Hippocampal tau pathology is related to neuroanatomical connections: an ageing population-based study


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Deposits of abnormally phosphorylated tau protein are found in numerous neurodegenerative disorders; the ‘tauopathies’, which include Alzheimer’s and Pick’s diseases, but tau pathology is also found in the ageing brain. Variation in tau pathology in brain ageing and its relationship to development of tauopathies and cognitive impairment remains unclear. We aimed to determine the extent and pattern of spread of tau pathology in the hippocampus, a susceptible region important in dementia and milder states of memory impairment, using hippocampal samples from the elderly population-based Medical Research Council Cognitive Function and Ageing Study neuropathology cohort. Tau deposition was assessed in hippocampal anatomical sub-regions using the AT8 antibody to phosphorylated tau and isoform-specific antibodies to 3 and 4-repeat tau (RD3 and RD4). Aβ pathology was also assessed. In this population sample, which includes the full ageing spectrum from individuals with no cognitive impairment to those with dementia satisfying clinico-pathology criteria for Alzheimer’s disease, we have demonstrated a high prevalence at death of tau pathology. AT8, Aβ, RD3 and RD4 showed similar regional distribution and increased RD3 was noted in late-stage ghost tangles. Aβ was shown to be a poor explanatory variable for tau pathology. Tau deposition progressed in a hierarchical manner. Hippocampal input regions and projection zones (such as lateral entorhinal cortex, CA1/subiculum border and outer molecular layer of dentate) were initially affected, with anterograde progression though the hippocampal circuitry. Six hippocampal tau anatomical stages were defined, each linking projectionally to their adjacent stages, suggesting spread of tau malfunction through neuroanatomical pathways in hippocampal ageing. These stages were significantly associated with dementia, and may provide a clinically useful tool in the clinico-pathological assessment of dementia and mild cognitive impairment.

Keywords: tau; neurodegeneration; dementia; ageing; Alzheimer’s disease

Abbreviations: Aβ = Amyloid-beta; DG = dentate gyrus; EC = entorhinal cortex; GT = ghost tangles; IML = inner molecular layer of the dentate; LEC = lateral entorhinal cortex; MEC = medial entorhinal cortex; NFT = neurofibrillary tangle; NTh = Neuropil threads; NT = tau+ve neurones; NP = Neuritic plaques; OML = outer molecular layer of the dentate; SLM = stratum lacunosum-moleculare; SO = stratum oriens; SP = stratum pyramidale; SR = stratum radiatum; TEC = transentorhinal cortex.
Introduction

Population-based studies indicate that approximately 0.5 million people in England and Wales meet criteria for dementia (MRC-CFAS, 1998). Given the demographics of an ageing population, not only in the UK but worldwide, this will be an increasing problem; recent estimates suggest that the number of demented individuals worldwide will rise from 24.3 million now to over 81.1 million by 2040 (Ferri et al., 2005). An understanding of the biology of dementia will be essential to devise both public health and therapeutic strategies to ameliorate this growing burden, but assessments of both cognitive impairment, causative and risk factors and its pathological correlates are highly complex (Brayne, 2007).

Brain ageing is the key risk factor for the development of cognitive impairment and the development of age-related degenerative pathologies. One of the most striking pathological findings in the ageing brain is the large extent of Alzheimer-type pathology, namely neurofibrillary tangles, neuritp threads and neuritic plaques. Classical cohort-based studies of clinically defined control and disease groups treat Alzheimer’s disease as a distinct disease entity, but there is a spectrum of pathology with considerable overlap between normal and abnormal in the ageing population (Brayne, 2007).

Abnormally phosphorylated tau is the major component of neurofibrillary tangles, neuritp threads and the neuritic component of plaques (Lace et al., 2007). Tau is a microtubule-associated protein involved in regulating cytoskeletal maintenance and axoplasmic transport. There are six isoforms of tau, generated by the alternate splicing of exons 2 and 3 (yields tau with 0, 1 or 2 N-terminal amino-acid repeat sequences) and exon 10 (yields tau with three or four microtubule-binding domains) of the tau gene. Tau with three or four microtubule-binding domains (3R and 4R tau, respectively) has been shown to be differentially deposited in lesions of specific disorders. For example, Pick bodies, which are characteristic of Pick’s disease, contain 3R tau (Arai et al., 2001), whereas the argyrophilic grains characteristic of argyrophilic grain disease contain 4R tau (Togo et al., 2002). Variation in 3R and 4R tau within the ageing population, however, currently remains unclear.

The Medical Research Council Cognitive Function and Aging Study (MRC-CFAS) is a prospective longitudinal population-based study of the ageing population with a strategy of necropsy, based around five UK centres. The unselected, population-based approach means that the cases collected include a spectrum of pathologies from normal brain ageing to individuals with dementia and individual variation in the ability of the brain to cope with pathological burden.

Within brain ageing and Alzheimer’s disease, tau deposition occurs in a stereotypical fashion, with the hippocampus, limbic structures, brainstem and the basal nucleus of Meynert being early sites of pathological involvement (Braak and Braak, 1991; Rub et al., 2000, 2001; Sassin et al., 2000). Other studies suggest that only selectively vulnerable neuronal populations become infiltrated with abnormal tau deposits in disease (for review, see Hof, 1997; Braak et al., 2006b; Esiri and Chance, 2006). The basis for the increasing recruitment of anatomical areas with increasing severity of tau pathology is unclear, as is the question of whether there is population variation in the way that phospho-tau is deposited and handled.

This study aimed to investigate the population variation in tau pathology and its patterns of spread across the neuropathological spectrum, from healthy ageing to Alzheimer’s disease. We focused on the hippocampus because of its involvement in memory (Tulving and Markowitsch, 1998; Eichenbaum, 2001; Burgess et al., 2002), a faculty which becomes impaired in both ageing and dementia, and because it is a region that is affected by tau pathology relatively early in ageing (Braak and Braak, 1997; Duyckaerts and Hauw, 1997). We sought to determine whether, with increasing severity of tau pathology, there is a stereotyped, hierarchical pattern of recruitment of hippocampal sub-regions that reflects the neuroanatomical connectivity and to explore the relationship between tau and pre-mortem indices of cognitive impairment. We have also sought to refine estimates of the population-prevalence at death of classical Alzheimer-type tau lesions such as tau-positive neurones, neuritp threads and neuritic plaques and investigate variation in 3R and 4R isoforms deposition within these lesions.

Materials and Methods

Case and tissue selection

MRC-CFAS is a longitudinal study of ageing and cognitive impairment and has been described in detail previously. The study is population-based and prevalence and incidence of dementia have been previously reported (MRC-CFAS, 1998; Matthews and Brayne, 2005; Brayne et al., 2006). The study has an on-going programme of brain donation (MRC-CFAS, 2001). Assessment in the study has been described previously. Briefly, a standardized assessment for psychiatric disorders in older people (GMS AGECAT) was performed and dementia status at death was based on a review of all the information provided by the respondent and informant in the last years of life, an informant interview after death and death certification (MRC CFAS, 1998; 2001). Median MMSE scores of 18 and 10.5 and median ages of 84 and 89 were seen in the non-dementia and dementia group, respectively. Additional basic clinical features in the demented and non-demented group are shown in Supplementary Table 1.

Cases from the Cambridge centre of the CFAS cohort were used in this study. All 93 cases from this cohort were used to maintain the unbiased nature of the sample. All cases were aged over 65 and consisted of 34 males and 59 females. Cases included a range of pathologies from normal ageing to neurodegenerative disease.
Eight cases satisfied CERAD criteria for definite Alzheimer’s disease, 14 for probable and 21 possible Alzheimer’s disease cases. All definite and probable Alzheimer’s disease cases were demented whereas 13 possible Alzheimer’s disease cases were demented, and 8 were nondemented (Supplementary Table 1). All analysis was performed blind to clinical data. The cases have been previously scored for global Alzheimer-type pathology using the CERAD and Braak AT8 immunohistochemistry scoring methods (Braak et al., 2006a). The cohort included 28 cases with entorhinal (Braak I–II) tau pathology, 44 with limbic (Braak III–IV) and 19 with isocortical (Braak V–VI) stage neurofibrillary pathology. Two cases had missing Braak scores due to tissue unavailability.

**Immunohistochemistry**

The posterior hippocampus of each case was stained for phospho-tau using AT8 (Pierce biotechnology Inc, 1:400), RD3 and RD4 (described previously) (de Silva et al., 2003) at 1:3000 and 1:100, respectively, and Aβ (Dako-cytomation, 1:100). Sections were dewaxed, rehydrated and incubated for 20 min in 3% H2O2 in methanol to block endogenous peroxidase activity. Aβ immunohistochemistry required a 4 h 98–100% formic acid pre-treatment, whereas the RD3 and RD4 antibodies required only 10 min formic acid pre-treatment. AT8 and AT8 sections were microwaved for 10 min in 0.01 M tri-sodium citrate (TSC) buffer (pH 6.5) to allow antigen retrieval. RD3 and RD4 experiments required a 20 min pressure cooker treatment in 0.01 M TSC buffer (pH 6.0). Sections were incubated in 1.5% normal blocking serum (Vector Labs) for 30 min, and incubated overnight at 4°C in the case of AT8 and Aβ primary antibodies, and for 1 h at room temperature when using RD3 and RD4. RD3 and RD4 also required a blocking treatment of 1 h. Sections were washed in tri-buffered saline (TBS) and then incubated at room temperature for 30 min in biotinylated 2nd anti-mouse antibody (1:200, Vector Labs) with 1% normal blocking serum (Vector Labs). After a TBS wash the sections were incubated for 30 min at room temperature in ABC (4:100, Vector Labs), and then diaminobenzidine/H2O2 (Vector Labs) was used to develop the peroxidase activity. Sections were counterstained with haematoxylin. Anterior hippocampal sections were stained only with AT8 to confirm that differences in density across the entorhinal cortex remained consistent, anteriorly to posteriorly.

**AT8, RD3 and RD4 analysis**

Slides were scored on a semi-quantitative 0–3 scale (no pathology, mild, moderate and severe, respectively) by two independent observers (G.L. and S.W.) for tau positive (tau+ve) neurite threads, tau+ve neurons (neurons demonstrating tangle and pre-tangle pathology), neuritic plaques and white matter neurite threads. Pick bodies, neuronal loss/infarcts and other notable pathologies were also recorded. For each case, one slide for each antibody was analysed in the posterior hippocampus and, additionally, an AT8 immunostained slide from the anterior hippocampus was examined to ensure pathological findings remained robust posteriorly to anteriorly (data not shown). Areas scored included the dentate gyrus (DG), CA4, CA3, CA2, CA1, subiculum, entorhinal cortex and transentorhinal cortex. The CA1/subiculum border was also rated independently since this region demonstrated different tau deposition behaviour compared with CA1 and the subiculum. Separate scores were also obtained for the lateral and medial entorhinal cortex (LEC and MEC, respectively) and cellular layers pre-α and pri-α. Differential hippocampal sub-regions were distinguished via their cellular characteristics which have been described previously (Braak and Braak, 1992).

**Amyloid analysis**

The hippocampus was scored by two independent researchers for the presence of Aβ using the same qualitative scale as tau. Areas scored included the DG, CA4, CA2, CA1, subiculum, white matter, entorhinal cortex and transentorhinal cortex.

**Image analysis**

The Olympus CellR analysis system was used to calculate the percentage area immunolabelling for tau and Aβ in the lateral and medial entorhinal cortex. Images were captured from two adjacent strips of cortex (multiple contiguous frames running deep to superficial to include all cortical layers) at 20× magnification in both lateral and medial entorhinal cortex sites. The percentage tau/Aβ values from each frame were added together and divided by the number of frames/region to obtain averages of tau/Aβ in both the lateral and medial entorhinal cortices. The mean tau and Aβ burden over both areas was also found.

**Statistics**

Donors within the Cambridge neuropathology cohort belong to the detailed assessment arm of the MRC CFAS, which was sampled to over-represent those with cognitive impairments (for more details see www.cfas.ac.uk). In order to estimate prevalence at death of each neuropathological finding, an inverse probability weight was generated and applied to all prevalence calculations to adjust for the oversampling of those with cognitive impairment as well the selection bias introduced during the accrual of the brain tissue resource. The weighting was calculated by a comparison of the age, sex, cognitive impairment and time between interview and death between those who donated brain tissue and those who died in the remainder of our initial population-based sample. Death information was obtained on the complete study population. We have also shown (MRC CFAS, paper in submission) that those who donated brain tissue differ little in terms of age, education, sex, cause of death or social class from the rest of the study who had died. Consequently our estimates represent the prevalence at death of each neuropathological finding in the population aged over 65.

Variation in tau pathology (in terms of density) within the different hippocampal sub-regions was assessed using chi-squared tests, and the relationship between specific areas of interest (e.g. lateral entorhinal cortex and outer molecular layer pathology) and the relationship between different tau lesions was assessed with Fisher’s exact test. The areas showing associations via Fisher’s exact and also demonstrating similar prevalences were grouped to generate two staging schemes describing tau pathological progression (NT and NTh) through the hippocampus and associated entorhinal cortices.

Loevinger’s H statistic was used to measure whether the progression of NT and NTh pathology could be represented by one-dimensional staging schemes. Loevinger’s H tests the extent to which a group of variables conforms to a single underlying scale and takes values between 0 and 1, with values <0.3 conventionally suggesting that a single scale is not appropriate, 0.3–0.4 suggesting weak consistency, 0.4–0.5 moderate consistency and >0.5 strong consistency (Loevinger, 1948).

Associations between dementia status, the NT/NTh stages defined in this work and Braak stage were tested using logistic regression. Correlation between tau and Aβ percentage immunolabelling in entorhinal cortex was assessed using Spearman’s rank correlation coefficient. Inter-rater agreement was tested using Cohen’s kappa to
compare the scores in the CA1 region from two observers (G.L. and S.W.). A high level of agreement was found (kappa = 0.83, 0.80 and 0.69 with respect to NT, NTh and NP). Statistical analyses were performed using SPSS 14 and Stata 9.2.

Results

AT8 immunohistochemistry

The population prevalence at death of NTh, NT and NP, estimated using AT8 immunolabelling, were 98%, 100% and 78%, respectively (Fig. 1B). AT8 also revealed a high prevalence of positively stained axons in the white matter deep to the entorhinal cortex (89%). Moderate to severe pathology was estimated to be present in 89%, 87% and 62% of the population with respect to NTh, NT and NP. Further analysis of AT8 immunohistochemistry was performed using the dataset obtained from cases with moderate/severe pathology since these cases more closely represent cases with significant pathology due to the high prevalence noted.

Regional analysis of scores demonstrated a statistically significant variation in tau distribution across the hippocampus (P < 0.001). High prevalence of pathology was most evident across the entorhinal cortex and transentorhinal cortex, CA1 and CA1/subiculum border (Fig. 1B). A laminar distribution of tau deposition was noted in several hippocampal/entorhinal regions. In CA1 an increased density of NTh was noted in stratum oriens and stratum lacunosum-moleculare (Fig. 2C). The entorhinal cortex also demonstrated varying degrees in pathology density across its entirety as well as between layers (Fig. 2A); therefore independent scores for lateral entorhinal cortex, medial entorhinal cortex, superficial layer pre-α and deep layer pri-α were obtained to further explore differences in tau distribution in these areas. Lateral entorhinal cortex pathology was more dense than the medial entorhinal cortex in 40% of cases, whereas the medial entorhinal cortex was more dense than the lateral entorhinal cortex in 4% of cases. Also in 32% of cases layer pre-α was more densely involved than layer pri-α, 4% of cases had increased density in pri-α compared to pre-α, and 64% had equal involvement of pre/pri-α. The differences in tau density across the entorhinal sub-regions suggest that some areas become affected before others. 17% of cases demonstrated a deviation from the usual anatomical hierarchy, with increased pathological severity in CA2 compared to CA1 (Fig. 2D).

The border region between CA1 and the subiculum showed such a marked increase in density compared to the rest of CA1 and the subiculum that it was rated as an independent area (Fig. 2B). This border region showed a similar prevalence of tau pathology to the lateral entorhinal cortex and entorhinal cortex pre-α. A significant association between lateral entorhinal cortex NTh and NT with CA1/subiculum border NTh and NT was found (P < 0.001), which combined with having similar prevalence suggests that these pathologies are usually found together and may develop at a similar time. The medial entorhinal cortex and entorhinal cortex pri-α were rarely more dense than lateral entorhinal cortex and pre-α suggesting these areas become pathologically recruited downstream of lateral entorhinal cortex and pre-α. Pathology in medial entorhinal cortex and pri-α was strongly associated (P < 0.001) and had similar prevalence supporting development at a similar time period.

The prevalence at death of dentate gyrus tau pathology was 85%, with 43% having pathology restricted to the outer molecular layer (OML), shown Fig. 2E. The remaining cases had pathology in both the inner molecular layer (IML) and the outer molecular layer (Fig. 2F). No cases had only inner molecular layer pathology, implying that outer molecular layer dentate gyrus involvement is a pre-requisite to inner molecular layer tau pathology.

Five cases were identified within the cohort that had neuro-pathological features of specific non-Alzheimer entities. One case showed classical Pick bodies characteristic of the neuropathology of Pick’s disease. One case demonstrated the features characteristic of a fronto-temporal dementia with ubiquitinated inclusions, including ubiquitinated neuronal inclusions in the dentate gyrus and cerebral cortex. Three cases showed a subcortical preference for phospho-tau deposition suggestive of progressive supranuclear palsy, though they did not satisfy all of its diagnostic criteria. These five cases were initially included in all analyses because of the population basis of the approach. However, because some of these represent distinct entities, not on the ageing spectrum, the analyses were repeated with the five cases excluded. The analyses and models presented were found to be robust to the inclusion...
of these cases, which did not affect levels of statistical significance for any of the analyses.

**RD3 and RD4 immunohistochemistry**

RD3 and RD4 failed to detect more than half of the NTh detected by AT8 and also much lower amounts of NP (Supplementary Table 2). RD3 and RD4 both detected 85% of NT detected by AT8. RD3 detected ghost tangles in 67% of cases, with greater sensitivity than AT8, but RD4 did not label these structures. Ghost tangles were most commonly located in layer pre-α of the medial entorhinal cortex, in layer pri-α of more lateral/transentorhinal regions and at the CA1/subiculum border. RD3 detected more NP than RD4. The regional distribution of staining was similar with these antibodies to that of AT8 with the entorhinal cortex, transentorhinal cortex and CA1 regions being most severely affected by tau burden. An ordinal logistic regression model was used to compare the density of expression of RD3 and RD4 labelling in each area. For NTh (P = 0.38) and NP (P = 0.49) there was no variation in the RD3/RD4 ratio by area, but in NT regional variation of borderline statistical significance (P = 0.09) was observed, driven primarily by higher 3R than 4R scores in CA1.

**Relationship of tau pathology to Aβ**

The prevalence at death of Aβ was 82%, with 74% of cases achieving moderate/severe scores. Tau pathology, in the form of threads or neuronal tau, had a higher prevalence than Aβ in all sub-fields of the hippocampus but plaques had a lower prevalence. In 20% of cases, tau was present without any Aβ as

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**Figure 2** Tau pathology shown with AT8 in the hippocampus and entorhinal cortex. (A) Laminar NT and NTh distribution in entorhinal cortex layers pre-α and pri-α. (B) Dense pathology (especially NT and NTh) shown across the CA1/subiculum border. (C) Laminar distribution of tau in CA1. Dense NTh were noted in stratum oriens (SO) and stratum lacunosum-moleculare (SLM), dense NT with moderate NP and fewer NTh were evident in stratum pyramidale (SP) and milder tau deposition noted in stratum radiatum (SR) (D) CA2 predominant case. Increased density of NTh, NT and NP were seen in CA2 in comparison to CA1. (E) Involvement of the outer molecular layer (OML) only of the dentate gyrus. Dense NTh shown in the OML. (F) Involvement of the inner (IML) and outer (OML) molecular layer of the dentate gyrus. Dense NTh with a NP in the OML. Scale bars in A–D represent 1000 µm and 100 µm in E–F.
detected by immunoreactivity. 1% of cases demonstrated Aβ pathology without tau pathology. Like tau, Aβ was most prevalent in the entorhinal cortex/transentorhinal cortex, and was also found in CA1. 19% also showed patchy, powdery Aβ deposition in the parasubiculum which is a region only very mildly involved with abnormal tau. Analysis of Aβ was performed in fewer areas than tau, since its characteristically extracellular deposition could not be pinpointed to specific cell layers.

To further explore the relationship between tau and amyloid in this cohort, the expression of tau and amyloid was assessed in the medial entorhinal cortex and lateral entorhinal cortex using image analysis. Increased deposition of tau \((P<0.001)\) and amyloid \((P=0.06)\) was found in the lateral entorhinal cortex compared to the medial entorhinal cortex, supporting the results from semiquantitatively scoring these areas for tau burden (Fig. 3A). There was a correlation between average entorhinal tau and β-amyloid immunolabelling (Spearman’s rho, \(P=0.036\)), but the \(r^2\) value of 0.028 indicates that amyloid is a poor explanatory variable for tau pathology at this site (Fig. 3B), with many highly tau burdened individuals demonstrating low amyloid and vice versa. By also analysing pathology within the anterior hippocampus we confirmed that the increased pathology seen in the lateral entorhinal cortex was maintained anteriorly to posteriorly (data not shown).

### Staging scheme for tau pathology in the hippocampus

Based on the prevalence, at death, of tau in hippocampal sub-regions as described above, we generated a detailed staging scheme for the hierarchical distribution of tau within the hippocampus (Fig. 4) by grouping areas demonstrating similar prevalences of tau burden. Sub-regions within a specific group were shown to demonstrate associations (using Fisher’s exact tests) in terms of degree of pathology. For example, Fisher’s exact tests looking at lateral entorhinal cortex and outer molecular layer showed that if the lateral entorhinal cortex demonstrates moderate/severe pathology then so does the outer molecular layer and vice versa, and if the lateral entorhinal cortex is devoid of pathology, then so is the outer molecular layer and vice versa.

Loevinger’s H statistic was 0.63 overall for NTh and 0.58 for NT, strongly supporting a one-dimensional progression of each of these tau pathologies as described in the NT/NTh staging scheme shown in Fig. 4. For each of the hippocampal sub-regions \(H>0.5\) for both NT and NTh indicating strong consistency of adherence to the scheme, apart from the prevalence of NT in CA2, where \(H=0.43\) indicates moderately good consistency with the rest of the hippocampus.

Cases were assigned both an NT stage and an NTh stage (number of cases in each stage shown in Supplementary Tables 3 and 4). NP distribution was not incorporated within the staging since, although the overall consistency of NP distributions was reasonable \((H=0.57)\), the prevalence in several sub-regions did not conform well to the rest of the hippocampus \((H<0.3\) in CA2 and subiculum, \(H=0.31\) in CA3).

Stage 0 represents cases with no, or only mild distribution of NT or NTh. In Stage 1 moderate/severe scores were notable in the transentorhinal cortex, lateral entorhinal cortex, pre-α of entorhinal cortex, CA1/subiculum border and the outer molecular layer of the dentate gyrus. In Stage 2 the medial entorhinal cortex and layer pri-α of the entorhinal cortex attained moderate/dense NT scores. Stage 3 sees moderate/dense scores extending to CA1 and CA2 and in Stage 4 the inner molecular layer of the dentate gyrus and the rest of the subiculum becomes recruited. In Stage 5, moderate/severe NT and NTh scores are seen in CA3 and CA4. The relationship between regional prevalence of tau and NTh/NT stage is shown in Tables 2 and 3. This staging scheme was found to significantly correlate with Braak stage \((P=0.01)\), and is related to the circuitry within the hippocampus and associated areas (summarized in Fig. 4).
Relationship of tau burden and cognition

Braak scores, and hippocampal NTh and NT stages were found to be significantly related to dementia status in univariate analyses (P<0.001, P<0.001 and P=0.004, respectively) (Fig. 5A, B and C, Table 1 and 2). However, NT and NTh stages were not predictive of dementia when adjusted for Braak stage (P=0.121 and P=0.902, respectively). There was no relationship between CA2 predominant pathology cases and dementia.

Discussion

The population based approach adopted in this study allows an investigation of the variation in pathology that is present in the ageing population and how this relates to impaired function. It also allows an examination of changes that can be inferred as occurring early in the neurodegenerative process. Here we have focused in detail on tau pathology in the hippocampus, a structure that appears to be an early target of neurodegenerative processes and whose involvement is key to the memory impairments seen in age-related cognitive disorders (Tulving and Markowitsch, 1998; Eichenbaum, 2001; Burgess et al., 2002). This cohort includes a full range of tau pathology from very little (Braak stage I–II) to abundant (Braak stages V–VI). Eight of the cases would have satisfied CERAD neuropathological criteria for definite Alzheimer’s disease, 14 for probable and 21 for possible Alzheimer’s disease. All definite and probable Alzheimer’s disease cases were demented, whereas 13 possible Alzheimer’s disease were demented, and 8 were non-demented (see Supplementary Table 1). The study was performed blind to the clinical information. Examination of the CFAS neuropathology cohort has previously demonstrated a high prevalence at death of Alzheimer-type cellular pathologies in the brain as a whole with a considerable overlap in pathology between individuals with and without dementia (MRC-CFAS 2001). Other studies have also found pathology in cognitively intact individuals (Braak and Braak, 1991; Knopman et al., 2003). Thus, it is difficult to obtain a precise definition and minimum diagnostic thresholds for Alzheimer’s disease because of pathological overlap between the diseased and normal brain, and dementia studies using control material need to take account of the cellular pathology that is likely to be present. Our approach, therefore, is to analyse the variation in the population as a whole and not to pre-assign subgroups. CFAS is a true population-based neuropathology sample and we used all of the cases from one of the CFAS centres to maintain the unbiased population-nature of the sample.

We have identified a high prevalence at death of tau pathology in the ageing hippocampus, with very few individuals having no tau pathology. NTh and NT were more frequently observed than NP, supporting the suggestion that they form earlier in the
Table 1 Basic clinical features in each Tau NTh stage

<table>
<thead>
<tr>
<th>NTh stage</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>8</td>
<td>36</td>
<td>33</td>
</tr>
<tr>
<td>Demented, n (%)</td>
<td>1 (20%)</td>
<td>0 (0%)</td>
<td>3 (43%)</td>
<td>4 (50%)</td>
<td>19 (53%)</td>
<td>29 (91%)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>2 (40%)</td>
<td>1 (25%)</td>
<td>3 (43%)</td>
<td>6 (75%)</td>
<td>22 (61%)</td>
<td>25 (76%)</td>
</tr>
<tr>
<td>Age at death (median, IQR)</td>
<td>76 (74–77)</td>
<td>72 (71–75.5)</td>
<td>88 (72–89)</td>
<td>85.5 (82.5–89.5)</td>
<td>85 (82–90.5)</td>
<td>90 (86–93)</td>
</tr>
<tr>
<td>MMSE at last interview (median, IQR)</td>
<td>25 (23–26)</td>
<td>22.5 (21–26.5)</td>
<td>19 (16–18)</td>
<td>21 (16–25.5)</td>
<td>19.5 (14.5–25.5)</td>
<td>6 (0–17)</td>
</tr>
<tr>
<td>Years between last interview and death</td>
<td>1.2 (1.0–2.0)</td>
<td>1.4 (0.9–2.6)</td>
<td>1.2 (0.2–2.1)</td>
<td>1.8 (0.8–3.0)</td>
<td>1.4 (0.8–1.8)</td>
<td>1.3 (0.7–2.4)</td>
</tr>
</tbody>
</table>

Number of demented participants, females, median age at death (with inter-quartile range in brackets), MMSE at last interview (with inter-quartile range) and median age between last interview and death (with inter-quartile) are shown across Tau NTh stages.

Table 2 Basic clinical features in each Tau NT stage

<table>
<thead>
<tr>
<th>NT stage</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>2</td>
<td>11</td>
<td>17</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>Demented, n (%)</td>
<td>3 (27)</td>
<td>1 (50)</td>
<td>8 (73)</td>
<td>8 (50)</td>
<td>11 (50)</td>
<td>25 (86)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>5 (42)</td>
<td>0 (0)</td>
<td>5 (45)</td>
<td>11 (65)</td>
<td>15 (68)</td>
<td>23 (79)</td>
</tr>
<tr>
<td>Age at death (median, IQR)</td>
<td>74.5 (72–77)</td>
<td>80, 80</td>
<td>88 (81–89)</td>
<td>88 (86–92)</td>
<td>84.5 (82–89)</td>
<td>90 (84–94)</td>
</tr>
<tr>
<td>MMSE at last interview (median, IQR)</td>
<td>24 (18–26.5)</td>
<td>18, 26</td>
<td>19 (13–21)</td>
<td>21 (15–26)</td>
<td>20 (18–25)</td>
<td>8 (1–15)</td>
</tr>
<tr>
<td>Years between last interview and death</td>
<td>1.1 (0.8–2.7)</td>
<td>1.6, 2.2</td>
<td>1.2 (0.3–2.1)</td>
<td>1.4 (0.6–2.0)</td>
<td>1.1 (0.6–1.8)</td>
<td>1.7 (0.9–3.2)</td>
</tr>
</tbody>
</table>

Number of demented participants, females, median age at death (with inter-quartile range in brackets), MMSE at last interview (with inter-quartile range) and median age between last interview and death (with inter-quartile) are shown across Tau NT stages.

Figure 5 The relationship between tau pathology and dementia. (A) Distribution of Braak stages and dementia status. (B) Distribution of Tau NT stage and dementia status. (C) Distribution of Tau NTh stage and dementia status. Significant relationships between Tau NT/ NTh stage and dementia was reported ($P=0.004$ and $P<0.001$, respectively)
evolution of pathology at this anatomical site (Schonheit et al., 2004). Analysis across a population spectrum demonstrates a hierarchy of tau deposition within the sub-fields of the hippocampus, and shows that the order of regions affected by tau burden can be mapped to the circuitry of the hippocampus and associated areas. This pattern was defined using AT8 immunohistochemistry on conventional sections. Isoform specific antibodies to 3R and 4R tau revealed similar patterns of spread, although with lower sensitivity for lesions. Although both detected tangles, RD4 was especially successful in staining pre-tangles, whereas RD3 preferentially stained ghost tangles, as previously reported (Iseki et al., 2006). This may suggest a shift in tau isoform ratio as disease progresses, or that there are differences in 3R and 4R metabolism leading to a persistence of 3R tau in late-stage lesions. However, it must be considered that RD3 and RD4 detected a lower proportion of lesions as compared to AT8 (see Supplementary Table 2), therefore only certain tau epitopes or tau within specific conformations may be detected. Both 3R and 4R demonstrated a similar hierarchy of spread to AT8 and did not reveal population subgroups showing preferential selective isoform expression within particular hippocampal sub-regions. This anatomical hierarchy has allowed the definition of a six-stage scheme for the progression of tau pathology through hippocampal subregions based on immunohistochemistry with AT8 (summarized in Fig. 4). High values (>0.5) of Loevinger’s H coefficients show that the progression of NT and NTh in the hippocampus can be satisfactorily represented by one-dimensional staging schemes.

The spread of disease process along neuronal pathways has been proposed by other authors (Pearson et al., 1985; German et al., 1987; Saper et al., 1987; Hyman et al., 1988; Pearson, 1996; Shukla and Bridges, 1999, 2001; Rub et al., 2000; Thal et al., 2000). In Alzheimer’s disease it has been suggested that tau+ve neurones are associated with brain regions containing Aβ deposits via their axonal projections (Shukla and Bridges, 1999) and the spread of pathogenesis occurs through neuronal connectivity. Our study shows that in the hippocampus, Aβ pathology largely mirrors that of tau, with its deposition seeming to shadow hippocampal inputs, yet the anatomical hierarchy of Aβ deposition in brain ageing demonstrates a different pattern to that of tangle formation when considering other brain regions (Braak and Braak, 1991; Thal et al., 2002). NTh and NT, but not NP, prevalences were much higher than Aβ in most hippocampal regions, supporting other data suggesting that tangles may precede Aβ deposition (Braak and Braak, 1997; Duyckaerts and Hauw, 1997).

In this study the quantitative analysis of the entorhinal cortex suggests Aβ and tau deposition occurs independently. Other work has also demonstrated, both biochemically and immunohistochemically, that tau and Aβ accumulate independently in the entorhinal cortex (Katsuno et al., 2005). These findings suggest that, in simplistic terms, Aβ deposition, as identified by immuno-staining methods, is a poor explanatory variable for tau deposition, particularly in tangles and threads, in mesial temporal cortex. However, oligomeric forms of protein aggregate are likely to be important biological species in Alzheimer’s disease development and memory loss (Gong et al., 2003) but are likely to be undetected with our immunohistochemical methods, as are certain epitopes with specific conformations and post-translational modifications.

We have also shown a high prevalence at death of immuno-staining with AT8 in white matter axons, particularly in white matter just deep to entorhinal cortex, which are likely to be projection fibres to the hippocampus. All six tau isoforms can be identified in white matter and may be transported along axons (Boutajangout et al., 2004). The presence of AT8 immunoreactive tau suggests that axonal tau is abnormally phosphorylated and raises the possibility that axonal dysfunction in projections to the hippocampus is a common, early change in ageing.

The model of anterograde spread of molecular pathology, as an explanation of the apparent pattern of tau pathology progression, is an alternative to a model based upon a hierarchy of cellular susceptibility, and is supported by the relationship of anatomical connectivity to spread. The circuitry within the hippocampus is briefly summarized in Fig. 4, and we show that significant tau burden is seen initially in Tau Stage 1 areas, and then progresses so as to affect areas of Stages 2, 3, 4 then finally Tau Stage 5 areas. There remains controversy regarding some aspects of hippocampal connectivity (for review, see Witter et al., 2000; Seward and Seward, 2003) yet much of this complex circuitry is well documented (Braak and Braak, 1992; Witter, 2007). The lateral entorhinal cortex (especially superficial cell layers) is thought to receive the majority of the information input of the hippocampus, which then goes on to be processed in the hippocampus proper (Seward and Seward, 2003). The lateral entorhinal cortex projects to other areas grouped in Tau Stage 1, including the outer molecular layer (Hjorth-Simonsen, 1972; Hjorth-Simonsen and Jeune, 1972; Canning and Leung, 1997) and distant CA1/proximal subiculum (termed CA1/subiculum border in this study) (Hjorth-Simonsen and Jeune, 1972). Pre-α cells also preferentially target the outer molecular layer (Van Hoesen and Pandya, 1975a; b; Van Hoesen et al., 1975). The CA1/subiculum border then projects to the Tau Stage 2 areas; medial entorhinal cortex and layer PrI-α of the entorhinal cortex (Kohler, 1985, 1986; van Groen and Wyss, 1990). The Stage 2 areas project to Stages 3, 4 and 5 areas. For example, medial entorhinal cortex (Stage 2) projects to proximal CA1/CA2 (Stage 3), subiculum (Stage 4), inner molecular layer (Stage 4) and CA3 (Stage 5) (Hjorth-Simonsen and Jeune, 1972; Kohler, 1986; Witter et al., 2000). Stage 3 areas project to Stage 4 areas (CA1 projects to the subiculum (Gigg, 2006) and axons from the inner molecular layer (Stage 4) extend to CA4/3 (Deller, 1998). Stage 5 CA3/4 neurones then project to CA1 as well as other hippocampal regions (Witter, 2007). In this model, therefore, the progressive involvement of different sub-regions within the hippocampus primarily reflects neuroanatomical connections rather than intrinsic cellular susceptibility, although combination of cellular susceptibility and pathological spread via connectivity is not excluded.

In this study, the spread of tau burden through circuitry is supported by the increased pathological severity within regions of the hippocampus containing the largest density of synapses, such as in the molecular layer of the dentate gyrus, the CA1/subiculum border, CA1 stratum oriens and lacunosum molecular. Other researchers have reported the apparent spread of pathology via connectivity but these studies did not examine in detail the hippocampal sub-regions described in this study. A possible explanation for pathological spread via connectivity is that a
biological substrate (e.g. Aβ, protein kinases) capable of inducing tau phosphorylation and aggregation is being transported along axons to the different projection sites. In this context, the micro-anatomical distribution of Aβ and tau oligomers would be of great interest.

We show a continuum of pathology within the population, rather than discrete discontinuous groups that might represent healthy ageing and disease. Most individuals conformed to this stereotypical staging pattern, but there was some departure. We identified a subpopulation of individuals with increased pathological severity in CA2 compared to CA1, an inversion of the usual pattern, though we did not demonstrate a specific relationship between a CA2 predominance pattern and dementia status.

The relationship between Tau NT/NTh stage and dementia supports previous findings in CFAS regarding a large degree of pathological overlap between demented and non-demented cases. Work by Thal and colleagues (2000) also showed an anatomical spread of tau pathology, and they found that outer molecular layer pathology was almost exclusively found in demented individuals. In our study analysis of the population ageing neuropathological spectrum identified the outer molecular layer as an early site of abnormal tau manifestation, with CA3/4 pathology being more associated with demented cases (Fig. 5B and C, and Tables 1 and 2). CA3/4 are major components of the perforant pathway, which is associated with memory formation, and so we hypothesize that although hippocampal tau stage gives no additional predictive value to dementia status once adjusted for Braak stage, it may prove a useful predictive tool in understanding the neuropathological correlate of mild cognitive impairment. Although mild cognitive impairment is considered to be a clinical precursor of Alzheimer’s disease, some cases improve and the pathological correlates of mild cognitive impairment are currently poorly understood (Gauthier et al., 2006). We plan to assess the relationship between hippocampal tau staging and mild cognitive impairment in future work on the cohort.

The new staging scheme of hippocampal tau pathology described here adds further detail to the current staging schemes available e.g. Braak and Braak (1991). By closely examining more novel ‘sub-regions’ of the hippocampus (including the CA1/subiculum border, inner molecular layer and medial entorhinal cortex) we have been able to show a relationship between the order of areas affected and the circuitry of the hippocampus, providing support for the hypothesis that disease processes may spread via neuronal connections. The mechanisms underlying the hierarchical patterns of progression of tau and other neurodegenerative proteins are key questions in the neurodegenerative field. They underlie the progress of the disease and offer the potential for novel therapeutic intervention to ameliorate progression of cognitive impairment by interrupting anatomical progression.

**Supplementary material**

Supplementary material is available at *Brain* online.


Gigg J. Constraints on hippocampal processing imposed by the connectivity between CA1, subiculum and subicular targets. Behav Brain Res 2006; 174: 265–71.


