et al., 2009). We applied this method to the single MPRAGE acquisition that had been processed using the routine Freesurfer stream; although the technique was developed based on multi-acquisition MPRAGE sequences to increase image resolution, the principle goal of the development of this method was for the possibility of fully reproducible and rapid routine analysis of hippocampal subfields in large imaging studies (Van Leemput, et al., 2009). The present study applies the subfield mapping technique to data acquired using similar imaging acquisition parameters and MRI systems as previous studies (Hanseeuw, et al., 2011; Li, et al., 2013; Lim, et al., 2012; Lim, et al., 2013; Pereira, et al., 2013; Teicher, et al., 2012). Seven regions of interest were delineated, including CA1, CA2/3, CA4/DG, fimbria, subiculum, presubiculum and hippocampal fissure. An atlas mesh of these regions were built from the manual delineation of the right hippocampus (mirrored for the left hippocampus) of ten control subjects from high resolution images, for which Dice overlap between manual and automated segmentation methods were approximately 0.7 for all structures (Van Leemput, et al., 2009). Supplementary Table 1 provides information on the anatomical borders of each subfield.

Surgery and histopathology

Of the 82 patients studied, 54 (65.9%) underwent standardised selective amygdalohippocampectomy (35 left, 19 right) by either a transsylvian, transcortical or subtemporal approach (Bien, et al., 2013), qualitative histopathological assessment and

neuronal density measurements of the excised hippocampus. For this sub-sample, mean age at surgery was 40.0 years (SD 14.1), mean age 42.8 years (SD 14.0), mean age of onset of epilepsy 17.4 years (SD 12.6), and mean epilepsy duration 25.4 years (SD 12.6). Twenty-two (40.7%) patients had a history of early childhood febrile seizures.

Biopsy samples were fixed with formaldehyde overnight, embedded into paraffin, cut into 4 µm sections and mounted on slides (HistoBond, Marienfelde, Germany). Neuropathological analysis comprised at least H&E-staining as well as immunohistochemistry for glial fibrillary acidic protein (GFAP, DakoCytomation, Glostrup, Denmark) and NeuN (Chemicon, Temecula, USA). Qualitative assessment of hippocampal cell loss and reactive gliosis was performed by experienced neuropathologists as part of diagnostic standard procedure, including a semi-quantitative classification of the extent of neuronal cell loss in the subfields CA1-4. In accordance with others (Thom et al., 2010), the CA4 sector was defined as the region within the arms of the DG. This region included cells of the polymorphic layer in addition to hilar pyramidal neurones. This semi-quantitative classification ranged from no/mild (0) cell loss, over moderate (+) and severe (++) to extensive cell loss (+++). Wyler grades (Wyler, et al., 1992) were also determined for each specimen. Additional quantitative neuronal density (number of neurons/μm^2) was obtained for regions CA1-4. To determine neuronal density, NeuN-immunostainings were scanned with a Mirax scanner (3DHistech, Hungary). Using the “Panoramic Viewer” (3DHistech, Hungary) rectangular regions of interest (ROI) were outlined in the pyramidal cell layer of each hippocampal subfield CA1-4 (range 243.425 μm^2 to 299.670 μm^2; mean 273.806 μm^2). All NeuN positive cells in every ROI were counted using a quantitative software solution (HistoQuant, 3DHistech, Hungary). Correct identification of all NeuN-positive neurons, regardless of size and morphology, were checked manually.

Statistical analysis

Analyses were performed using SPSS (IBM Corp. Released 2012. IBM SPSS Statistics, Version 21.0. Armonk, NY). Hippocampal subfield volume and neuronal density were analysed using one-way ANOVAs including group (control, left mTLE, right mTLE) as a fixed factor, and subfield volume and neuronal density as dependent variables, including age and sex as nuisance variables. Given the large number of between-group comparisons (8 x MRI hippocampal subfield volume, 4 x CA neuronal density, 2 x hemispheres), we incorporated a post-hoc Bonferroni correction on our data. Statistically significant findings are reported at $p<0.05$ with Bonferroni correction. Although we report

on data uncorrected for intracranial volume (ICV), we also ran the same analyses on volumes and densities corrected for ICV, which was automatically estimated from the routine Freesurfer processing pipeline. To determine left-right differences in controls a two-tailed paired samples t test was used. Given that group comparison statistical approaches may mask inter-individual patterns of hippocampal subfield atrophy, we analysed individual patient profiles of subfield volume loss relative to the lower 95th percentiles of volume in controls, which represents the limits of normality (Scott, et al., 2003). Pearson's correlations were used to examine relationships between volume and neuronal density. For correlational analyses, patients with left and right mTLE were combined and neuroimaging data were treated as ipsilateral or contralateral to the epileptogenic focus.

RESULTS

MRI mapping: visual assessment

The automated subfield mapping approach initially appeared to differentiate hippocampal and non-hippocampal tissue reasonably well in our sample of patients and controls. On closer inspection, there appeared to be subtle misclassifications of subfields in some cases. Figure 1A shows results in a patient with right HS with small hippocampal asymmetry, and indicates that the choroid plexus (white arrows), located dorsal to the alveus (black arrows), is included in the map of CA2-3. There also appeared to be an over- and under-estimation of subfield area in some cases, although we were unable to quantify the extent to which each area was misclassified. In particular, CA2-3 appeared overestimated in size relative to CA1, which is generally the largest CA region.

MRI mapping: volume quantification

In controls rightward volume asymmetry was observed for the whole hippocampus ($t=3.65$, $p<0.001$), CA1 ($t=6.52$, $p=0.006$), CA2-3 ($t=7.73$, $p<0.001$), CA4-DG ($t=5.66$, $p<0.001$), and fimbria ($t=7.77$, $p<0.001$). Leftward asymmetry was observed for the presubiculum ($t=3.66$, $p<0.001$). There were no significant asymmetries of the subiculum ($t=0.18$, $p=0.86$) or hippocampal fissure ($t=1.70$, $p=0.10$). For patient-control comparisons, descriptive and inferential statistics are provided in Supplementary Tables 2-4. There was a significant main effect of group on all subregions (at $p<0.001$) other than hippocampal fissure. Post-hoc analysis with Bonferroni correction revealed group-wise statistically significant volume loss of the ipsilateral whole hippocampus, CA1, CA2-3, CA4-DG, subiculum, and presubiculum in both patient groups relative to controls at $p<0.001$. Only patients with right TLE had significant volume loss of the ipsilateral fimbria ($p<0.001$), although there was the same trend in patients with left TLE ($p=0.095$). Patients with left TLE had additional significant volume loss of the contralateral whole hippocampus ($p=0.025$) and contralateral fimbria ($p<0.001$). The distribution of volume loss of hippocampal subfields is shown in Figure 2. When patients were analysed according to ipsilateral and contralateral volumes, patients with right mTLE had significantly smaller contralateral CA1 volumes compared to patients with left mTLE ($F=6.96$, $p=0.01$). The above results were retained when data were corrected for ICV.

The lower 95th percentile of control subfield volumes and the proportion of patients with volumes lower than this value for each subfield are shown in Table 1. For individual patients with left and right mTLE respectively, 59/81% had ipsilateral volume abnormalities

of the whole hippocampus, 73/74% of CA1, 73/81% of CA2-3, 76/81% of CA4-DG, 82/84% of the subiculum, 76/74% of the presubiculum, and 16/42% of the fimbria.

Post-surgical histopathology

Abnormalities were reported in all 54 resections. Using qualitative assessment, 51 cases (94.4%) were diagnosed with HS. Classical HS with relative sparing of the CA2 region (corresponding to a Wyler grade III) was reported in 26 cases (41.1%); an example specimen is shown in Figure 3. Twenty biopsy samples (37.0%) were classified as a Wyler grade IV with severe to extensive neuronal cell loss in all CA subfields, indicating a high incidence of severe HS in our sample. An atypical pattern of cell loss was observed in the remaining five cases (9.3%) with diagnosis of HS. HS could not be confidently attributed to three cases (5.6%), but all showed at least focal neuronal cell loss and astrocytic gliosis. Semi-quantitative classification of neuronal loss of the four CA regions is provided in Table 2. Extensive damage (+++, over 75% neuronal loss) was observed in CA1 (94.1% of all patients). Over half of the patients had extensive cell loss in CA3 (57.7%) and CA4 (59.6%), and an increasingly modest 23.1% having the same extent of damage in CA2. Virtually all samples showed either fibrillary or cellular gliosis. Combination of both was observed in several cases.

Relation between MRI and neuronal density

For the sample who had undergone surgery, patients with left mTLE again had significantly smaller MRI-determined volumes of the contralateral fimbria ($F=11.66$, $p=0.001$) relative to patients with right mTLE. Unlike analysis of the total sample, left-sided patients also had significantly smaller contralateral presubiculum volumes relative to right-sided patients ($F=4.76$, $p=0.03$).

Quantitative neuronal density measures were acquired for regions CA1-4. There were no significant differences between patients with left and right mTLE. Neuronal density of CA1 was significantly positively correlated with MRI-determined volume of CA1 ($r=.38$, $p=0.004$), CA2-3 ($r=.42$, $p<0.001$), and CA4-DG ($r=.47$, $p<0.001$). These relationships are shown in Figure 4. However, there were no significant correlations between neuronal density of CA2 or CA3 and MRI-determined region CA2-3, or between neuronal density of CA4 and MRI-determined region CA4-DG, respectively. There were also no significant correlations between the sum of CA2 and CA3 neuronal densities and MRI region CA2-3. These correlations were identical when volumes and densities were corrected for ICV.

Following the work of Davies et al. (1996), we separated patients according to severity of HS, and performed statistical comparisons based on this dichotomy. Patients were determined to have increased HS damage (HSi group) if they had Wyler gradings of III / IV and reduced HS damage (HSr group) if Wyler gradings were I or II. Patients with HSi had significantly smaller ipsilateral whole hippocampal volumes ($F=4.00$, $p=0.05$), and a strong tendency to have smaller ipsilateral CA1 ($F=3.12$, $p=0.06$) and CA4-DG volumes ($F=2.84$, $p=0.09$) relative to patients with HSr.

DISCUSSION

There were two primary objectives of the present study. Firstly, we sought to compare the MRI-determined hippocampal subfield volumes between a relatively large sample of patients with mTLE who had unilateral HS on routine clinical neuroimaging evaluation and age- and sex-matched healthy controls. With respect to this objective, we report three findings: (i) loss of all MRI-determined ipsilateral hippocampal subfields (except hippocampal fissure) in *group comparisons* between patients and controls; (ii) patients with left mTLE had bilateral volume loss (including contralateral whole hippocampus, fimbria and presubiculum) whereas patients with right mTLE only had volume loss of ipsilateral hippocampal structures; (iii) approximately three quarters of individual patients had MRI volume abnormalities of CA1, CA2-3, CA4-DG, subiculum and presubiculum ipsilateral to HS. Secondly, we sought to compare the presurgical MRI-determined subfield volumes with subfield neuronal density measures in patients who underwent amygdalohippocampectomy. We report that CA1 neuronal density was significantly correlated with MRI-determined volume of CA1, CA2-3 and CA4-DG, and that patients with increased HS based on Wyler gradings had greater volume loss of the ipsilateral whole hippocampus, CA1 and CA4-DG relative to patients with reduced HS. We address these findings with respect to methodological and clinicopathological issues.

MRI mapping of hippocampal subfields indicated significant volume loss of all subfields other than the hippocampal fissure ipsilateral to HS in patients with mTLE relative to controls in group comparisons. However, classical HS is characterised by preferential neuron loss and gliosis in region CA1, and also to a lesser extent of regions CA4 and CA3, with relative resistance of CA2 neurons (Bratz, 1899; de Lanerolle, et al., 2003). Our histopathological data are consistent with these earlier reports insomuch that we observed the most severe neuronal loss in CA1 (94.1%), then CA4 (59.6%), CA3 (57.7%) and CA2 (23.1%). We have no healthy control population to quantify the extent of neuronal loss in our patients, and therefore we are unable to provide a direct comparison with the imaging data. However, our imaging data indicated that the CA2-3 subfield was as damaged as the CA1 subfield in our sample relative to controls. At first consideration, we may have suggested that this was due to a large proportion of our patients having ‘total’ HS (i.e. significant neuronal loss throughout all CA regions). Indeed, total HS is more common that previously thought; one study reported total HS in 53%, and resistance of CA2 neurons in only 19% of well-preserved post-surgical specimens (Blumcke, et al., 2007). However, the visually graded histopathological data from our study indicated that region CA1 was

substantially more damaged than CA2. We therefore need to reconcile our MRI and histopathological findings, and there are two likely contributory reasons for the inconsistency.

Firstly, CA2 and CA3 were sampled together using the automated morphometric analysis, and therefore a direct comparison with CA2 alone is not permitted. We were aware of the lack of differentiation between CA2-3 at the onset of this study, and we chose to employ routines incorporated into Freesurfer software to estimate hippocampal subfield volume given the software's free availability, fully automated image processing pipelines, and independently determined consistency between Freesurfer automatically determined volumes and 'gold standard' manually determined volumes of hippocampal (Dewey, et al., 2010; Morey, et al., 2009; Pardoe, et al., 2009) and extrahippocampal (Dewey, et al., 2010; Keller, et al., 2012a) structures. More recent techniques have offered the possibility of automatically differentiating CA2 and CA3 (Bonnici, et al., 2012). Secondly, the morphometric technique used in the present study quantifies the volume of an area approximating a hippocampal subfield region based on *a priori* probabilistic information of the spatial distribution of subfields obtained from a training set determined using high resolution MRI *and* local tissue voxel information from individual MR images (Van Leemput, et al., 2009). It is not a method that determines the transition from one cellular region to another, which is currently beyond the resolution of conventional MRI. Instead, the method used here is a fully automated reproducible technique that provides novel information on the approximate location of hippocampal subfields. These locations are determined based on macroscopic anatomical geometry, where the morphology of a given area of the hippocampus (e.g. medial aspect of fimbria, dorsomedial crown of parahippocampal gyrus, point of highest curvature on the dorsolateral and ventrolateral edges of hippocampus; see Supplementary Table 1) delineates one subfield from another. The hippocampal subfield technique provides a geometrical subdivision of the hippocampal formation that is based on gross approximations, which may not reflect anatomical reality in a given subject. Whilst there will inevitably be errors in the automated estimation of cellular subfields, automated approaches like the one used this study are fully reproducible and fast enough to enable routine analysis of subfields in large imaging studies (Van Leemput, et al., 2009). This is particularly appealing given that the manual labelling of all hippocampal subfields can take between two to four hours (Yushkevich, et al., 2010). However, whether the approximate MRI-based localisation of subfields is an

accurate reflection of the anatomical topology of subfields is an important and so far unaddressed issue.

We show significant relationships between neuronal density of CA1 and volume of CA1 determined by MRI. Furthermore, patients with increased histopathologically determined HS had smaller ipsilateral whole hippocampal, CA1 and CA4-DG volumes relative to patients with decreased HS. On the one hand, the correlations of CA1 would seem particularly important for evaluation of patients with mTLE and Alzheimer's disease, given that CA1 is most severely affected in these conditions (Blumcke, et al., 2007; Bratz, 1899; Margerison and Corsellis, 1966; Sommer, 1880; West, et al., 1994; West, et al., 2004). On the other hand, these findings must be tempered with (i) the significant relationships also observed between neuronal density of CA1 and MRI volume of CA2-3 and CA4-DG and (ii) that two outliers appear to have an influence on the extent of these correlations (Figure 4). Although the subfield MRI volume estimates appear sensitive to the detection of large field cell densities, the multiple correlations suggest that this may be a non-specific subfield effect. In other words, changes in a given hippocampal subfield volume may not be exclusive to the same anatomical CA region and could be a marker of overall hippocampal pathology. The correlations between neuronal density of CA1 and volume of multiple CA regions observed in the present study may be influenced by an underestimation of the size of CA1 in the MRI probabilistic atlas used in this study (Lim, et al., 2013; Pluta, et al., 2012). Figure 1 suggests that the classification of CA1 appears smaller than what would have been expected, particularly as this region is the largest of the CA regions (West, et al., 1994). Yushkevich et al. (2010) demonstrated excellent agreement between manual and automated segmentation of CA1, which encompassed a much larger area of the hippocampus relative to the method used in the present study.

High-resolution images of the hippocampal formation are not necessarily obtained by long acquisition period on high field scanners. Several manual parcellation studies have acquired a small number of thick T2-weighted MRI sections with high in-plane resolution perpendicular to the long axis of the hippocampus (Mueller, et al., 2009; Mueller, et al., 2007; Mueller and Weiner, 2009; Small, et al., 2000), which is obtained in clinically acceptable time periods (~5-6 minutes). Based on focal T2-weighted imaging of the hippocampal formation with an acquisition time under five minutes, Yushkevich et al. (2010) developed a method of subfield segmentation that was almost fully automated. In particular, after manual delineation of MR sections into those that lie within the

hippocampal head and tail, the technique automatically classifies CA1, CA2, CA3, DG and subiculum in the sections containing the hippocampal body. The subfields are not delineated in the hippocampal head and tail because the increased convolutions in these regions cause problematic partial volume effects, making subfield segmentation unreliable (Yushkevich, et al., 2010). Nevertheless, excellent consistency between manual and automated measures was achieved for the subfield regions within the hippocampal body, particularly the larger areas including CA1 and DG, which were generally higher than the ones reported by Van Leemput et al. (2009). Developing this technique to delineate subfields throughout the entire extent of the hippocampal formation would represent an important advancement, and may be achieved by incorporating a model derived from a postmortem hippocampal atlas (Yushkevich, et al., 2009). A feasible focal T2-weighted MR sequence for prospective hippocampal subfield analysis may offer supplementary diagnostic opportunities in the clinical evaluation of patients with mesial temporal pathology.

There are methodological issues with respect to histopathological assessment that merit discussion. We performed quantitative cell density measurements on only single histological sections as per routine protocol. A more rigorous examination of the relationship between MRI and histopathological measures of HS would necessitate the acquisition of cell densities measurements throughout the extent of the hippocampal axis (Thom, et al., 2012). Furthermore, we were lacking histological control data. Examination of control cell densities would permit a more rigorous examination of the reliability of subfield mapping for the assessment of histopathology profiles in individual patients. As applied in the present study, it may be unwise to consider that the MRI subfield mapping technique has reliable utility for clinical classification. It is important to examine the technique's subfield classifications based on ultra-high resolution MRI data, rather than conventionally acquired scans at 1.5 or 3 Tesla. At present, the acquisition of such high-resolution data on clinically utilised MRI systems requires (i) at least multiple whole-brain acquisitions over longer time periods (e.g. ~35 minutes as per Van Leemput et al. (2009)) or (ii) prospective focal T2-weighted acquisitions of the hippocampal formation, which is achievable in clinically-acceptable time frames (Mueller, et al., 2009; Mueller, et al., 2010; Mueller and Weiner, 2009; Yushkevich, et al., 2010).

The present study is the first to offer histopathological clarification of a frequently applied MRI subfield approach. The automated subfield technique offers a fully reproducible, time

efficient and opportunistic way of obtaining volume estimates of hippocampal subfields from conventionally acquired T1-weighted data. Our results, in particular the limited MRI-histopathological specificity, should be considered in future applications of this method. Future work should compare the performance of the subfield approach applied to single acquisition conventional T1-weighted data and multi-acquisition high-resolution T1-weighted data in the same participants.

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Disclosure of Conflicts of Interest

The authors have no conflicts of interest.

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Figure Legend

Figure 1

Illustration of the hippocampal subfield mapping technique in three patients with unilateral HS. Coronal and axial sections are shown for each patient with subfields (i) labelled and (ii) transparentised to reveal the underlying anatomy. Sections are in radiological convention (left=right). (A) Patient with right HS and small hippocampal asymmetry. (B) Patient with increasingly severe right HS and hippocampal asymmetry. (C) Patient with severe left HS and hippocampal asymmetry. In case A, the choroid plexus (white arrows) that is located immediately dorsal to the alveus (black arrows) is included in the map of CA2-3. CA1 appears consistently small. Abbreviations: DG, dentate gyrus; fim, fimbria; hf, hippocampal fissure; presub, presubiculum; sub, subiculum.

Figure 2

Distribution of hippocampal subfield volume loss in patients with mTLE determined using MRI. Significant volume loss of ipsilateral CA1 (a), CA2-3 (b), CA4-DG (c), subiculum (d), presubiculum (e) and fimbria (f) is observed in patients with left and right mTLE (L/R TLE). Significant volume loss of the contralateral fimbria is also seen in patients with left mTLE.
***$p<0.001$

Figure 3

A stained coronal hippocampal cross section indicating classical HS in one patient. Analysis with antibodies against the neuronal antigen NeuN shows a typical pattern of HS with distinctive neuronal cell loss in the CA1, CA3 and CA4 regions. Note the relative neuronal preservation of the CA2 subfield. Abbreviation: sub, subiculum.

Figure 4

Significant positive correlations between neuronal density of CA1, and MRI-determined volume of CA1 (A), CA2-3 (B) and CA4-DG (C). Volume is cm^3 and neuronal density is x $10^{4}/\mu m^2$.

Structure	Lower 95th Percentile	Left mTLE	Right mTLE
L Hippocampus	2084.96	30 (59%)	5 (16%)
L Presubiculum	331.90	39 (76%)	6 (19%)
L CA1	200.95	37 (73%)	0
L CA2-3	645.90	37 (73%)	0
L Fimbria	35.88	8 (16%)	0
L Subiculum	453.13	42 (82%)	7 (23%)
L CA4-DG	362.55	39 (76%)	0
L Hipp Fiss	21.97	4 (8%)	2 (6%)
R Hippocampus	2320.57	8 (16%)	25 (81%)
R Presubiculum	322.08	10 (20%)	23 (74%)
R CA1	233.86	9 (18%)	23 (74%)
R CA2-3	666.82	11 (22%)	25 (81%)
R Fimbria	46.46	19 (37%)	13 (42%)
R Subiculum	443.66	9 (18%)	26 (84%)
R CA4-DG	379.60	10 (20%)	25 (81%)
R Hipp Fiss	19.62	1 (2%)	3 (10%)

Table 1. Number (and percentage) of patient MRI volumes under the 95th lower percentile of hippocampal subfield volume in controls.

	CA1	CA2	CA3	CA4
Neuronal Loss: **n**	**51**	**52**	**52**	**52**
0 (little / no significant loss)	1 (2.0%)	12 (23.1%)	8 (15.4%)	1 (1.9%)
I (≤ 50% loss)	1 (2.0%)	19 (36.5%)	4 (7.7%)	7 (13.5%)
II (≤ 75% loss)	1 (2.0%)	9 (17.3%)	12 (23.1%)	13 (25.0%)
III (> 75% loss & dispersion of granula cell layer)	48 (94.1%)	12 (23.1%)	30 (57.7%)	31 (59.6%)

Table 2. Graded levels of neuronal loss in CA1 – CA4 for patients who went to surgery.