Title

CSF neurogranin as a neuronal damage marker in CJD: a comparative study with AD.

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ABSTRACT

Objective: To investigate whether cerebrospinal fluid (CSF) neurogranin concentrations are altered in sporadic Creutzfeldt-Jakob disease (CJD), comparatively with Alzheimer's disease (AD), and associated with neuronal degeneration in brain tissue.

Methods: CSF neurogranin, total-tau(tau), neurofilament light(NFL) and 14-3-3 protein were measured in neurological controls (NC,n=64), (AD (n=46) and CJD (n=81). The accuracy of neurogranin discriminating the three diagnostic groups was evaluated. Correlations between neurogranin and neurodegeneration biomarkers, demographic, genetic and clinical data were assessed. Additionally, neurogranin expression in post-mortem brain tissue was studied.

Results: Compared to NC, CSF neurogranin concentrations were increased in CJD (4.75 times of NC; $p<0.001$, AUC (95%CI)=0.96 (0.93-0.99) and AD (1.94 times of NC; $p<0.01$, AUC (95%CI)=0.73 (0.62-0.82), and were able to differentiate CJD from AD ($p<0.001$, AUC (95%CI)=0.85 (0.78-0.92)). CSF tau was increased in CJD (41 times of NC) and in AD (3.1 times of NC), both at $p<0.001$. In CJD, neurogranin positively correlated with tau (rho=0.55,$p<0.001$) and was higher in 14-3-3-positivity ($p<0.05$), but showed no association with NFL (rho=0.08,p=0.46). CJD-MM1/MV1 cases displayed higher neurogranin levels than VV2 cases. Neurogranin was increased at early CJD disease stages and was a good prognostic marker of survival time in CJD. In brain tissue, neurogranin was detected in the cytoplasm, membrane and post-synaptic density fractions of neurons, with reduced levels in AD, and more significantly in CJD, where they correlated with synaptic and axonal markers.

Conclusions: Neurogranin is a new biomarker of prion pathogenesis with diagnostic and prognostic abilities, which reflects the degree of neuronal damage in brain tissue in a CJD subtype manner.

Keywords

Neurogranin, cerebrospinal fluid; neurodegenerative dementias; Creutzfeldt-Jakob disease, Alzheimer's disease, tau, neurofilament light.

INTRODUCTION

Neurogranin is a calmodulin-binding protein abundantly expressed in the soma and dendrites of neurons of the telencephalon[1,2] involved in synaptic plasticity and long-term potentiation[3,4]. Neurogranin has been suggested to be a specific cerebrospinal fluid (CSF) Alzheimer's disease (AD) biomarker, since its concentration is increased in AD, but not in other neurodegenerative diseases (*i.e.*, frontotemporal dementia, Lewy body dementia, Parkinson's disease, progressive supranuclear palsy, multiple system atrophy and Huntington's disease)[5–7]. Although CSF neurogranin presents only moderate diagnostic value for AD[5,8], this can be improved when combined with other CSF biomarkers of AD such as tau and neurofilament light (NFL)[9]. In AD, CSF neurogranin displays strong positive correlation with other AD biomarkers such as tau and phospho-tau[5,10–13], while

weak or no correlations were detected with amyloid-beta42, a biomarker of amyloid plaques load[5,10,13].

A prognostic value for neurogranin in AD has been proposed, as its CSF concentration is differentially elevated in mild cognitive impairment (MCI) patients with biomarker AD-signature[11] as well as in MCI patients who progress to AD dementia compared to those who remain cognitively stable[10,13]. Similarly, CSF neurogranin correlates with rate of cognitive decline in MCI[14] and with reduction of brain volume in AD[8]. In cognitively normal individuals, CSF neurogranin is also useful in predicting future cognitive impairment[8]. Regrettably, neurogranin analysis in paired plasma-CSF samples indicated that the AD-specific increased CSF levels are not reproduced in plasma, discarding the potential use of blood neurogranin measurements for diagnostic or prognostic purposes[15].

Although extensive work has been done in AD, data is lacking regarding neurogranin levels in other diseases presenting substantial synaptic and neuronal loss. This is the case of prion diseases, one of whose fundamental characteristics is synaptic degeneration and disorganization, which leads to neuronal loss and spongiform changes. Indeed, over a 30% reduction in the relative synaptic index has been reported in prion disease-affected brains compared to controls[16]. Similarly to AD, synaptic loss occurs at early stages of prion diseases[17], and it is suggested that synaptic pathology is initiated at the synaptic spine[18]. Experiments conducted in prion disease mouse models revealed that axon terminal degeneration and synaptic loss precede neuronal death and are associated with the onset of clinical symptomatology[19]. Sporadic Creutzfeldt-Jakob disease (CJD) is the most prevalent human prion disease characterized by rapidly progressive dementia and short disease duration [20]. The combination of genotype at codon 129 (methionine or valine) and PrPSc type (1 or 2 based on the size of protease resistant PrP fragments) gives rise to different CJD subtypes with characteristic disease phenotype and neuropathological features. Thus, synaptic and neuronal damage, neuroinflammation, deposition of pathogenic prion protein (PrPSc) and lesion profile occur in a well-defined regional- and subtype-specific manner[17,21–23].The most prevalent subtypes are CJDMM1/CJDMV1 (60-70% of the cases) with predominant cortical affection and, CJD VV2 (~16% of the cases), with prominent cerebellar affection [22]. Several pathological mechanisms are suggested to contribute to CJD synaptic pathology, including the accumulation of the abnormal form of prion protein in synaptic structures[24].

In the present study, we quantified CSF neurogranin in CJD and AD cases in order to comparatively unveil its diagnostic and prognostic potential. We also characterized the presence of neurogranin in CJD and AD brains to investigate the underlying pathological conditions in the central nervous system that may lead to the observed disease-specific CSF signatures.

METHODS

Antibodies

The monoclonal neurogranin antibody Ng2 was produced using KLH-conjugated peptide Ng52–75 as immunogen, as described previously[14] and was used (1:400) for immunohistochemistry (IHC). The neurogranin antibody Ng36 was generated using the same protocol, but with KLH-conjugated peptide Ng63-75 as immunogen and was used for western blot (1:6000). Antibodies against sodium-potassium adenosine triphosphatase (ATPaseNa/Kβ, Affinity-MA3-930;1:2000), glyceraldehyde3-phosphate dehydrogenase (GAPDH, Abcam ab9485;1:2500), postsynaptic density protein 95 (PSD-95, Thermo-Fisher-7E3-1B8;1:1000), synaptophysin (SYNP, Novocastra-NCL-L-SYNAP-299;1:4000), total-tau (tTau, Sigma-T5530;1:500) and beta-actin (β-actin, Sigma-A5316;1:30000) were used in the western blot experiments.

Patients and CSF sampling

Neurological controls (NC) were composed of patients diagnosed with a neurological or psychiatric disease non-associated with a primarily neurodegenerative disease, and were diagnosed according to acknowledged standard neurological clinical and para-clinical findings based on the 10th revision of the International Statistical Classification of Diseases definitions. NC include the following diagnoses: alcohol abuse, astrocytoma, bipolar disorder, cerebral lymphoma, cerebral vasculitis, depression, epilepsy, Graves' disease, acute or chronic headache, acute hypoxia, ischemic stroke, meningitis, multiple infarct, pain syndromes, paraneoplasia, paranoid psychosis, peripheral polyneuropathy, psychosis, schizophrenia, vascular encephalopathy, vasculitis and vertigo. AD was diagnosed according to the National Institute on Aging-Alzheimer's Association workgroups(NIA-AA) criteria[25]. CJD was diagnosed according to consensus criteria[26], 60 definite and 21 probable CJD cases were included. All CSF samples were collected at the Clinical Dementia Center and the National Reference Center for CJD Surveillance in the Department of Neurology of the University Medical Center of Göttingen, Germany.

Lumbar punctures (LPs) were performed for diagnostic purposes at the first evaluation. For disease stage, samples were stratified in three categories according to whether CSF was collected in the first (early) (time of LP to disease onset/total duration of the disease < 0.33), second (middle) (0.33–0.66) or third (last) (> 0.66) stage of the disease. Disease duration was recorded as the time (in months) from symptom onset to the death of the patient.

Brain samples

Brain tissue was obtained from the Institute of Neuropathology HUB-ICO-IDIBELL-Biobank following the guidelines of Spanish legislation on this matter (Real Decreto de Biobancos 1716/2011). Control cases had not suffered from neurologic or psychiatric diseases, infections of the nervous system, brain neoplasms, or systemic and central immune diseases, and did not have abnormalities in the neuropathological examination. Neurofibrillary tangles stages were categorized according to Braak and Braak modified for paraffin sections[27]. CJD cases underwent neuropathological

diagnosis according to established neuropathological criteria[28]. Information about brain cases used in this study is detailed in Supplementary Table 1. CSF was not available for study in any of the post-mortem brain series.

CSF analyses

Neurogranin and NFL were quantified using two in-house enzyme-linked immunosorbent assay (ELISA) as described before[13,29]. Total-tau (tau) was quantified using the ELISA kit INNOTEST®hTAU-Ag (Fujirebio Europe, Ghent, Belgium). CSF was analyzed for the presence of 14-3-3 protein by Western blot according to established CJD diagnostic protocol[30]. The analysts were blinded to clinical data.

Immunohistochemistry

De-waxed sections, 4 micrometer thick, were processed for immunohistochemistry and incubated at 4ºC overnight with one of the primary antibodies and then incubated with R.T.U. Biotinylated Universal Antibody (Vector,BP1400) for 30 min at room temperature followed by R.T.U. HRP-Streptavidin (Vector,SA-5704). The peroxidase reaction was visualized with diaminobenzidine and hydrogen peroxidase. Control of the immunostaining included omission of the primary antibody. Immunostaining of neurogranin levels were quantified using Image J software, using thresholding tool settings to subtract background and allow quantification of neuronal neurogranin.

Brain homogenates, subcellular fractionation and western blot.

The purification of PSD fractions from human post-mortem brain tissue was performed as published before[31]. Brain homogenates and fractions were mixed with SDS-PAGE sample buffer, boiled, and subjected to 8-15% SDS-PAGE. Gels were transferred onto nitrocellulose membranes and probed for specific immunodetection by chemiluminescence (ECL-Amersham) using the indicated antibodies. Densitometries were carried out with the ImageJ software and for brain homogenates values were normalized using β-actin or GAPDH levels. Since Neurogranin was expressed in all subcellular fractions, difference among NC, AD and CJD cases was determined in the input. Brain homogenates were mixed with NuPAGE (Thermo-Fisher) LDS buffer and Reducing Agent, boiled and subjected to electrophoresis in NuPAGE Bis-Tris 4-12% gels (Thermo-Fisher). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and immunodetection was performed as mention above. Densitometries were determined with the ImageJ software and were normalized using β-actin levels.

Statistical tests

According to distributional features, Mann-Whitney U tests or unpaired t-tests were used to compare two groups of samples; Kruskal-Wallis test followed by Dunn's post-hoc tests or ANOVA test followed by Tukey's post-hoc tests was applied for multiple comparisons. To assess the diagnostic accuracy of neurogranin in the discrimination of the diagnostic groups, receiver operating characteristic (ROC) curve analyses were carried out and areas under the curve (AUC) with 95% confidence intervals (95%CI) were calculated using GraphPad-Prism6.01. The best cut-off values were estimated based on the Youden index. Spearman rank and Pearson correlation coefficients were

used to assess associations between continuous biomarker levels. Comparison between AUC was performed using the DeLong's test[32], available in the R package pROC[33]. To determine the association between neurogranin, NFL and tau concentrations and total disease duration we used a fractional polynomial approach based on linear regression methodology as provided in the Stata package "mfp". The prognostic capacity of potential biomarkers was assessed using Somers' D, Harrells's C (the higher the better the prognosis) and Brier Scores at 12 months (the lower the score, the better the prognosis) based on Cox regression models.

RESULTS

CSF neurogranin in AD and CJD

The study population included NC (n=64), AD (n=46) and CJD (n=81) cases. CSF NFL showed a mild increase in AD (1.3 times of NC;$p<0.05$) and a marked increase in CJD (4.3 times of NC;$p<0.001$). CSF tau showed a moderate increase in AD (3.1 times of NC; $p<0.001$) while levels in CJD were very markedly (41 times) higher than in NC ($p<0.001$). Additionally, increased tau and NFL concentrations were detected in CJD compared to AD ($p<0.001$) (Figure 1A) in agreement with previous studies [34,35].

Highest neurogranin concentrations were detected in CJD (571±291 pg/mL), followed by AD (233±191pg/mL) and NC (120±65pg/mL) (Figure 1A). Neurogranin was significantly different in NC vs. AD ($p<0.01$), NC vs. CJD ($p<0.001$) and AD vs. CJD ($p<0.001$) (Figure 1B). To determine the diagnostic accuracy of neurogranin in discriminating the three diagnostic groups, pairwise AUCs were calculated. Neurogranin poorly discriminated AD from NC (AUC=0.73, 95%CI=0.62-0.82), but displayed high accuracies distinguishing CJD from NC (AUC=0.96, 95%CI=0.93-0.99) and CJD from AD (AUC=0.85, 95%CI=0.78-0.92) (Figure 1C). In agreement to this, pROC analysis for the comparison of AUC values indicate that the AUC for the NC vs CJD comparison was significantly higher than the AUC for the NC vs AD ($p<0.001$). A cut-off of 285 pg/mL revealed 89% sensitivity and 92% specificity for the discrimination of CJD from NC in the study population. In comparison, diagnostic accuracy of 14-3-3 and tau in the discrimination of CJD from NC was 89% sensitivity/95% specificity (14-3-3) and 91% sensitivity/98% specificity (tau).

The diagnostic value of neurogranin in the discrimination of CJD from NC (AUC=0.96) was statistically lower than the one for tau (AUC=0.99, 95%CI=0.97-1, pROC neurogranin vs tau, $p=0.012$), but higher than the one for NFL (AUC=0.89, 95%CI=0.83-0.95, pROC neurogranin vs NFL $p=0.041$).

The diagnostic value of neurogranin in the discrimination of CJD from AD (AUC=0.85) was lower than the one for tau (AUC=0.94, 95% CI=0.91-0.99, pROC neurogranin vs tau, $p=0.001$) and not significantly different than the one for NFL (AUC=0.84, 95%CI=0.76-0.91, pROC neurogranin vs NFL, $p=0.84$).

Next, we compared the accuracy of neurogranin in the discrimination of CJD from rapidly progressive AD(rpAD), which turns to be challenging in clinical scenario. AD cases with available data on disease duration (n=32) were stratified in those with disease survival shorter (rpAD, n=11) and longer (AD, n=21) than 2 years following the definition of Grau-Rivera et al. for rapidly progressive dementia [36]. Neurogranin concentrations were higher in rpAD (256pg/mL) than in AD (214pg/mL), but those were not significantly different (p=0.47). Similarly, neurogranin was not significantly different for the CJD vs. AD (p<0.001) and CJD vs. rpAD (p<0.001) comparisons.

Influence of demographic and genetic parameters on neurogranin concentrations

Neurogranin concentrations in CJD were neither affected by age at LP (ranging from 43 to 90 years old, rho=0.05, p=0.64) (Figure 2A) nor by the sex of the patients (p=0.80) (Figure 2B). Similarly, no strong associations between neurogranin and age at LP and sex were detected in NC (age at LP:p=0.27, sex:p=0.16), and AD (age at LP:p=0.18, sex:p=0.77) (Figure 2A and Figure 2B). To test whether genetic characteristics of the patients were associated with differential neurogranin concentrations, we stratified CJD samples by prion protein gene (*PRNP*) codon 129 genotype (data available for 65 cases), a well-known CJD risk factor and disease modifier[37]. Mean neurogranin concentrations were significantly lower in valine/valine [VV] (n=14, 384±172pg/mL) compared to methionine/methionine [MM] (n=38, 630±318pg/mL) and methionine/valine [MV] (n=13, 640±249pg/mL) cases (p<0.05) (Figure 2C). To explore whether neurogranin was associated with prion disease subtype, we further stratified CJD cases with known prion subtype achieved through post-mortem brain tissue analysis (n=28). CJD MM1/MV1 (n=15) and VV2 (n=8) cases, representing the two most prevalent CJD subtypes were studied. Due to their low number, other subtypes were not included in the analysis. Neurogranin concentrations were significantly higher in CJD MM1/MV1 (718±306 pg/mL) compared to CJD VV2 (373±160 pg/mL) (p<0.01) (Figure 2D).

Correlations between neurogranin, surrogate prion biomarkers and clinical data

In CJD, CSF neurogranin showed a good correlation with tau (rho=0.55, p<0.001), but did not correlate with NFL (rho=0.08, p=0.46) (Figure 3A). Additionally, tau and NFL displayed a positive but weak correlation (rho=0.26, p=0.01), in agreement with previous reports[34]. CJD cases displaying positive 14-3-3 test presented higher neurogranin levels than those showing no 14-3-3 (or traces) signal in the western blot test (p<0.05) (Figure 3B).

To study a potential association between neurogranin levels at the time of lumbar puncture and the timeliness of the disease in CJD patients, samples were stratified in early, middle and late stages. Neurogranin concentrations were not significantly different between early (n=9, 510±292 pg/mL), middle (n=26, 576±294 pg/mL) or late (n=28, 635±319 pg/mL) disease stages (Figure 3C).

Next we assessed the potential role of neurogranin as a biochemical marker of disease survival in 63 CJD cases where disease duration was available, and compared it with the performance of tau and NFL. When allowing for non-linear associations between biomarker levels and disease duration, neurogranin was able to explain more of the variability in disease duration (R^2=0.19) than tau

(R^2=0.10) and NFL (R^2=0.07). All three biomarkers showed a log-linear decrease with increasing disease duration (Figure 3E for neurogranin). For neurogranin, the association with survival time can be modelled using a linear combination of the terms: neurogranin (in g/ml) =533+1/(47*[survival time in months-1.6])-28*[survival time in months-0.6]; it showed a good ability as a prognostic marker, represented by Somers' D value of 0.32; Harrell's C value of 0.66 and a Brier score at 12 months of 0.09. For tau and NFL, similar values were achieved (tau: Somers' D=0.27, Brier score=0.11; NFL: Somers' D=0.16, Brier score=0.09). In AD, total disease duration was available in 32 cases, in which neurogranin values were also associated with disease (as well via a log-linear decline, R^2=0.32).

Neurogranin expression in brain tissue

In human brain tissue of control cases, neurogranin was highly expressed in the neuronal soma of the cerebral cortex (n=13) and hippocampus (n=6), but absent in the white matter (n=13) and cerebellum (n=8) (Figure 4A). To further study neurogranin subcellular levels, different brain fractions from control cases (n=4) were purified. Neurogranin was detected in the cytoplasmic (41 ± 5%), membrane (32 ± 4%) and post-synaptic density (PSD) (27 ± 2%) fractions. As control proteins for each fraction we used PSD-95 (post-synaptic), ATPase Na/Kβ (plasma membrane) and synaptophysin (pre-synaptic) for membrane fraction and GAPDH (cytoplasm) (Figure 4B).

Neuronal neurogranin levels were analyzed in the cerebral cortex (control,n=10, AD,n=10, CJD,n=9) and hippocampus (control,n=6, AD,n=7, CJD,n=5) (Figure 5A). A multiple-comparative tests analysis of neurogranin expression from immunohistochemical analysis revealed a significant decrease in CJD ($p<0.001$) and AD ($p<0.001$) compared to controls in both brain regions (Figure 5B). Additionally, neurogranin immunostaining in CJD was significantly lower than in AD in both brain regions ($p<0.01$ in cerebral cortex and $p<0.05$ in hippocampus). No statistical differences were detected in neurogranin levels between Braak stages IV (n=3), V (n=4) and VI (n=3), indicating that alterations in neurogranin expression were not an end-stage feature on AD pathology (Figure 5A).

Reduction of neurogranin levels in the frontal cortex of CJD MM1 (n=10) and VV2 (n=10) cases compared to controls (n=8) was validated by western blot analysis and accompanied by decreased levels of post-synaptic (PSD-95), pre-synaptic (synaptophysin) and axonal (tau) markers (Figure 6A and 6B). Compared to controls, and similar to PSD-95, synaptophysin and tau, decreased neurogranin levels were more severe in CJD MM1 ($p<0.001$) than VV2 cases ($p<0.05$) (Figure 6B). Neurogranin in CJD (n=20) correlated significantly with tau and PSD-95 ($p<0.001$) and with synaptophysin ($p=0.01$). All four proteins presented close correlations with each other (Figure 6C).

Neurogranin levels by means of western blot analysis in the frontal cortex region of AD cases (n=18) were also reduced significantly compared to controls (n=23, $p<0.01$). Moderate decreases in synaptic proteins PSD-95 ($p<0.01$) and synaptophysin ($p<0.01$) were detected, while tau levels were not altered (Figure 7A and 7B). Neurogranin in AD (n=18) significantly correlated with synaptophysin ($p<0.001$) and PSD-95 ($p<0.05$) but not with tau ($p>0.05$). An additional correlation was detected

between PSD-95 and synaptophysin (p=0.01) (Figure 7C). No significant associations between age, sex, post-mortem time delay and neurogranin levels measured by western-blot were found in controls, CJD and AD cases.

DISCUSSION

In this study, we demonstrate that CSF neurogranin is increased in CJD compared to NC (4.75 fold change) and AD (2.5 fold change), reaching good diagnostic accuracies in the discrimination of CJD from AD (AUC=0.85, 95% CI=0.78-0.92). The increased CSF neurogranin concentrations detected in CJD compared to AD is in line with the lower neurogranin levels detected in the cerebral cortex and hippocampus of CJD cases, and with the well-known higher neuronal damage present in CJD compared to AD.

In CJD, CSF neurogranin concentrations at early disease stages were not different from those detected at middle and late stages, indicating that synaptic damage is an early event in CJD, similar to what previously has been found for AD[8]. Indeed, the observation that neurogranin levels in AD brain tissue were not different between early and late Braak stages further supporting that synaptic loss, as measured by neurogranin, is not a late stage pathological event. In this regard, it is well known that synaptic damage is an early event in AD [38].

In our study population, CSF neurogranin correlated neither with age nor with sex in any of the diagnostic groups but we detected differences in CJD cases regarding codon 129*PRNP* polymorphism and subtype with potential clinical implications. First, neurogranin concentrations were significantly higher in CJD MM and MV compared to VV cases, in contrast to tau, which shows higher concentrations in MM and VV, compared to MV cases [39]. Since codon 129*PRNP* data are pre-mortem available, the combined analysis of tau and neurogranin could led to specific codon 129*PRNP* polymorphism-dependent cut-offs enhancing the discriminatory value of single biomarker measurements. Second, CJD MM1/MV1 cases, two subtypes with similar clinco-pathological phenotype, displayed higher CSF neurogranin concentrations than VV2. As described before[21] and in the present study, synaptic and neuroaxonal damage is higher in CJD MM1/MV1 than in VV2 in cortical regions, where neurogranin is highly expressed. Thus, it is tempting to speculate that CSF neurogranin levels reflect the neuropathological heterogeneity of CJD prion subtypes regarding synaptic and neuronal loss. In this regard, biomarkers such as neurogranin, able to recapitulate the heterogeneity of CJD pathology, may turn into valuable markers for disease diagnosis, prognosis and for, monitoring potential therapeutic approaches and inclusion of patient populations in clinical trials. Limitations of this study were the low number of CJD cases with subtype available and the absence of CSF-brain paired cases. Thus, further analysis including less prevalent subtypes and paired cases should be carried out to determine the complete neurogranin profile in the spectrum of CJD cases and its association with neuropathological correlates.

Compared to 14-3-3, one of the gold standards CSF biomarkers for CJD, neurogranin presented similar diagnostic accuracies in the discrimination of CJD from controls. In contrast, tau showed a much more fold change (41 times as compared with 4.75 times for neurogranin) and higher diagnostic accuracy than neurogranin in the discrimination of CJD cases from NC and AD. However, neurogranin explained more of the variance in disease duration than tau and NFL. Further studies should clarify the precise value of neurogranin over tau and other described prognostic markers for CJD[34,40] and its precise context of use in disease monitoring and evaluation of eventual therapeutic therapies. Similarly, in the AD cases, neurogranin was also associated with disease survival, validating previous reports in which neurogranin was proposed as a marker of AD outcome[8,41].

An interesting finding from our study is the observation that neurogranin is broadly present in different neuronal fractions/compartments. Immunohistochemical analysis was supported by biochemical studies where we detected similar neurogranin levels in the cytoplasmic, membrane and post-synaptic fractions. The fact that only a percentage (27%) of total neurogranin is expressed in the post-synaptic fraction calls attention to its proposed use as post-synaptic damage marker, and suggests a dual role as a synaptic and neuroaxonal damage marker.

Our studies in brain tissue also indicated a major overlap between neurogranin and tau expressing neurons in the cerebral cortex (data not shown), which explains the high degree of association between both proteins in the CSF of CJD cases, where major neuronal damage occurs. Likewise, the absence of a clear correlation between CSF neurogranin and NFL in CJD can be explained by the lack of overlap between the levels of both proteins in the brain tissue. In this regard, NFL expression is mainly reported in the axons of the white mater region[42] where neurogranin staining was undetectable in our cases. Additionally, these results are in agreement with the recent observation that NFL in the CSF, in contrast to neurogranin, is more increased in CJD VV2 cases than in MM1[34], with VV2 cases showing higher subcortical pathology compared with other CJD subtypes[43]. Indeed, neurogranin paralleled the CJD subtype-dependent reduced expression levels of PSD-95, synaptophysin and tau, showing a significant correlation with all the studied proteins, especially with tau and PSD-95. Whether these associations are relevant for the neurodegenerative process in CJD remains unknown due to the rapid and massive synaptic and neuronal damage occurring in this pathology. In contrast, reduction of synaptic markers was only moderate in AD brain, while tau levels were unchanged, most likely due to its aggregation in the brain tissue. Moderate decline on synaptic markers in AD tissue observed in our study was not surprising. While synaptophysin was reported to be decreased (≈25%) in the cortex of mild AD patients[44], recent studies revealed only a moderate decline in synaptic markers, including PSD-95 and synaptophysin in the prefrontal cortex (BA9) of patients with AD at advanced cognitive deterioration[45].

Similar to CJD, neurogranin levels in AD correlated with both synaptic markers. On the one hand, this indicates that neurogranin, while not specifically expressed in synapsis, but rather in several neuronal compartments, could be a synaptic dysfunction marker in AD and CJD. On the other hand, our data

also suggest that both pre and post-synaptic dysfunction can be surveyed through the evaluation of biological fluids. In this regard, it would be interesting to determine whether novel biomarkers that may be more specific to the synapse[46–48] are differentially altered in AD and CJD and better reflect synaptic damage than neurogranin.

Recently, the presence of increased neurogranin processing peptides and decreased full-length protein has been reported in AD brain tissue[49]. These observations suggest that neurogranin processing in AD may reflect both synaptic and axonal damage. Since neurogranin was associated with tau and amyloid pathology, it would be interesting to study whether a similar proteolytic pattern is observed in CJD, where neurogranin levels are altered in brain and CSF tissue without the presence of AD pathological hallmarks.

In total, this study evaluates for the first time the diagnostic and prognostic value of CSF neurogranin in CJD in comparison to AD. Additionally, we show a striking correlation between brain and CSF findings regarding different diseases (CJD vs AD) and CJD subtypes (MM1/MV1 vs VV2). This strongly supports the usefulness of comparative analysis between brain and biological fluids to comprehensively understand the molecular mechanisms underlying neurodegenerative dementias and the associate value of their study as diagnostic and prognostic markers for these conditions.

Author contributorship:

IZ, IF and FL designed the study. KB, DD-L, HZ, IF, and FL performed experiments. KB, DD-L, HZ, AV-P, AK, MS, IF and FL analyzed data and interpreted the results. EV provided reagents and technical expertise. FL wrote the manuscript draft. All authors critically revised the manuscript and approved its content before submission.

Competing interest and funding:

KB has served as a consultant or at advisory boards for Alzheon, CogRx, Biogen, Novartis, and Roche Diagnostics, unrelated to this work. HZ has served at scientific advisory boards for Eli Lilly, Roche Diagnostics, Samumed, CogRx and Wave and has received travel support from Teva. KB and HZ are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. The other authors report no conflicts of interest related to the present study.

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Ethical approval:

The study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines, and approved by local Ethics committees (Reference numbers 11/11/93, 9/06/08, Universitaetsmedizin Göttingen, Germany).

Data sharing:

All data relevant to the study are included in the article or uploaded as supplementary information.

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FIGURE LEGENDS

Figure 1. Analysis of CSF neurogranin levels in the differential diagnosis of AD and CJD.

(A) Demographic and biomarker characteristics of the CSF cases used in the present study. Number of cases, sex (f: female, m: male), age, semi-quantitative analysis of 14-3-3 protein (pos: positive, neg: negative) and quantitative analysis of neurogranin, total tau (tau) and neurofilament light (NFL) (mean ± standard deviation (SD)) are indicated. NC: Neurological controls, AD: Alzheimer's disease and CJD: sporadic Creutzfeldt-Jakob disease. (B) Neurogranin concentrations in NC, AD, and CJD. Neurogranin was significantly different in ND vs AD ($p<0.01$), NC vs CJD ($p<0.001$) and AD vs CJD ($p<0.001$) comparisons. Statistical significance derived from a multi-comparison analysis for tau, NFL and neurogranin among the diagnostic groups is indicated. Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) was applied. (C) Diagnostic accuracy of CSF neurogranin in the discrimination of NC, AD and CJD groups. Area Under the Curve (AUC) with Standard Error (Srtd. Error) and 95% Coefficient of Interval (CI) derived from Receiver Operating Characteristic curves for the comparisons between pairs of diagnostic groups is shown. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Figure 2. Association between neurogranin, demographic and genetic factors in the study population in CJD.

(A) No correlation was found between neurogranin levels and age at disease onset in CJD cases. (B) Neurogranin concentrations did not correlate with sex distribution in CJD cases. Spearman rank correlation and unpaired t-test analysis were used respectively. (C) Neurogranin concentrations in CJD stratified by prion protein gene (*PRNP*) codon 129 polymorphism (M = Methionine, V = Valine, MM, n=38, MV: n=13, VV: n=14). Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) was applied (*$p<0.05$ for MM vs VV and MV vs VV comparisons). (D) Neurogranin concentrations in sCJD MM1/MV1 (n=15) and VV2 (n=9) subtypes. Unpaired t-test analysis was applied (**$p<0.01$ for MM1/VV1 vs VV2 comparison).

Figure 3. Association between neurogranin, prion biomarkers and clinical data in CJD.

(A) Correlation analysis between neurogranin, tau and NFL concentrations in CJD cases. Spearman's rho and p values are indicated for each comparison. Positive significant associations were detected between neurogranin and tau ($p<0.001$) and between tau and NFL ($p<0.01$). (B) Neurogranin concentrations in CJD stratified by 14-3-3 protein testing outcomes. Negative test was considered when absence or trace of 14-3-3 protein was detected in the western blot analysis. Mann-Whitney U test was used. CJD cases with positive 14-3-3 test displayed higher neurogranin concentrations than CJD cases with negative 14-.3-3 test (*$p<0.05$). (C) Neurogranin concentrations stratified by disease stage (early, middle and late) in CJD cases. No statistical differences between disease stages were

detected. Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) was applied. (E) Association between neurogranin concentrations and disease duration (months) in CJD patients using a fractional polynomial approach based on a linear regression model. Disease duration can be modelled as a function of neurogranin values based on the formula: neurogranin (in g/ml) = 533 + 1/(47*[survival time in months-1.6]) -28*[survival time in months-0.6].

Figure 4. Neurogranin expression in control brain tissue.

(A) Immunohistochemical analysis of neurogranin expression in the cerebral cortex (n=13), white matter (n=13), cerebellum (n=8) and hippocampus (n=6) of control brain tissue. Neurogranin immunoreactivity was present in the cerebral cortex and hippocampus and absent in white matter and cerebellum regions. Bar: 50 μm. (B) Cell fractionation analysis of human frontal cortex cases (n=4) by differential centrifugation. Input and cell fractions (Cyt: cytoplasm, Memb: membrane, PSD: post-synaptic-density) were separated by SDS–PAGE, followed by immunoblotting with neurogranin, PSD-95, ATPase Na/Kβ, GAPDH and synaptophysin antibodies as specific markers of each cellular fraction (left panel). Quantification analysis relative to the % of protein detected in each cell fraction is indicated (right panel).

Figure 5. Neurogranin expression in AD and CJD brain tissue.

(A) Immunohistochemical analysis of neurogranin expression in the cerebral cortex and hippocampus of control, CJD and AD brain tissue. Bar: 50 μm. (B) Quantification of immunohistochemical staining of neuronal neurogranin from figure 5A. Cerebral cortex: control; n=10, AD; n=10, CJD; n=9. Hippocampus: control; n=6, AD; n=7, CJD; n=5. Neurogranin expression in both regions was decreased in controls compared to AD and CJD ($p<0.001$ for all the comparisons) and in AD compared to CJD ($p<0.01$ in cerebral cortex and $p<0.05$ in hippocampus). ANOVA test followed by Tukey's post-hoc was applied. *$p<0.05$, **$p<0.01$ and ***$p<0.001$. (C) Quantification of immunohistochemical analysis from AD cases according to Braak stage. AD IV; n=3, AD V; n=4; AD VI; n=3. ANOVA test followed by Tukey's post-hoc was applied.

Figure 6. Neurogranin expression in CJD and association with synaptic and axonal markers. (A) Western blot analysis of PSD-95, tau, synaptophysin, neurogranin and β-actin in the frontal cortex of control, sCJD MM1 and sCJD VV2 cases. A representative image (4 controls, 5 CJD MM1 and 5 CJD VV2) is shown. (B) Quantification of the western blot analysis from the complete cohort of cases analyzed, which included: controls; n=8, CJD MM1; n=10 and CJD VV2; n=10. ANOVA test followed by Tukey's post-hoc was applied. PSD-95, tau, synaptophysin and neurogranin levels was reduced in CJD cases compared to controls (*$p<0.05$, **$p<0.01$ and ***$p<0.001$). (C) Correlation analysis of Neurogranin with tau, synaptophysin and PSD-95 in CJD cases (n=20) (left panel) and correlation values (rho, 95% CI and p value) for each comparison between pair of proteins (right panel).

Figure 7. Neurogranin levels in AD and association with synaptic and axonal markers.

Western blot analysis of PSD-95, tau, synaptophysin, neurogranin and β-actin in the frontal cortex of control, and AD cases. A representative image (4 controls and 4 AD) is shown. (B) Quantification of the western blot analysis from the complete cohort of cases analyzed (controls; n=23, AD; n=18). ANOVA test followed by Tukey's post-hoc was applied. PSD-95synaptophysin and neurogranin expression was reduced in AD cases compared to controls (*$p<0.05$, **$p<0.01$ and ***$p<0.001$). (C) Correlation analysis of Neurogranin with tau, synaptophysin and PSD-95 in AD cases (n=18) (left panel) and correlation values (rho, 95% CI and p value) for each comparison between pair of proteins (right panel).

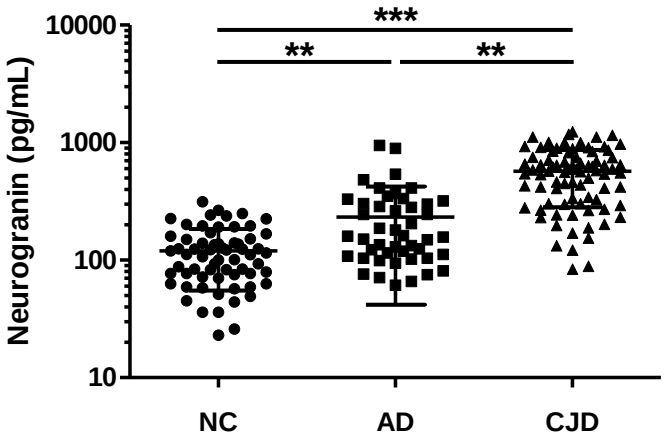
Supplementary Table 1. Demographic, neuropathological genetic characteristics of the brain cases used in the present study. (A) Controls, (B) AD and (C) CJD. Number of cases, age at onset, sex (f: female, m: male), and post-mortem time delay (PMT) is indicated. Braak neurofibrillary tangle (NFT) stage in AD cases and CJD subtype in CJD cases is indicated. FC(R8): frontal cortex Brodmann region 8, HPC: hippocampus, CB: cerebellum. IHC: Immunohistochemistry, WB: Western blot, PSD: Post-synaptic density. 0 and B refers to amyloid stage.

Figure 1

A

	NC	*AD*	*CJD*
Number of cases	64	46	81
Sex (number, f/m)	33/31	27/19	47/34
Age (mean ± SD, years)	65 ± 11	66 ± 9	67 ± 10
14-3-3 (number, pos/trace/neg)	3/4/57	9/3/34	72/4/5
Neurogranin (mean ± SD, pg/mL)	120 ± 65	233 ± 191	571 ± 291
tau (mean ± SD, pg/mL)	243 ± 204	747 ± 606	10017 ± 8541
NFL (mean ± SD, pg/mL)	1391 ± 2112	1851 ± 1537	5919 ± 4229

B



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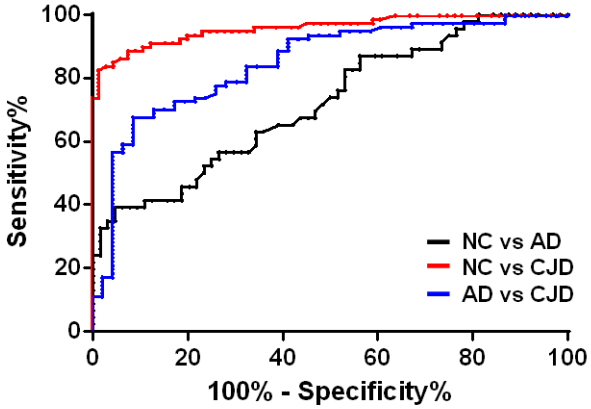


	NC vs AD	NC vs CJD	AD vs CJD
Area	0.73	0.96	0.85
Std. Error	0.05	0.01	0.03
95% CI	0.62-0.82	0.93-0.99	0.78-0.92

Figure 2

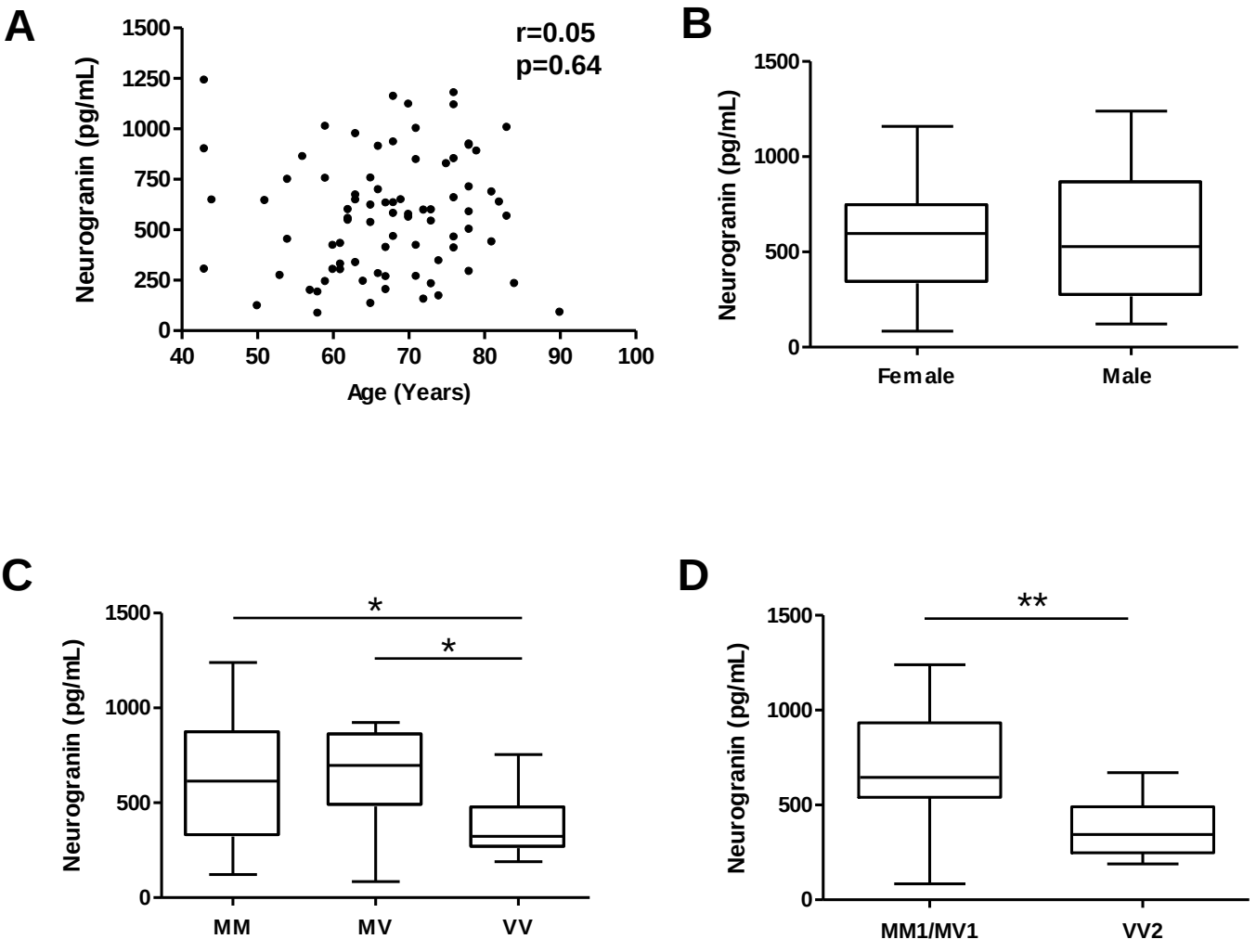
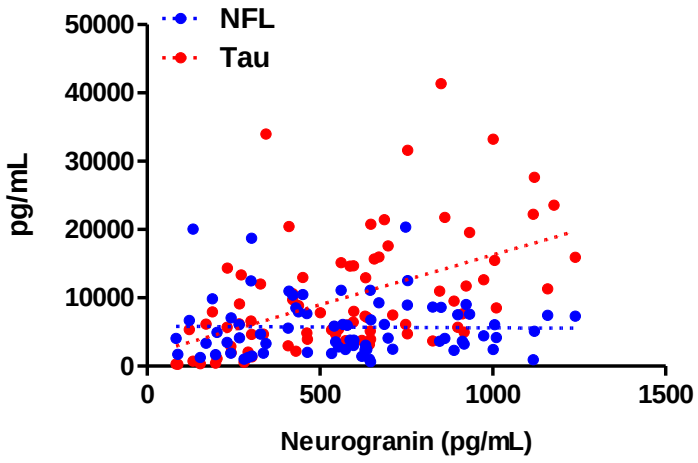

A
r=0.05
p=0.64
Neurogranin (pg/mL)
1500
1250
1000
750
500
250
0
40
50
60
70
80
90
100
Age (Years)
B
Neurogranin (pg/mL)
1500
1000
500
0
Female
Male
C
Neurogranin (pg/mL)
1500
1000
500
0
*
*
MM
MV
VV
D
Neurogranin (pg/mL)
1500
1000
500
0
**
MM1/MV1
VV2

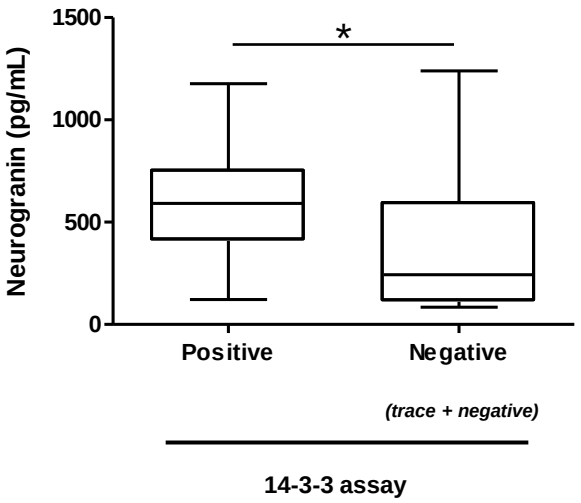
Figure 3

A



	rho	*95% CI*	*p value*
Neurogranin vs Tau	0.55	0.37-0.69	< 0.0001
Neurogranin vs NFL	0.09	-0.15-0.31	0.46
Tau vs NFL	0.28	0.05-0.49	0.01

B



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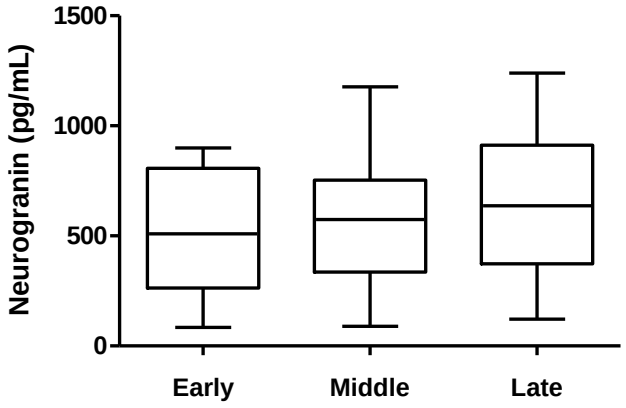

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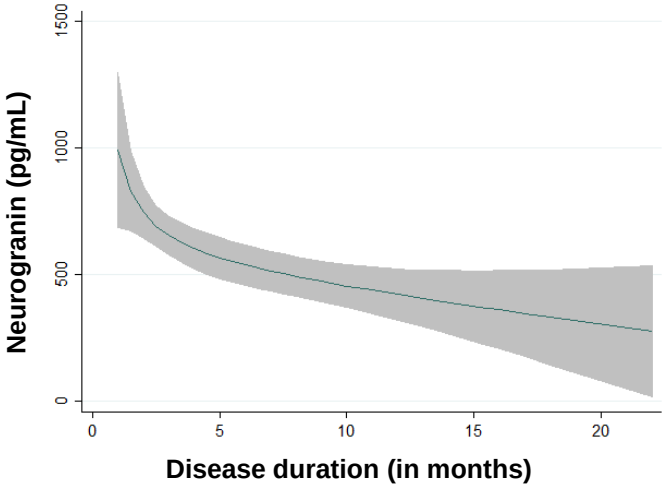
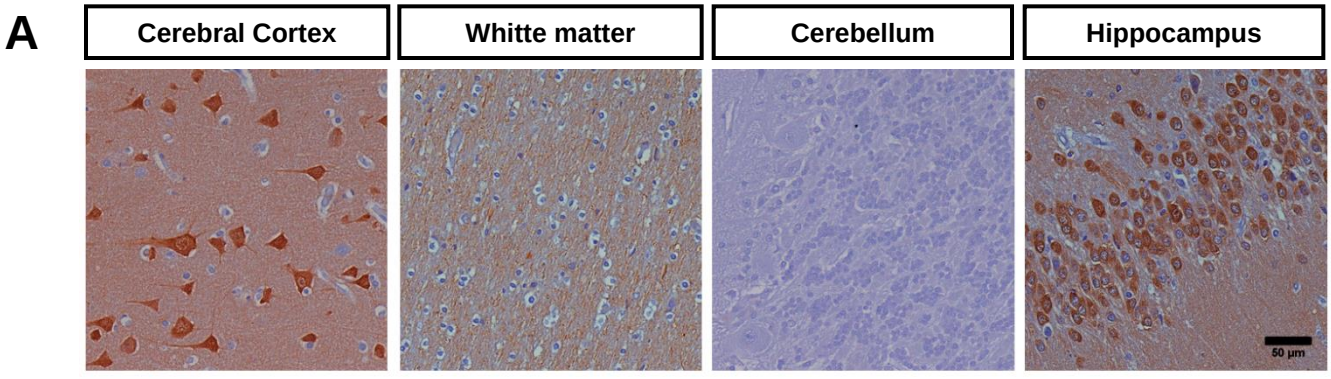


Figure 4


A
Cerebral Cortex
Whitte matter
Cerebellum
Hippocampus
50 μm

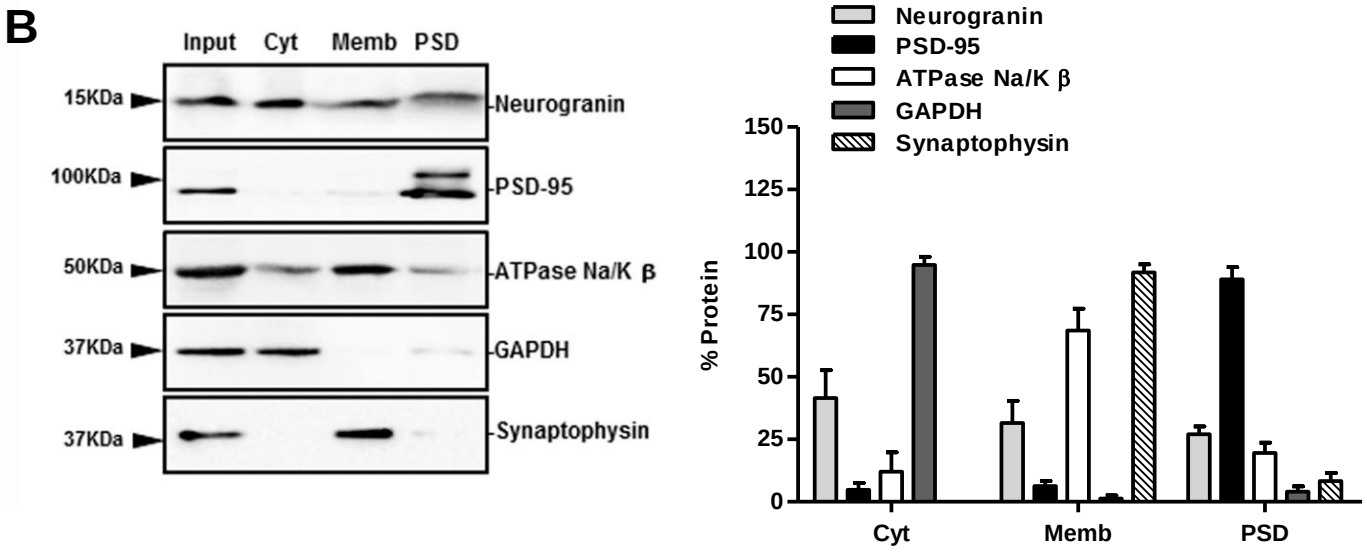

B
Input
Cyt
Memb
PSD
15KDa
Neurogranin
100KDa
PSD-95
50KDa
ATPase Na/K β
37KDa
GAPDH
37KDa
Synaptophysin
Neurogranin
PSD-95
ATPase Na/K β
GAPDH
Synaptophysin
% Protein
150
125
100
75
50
25
0
Cyt
Memb
PSD

Figure 5

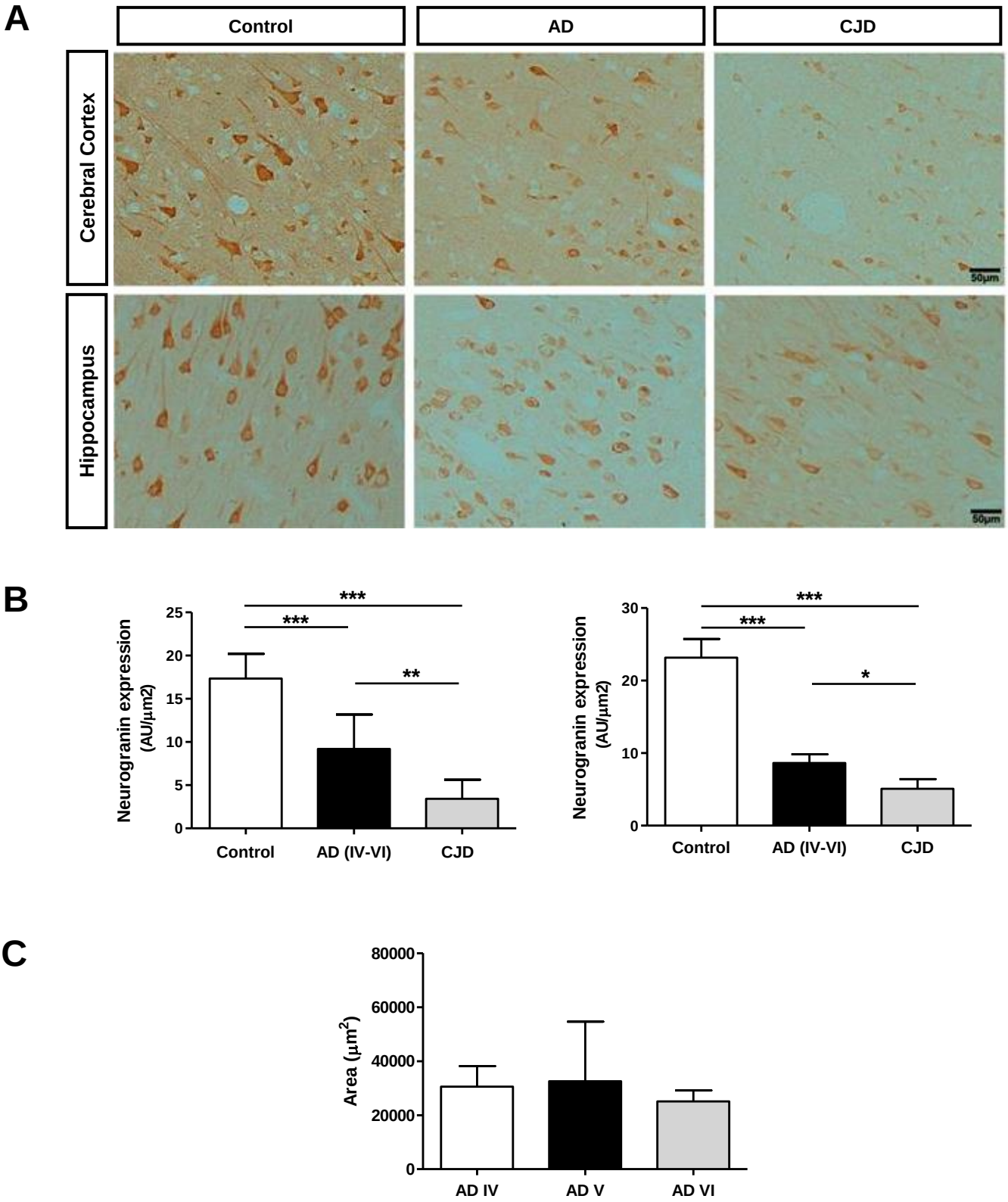
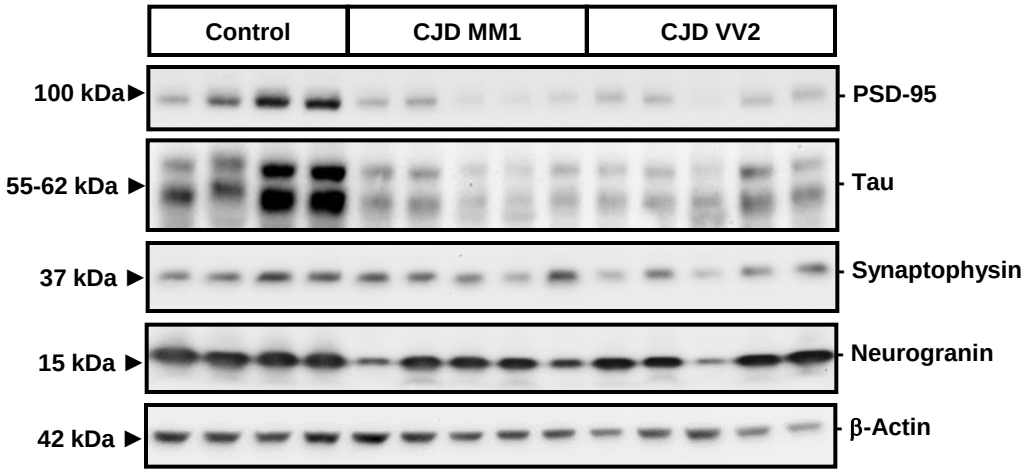

A
Control
AD
CJD
Cerebral Cortex
Hippocampus
50µm
50µm
B
Neurogranin expression (AU/µm2)
25
20
15
10
5
0
***
***
**
Control
AD (IV-VI)
CJD
Neurogranin expression (AU/µm2)
30
20
10
0
***
***
*
Control
AD (IV-VI)
CJD
C
Area (µm2)
80000
60000
40000
20000
0
AD IV
AD V
AD VI

Figure 6

A



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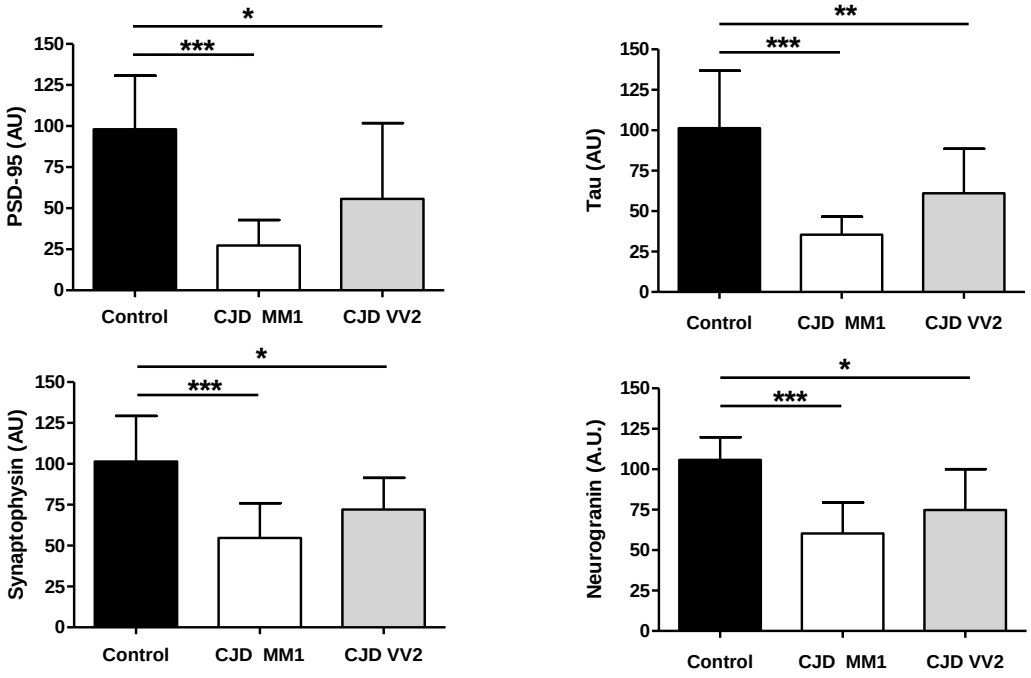

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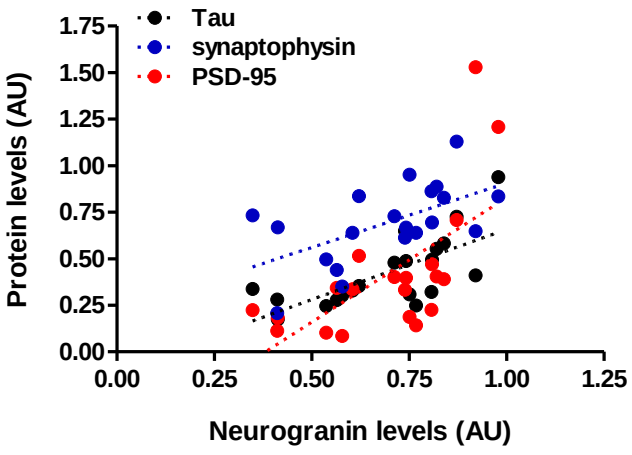
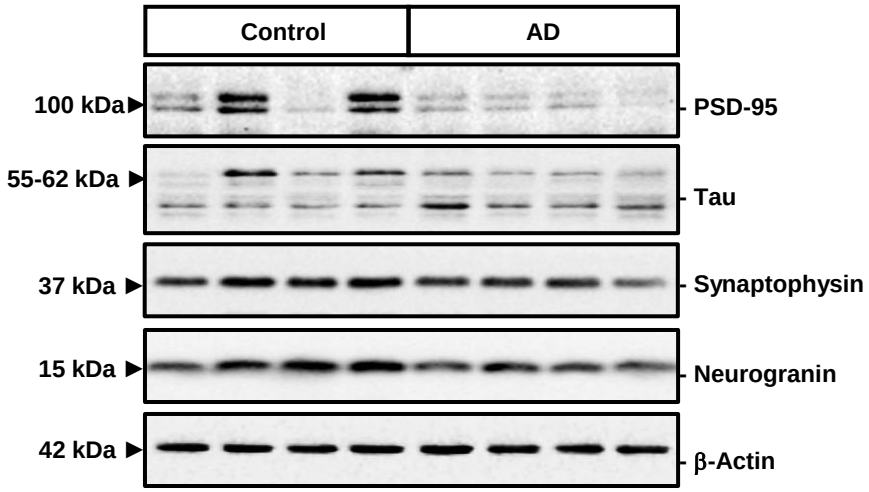


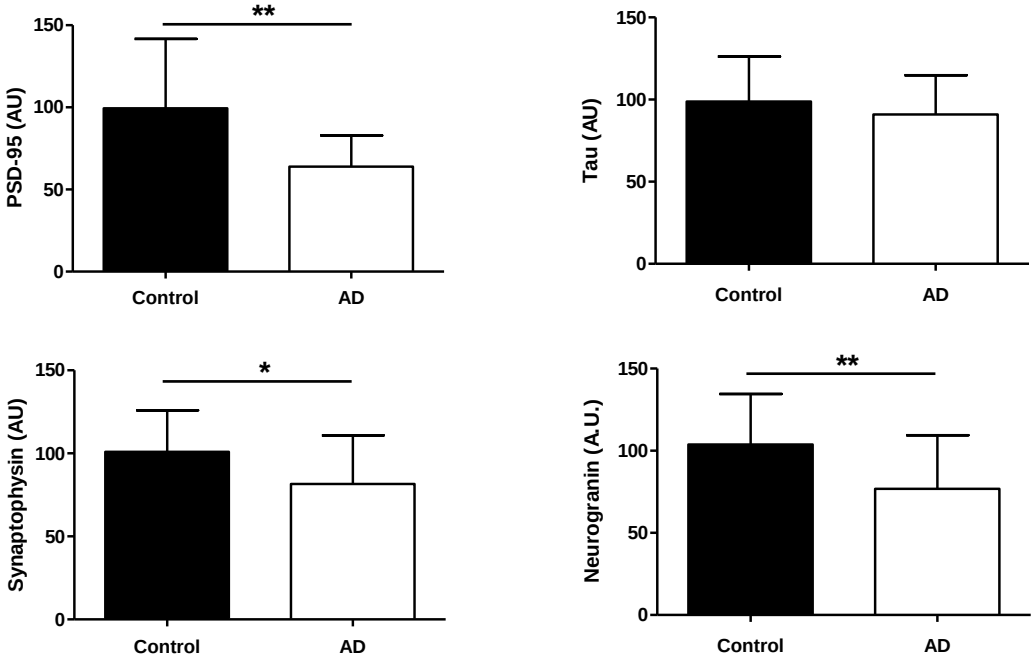
	rho	*95% CI*	*p value*
Neurogranin vs Tau	0.69	0.34-0.87	< 0.001
Neurograninvs synaptophysin	0.55	0.14-0.80	0.01
Neurogranin vs PSD-95	0.69	0.35-0.87	< 0.001
Tau vs synaptophysin	0.52	0.09-0.79	0.02
Tau vs PSD-95	0.76	0.47-0.90	< 0.001
PSD-95 vs synaptophysin	0.53	0.10-0.79	0.02

Figure 7

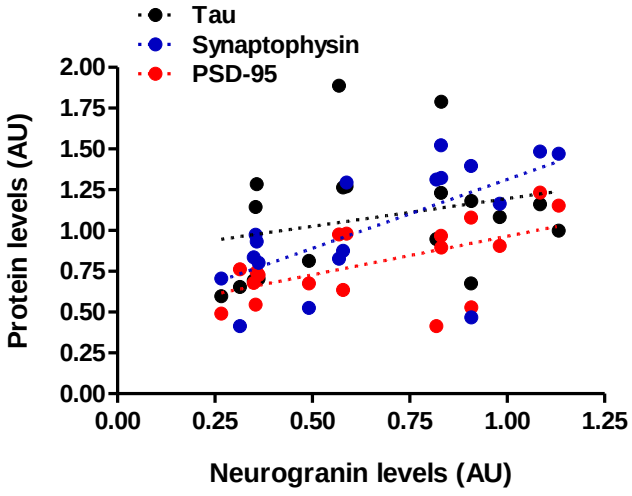
A



B



C



	rho	*95% CI*	*p value*
Neurogranin vs Tau	0.32	-0.19-0.69	0.19
Neurograninvs synaptophysin	0.64	0.24-0.86	0.004
Neurogranin vs PSD-95	0.53	0.08-0.81	0.02
Tau vs synaptophysin	0.29	-0.21-0.68	0.23
Tau vs PSD-95	0.36	-0.14-0.71	0.14
PSD-95 vs synaptophysin	0.56	0.11-0.82	0.01

A

Supplementary Table 1

Frontal Cortex	ID	Age (years)	Sex	PMT (hours)	Neuropathological findings	IHC	WB	PSD
	1	41	F	12h	Not available		X	
	2	53	M	3h	No neuropathological lesions	X	X	
	3	70	M	12h	No neuropathological lesions	X	X	
	4	55	M	5h 40m	Not available		X	
	5	59	M	7h 5m	No neuropathological lesions			X
	6	39	M	9h 15m	Infarction, lacunar	X		X
	7	46	F	14h 5m	Not available		X	
	8	59	M	6h 25m	No neuropathological lesions		X	
	9	71	F	8h 30m	Neurofibrillary tangle pathology I, 0		X	
	10	43	M	5h 55m	No neuropathological lesions		X	
	11	39	M	3h 30m	Argyrophilic grain disease III	X	X	X
	12	56	M	3h 50m	Multi-infarct		X	
	13	71	M	15h	Neurofibrillary tangle pathology I, 0		X	
	14	79	F	3h 35m	Neurofibrillary tangle pathology I, 0			X
	15	55	M	2h 45 m	Infarction, lacunar	X		
	16	49	M	09h 25 m	Neurofibrillary tangle pathology, scant	X		
	17	62	M	19h 55 m	No neuropathological lesions	X		
	18	50	F	14h 30 m	Hyphoxia, hippocampus	X		
	19	72	F	8h 30m	Neurofibrillary tangle pathology II, 0		X	
	20	50	M	17h 15m	No neuropathological lesions	X		
	21	54	F	6h 45m	Ischaemic changes hippocampus			
	22	52	M	4h 40m	No neuropathological lesions	X	X	
	23	52	F	5h 45m	No neuropathological lesions		X	
	24	61	M	4h 30m	Neurofibrillary tangle pathology I, 0		X	
	25	45	M	4h 5m	Cerebral infarction		X	
	26	77	M	6h 55m	No neuropathological lesions		X	
	27	73	M	9h 35m	Neurofibrillary tangle pathology II, 0		X	
	28	65	F	15h	Neurofibrillary tangle pathology II, B		X	
	29	60	F	11h 30m	No neuropathological lesions		X	
	30	72	M	15h 55m	Argyrophilic grain disease III		X	
	31	63	M	4 h 5 min	Hypoxia		X	
	32	41	M	11h 35m	Small vessel disease	X	X	
	33	78	M	12h	Small vessel disease		X	
	34	72	F	4h	Status cribosus		X	
	35	59	M	21h 35m	No neuropathological lesions	X	X	
	36	59	M	8h 30m	Status cribosus	X	X	
	37	54	M	8h 45m	No neuropathological lesions		X	
	38	72	M	4h 20m	No neuropathological lesions		X	
	39	70	M	13h	No neuropathological lesions		X	
	40	63	M	17h	No neuropathological lesions		X	
	41	59	M	7h	No neuropathological lesions		X	

Hippocampus	ID	Age (years)	Sex	PMT (hours)	Neuropathological findings	IHC	WB	PSD
	1	53	M	15h 20m	Neurofibrillary tangle pathology, scant	X		
	2	63	M	17h	No neuropathological lesions	X		
	3	53	M	3h	No neuropathological lesions	X		
	4	55	M	2h 45m	Infarction, lacunar	X		
	5	40	M	5h 10m	Neurofibrillary tangle pathology, scant	X		
	6	54	M	8h 45m	Calcifications	X		

Cerebellum	ID	Age (years)	Sex	PMT (hours)	Neuropathological findings	IHC	WB	PSD
	1	39	M	9h 15m	No neuropathological lesions	X		
	2	23	M	12h 55m	No neuropathological lesions	X		
	3	52	M	04h 40 m	No neuropathological lesions	X		
	4	52	F	05h 45 m	No neuropathological lesions	X		
	5	62	M	3h 30m	Hematoma	X		
	6	42	M	4h 20m	No neuropathological lesions	X		
	7	54	M	8h 45m	Calcifications	X		
	8	40	M	18h 30m	No neuropathological lesions	X		

B

Supplementary Table 1

Frontal Cortex	ID	Age (years)	Sex	PMT (hours)	NFT (Braak Stage)	IHC	WB
	1	75	M	6h 10m	IV		X
	2	79	M	5h	IV	X	
	3	79	M	4h 15m	IV		X
	4	89	M	3h 20m	IV	X	X
	5	84	M	26h	IV	X	
	6	72	F	9h 30m	V		X
	7	86	M	4h 15m	V		X
	8	82	F	1h 45m	V		X
	9	73	M	4h 30m	V	X	
	10	85	F	16h 15m	V	X	
	11	93	M	3h	V		X
	12	75	M	11h 30m	V		X
	13	81	F	5h 15m	V		X
	14	77	M	NA	V	X	
	15	74	F	9h	V		X
	16	50	M	9h 15m	V	X	
	17	69	M	13h 10m	V-VI		X
	18	64	M	6h 10m	V-VI		X
	19	67	F	6h 10m	V-VI		X
	20	82	M	5h	V-VI		X
	21	86	F	20 h 35 m	VI	X	X
	22	67	F	8h	VI		X
	23	56	F	7 h	VI	X	X
	24	88	M	4h 45m	VI	X	X

Hippocampus	ID	Age (years)	Sex	PMT (hours)	NFT (Braak Stage)	IHC	WB
	1	72	F	16h 10m	IV	X	
	2	84	M	26h	IV	X	
	3	75	M	11h 30m	V	X	
	4	88	M	4h 45m	V	X	
	5	50	M	9h 15m	V	X	
	6	84	F	21h	V	X	
	7	56	F	7h	VI	X	

C

Supplementary Table 1

Frontal Cortex	ID	Age (years)	Sex	PMT (hours)	CJD Subtype	IHC	WB
	1	44	M	6h 30 m	MM1	X	
	2	46	M	4h 45m	MM1	X	X
	3	59	M	21 h	MM1	X	X
	4	57	M	4h 15 m	Not available	X	
	5	56	F	13h 30m	MV2	X	
	6	61	M	30h 30m	MM1	X	
	7	64	M	40h	Not available	X	
	8	66	M	NA	VV2	X	
	9	59	M	10h	MV2	X	
	10	72	F	8h	MM1		X
	11	85	F	NA	MM1		X
	12	59	F	15h	MM1		X
	13	60	F	5h 30m	MM1		X
	14	65	F	5h 30m	MM1		X
	15	76	M	18h 30m	MM1		X
	16	78	M	23h	MM1		X
	17	25	M	4h	MM1		X
	18	66	M	5h	VV2		X
	19	71	M	9h	VV2		X
	20	76	F	5h	VV2		X
	21	76	F	5h 30m	VV2		X
	22	51	F	6h	VV2		X
	23	65	M		VV2		X
	24	73	F	24h	VV2		X
	25	47	F	5h 30m	VV2		X
	26	54	M	9h	VV2		X
	27	65	F	7h	VV2		X

Hippocampus	ID	Age (years)	Sex	PMT (hours)	CJD Subtype	IHC	WB
	1	57	M	4 h 15 m	MM1	X	
	2	56	F	13h 30m	MM2	X	
	3	72	F	17h	MM1	X	
	4	74	M	2h	MM1	X	
	5	61	M	30h 30m	Not available	X	