

Accumulation of Prion Protein in the Vagus Nerve in Creutzfeldt–Jakob Disease

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Disease-associated proteins are thought to propagate along neuronal processes in neurodegenerative diseases. To detect disease-associated prion protein (PrP^{Sc}) in the vagus nerve in different forms and molecular subtypes of Creutzfeldt–Jakob disease (CJD), we applied 3 different anti-PrP antibodies. We screened the vagus nerve in 162 sporadic and 30 genetic CJD cases. Four of 31 VV-2 type sporadic CJD and 7 of 30 genetic CJD cases showed vagal PrP^{Sc} immunodeposits with distinct morphology. Thus, PrP^{Sc} in CJD affects the vagus nerve analogously to α -synuclein in Parkinson disease. The morphologically diverse deposition of PrP^{Sc} in genetic and sporadic CJD argues against uniform mechanisms of propagation of PrP^{Sc}.

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Creutzfeldt–Jakob disease (CJD) is a lethal and transmissible neurodegenerative disease. CJD has 3 major etiological forms: acquired, genetic, and the most frequent, sporadic. In sporadic CJD (sCJD), at least 6 different molecular subtypes are distinguished based on the polymorphism at codon 129 (methionine [M] or valine [V]) of the prion protein gene (*PRNP*) and the type of proteinase K–resistant prion protein (PrP) fragments detected by Western blot.¹ Importantly, neuropathological examination, based on the analysis of the distribution of characteristic spongiform change and immunomorphology of disease-associated PrP (PrP^{Sc}), reliably allows the recognition of molecular subtypes.² Lesion profiles and clinical phenotypes are significantly different between molecular subtypes.² Interestingly, VV-2 and MV-2 cases show prominent involvement of subcortical areas, including the brainstem.³

In acquired animal prion diseases, including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease in deer, the medulla oblongata is thought to be a location where PrP^{Sc} reaches the central

nervous system after being transported through the vagus nerve from the gastrointestinal tract.^{4,5} In humans, the detection of PrP^{Sc} in the lymphoreticular system of the gastrointestinal tract supports the spreading concept from the periphery to the brain in variant CJD, which is associated with BSE.⁶ Furthermore, Iacono et al have suggested that involvement of the dorsal motor nucleus of the vagal nerve might indicate an “environmental exposure” in sCJD.⁷

Prion diseases are model disorders for other, more frequent, neurodegenerative conditions. Prion-like propagation of disease-associated α -synuclein and hierarchical involvement of brain regions is thought to be a central event in the pathogenesis of Parkinson disease (PD).⁸ Interestingly, α -synuclein can be found in the gastrointestinal tract; therefore, the vagus nerve has been suggested as a route of α -synuclein spreading to the brain.⁹ However, the direction of α -synuclein propagation in the vagus nerve is still debated.¹⁰ In human prion disease, it has been suggested that accumulation of PrP^{Sc} in the dorsal motor nucleus of the vagus nerve could be an indication for disease propagation outside of the brain.^{7,11} To expand previous findings, we assessed the peripheral medullary segments of the vagus nerve in individuals suffering from sporadic and genetic forms of CJD.

Materials and Methods

Selection of the Cases

We recruited brain samples of definite CJD cases from 3 different cohorts. In 2 cases (1 sporadic and 1 T188K mutation; genetic CJD [gCJD]), postmortem samples from the gastrointestinal tract were also available. Samples derived from the Austrian Surveillance Center for Prion Diseases, Vienna, Austria, the Hungarian Surveillance Center for Prion Diseases, Budapest, Hungary, and the Neurological Tissue Bank of the Biobanc Hospital Clinic, August Pi i

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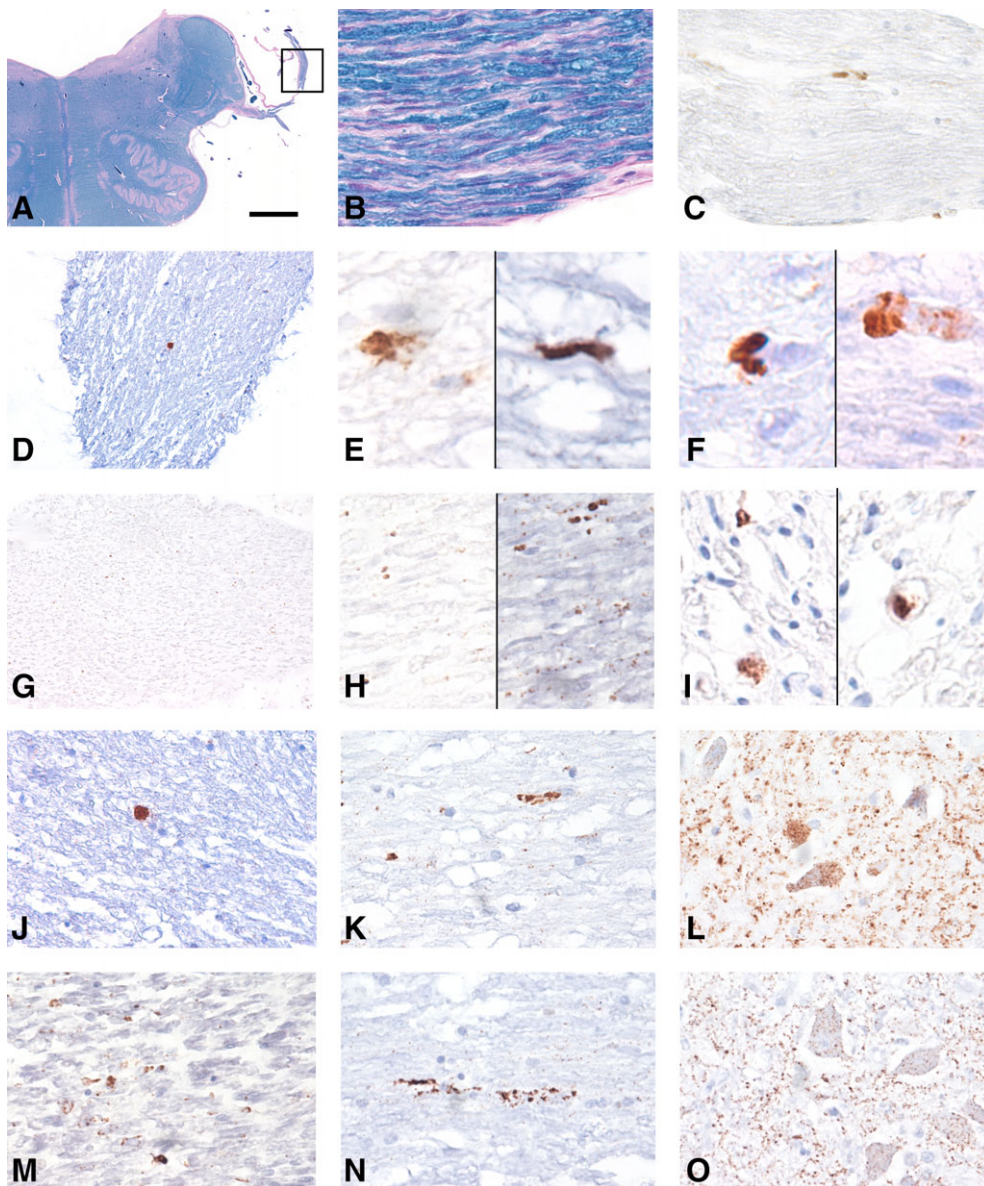


FIGURE 1: Involvement of the vagus nerve in sporadic Creutzfeldt–Jakob disease (sCJD) and genetic CJD (gCJD). (A) Cross-section of the brainstem at the level of the medulla oblongata using Klüver–Barrera staining to show the peripheral emerging part of the vagus nerve (marked and enlarged in B) evaluated in this study. (C, D) Disease-associated prion protein (PrP^{Sc}) deposits (antibody 12F10) in the vagus nerve in representative VV-2 sCJD cases. (E, F) Coarse PrP^{Sc} deposits in different VV-2 sCJD cases (all with antibody 12F10 except right image of E, which represents KG9 antibody). (G) Fine granular deposits of PrP^{Sc} in the vagus nerve of an E200K gCJD case (enlarged in H; all with antibody 12F10 except right image of H, which represents KG9 antibody). (I) PrP^{Sc} deposits in the vagus nerve of a T188K gCJD case (the left image is stained for 12F10 and the right for 6H4). (J–O) Immunostaining for 12F10 antibody in a VV-2 sCJD (J–L) and an E200K gCJD (M–O) case showing the peripheral segment (J, M) and intracerebral course of the vagus nerve (K, N) and its dorsal motor nucleus (L, O). The bar in A represents 0.4cm for A; 20 μ m for B, C, and J–O; 40 μ m for D and G; and 10 μ m for E, F, H, and I. [Color figure can be viewed at www.annalsofneurology.org]

Sunyer Biomedical Research Institute, Barcelona, Spain. We also included 5 non-CJD control cases from the Neurobiobank of the Institute of Neurology, Vienna, Austria. Samples were collected in the framework of National Prion Disease Surveillance systems with appropriate institutional board approvals.

Neuropathology

Five-micrometer-thick slices from formalin-fixed, paraffin-embedded tissue blocks of the medulla oblongata were examined. Only those cases were included in the study where the peripheral

(extracerebral and intracranial) segment of vagus nerve was present on Klüver–Barrera–stained sections (Fig 1A, B). We first screened the sections using the 12F10 anti-PrP antibody (Epitope: aa. 142–160; 1:1,000; Cayman Chemical, Ann Arbor, MI). In addition, we evaluated the peripheral segment of the hypoglossal nerve as well as the hypoglossal nucleus, the nucleus ambiguus, and the dorsal motor nucleus of the vagal nerve for the presence of intraneuronal PrP deposits. For epitope retrieval, we applied autoclaving at 121°C for 10 minutes, immersion in 96% formic acid for 5 minutes, and treatment with proteinase K (5 μ g/ml in

TRIS buffer) at 37°C for 5 minutes, to eliminate the cellular PrP. In the case of a positive signal for PrP^{Sc}, we expanded immunostainings using 2 additional antibodies targeting different epitopes of the PrP (6H4; epitope: aa. 144–152; 1:500; Prionics, Schlieren, Switzerland; and KG9, epitope: aa. 140–180; 1:1,000; TSE Resource Centre, Roslin Institute, Edinburgh, UK). We defined a positive signal as a dark brown coarse aggregate inside the peripheral nerve fibers with at least 50 µm distance from the edge of the nerve parenchyma.

Statistical Analysis

We used a chi-squared test to compare the frequencies of PrP^{Sc} immunoreactivity in the vagus nerves among the different molecular subtypes of sCJD and gCJD cases.

Results

We screened a total of 405 sCJD and gCJD cases; 192 contained the peripheral segment of the vagus nerve at the level of the medulla oblongata. From these cases, 127 sections (66%) contained the peripheral segment of the hypoglossal nerve, and in 169 (88%) the hypoglossal nucleus, 166 (86%) the dorsal motor nucleus of the vagal nerve, and 158 (82%) the nucleus ambiguus were evaluable. Following consensus classification,² 106 of 192 with peripheral vagus nerve were MM/MV-1 sCJD, 31 VV-2, 15 MV-2, 9 MM-2, and a single case was VV-1. Thirty cases were classified as genetic prion diseases (19 E200K, 6 D178N-FFI, 2 cases of 4 octapeptide repeat, and single cases of 5 octapeptide repeat, T188K, and P102L mutations). Fifty percent were female, and the median age at death was 66.2 years.

Eleven cases (5.7% of the cohort) showed PrP^{Sc} immunoreactivity in the peripheral segment of the intracranial portion of the vagus nerve. This included 4 of 31 (12.9%) VV-2 sCJD and 7 of 30 (23.3%) gCJD cases. Six carried the E200K *PRNP* mutation (31.6% of E200K mutation cases). The single case with the T188K mutation was also PrP^{Sc} positive. Fine somatosynaptic PrP immunoreactivity was seen in the brainstem in different molecular subtypes, and coarser granular intraneuronal deposits were observed in the hypoglossal nucleus, dorsal motor nucleus of the vagal nerve, and the nucleus ambiguus only in VV-2 (67.7–74.2% of cases in these nuclei), MV-2 K (56.3–75%), and gCJD (54.8–61.3%) cases. Although the presence of PrP^{Sc} immunoreactivity in the vagus or hypoglossal nerve (only 2 cases with the E200K *PRNP* mutation) was associated with intraneuronal PrP^{Sc} in the ipsilateral corresponding nuclei (Fig 2), not all cases with intraneuronal PrP^{Sc} in the nuclei contained simultaneous immunoreactivity in the corresponding nerves (Table 1).

The morphology of the immunoreactivity in the vagal nerve differed between CJD subtypes. In the VV-2 sCJD cases, we observed coarse aggregates within the extracerebral

portion of the vagus nerve with all anti-PrP antibodies (see Fig 1). In contrast, in the gCJD cases, the PrP^{Sc} immunoreactivity consisted of fine granular dots along the nerve in particular with the 12F10 and KG9 antibodies. The 6H4 antibody did not show unequivocal PrP^{Sc} immunoreactivity in E200K mutation cases. Importantly, we detected PrP^{Sc} deposits within the axons surrounded by the myelin sheath. The gastrointestinal samples of 2 of the genetic cases did not show PrP^{Sc} deposits. Finally, in control cases, we observed no PrP^{Sc} staining in the vagus nerve. In cases with positive PrP^{Sc} in the peripheral segment of the vagus nerve, deposits could be followed along its intraparenchymal medullary course up to the dorsal motor nucleus in both sporadic and genetic cases.

Chi-squared test revealed significant differences between the molecular groups ($p < 0.001$). The frequency of PrP^{Sc} immunoreactivity in the vagus nerve was significantly higher in VV-2 sCJD than in MM/MV-1 ($p = 0.002$), but not after comparisons with VV-1, MV-2, MM-2 sCJD and gCJD cases ($p > 0.1$). The frequency was higher in gCJD cases when compared to the MM/MV-1 ($p < 0.0001$) and MV-2 ($p = 0.045$) sCJD groups, but not when comparing with VV-1 or MM-2 groups ($p > 0.1$). No clinical data on autonomic disturbances were available.

Discussion

We show here that in CJD, PrP^{Sc} accumulates in the intracranial extracerebral segment of the vagus nerve. This finding expands previous observations on PrP^{Sc} deposition in the dorsal motor nucleus of the vagus nerve.⁷ We had no access to extracranial portions of the nerve or peripheral organs, except for 2 genetic cases that did not exhibit disease-associated PrP aggregates.

Abnormal protein processing and propagation are a common denominator of neurodegenerative diseases. Peripheral involvement of the autonomic nervous system has been studied in PD, where peripheral α -synuclein aggregates have been detected throughout the body.¹² Previous studies demonstrated limited or no peripheral involvement in human sporadic CJD with only discrete PrP^{Sc} deposits found in a few dorsal root ganglia, associated to Schwann cells of myelinated and unmyelinated fibers in the superficial peroneal nerve;¹³ they were also identified adaxonally in posterior root nerve fibers, but no labeling of the enteric nervous system has been reported.^{6,14} Moreover, contrasting with α -synuclein in PD, the optic nerve does not show PrP^{Sc} deposits in sCJD despite the involvement of further stations of the optic pathway.¹⁵

The higher frequency of PrP^{Sc} immunoreactivity in vagus nerve in VV-2 cases compared with MM/MV-1 cases could be attributed to the prominent subcortical pathology

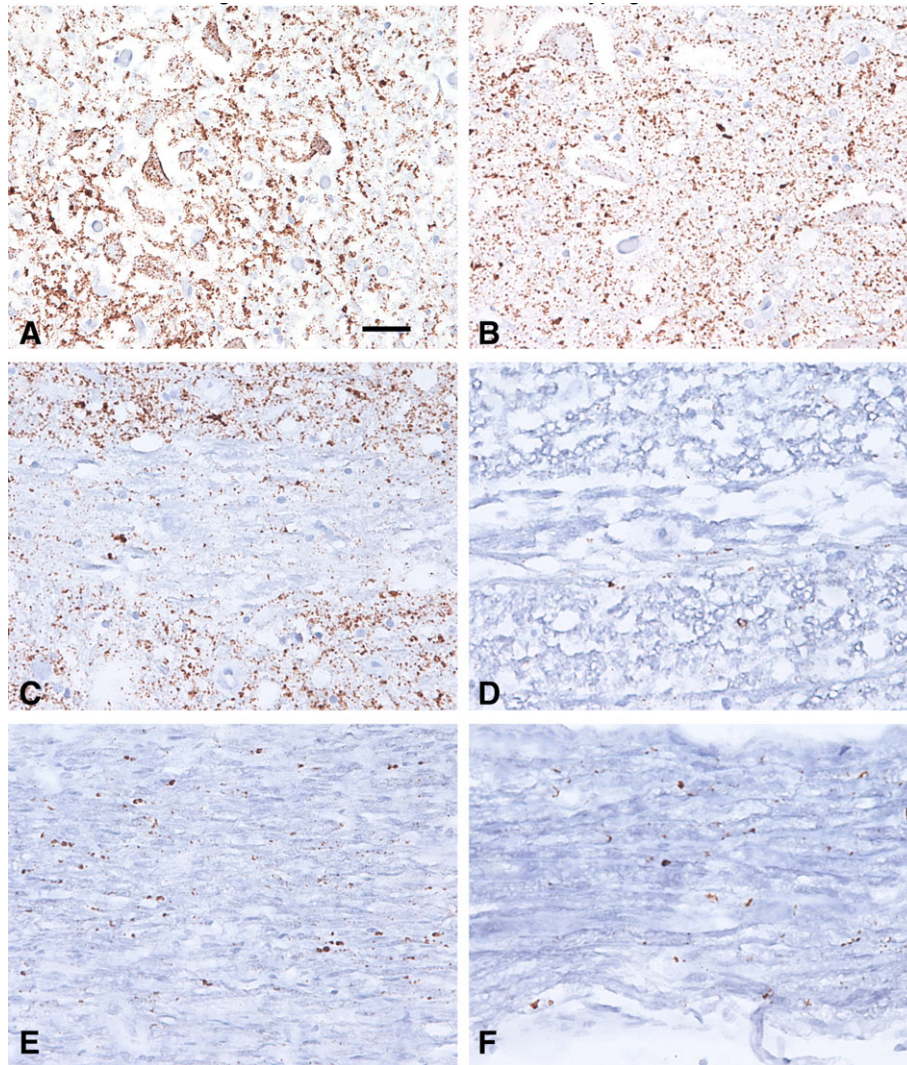


FIGURE 2: Representative microphotographs of the dorsal motor vagal and hypoglossal nuclei and nerves in a genetic Creutzfeldt-Jakob disease case with the E200K *PRNP* mutation. (A, B) Immunohistochemistry for prion protein (antibody 12F10) reveals diffuse synaptic neuropil staining and a granular intraneuronal immunoreactivity in the neurons of the dorsal nucleus of the vagal nerve (A) and the hypoglossal nucleus (B). (C–F) A granular pattern of immunoreactivity is also observed along the intramedullary (C, D) and extramedullary (E, F) segments of the vagal (C, E) and hypoglossal (D, F) nerves. The bar in A represents 35 μm for A and B and 50 μm for C–F. [Color figure can be viewed at www.annalsofneurology.org]

usually observed in this molecular subtype. The coarse morphology of PrP^{Sc} aggregates in VV-2 CJD is somewhat different from the adaxonal and Schwann cell-associated morphologies described previously^{13,14} and may be associated with failure of either directional components of axonal transport. This morphology suggests similarities with α -synuclein deposits in PD, where the olfactory and gastrointestinal tract have been discussed as potential catalysts of α -synuclein misfolding and spread.⁹ The involvement of sympathetic nerves in the process of neuroinvasion has been proposed in experimental scrapie.¹⁶ In sCJD, involvement of peripheral organs, including the spleen and muscle¹⁷ and dorsal roots,¹⁴ has been interpreted as a spillover of cerebral PrP^{Sc} or centrifugal spread.^{14,17} Whether PrP^{Sc} in the vagus nerve moves centrifugally toward the periphery or

is transported centripetally toward the brain is still unclear. Noteworthy is the observation of α -synuclein deposits in PD in further cranial nerves and their nuclei,^{18,19} arguing for the concept that α -synuclein accumulation in the brain can also spread to the periphery. Interestingly, the morphology with fine granular vagal PrP^{Sc} deposits was distinct in gCJD and also resembled adaxonal deposition (see Fig 1I). Although the accumulation of PrP^{Sc} in gCJD cases with E200K and T188K *PRNP* mutations most likely starts in the central nervous system, rarely the E200K mutation can be associated with early peripheral neuropathy.^{20–22} Together with recent reports on *PRNP* mutations associated with autonomic neuropathy and involvement of the gastrointestinal system,^{23,24} an out to inward direction of propagation can be also considered, at least in some cases.

TABLE 1. Number of Cases Showing PrP^{Sc} Immunoreactivity or Lack of PrP^{Sc} Immunoreactivity in Different Medullary Nuclei and the Vagal and Hypoglossal Nerves

PrP IHC Positive					
PrP IHC Positive	Dorsal Vagus NU	Ambiguus NU	Hypoglossal NU	Hypoglossal Nerve	Vagus Nerve
Dorsal vagus NU		51	50	2	11
Ambiguus NU	55		48	2	11
Hypoglossal NU	47	45		2	11
Hypoglossal nerve	2	2	2		2
Vagus nerve	11	11	11	2	
PrP IHC Negative					
PrP IHC Positive	Dorsal Vagus NU	Ambiguus NU	Hypoglossal NU	Hypoglossal Nerve	Vagus Nerve
Dorsal vagus NU		1	8	30	44
Ambiguus NU	0		7	28	41
Hypoglossal NU	0	0		27	37
Hypoglossal nerve	0	0	0		0
Vagus nerve	0	45	0	1	
PrP IHC Negative					
PrP IHC Negative	Dorsal Vagus NU	Ambiguus NU	Hypoglossal NU	Hypoglossal Nerve	Vagus Nerve
Dorsal vagus NU		103	111	82	111
Ambiguus NU	100		104	79	106
Hypoglossal NU	108	101		87	119
Hypoglossal nerve	80	77	85		124
Vagus nerve	108	103	121	124	

IHC = immunohistochemistry; NU = nucleus; PrP^{Sc} = disease-associated prion protein.

Because our study was retrospective and on archival tissue samples, there are inherent limitations. First, not all cases showed the segments of the vagus nerve emerging from the medulla oblongata and for some molecular subtypes the case numbers might be low; second, the entire course of the vagus nerve from the central nervous system to the periphery was not available; third, no peripheral organs innervated by the vagus had been systematically collected in our series; fourth, we could not evaluate specific autonomic symptoms or whether longer duration of illness was associated with more intense PrP^{Sc} immunoreactivity; and finally, we did not have the appropriate methodology to determine the anterograde or retrograde transport of PrP^{Sc}. However, our study expands previous observations restricted to medullary nuclei^{3,7} and provides proof that PrP^{Sc} accumulation not only represents a local phenomenon but also involves corresponding cranial nerves. Furthermore,

we suggest that the morphologically diverse deposition of PrP^{Sc} argues against a uniform propagation mechanism. These observations have implications for studies on other neurodegenerative conditions sharing prionlike pathogenic mechanisms, including α -synucleinopathies.

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Author Contributions

All authors contributed to the conception and design of the study. P.K., J.R., E.G., I.A., K.D., and G.G.K. contributed to the acquisition and analysis of data. P.K., J.R., E.G., and G.G.K. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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