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Hippocampal pathology in Amyotrophic Lateral Sclerosis: selective vulnerability of subfields and their associated projections

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Highlights

- Amyotrophic lateral sclerosis is associated with considerable hippocampal pathology
- Hippocampal subfields and their projections are selectively affected in ALS
- The imaging features of hippocampus are consistent with post mortem findings in ALS
- The hippocampal profile of ALS is different from AD and healthy ageing
- Memory deficits in ALS are notoriously under recognised despite their clinical relevance

ABSTRACT

While hippocampal involvement in ALS has been consistently highlighted by post-mortem studies, memory impairment remains under recognised and the involvement of specific hippocampal subfields and their connectivity patterns are poorly characterised in vivo. A prospective multimodal neuroimaging study has been undertaken with 50 well-characterised ALS patients, 18 patients with Alzheimer's disease and 40 healthy controls to evaluate their mesial temporal lobe profile. Patients with ALS and AD have divergent hippocampal signatures. The Cornu Ammonis 2/3 subfield and the hippocampus-amygdala transition area are the most affected regions in ALS in contrast to AD, where the presubiculum and subiculum are the most vulnerable regions. Tractography reveals considerable fornix and perforant pathway pathology in both patient groups. Mesial temporal lobe structures in ALS have a selective and disease-specific vulnerability profiles and their white matter projections exhibit concomitant degeneration. Our combined grey and white matter analyses indicate a connectivity-based, network-defined involvement of interconnected temporal lobe structures as opposed to contiguous involvement of adjacent structures. Our findings underline the importance of screening for memory deficits and personalised management strategies in ALS.

Glossary

AD = Alzheimer disease; **ALS** = amyotrophic lateral sclerosis; **ALSFRS-r** = The revised ALS functional rating scale; **ANCOVA** = Analysis of covariance; **BSRT** = Babcock Story Recall Test; **CA** = Cornu Ammonis; **Dax** = axial diffusivity; **Drad** = radial diffusivity; **DTI** = Diffusion Tensor Imaging; **EMM** = estimated marginal mean; **FA** = fractional anisotropy; **FDR** = false discovery rate; **FLAIR** = Fluid-attenuated inversion recovery; **FOV** = Field-of-view; **Fx** = fornix; **FWE** = Family-wise error; **GC-DG** = granule cell layer of dentate gyrus; **GM** = Grey matter; **HARDI** = High angular resolution diffusion imaging; **HATA** = hippocampus-amygdala transition area; **HC** = healthy controls; **ICC** = intra-class Correlation; **Lt** = left; **MRS** = Magnetic resonance spectroscopy; **MANCOVA** = Multivariate analysis of covariance; **NIA-AA** = National Institute on Aging and Alzheimer's Association; **NODDI** = Neurite orientation dispersion and density imaging; **PPZ** = perforant pathway zone; **QSM** = Quantitative susceptibility mapping; **RAVLT** = Rey Auditory Verbal Learning Test; **ROCFT** = Rey-Osterrieth Complex Figure Test; **ROI** = region-of-interest; **Rt** = right; **SE** = standard error; **TIV** = total intracranial volume; **TE** = echo time; **TFCE** = threshold-free cluster-enhancement; **TR** = time of repetition; **WM** = white matter

1. Introduction

ALS is now universally recognised as a multisystem condition affecting frontotemporal, subcortical and cerebellar regions beyond its hallmark motor cortex and spinal cord pathology. In contrast to the extensive literature of frontal and temporal changes in ALS, hippocampal involvement and memory deficits remain relatively under recognised, despite pioneering longitudinal neuropsychology and neuroimaging studies (Christidi, et al., 2018b).

Early neuropsychology studies in ALS have overwhelmingly focused on executive dysfunction (Abrahams, et al., 1995a, Abrahams, et al., 1995b), which were subsequently complemented by the characterisation of behavioural, visuospatial and language deficits (Burke, et al., 2016, Christidi, et al., 2018a, Finegan, et al., 2019a, Montuschi, et al., 2015, Phukan, et al., 2012, Pinto-Grau, et al., 2018). Memory deficits have only recently been recognised in ALS (Beeldman, et al., 2016, Christidi, et al., 2018b) and are thought to impact on compliance with assistive devices, participation in clinical trials, adherence to therapy, and activities of daily living (Burke, et al., 2017a, Caga, et al., 2019).

Pharmacological studies of ALS continue to rely on survival and clinical scores as key outcomes measures overlooking the biomarker potential of quantitative neuroimaging measures (Bede, et al., 2018b, Chipika, et al., 2019, Schuster, et al., 2016). Furthermore, clinical trials in ALS overwhelmingly focus on motor disability and respiratory function and do not take extra-motor manifestations of the disease into account. Extra-motor involvement is an important contributor to clinical heterogeneity in ALS (Burke, et al., 2017b, Feron, et al., 2018, Omer, et al., 2017) which is recognised as a key barrier to successful clinical trials (Mitsumoto, et al., 2014).

Despite the co-occurrence of ALS and AD (Frecker, et al., 1990, Segers, et al., 2012) and similar memory profiles (Machts, et al., 2014), it is unclear if ALS is associated with a characteristic hippocampal signature distinct from AD. While the majority of hippocampal studies in ALS focus on grey matter pathology (Bede, et al., 2013b, Westeneng, et al., 2015), hippocampal subfields and their respective white matter projections are ideally studied together to test emerging concepts of trans-synaptic spread and connectivity based disease propagation.

Accordingly, we sought to characterise the hippocampal profile of ALS in contrast to healthy-controls and disease-controls using multimodal neuroimaging and test the hypothesis that ALS is associated with a unique hippocampal signature.

2. Methods

2.1 Ethics approval

This prospective neuroimaging study has been approved by the institutional review board of the Aeginition University Hospital and all participants provided informed consent prior to inclusion.

2.2 Participants

Fifty ALS patients with a diagnosis of ‘definite’ or ‘probable’ ALS based on the revised El Escorial criteria (Ludolph, et al., 2015) have been included. A group of 18 unselected Alzheimer’s disease (AD) patients were included as disease controls, and 40 healthy controls (HC) were also included. The National Institute on Aging and Alzheimer’s Association (NIA-AA) criteria were used to establish the diagnosis of AD (McKhann, et al., 2011). All AD patients were “probable AD” by the current categorisation guidelines. AD-mimics have been reassuringly out ruled by standardised assessments for structural, vascular and inflammatory brain changes (MRI), endocrine, infectious and haematological profiling, and screening for depression by the Geriatric Depression Scale (Yesavage, et al., 1982). **The majority of AD patients (n = 11) had CSF analyses for beta-amyloid and tau, and their CSF profile was consistent with the diagnosis of AD.** Exclusion criteria for study participation included comorbid neurological conditions, established psychiatric illness, psychoactive medications that may affect memory performance, and a clinical diagnosis of FTD. Demographic and representative clinical data are shown in **Table 1.**

----- Table 1 here -----

2.3 Memory assessment

Memory performance was evaluated using the Rey Auditory Verbal Learning Test (RAVLT); Babcock Story Recall Test (BSRT) and the Rey-Osterrieth Complex Figure Test (ROCFT) in the ALS group (**Table 2**), as part of a larger cognitive battery assessing other cognitive domains (Christidi, et al., 2012). To exclude the confounding effect of depression or low-mood, the ALS Depression Inventory (ADI) (Ferentinos, et al., 2011, Hammer, et al., 2008) was also administered.

----- Table 2 here -----

2.4 MRI data acquisition

All participants underwent standardised brain imaging on a 3 Tesla Philips Achieva-Tx MR scanner. A 3D-T1-weighted sequence was acquired with a time of repetition (TR): 9.9ms, echo time (TE): 3.7ms, flip angle: 7°, voxel-size 1×1×1mm, matrix size 244×240, 170 slices. DTI data were acquired with an axial single-shot spin-echo echo-planar imaging sequence with 30 diffusion encoding directions and the following parameters: TR: 7299ms, TE: 68ms, flip angle: 90°, field of view: 256×256 mm, voxel size: 2×2×2 mm, 70 slices. FLAIR data were acquired with TR: 11000ms, TI: 2800ms, TE: 125ms, acquisition matrix 384×186, slice thickness 4mm.

2.5 MRI data analysis

2.5.1 Grey matter analyses

A multimodal approach was implemented to comprehensively characterise mesial temporal lobe pathology in the three study groups. Total hippocampal volumes were estimated, hippocampal segmentation was performed to estimate volumes of specific subfields, vertex analyses were undertaken to reflect on shape deformation, and supplementary brain morphometry was carried out to confirm disease-specific cerebral grey matter signatures.

First, total intracranial volume (TIV) was estimated for each participant to be used as a covariate for volumetric analyses. TIV was estimated by linearly aligning each subject's skull-stripped brain to the standard MNI152 brain image in MNI space, calculating the inverse of the determinant of the affine registration matrix and multiplying it by the size of the template. Registration to template was undertaken using FSL-FLIRT (Jenkinson and Smith, 2001), and FSL-FAST was used for tissue type segmentation (Zhang, et al., 2001).

The hippocampus was segmented into cytologically-defined subfields (**Figure 1A**) using version 6.0 of the FreeSurfer image analysis suite (Fischl, 2012). The pre-processing pipeline included the removal of non-brain tissue, segmentation of the subcortical white matter and deep grey matter structures, intensity normalization, tessellation of the grey matter-white matter boundary, and automated topology correction. The hippocampal stream of the FreeSurfer package was used for the accurate segmentation of the following hippocampal subfields: CA1, CA2/3, CA4, fimbria, hippocampal fissure, presubiculum, subiculum, hippocampal tail, parasubiculum, molecular layer; granule cell layer of the dentate gyrus (GC-DG), hippocampal-amygdala transition area (HATA) (Iglesias, et al., 2015).

Vertex analyses were performed using FMRIB's subcortical segmentation and registration tool FIRST to provide quantitative, surface-projected information about focal hippocampal involvement. Vertex locations of individual subjects were projected on the surface of an average template shape as scalar values, positive value being outside the surface and negative values inside. Design matrices included age, gender and education.

To confirm disease-specific grey matter signatures beyond the hippocampus, supplementary morphometric analyses were carried out in the FSL environment (Smith, et al., 2004). Following brain extraction and tissue-type segmentation, grey-matter partial volume images were aligned to the MNI152 standard space using affine registration (Good, et al., 2001). The resulting images were averaged and flipped along the x-axis to create a left-right symmetric, study-specific grey matter template. All native grey matter images were non-linearly co-registered to the study-specific template, modulated by a Jacobian field warp and smoothed with an isotropic Gaussian kernel with a sigma of 3 mm. The threshold-free cluster-enhancement (TFCE) method (Smith and Nichols, 2009) and permutation-based nonparametric inference were used for the comparison of study groups controlling for age, gender and education.

2.5.2 White matter analyses

White matter tractography was conducted using the Brainance DTI Suite (Advantis Medical Imaging, Eindhoven, the Netherlands). First, the raw DWI datasets were motion and eddy-current corrected. Three ROIs were used for the reconstruction of perforant pathway zone (PPZ); (1) on a coronal slice at the middle of the splenium of the corpus callosum the perforant pathway was identified subjacent the corpus callosum, (2) on a coronal slice anterior to the first one at the level of the cerebellar dentate nucleus and (3) the white matter area of the mesial temporal lobe just underlying the hippocampus on a coronal slice anterior to the pons at the level where the projections of the posterior limb of internal capsule are prominent (**Figure 1B**). For the reconstruction of the fornix, three ROIs were placed along the fornix in three coronal slices where the fornix was visible as one bundle (Figure 1C). The following parameters were used for tracking: PPZ: FA threshold: 0.20 and angle threshold: 6; Fornix: FA threshold: 0.25 and angle threshold: 60. In order to provide a multifaceted characterisation of white matter integrity changes, the following diffusivity metrics were extracted for each tract: fractional anisotropy (FA); axial diffusivity (Dax); and radial diffusivity (Drad). Intra- and inter-rater reliability were assessed for all tracts in each participant and showed high intra-class correlation [(ICC) > 0.8].

2.6 Statistical analyses

Assumptions of normality were examined using the Kolmogorov-Smirnov test. Since all variables followed a normal distribution, parametric statistics were applied. Considering the patients' heterogeneous memory performance, a hierarchical cluster analysis using Ward's method of minimum variance with a squared Euclidean distance measure was conducted within the ALS cohort to identify clusters of memory performance (i.e. 'ALS-high performers'; 'ALS-low performers') based on episodic memory measures (i.e. RAVLT-Total Learning, Immediate Recall, Delayed Recall; BSRT-Immediate Recall, Delayed Recall). Ward's method was chosen because of its efficiency to model quantitative variables and its resilience to outlier effects. The squared Euclidean distance coefficient was calculated as it is the most widely used distance coefficient in neuropsychological studies and detects performance levels and patterns within the clusters (Allen and Goldstein, 2013). The following multivariate analyses of covariance (MANCOVAs) were conducted to examine differences in hippocampal subfields and tractography measures (1) HC, ALS and AD and (2) HC, ALS-High, ALS-Low and AD using age, gender, education and TIV (for subfield volumes) as covariates. If a significant main effect of group membership was found ($p < 0.05$), post hoc group comparisons were performed using the false discovery rate (FDR) correction for multiple comparisons. Correlation analyses (Pearson r) were also conducted within ALS group between MRI metrics and patients' clinical data. The significance level was set at $p < 0.05$.

3. Results

3.1 The volumetric profile of hippocampal subfields

The adjusted estimated marginal means and standard error of hippocampal subfield volumes are presented in **Table 3**. Significant differences were observed in total hippocampal volumes between the ALS and AD ($p < 0.001$), and between HC and AD ($p < 0.001$). Significant main effects of group were observed for all subfields with the exception of the parasubiculi. Compared to HC, patients with ALS showed significant volume reductions in bilateral CA2/3, left fimbria, bilateral molecular layer, left GC—DG, left hippocampus-amygdala transition area (HATA) and increased bilateral hippocampal fissure volumes. Compared to AD patients, patients with ALS showed higher volumes in all CA subfields, fimbria, presubiculum and subiculum, hippocampal tail, molecular layer, GC-DG and hippocampal fissure. With the

exception of the hippocampal fissures and hippocampal tail, the AD group exhibited widespread hippocampal atrophy in all hippocampal subfields compared to HC.

Regarding the neuropsychologically-defined ALS subgroups, no significant hippocampal volume differences were identified between HC and ALS-High. ALS-Low performers showed significantly reduced volume in right hippocampus compared to ALS-High ($p = 0.018$) and higher volume bilaterally compared to AD ($p < 0.001$). With regards to hippocampal subfields, the ALS-High cohort exhibited higher bilateral hippocampal fissure volumes (left: $p = 0.013$; right: $p = 0.026$) and decreased left HATA volumes ($p = 0.016$) compared to HC. The direct comparison on subfields between the two ALS groups revealed volume reductions in the ALS-low group in the left fimbria, bilateral hippocampal tails, right CA1, right molecular layer and right GC-DG.

The most affected hippocampal subfields in the study groups were identified using percentage of change with reference to HC and averaging left and right volumes of respective subfields. **Figure 2** illustrates the hippocampal profile of the study groups using estimated marginal means adjusted for age, gender, education and TIV. Based on normative reference values, the HATA and CA2/3 are the most affected subfields in ALS, the presubiculum and subiculum in AD, and the fimbria are affected in both ALS and AD. In the neuropsychologically-defined ALS groups, fimbria and HATA are particularly atrophic in the ALS-Low group and HATA and CA2/3 are the most affected subfields in the ALS-High cohort.

----- Table 3 here -----

3.2 Vertex analyses

Vertex analyses demonstrated that the inferior and superior aspects of both left and right hippocampi are more affected in AD than ALS. No significant differences were identified between ALS-High performers and HCs. Compared to ALS-Low performers, the AD group revealed considerable surface projected atrophy in the right hippocampus at $p < 0.05$ and a trend of atrophy at $p < 0.07$ in the left hippocampus (**Figure 3**). The contrast between the neuropsychologically defined ALS-High and ALS-Low groups also revealed significant shape differences in the lateral aspect of the left hippocampus.

3.3 Whole-brain morphometric analyses

Our VBM-type analyses confirmed disease-associated cerebral signatures. The AD group exhibited widespread bi-temporal and bi-parietal atrophy, while the ALS cohort showed degenerative changes in the bilateral motor cortices (**Figure 4**).

3.4 DTI metrics

The adjusted estimated marginal means of white matter tracts for HC, ALS and AD are summarized in **Table 3**. No significant group effect was identified for FA values in bilateral PPZ. Compared to HC, patients with ALS exhibit significantly lower FA in fornix, higher Dax in right PPZ and higher Dax and Drad in fornix. Compared to AD, ALS patients also exhibited significantly higher FA in fornix, lower Dax and Drad in the bilateral PPZ and fornix. The comparison between HC and AD confirmed widespread white matter pathology in both the PPZ and fornix.

The direct comparison of DTI metrics in ALS-High and ALS-Low performers did not reach statistical significance using FDR corrections. Compared to AD patients, ALS-Low performers exhibited higher FA in the fornix and lower Dax and Drad in both the PPZ and fornix.

Figure 5 depicts the DTI profile of the PPZ and fornix in ALS, AD, ALS-High and ALS-Low performers with reference to HCs. Fornix (specifically on Drad) was the most affected tract in both ALS and AD. Drad and Dax indices of the right PPZ were the second most affected metrics in the study groups.

3.5 Correlation with clinical and memory variables

To avoid type II error, correlation analyses were restricted to imaging metrics showing significant differences between HC and ALS. Following correction for multiple comparisons, neither disease duration, ALSFRS-r, nor memory scores correlated with grey or white matter metrics.

4. Discussion

Our findings indicate that ALS is associated with widespread hippocampal pathology which is independent from disease duration and motor disability and is distinct from AD. The novelty of our study lies in the integration of grey and white matter metrics and the ascertainment of divergent hippocampal signatures in ALS and AD.

4.1 Radiological insights into hippocampal pathology in ALS and AD

There is ample pathological evidence of hippocampal pathology in ALS, but the neuroimaging literature is relatively sparse (Christidi, et al., 2018b). Hippocampal atrophy has been demonstrated in both *C9orf72*-negative and *C9orf72*-positive ALS patients (Bede, et al., 2013b, Floeter, et al., 2018) and is not due to the inclusion of patients with comorbid FTD (Abdulla, et al., 2014, Westeneng, et al., 2015). The majority of existing MRI studies in ALS evaluate entire hippocampal volumes and rely on healthy controls alone as opposed to disease controls (Abdulla, et al., 2014, Bede, et al., 2013b, Bueno, et al., 2018, Christidi, et al., 2018b, Machts, et al., 2018, Westeneng, et al., 2015). We identified selective subfield involvement in ALS in bilateral CA2/CA3, left fimbria, with the relative sparing of the subiculum and presubiculum. A more widespread anatomical pattern of hippocampal change was identified in AD with the preferential involvement of the subiculum and presubiculum. The independence of hippocampal pathology from motor disability is an important finding, as clinical trials of ALS continue to overwhelmingly rely on motor metrics (Mitsumoto, et al., 2014) despite evidence of considerable disease burden in non-motor regions (Christidi, et al., 2018a). Hippocampal subfields are routinely assessed in studies of normal aging, mild cognitive impairment (MCI) and AD (de Flores, et al., 2015) but seldom evaluated in ALS (Westeneng, et al., 2015). Our findings highlight the utility of subfield analyses versus whole-hippocampal volumetry and underlines the selective vulnerability of specific subfields, with distinct cytoarchitecture, dedicated functions and distinct connections (Duvernoy, et al., 2013). The entire ALS group, irrespective of neuropsychological performance, exhibited hippocampal atrophy. Following the stratification of ALS patients based on memory performance, vertex-wise and volumetric differences were identified between ALS-High and ALS-Low performers underscoring the detection sensitivity of neuropsychological tests to capture hippocampal pathology.

The most affected subfields in ALS are CA2/CA3 and HATA, the presubiculum and subiculum in AD and the fimbria is affected in both groups. Fimbria is a white matter subfield which runs along the superomedial edge of the hippocampus and then at the posterior part of the

hippocampus and transitions into the crux of the fornix. Fimbria and HATA have not been previously studied in ALS as a distinct hippocampal subfield due to the methodological constraints of older segmentation approaches (Westeneng, et al., 2015). The involvement of the fimbria in ALS, particularly its atrophy in the ALS-Low cohort, is in line with its role (Zheng, et al., 2018) in memory function and consistent with previous studies suggesting that fornix metrics may indicate AD risk in cognitively normal persons and may also act as prognostic indicators for conversion from MCI and AD (Nowrangi and Rosenberg, 2015).

Our findings also need to be discussed from a ‘tau’ vs ‘TDP’ perspective. The AD cohort in this study is likely to represent a relatively homogenous group pathologically as they had no evidence motor involvement, their VBM analysis confirmed disease specific bi-temporal and bi-parietal pathology, and fulfilled clinical criteria, therefore representing an archetypic AD cohort without clinical and radiological features suggestive of TDP. The hippocampal imaging signature of our AD cohort is also consistent with larger imaging studies (Evans, et al., 2018). Conversely, our ALS cohort is less likely to be pathologically homogenous and may include patients with co-existing tau pathology (Freckler, et al., 1990, Segers, et al., 2012). Thus, fimbria changes detected in the ALS cohort may, in part, be manifestations of coexisting AD pathology.

4.2 Network-wise degeneration in ALS

Our study demonstrates the benefit of using multiple complementary imaging techniques and systematically evaluating grey and white matter structures. (Bede and Hardiman, 2014) Subfield volumetrics and shape analysis provided complementary insights; vertex analyses captured differences between ALS-High and ALS-Low performers, whereas subfield volumetrics revealed selective hippocampal vulnerability. The selective degeneration of cell-specific hippocampal regions is also well recognised in healthy aging (Haigler, et al., 1986).

An additional advantage of appraising multiple grey and white matter metrics is the establishment of their respective sensitivity profiles. From a motor perspective, corticospinal white matter degeneration is invariably detected irrespective of disease duration (Bede and Hardiman, 2018, Pyra, et al., 2010), but the detection of cortical grey matter involvement depends on disease stage, UMN predominance and imaging techniques (Bede, et al., 2013a). In contrast, our study indicates that hippocampal grey matter metrics (subfields volumes and vertex metrics) readily detect mesial temporal lobe pathology. The ranking of the detection sensitivity of various imaging metrics is indispensable to establish which index may be used in diagnostic, monitoring or prognostic applications. (Schuster, et al., 2015, Schuster, et al., 2017)

Our findings also confirm the concomitant degeneration of associated grey and white matter structures in ALS (Bede, et al., 2018a, Nasserolelami, et al., 2017). The fimbria was the most affected hippocampal subfield in both ALS and AD which is consistent with the widespread diffusivity changes identified in the fornix. The perforant pathway linking the entorhinal cortex, dentate gyrus, CA and subiculum is preferentially affected in our AD cohort, which is consistent with the selective degenerative changes observed in the hippocampal subfields it connects. The evaluation of multiple diffusivity metrics helps the biological characterisation of white matter degeneration. While FA is widely regarded a composite proxy of white matter integrity, Dax is primarily regarded as an axonal marker and Drad also reflects on myelin related pathology (Song, et al., 2005, Sun, et al., 2006).

4.3 Pathological correlates

The lack of correlations between clinical measures and radiological findings do not directly support a sequential model of pathological spread in ALS and highlight a possible inconsistency between radiological observations and the pathological staging. In ALS, pathological TDP-43 can be detected in the hippocampal CA4-CA1/subiculum in 25% of post mortem samples (Geser, et al., 2008). Pathological TDP-43 burden in the anteromedial temporal lobe and the hippocampal formation is now regarded as a stage-defining feature of a widely used pathological staging system in ALS (Brettschneider, et al., 2013), corresponding to ‘Stage 4’ of the disease.

According to the three-stage model of PPZ degeneration, stage I (“inclusion stage”) is characterized by TDP-43-positive cytoplasmatic inclusions in the granular cells of the dentate gyrus, stage II (i.e. “early perforant stage”) by gliosis and neuronal loss in the transentorhinal cortex and stage III (i.e. “advanced perforant stage”) by the degeneration of the molecular layer of the dentate gyrus (Takeda, et al., 2009). These stages of hippocampal pathology are closely linked to its white matter architecture, suggestive of connectivity-based disease propagation (Schmidt, et al., 2016) and supports the notion that interconnected grey matter regions become gradually affected as opposed to contiguous spread (Braak, et al., 2013). This is further supported by the observation the despite anatomical proximity certain subfields, such as the subiculum are not affected in ALS.

4.4 Clinical relevance

Our findings may have implications for caregiver support, screening strategies and clinical trial designs. Memory impairment in ALS remains relatively under recognised despite its

quality of life implications and potential effect on compliance with assistive devices, engagement in rehabilitation efforts, and participation in clinical trials (Lomen-Hoerth, et al., 2003, Olney, et al., 2005). Pharmacological studies of ALS continue to rely on survival and clinical scores as key outcomes measures overlooking the biomarker potential of quantitative neuroimaging measures which are widely used in studies of other neurological disorders. (Chipika, et al., 2019) Furthermore, current clinical trials overwhelmingly focus on mobility, motor deficits, and respiratory dysfunction as endpoints despite irrefutable evidence of widespread extra-motor involvement in ALS (Beeldman, et al., 2016, Christidi, et al., 2018a). Frontotemporal involvement is a key contributor to clinical heterogeneity in ALS, it has survival (Elamin, et al., 2011, Ishaque, et al., 2018) and caregiver burden implications (Caga, et al., 2018), yet it is not evaluated in most clinical trials. At a time when ALS-specific cognitive instruments have been extensively validated (Abrahams, et al., 2014) and non-invasive neuroimaging techniques readily capture motor and extra-motor changes, the incorporation of cognitive testing and quantitative imaging protocols into pharmacological trials seems overdue.

4.5 Study limitations and future directions

This study is not without limitations. The biggest shortcoming of this report is the the small sample size of our AD group which was included as a disease control group. Despite the sample size limitation of the AD cohort, this group exhibits disease-associated atrophy patterns typical of AD. Due to disease-specific characteristics, the AD cohort of our study is older than the ALS patients and healthy controls included in our imaging analyses. While age was strictly included as a covariate in all of our statistical models, in order to ensure that age-differences have not influenced our findings, an additional sub-analysis was conducted with an age ($p = 0.135$), education ($p = 0.078$) and gender ($p = 0.334$) matched sub-cohort of AD patients and controls. The structural differences identified by this age-matched supplementary analysis were consistent with our main findings indicating that the identified hippocampal patterns are disease related and not driven by age. This study focused on ALS, but as relatively little is known of the hippocampal profile of other motor neuron disease phenotypes, the inclusion of additional “disease controls”, such as primary lateral sclerosis, Kennedy’s disease or spinal muscular atrophy patients would have potentially contributed important additional insights (Finegan, et al., 2019b, Querin, et al., 2018, Querin, et al., 2019). Our choice of a two-cluster model to stratify our ALS cohort for memory performance was also driven by sample size considerations. Another potential limitation of our study is the risk of partial volume effects on the tractographic reconstruction of the fornix. CSF contamination however typically affects axial and radial

diffusivity metrics, and FA is thought to be less influenced by it (Douet and Chang, 2014). Dedicated acquisition methods (Baron and Beaulieu, 2015) and post-processing approaches (Chad, et al., 2018, Metzler-Baddeley, et al., 2012) would be required if the primary focus of the study would be the characterisation of fornix integrity. Our fornix findings however are in line with previous studies of ALS and AD and consistent with recent reports in neuropsychologically unaffected subjects with elevated A β burden (Rabin, et al., 2019). An additional drawback of the study is the lack of comprehensive genetic profiling to explore whether *C9orf72* hexanucleotide repeats may contribute to the hippocampal disease burden observed in this study. However, the potential influence of *C9orf72* hexanucleotide carriers in our sample is not considered substantive since our sample only included sporadic ALS cases, and the prevalence of *C9orf72* hexanucleotide carriers in the Greek population is 8% in sporadic cases (Mok, et al., 2012). Previous studies indicate that while *C9orf72* negative ALS patients exhibit overall hippocampal pathology (Machts, et al., 2015), which may be more marked in hexanucleotide carriers (Bede, et al., 2013b). Finally, spinal fluid panels for tau, amyloid and neurofilaments in the ALS cohort would have provided important additional insights to reflect on the molecular substrate of hippocampal pathology. Notwithstanding these limitations, our study demonstrates the divergent mesial temporal lobe profile of ALS and AD highlighting the need for further wet-biomarker supported and histopathologically validated studies. Emerging imaging modalities such as neurite orientation dispersion and density imaging (NODDI) (Barritt, et al., 2018) and high field MR imaging (Cardenas, et al., 2017) may help to characterise mesial temporal lobe degeneration in ALS further.

5. Conclusions

Patients with ALS exhibit disease-specific patterns of hippocampal pathology which is distinct from AD. The preferential involvement of specific hippocampal subfields connected by selectively affected white matter projections suggests network-based disease propagation as opposed to contagious spread. The comprehensive characterisation of mesial temporal lobe degeneration in ALS has implications for screening practices, supportive interventions, clinical trial designs and expanding the extra-motor profile of ALS.

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Figure Legends

Figure 1. Left: Hippocampal segmentation and resulting subfields in coronal (1), sagittal (2) and axial views (3) Right: the tractographic reconstruction of the right perforant pathway (4) and the fornix (5) with their respective region-of-interest gates on fractional anisotropy maps.

Figure 2. The regional hippocampal profile of each study group: ALS (A), AD (B), ALS-High (C) and ALS-Low (D), and their comparative volumetric profile (E) with reference to healthy controls/ Subfield volumes are averaged across left and right. CA = Cornu Ammonis; GC-DG = granule cell layer of dentate gyrus; HATA = hippocampus-amygdala transition area; ALS = Amyotrophic Lateral Sclerosis; AD = Alzheimer disease; HCs = healthy controls.

Figure 3. The comparative hippocampal atrophy profile of the AD, ALS-High and ALS-Low cohorts. Surface projected patterns of atrophy are highlighted in orange and yellow and the underlying hippocampal mesh volumes are represented in green, pink and blue. Comparisons are adjusted for age, gender and education. ALS = Amyotrophic Lateral Sclerosis; AD = Alzheimer's disease.

Figure 4. Cortical atrophy patterns in ALS and AD identified by voxel-based morphometry using age, gender and education as covariates. ALS = Amyotrophic Lateral Sclerosis; AD = Alzheimer's disease.

Figure 5. The diffusivity profile of the perforant pathway and fornix in each study group: ALS (A), AD (B), ALS-High (C) and ALS-Low (D), and their comparative profile (E) with reference to healthy controls. L = Left; R = Right; PPZ = perforant pathway zone; Fx = fornix; FA = fractional anisotropy; Dax = axial diffusivity; Drad = radial diffusivity; ALS = Amyotrophic Lateral Sclerosis; AD = Alzheimer disease; HCs = healthy controls

Tables

Table 1. The demographic and clinical profile of study participants

	ALS (n = 50)	AD (n = 18)	HC (n = 40)	Statistical differences
Age (years)	61.70 ± 10.22	76.28 ± 6.37	59.00 ± 6.28	AD>HC (p<0.001) AD>ALS (p<0.001)
Gender (M / F)	28 / 22	2 / 16	18 / 22	AD vs HC (p=0.012), AD vs. ALS (p=0.001)
Education (years)	10.62 ± 3.53	7.89 ± 5.06	14.00 ± 2.43	HC>ALS,AD (p<0.001) ALS>AD (p=0.014)
Handedness (Rt / Lt)	50 / 0	18 / 0	40 / 0	Ns
Disease duration from symptom onset (m)	17.72 ± 18.34	33.67 ± 21.60	-	ALS<AD (p=0.004)
ALSFRS-R	39.80 ± 6.10	-	-	-
ALS onset (S / B)	38 / 12	-	-	-
Riluzole (Y / N)	14 / 36			
MMSE	26.08 ± 2.22	23.00 ± 3.07	28.50 ± 1.06	HC>ALS, AD (p < 0.001) ALS>AD (p = 0.001)

ALS = amyotrophic lateral sclerosis; **AD** = Alzheimer's dementia; **HC** = healthy controls; **M / F** = male / female; **Rt / Lt** = right / left; **m** = months; **ALSFRS-R** = Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; **S / B** = spinal / bulbar; **Y / N** = Yes / No; **MMSE** = Mini-Mental State Examination. Differences in MMSE are adjusted for age, gender and education.

Table 2. Memory profile of the ALS groups and ALS-High and ALS-Low subgroups

	ALS		ALS-High		ALS-Low	
	Raw	Z-score	Raw	Z-score	Raw	Z-score
RAVLT-Learning	38.44±9.03	-0.49±1.95	46.22±5.97	0.67±1.84	31.81±4.94	-1.47±1.45
RALVT-IR	7.14±3.00	-0.34±2.98	9.35±2.27	1.36±2.54	5.26±2.16	-1.79±2.55
RAVLT-DR	5.84±2.96	-1.86±3.15	7.48±2.71	-0.76±3.06	4.44±2.49	-2.79±2.96
BSRT-IR	10.02±2.17	-0.82±1.22	10.87±2.24	-0.56±1.32	9.30±1.86	-1.03±1.11
BSRT-DR	8.22±3.50	-1.53±1.78	9.96±3.88	-0.81±1.91	6.74±2.31	-2.14±1.43
ROCFT-IR*	13.32±6.11	-0.69±1.17	16.56±5.94	-0.25±1.16	10.86±5.09	-1.02±1.08

ALS = amyotrophic lateral sclerosis; RAVLT = Rey Auditory Verbal Learning Test; BSRT = Babcock Story Recall Test; ROCFT = Rey Osterreith Complex Figure Test; IR = Immediate Recall; DR = Delayed Recall. Z-scores were calculated based on available normative data ((Messinis, et al., 2007, Meyers JE, 1995, Zalonis I, 2008) (Messinis et al., 2007; Zalonis et al., 2008; Meyers *Available for 16 ALS-High and 21 ALS-Low performers.

Table 3. Volumes of specific hippocampal subfields and the entire hippocampus. The white matter profile of the PPZ and the fornix in ALS, AD and HC.

	HC (N = 40)	ALS (N = 50)	AD (N = 18)	ALS-High (N = 23)	ALS-Low (N = 27)	ALS vs HC	ALS vs AD	AD vs HC	ALS- High vs HC	ALS- High vs ALS- Low	ALS- Low vs AD
	Mean ± SD					p-values					
Total hippocampal volumes											
Left	3423.84±422.85	3223.46±458.38	2415.54±342.73	3305.07±396.81	3153.95±501.84		<0.001	<0.001			<0.001
Right	3506.29±444.01	3308.92±446.37	2542.70±393.01	3412.04±396.29	3221.09±474.55		<0.001	<0.001		0.018	<0.001
Subfields in the left hemisphere											
CA1	632.03±91.25	594.36±97.22	467.90±73.10	607.49±98.07	583.17±96.91		<0.001	<0.001			0.016
CA2/CA3	202.25±28.90	189.14±33.09	143.51±27.90	187.66±21.51	190.40±40.85	0.049	<0.001	<0.001			0.005
CA4	251.70±31.25	237.39±35.51	176.04±30.25	240.23±27.62	234.97±41.44		<0.001	<0.001			0.001
Fimbria	72.98±12.41	61.73±19.64	34.67±10.65	69.27±19.77	55.31±17.40	0.007	0.002	<0.001		0.013	0.037
Hippocampal fissure	150.35±27.08	178.57±35.13	169.57±37.40	172.17±40.14	184.02±29.93	<0.001	0.040		0.013		
Presubiculum	313.52±47.34	299.03±47.62	219.01±32.57	303.36±42.99	295.35±51.76		<0.001	<0.001			0.001
Subiculum	448.95±61.43	425.82±62.08	310.31±48.79	434.56±55.44	418.37±67.31		<0.001	<0.001			0.001
Hippocampal tail	520.62±74.85	508.34±89.37	390.52±55.91	535.10±78.09	485.54±93.37		0.010			0.035	
Parasubiculum	56.33±12.56	53.37±13.65	48.58±9.48	51.10±10.37	55.31±15.86						
Molecular layer	571.55±73.67	531.82±81.33	388.06±59.30	546.62±72.15	519.22±87.77	0.020	<0.001	<0.001			0.001
GC-DG	293.12±37.40	270.44±42.22	194.88±32.86	276.19±33.44	265.54±48.56	0.021	<0.001	<0.001			0.001
HATA	60.77±11.94	52.02±8.52	42.07±5.10	53.49±7.84	50.76±9.01	<0.001		0.002	0.016		
Subfields in the right hemisphere											
CA1	648.04±92.79	618.67±84.67	489.56±83.20	639.21±80.30	601.18±85.81		<0.001	<0.001		0.041	0.005
CA2/CA3	224.59±36.50	207.10±35.01	164.06±28.12	211.76±31.46	203.14±37.91	0.026	0.006	<0.001			0.041
CA4	265.54±35.90	249.90±36.22	197.82±32.50	256.37±32.21	244.40±39.06		<0.001	<0.001			0.005
Fimbria	70.31±15.10	63.92±20.17	40.05±16.89	69.91±18.28	58.82±20.62		0.021	0.009			
Hippocampal fissure	162.18±29.38	191.63±36.39	180.33±42.81	182.63±37.29	199.30±34.45	<0.001	0.002		0.026		0.001
Presubiculum	298.44±42.44	283.27±47.40	211.78±39.68	287.14±47.40	279.97±48.05		<0.001	<0.001			0.001
Subiculum	443.93±59.09	426.36±62.48	319.66±58.91	434.01±53.34	419.84±69.66		<0.001	<0.001			0.001
Hippocampal tail	545.56±74.13	518.59±84.42	400.22±60.40	542.76±77.47	498.01±85.99		0.002			0.026	0.036
Parasubiculum	55.57±12.31	53.54±13.82	43.53±8.99	51.63±12.94	55.16±14.56						

Molecular layer	584.41±76.96	544.43±76.24	408.41±69.53	563.54±66.92	528.15±81.04	0.020	<0.001	<0.001		0.026	0.001
GC- DG	307.73±41.36	286.59±43.15	221.56±39.13	296.41±37.63	278.22±46.40		<0.001	<0.001		0.041	0.005
HATA	62.17±9.84	56.55±11.34	46.05±8.83	59.29±9.40	54.22±12.46			0.013			
White Matter tract DTI metrics											
Lt Hippocampal PPZ											
FA	0.39±0.02	0.39±0.02	0.39±0.02	0.40±0.02	0.39±0.02						
Dax (×10⁻³)	1.18±0.05	1.19±0.04	1.25±0.05	1.19±0.04	1.18±0.05		<0.001	<0.001			0.001
Drad (×10⁻³)	0.63±0.03	0.63±0.03	0.68±0.04	0.63±0.03	0.63±0.03		<0.001	<0.001			0.001
Rt Hippocampal PPZ											
FA	0.40±0.02	0.39±0.02	0.38±0.02	0.39±0.02	0.39±0.03						
Dax (×10⁻³)	1.16±0.05	1.19±0.05	1.29±0.09	1.18±0.05	1.19±0.06	0.024	<0.001	<0.001			0.005
Drad (×10⁻³)	0.61±0.003	0.63±0.04	0.71±0.06	0.63±0.03	0.64±0.04		<0.001	<0.001			0.005
Fornix											
FA	0.37±0.02	0.35±0.04	0.30±0.04	0.36±0.03	0.34±0.04	0.036	0.008	<0.001			0.042
Dax (×10⁻³)	1.92±0.01	2.04±0.02	2.28±0.02	1.97±0.02	2.09±0.02	0.002	0.002	<0.001			0.027
Drad (×10⁻³)	1.10±0.09	1.21±0.02	1.47±0.02	1.16±0.01	1.26±0.02	0.004	<0.001	<0.001			0.005

HC = healthy controls; **ALS** = amyotrophic lateral sclerosis; **AD** = Alzheimer disease; **CA** = Cornu Ammonis; **GC-DG** = granule cell layer of dentate gyrus; **HATA** = hippocampus-amygdala transition area; **WM** = white matter; **DTI** = diffusion tensor imaging; **PPZ** = perforant pathway zone; Lt / Rt = left / right; **FA** = fractional anisotropy; **Dax** = axial diffusivity; **Drad** = radial diffusivity; **SD** = standard deviation. Absolute hippocampal subfields volumes (mm³) and DTI metrics (FA, Dax, Drad) are presented for each group. Age, gender, education and TIV (for hippocampal subfields) were used as covariates in the MANCOVA analyses. n/a: post-hoc comparisons were not conducted due to non-significant ($p > 0.05$) F-test. Bold post-hoc comparisons are significant after false-discovery rate (FDR) correction for multiple comparisons.

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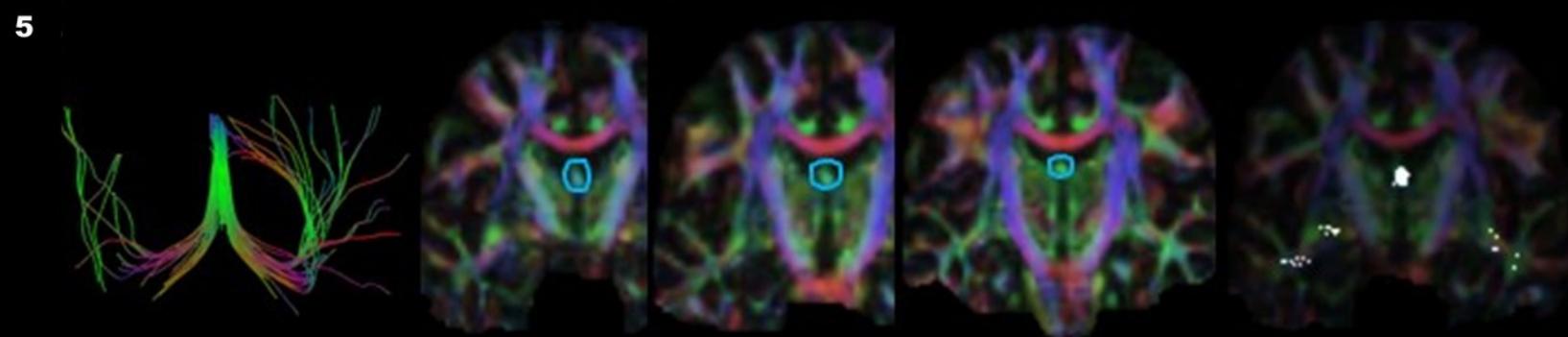
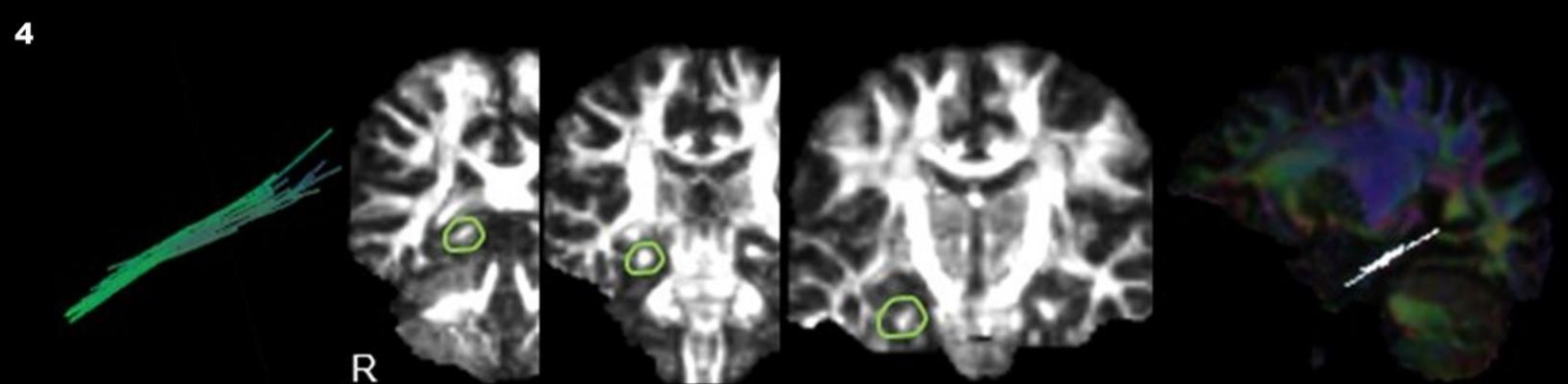
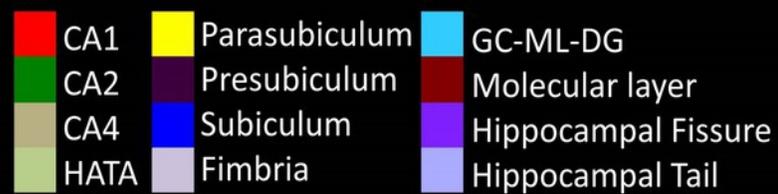
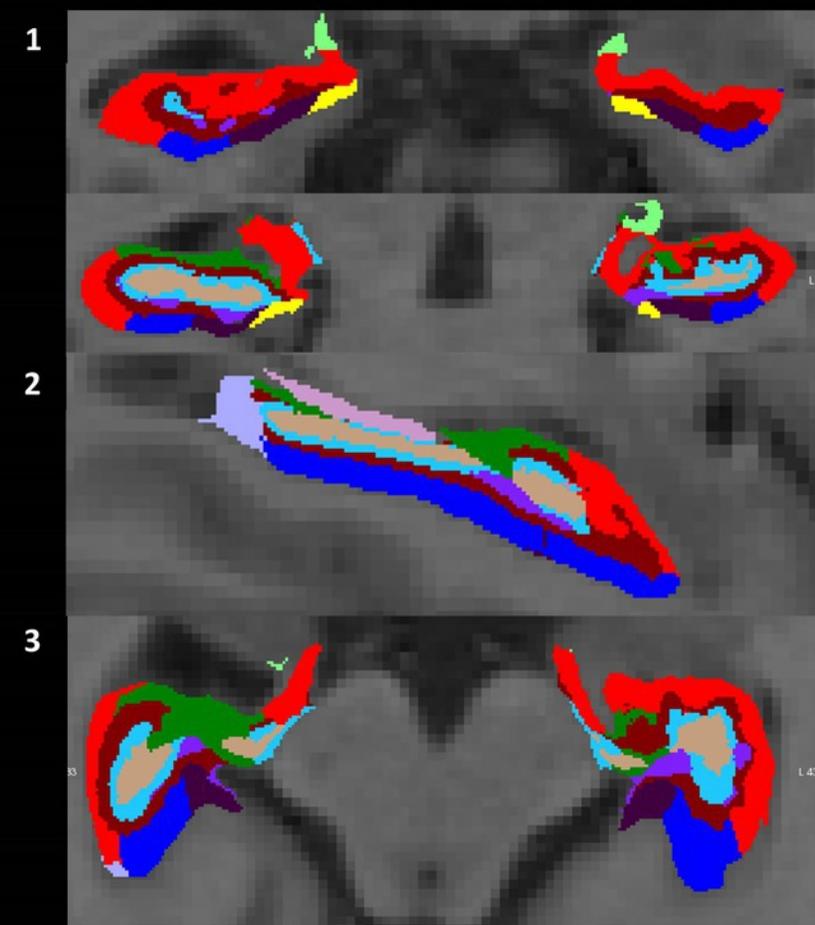
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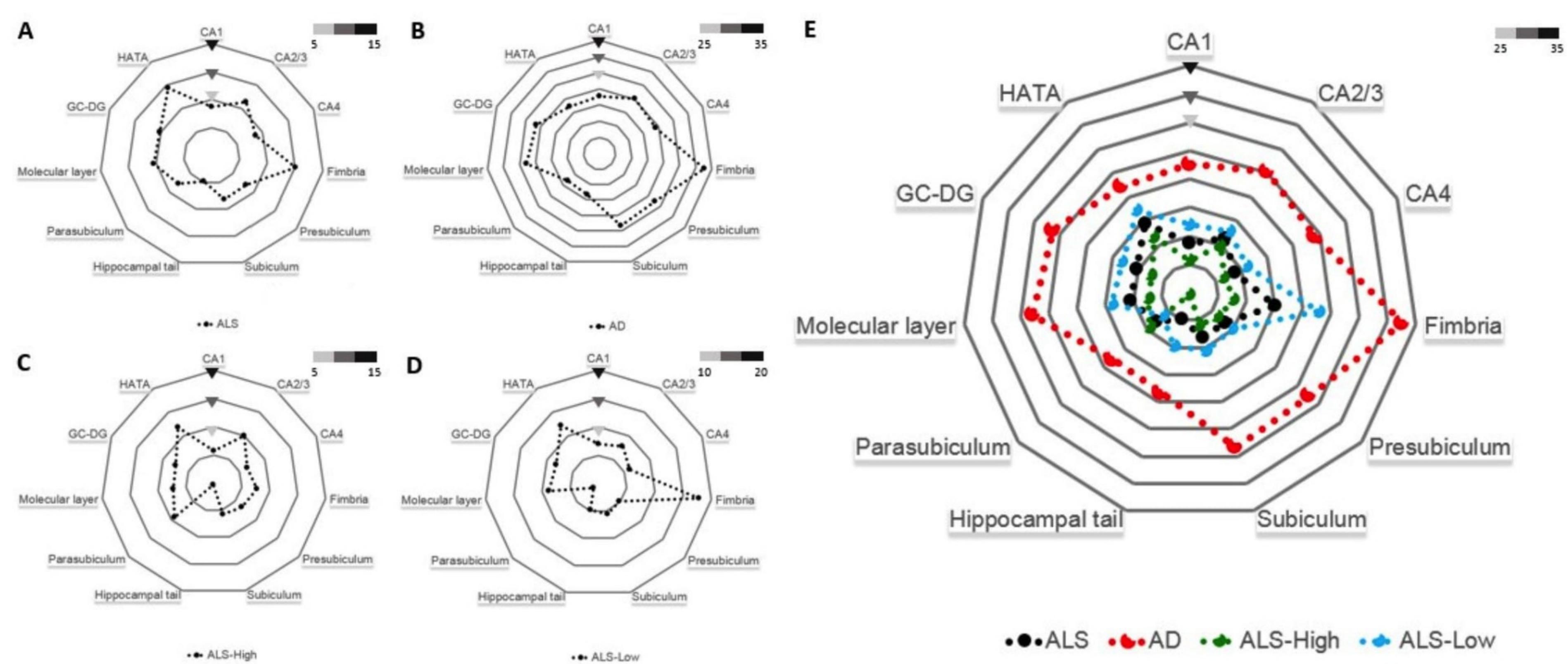
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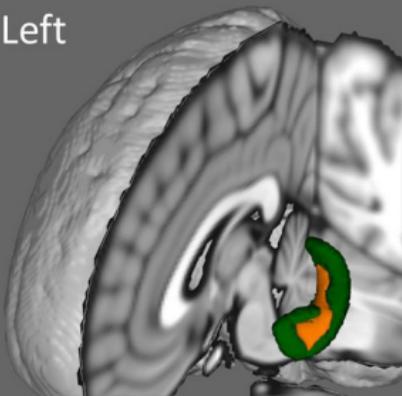
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AD < ALS-High

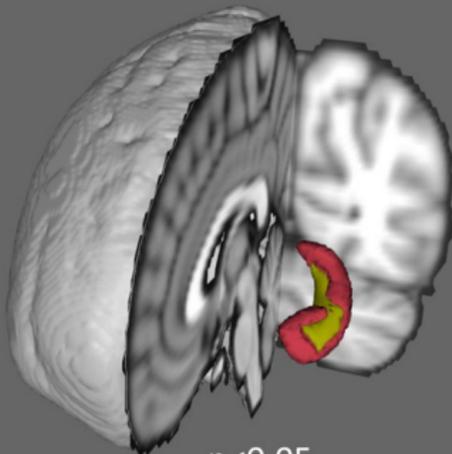
AD < ALS-Low

ALS-Low < ALS-High

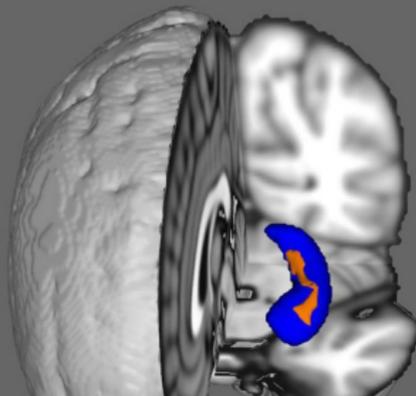
Left



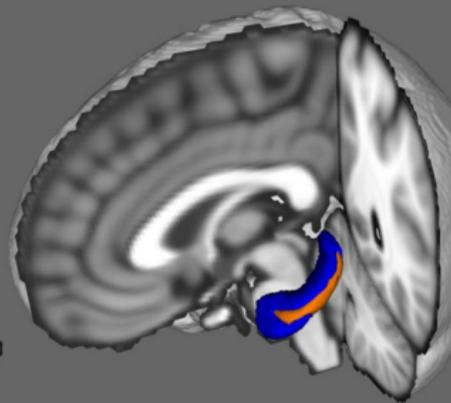
$p < 0.05$



$p < 0.05$

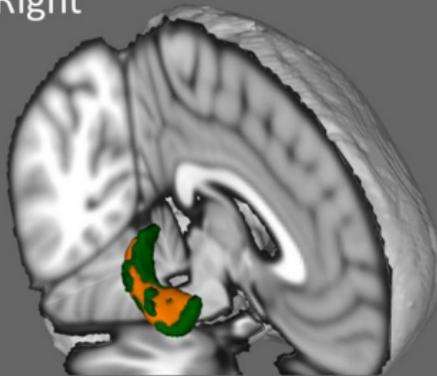


$p < 0.07$



$p < 0.05$

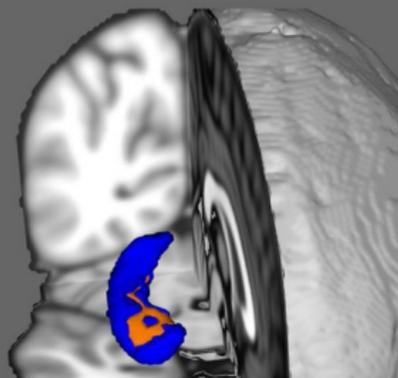
Right



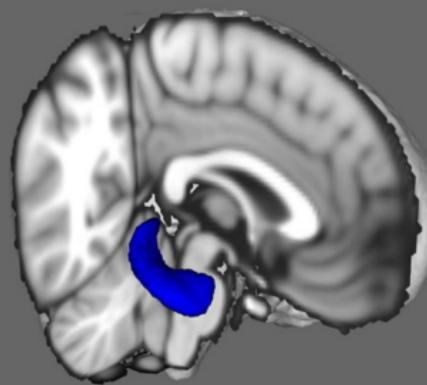
$p < 0.05$



$p < 0.05$



$p < 0.05$



$p < 0.05$

AD < ALS $p < 0.001$

ALS < HC $p < 0.01$

AD < HC $p < 0.001$

