Thalamo-striatal diffusion reductions precede disease onset in prion mutation carriers

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Human prion diseases present substantial scientific and public health challenges. They are unique in being sporadic, infectious and inherited, and their pathogen is distinct from all other pathogens in lacking nucleic acids. Despite progress in understanding the molecular structure of prions, their initial cerebral pathophysiology and the loci of cerebral injury are poorly understood. As part of a large prospective study, we analysed early diffusion MRI scans of 14 patients with the E200K genetic form of Creutzfeldt–Jakob Disease, 20 healthy carriers of this mutation that causes the disease and 20 controls without the mutation from the same families. Cerebral diffusion was quantified by the Apparent Diffusion Coefficient, and analysed by voxel-wise statistical parametric mapping technique. Compared to the mutation-negative controls, diffusion was significantly reduced in a thalamic-striatal network, comprising the putamen and mediodorsal, ventrolateral and pulvinar thalamic nuclei, in both the patients and the healthy mutation carriers. With disease onset, these diffusion reductions intensified, but did not spread to other areas. The caudate nucleus was reduced only after symptomatic onset. These findings indicate that cerebral diffusion reductions can be detected early in the course of Creutzfeldt–Jakob Disease, and years before symptomatic onset in mutation carriers, in a distinct subcortical network. We suggest that this network is centrally involved in the pathogenesis of Creutzfeldt–Jakob Disease, and its anatomical connections are sufficient to account for the common symptoms of this disease. Further, we suggest that the abnormalities in healthy mutation-carrying subjects may reflect the accumulation of abnormal prion protein and/or associated vacuolation at this time, temporally close to disease onset.

Keywords: prion; MRI; Creutzfeldt–Jakob disease; E200K; diffusion

Abbreviations: ADC = apparent diffusion coefficient; BSE = bovine spongiform encephalopathy; CJD = Creutzfeldt–Jakob disease; DWI = diffusion weighted imaging; fCJD = familial CJD; sCJD = sporadic CJD; vCJD = variant CJD
Introduction

Human prion diseases constitute a group of rare and invariably fatal diseases, presumed to be caused by the accumulation of an abnormally folded prion protein, designated PrPSc, in the brain. The most common form, Creutzfeldt–Jakob disease (CJD), is characterized by rapidly progressive dementia, ataxia and myoclonus, leading to akinetic mutism and death within a year of onset. Despite their current rarity, these diseases present unique public health and scientific challenges. Public health concerns have arisen because the disease is infectious and transmissible, both within and between species. These concerns have gained attention with the variant CJD outbreak due to consumption of beef contaminated by Bovine Spongiform Encephalopathy (BSE), and the recent evidence that infection can occur iatrogenically from invasive medical procedures, as well as blood transfusion, even from asymptomatic and undiagnosed donors (Brown, 2007). Scientifically, transmissible encephalopathies have introduced a novel disease mechanism, based on abnormal spatial conformation, which is still incompletely understood and is being intensely investigated.

CJD can be inherited, in addition to the sporadic and infectious forms. The Israeli cluster of hereditary CJD among Libyan Jews is the largest in the world, and it is associated with a specific G to A mutation at codon 200 of the prion protein gene (PRNP) on chromosome 20, the most common PRNP mutation (Goldfarb et al., 1990; Kovacs et al., 2005). This familial variant (fCJD) with the E200K mutation was noted to be clinically similar to sporadic CJD (sCJD) upon its original discovery (Kahana et al., 1974). The risk of developing CJD among carriers of this mutation rises gradually during middle age and reaches a cumulative penetrance of 80–100% by age 80 (Chapman et al., 1994).

The early stages of CJD are not well understood, due to the difficulty of early diagnosis and the very rapid deterioration of patients after onset. In particular, the initial site of cerebral insult, if any, is obscure. Healthy carriers of the E200K mutation, at high risk to develop the disease, provide a unique opportunity to study the brain during conversion to symptomatic disease, and even prior to onset, to investigate possible antecedents. In this article, we report the discovery of brain abnormalities in those healthy individuals. We demonstrate this finding by measuring water diffusion in the brain, a method that is known to offer optimal sensitivity for CJD-associated degeneration (Young et al., 2005; Kallenberg et al., 2006).

Methods

Subjects

The study included 54 participants (Table 1). The patients (group S+) fulfilled World Health Organization criteria for probable fCJD (Brown et al., 2003), were all positive for the E200K mutation, and were followed up until death to confirm the diagnosis. They demonstrated, as expected, significant neurological symptomatology and cognitive deficits, although they were studied early in the course of the disease. Healthy participants were recruited from the same families. They were healthy according to history, medical and neurological examinations and neuropsychological testing, and had no significant history of drug or alcohol abuse. Both mutation positive (group C+) and mutation negative (group C−) healthy subjects were included. All evaluations were double-blind to genotypes: staff examining the subjects did not know whether they were positive or negative for the mutation, nor did the subjects themselves. As expected, all healthy controls had normal cognition by the Mini-Mental Status Examination (MMSE) (Folstein et al., 1975), whereas the patients exhibited significant deficits. Specific neurological symptoms were rated by the Clinical Neurological Scale (Chapman et al., 2007).

Genotyping

DNA was extracted from peripheral lymphocytes according to standard procedures, and a 402 bp fragment within the PRNP gene was amplified by polymerase chain reaction (PCR). The PCR fragment was analysed by digestion with each of the following restriction enzymes: BsmA1 and Alw26I, both cleave the normal allele to yield fragments of 274 and 128 bp. This procedure identifies the E200K mutation characteristic of familial CJD in this ethnic group (Goldgaber et al., 1989; Goldfarb et al., 1990).

MRI acquisition

Scanning was performed on 1.5 T General Electric Signa Excite system with a standard quadrature head coil. MRI sequences included single-shot echo-planar spin-echo diffusion weighted sequence (DWI) and high resolution SPGR T1-weighted sequence. The imaging parameters in DWI were 48 contiguous slices, FOV = 240 mm, TR/TE/5000 ms/85 ms, 3.0 mm slice thickness with acquisition matrix 128 × 128, and averaged diffusion weighted image at b = 1000 s/mm² from the three orthogonal directions. The diffusion images were reconstructed at 256 × 256, yielding in-plane resolution of 0.94 mm × 0.94 mm. High resolution anatomical T1-weighted images (SPGR) were acquired with 104 axial slices yielding reconstructed voxel dimensions of 0.94 mm × 0.94 mm × 1.5 mm.

MRI analysis

We defined parenchymal voxels by the brain extraction tool (BET from FSL 4.0, http://www.fmrib.ox.ac.uk/fsl/; Smith, 2002) in the b = 0 s/mm² DWI image, and used the equation published by Stejskal and Tanner (1965) to calculate apparent diffusion coefficient (ADC) in each voxel. Two slices (out of 2592 total slices) in apparent diffusion coefficient maps suffered from signal loss in the DWI images.
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Spatial normalization to warp individual apparent diffusion coefficient maps into a common anatomical space was performed in a standard procedure by first deriving normalization parameters from anatomical (SPGR) scans, and then applying them onto diffusion scans using SPM5 (Wellcome Department Cognitive Neurology, London, UK, http://www.fil.ion.ucl.ac.uk/spm/). Normalization parameters were derived by the unified segmentation approach, which combines segmentation, bias correction and registration into a single generative model (Ashburner and Friston, 2005). Default registration parameters were used to match the anatomical scan with the ICBM Tissue Probabilistic Atlas. Head movement between the apparent diffusion coefficient and anatomical scans was corrected retrospectively by aligning non-diffusion $b=0\text{s/mm}^2$ image with the SPGR image using rigid body registration, and derived registration parameters were applied onto the corresponding apparent diffusion coefficient maps, which were smoothed by isotropic $4\text{mm}$ full width half maximum Gaussian smoothing kernel. These images were submitted to a group level random effect model ANCOVA, consisting of the diagnostic grouping ($C_-, C+, S+$) with individual age and gender as covariates. A custom explicit mask was also created to eliminate voxels likely to contain largely cerebrospinal fluid by setting the upper limit of the voxel to $2500\mu\text{m}^3/s$. In each voxel, if any of the subjects’ apparent diffusion coefficient values exceeded this upper limit, the voxel was discarded from further analysis. Because the differences between the two healthy groups were expected to be stronger than the effects of disease, voxels were considered significant at $P$-value of less than 0.005 ($C_-$ versus $C+$) or 0.001 ($C_-$ versus $S+$ and $C+$ versus $S+$), with an extent cluster threshold of five contiguous voxels. Note, however, that all our hypotheses were one-tailed (predicting reduced diffusion due to the mutation or disease), whereas the significance thresholds used by SPM are two-tailed. Therefore, the true significant thresholds in these data are 0.0025 for the healthy group contrast and 0.0005 for contrasts involving patients. Finally, voxels surviving the threshold were overlaid onto an anatomical image for a visualization purpose, and the corresponding anatomical locations were estimated in Talairach coordinate using MSU (http://www.ihb.spb.ru/~pet_lab/). The anatomical image depicted in Fig. 1 was created by averaging the normalized T$_1$-weighted anatomical SPGR scans of all 54 subjects.

Results

All three samples were matched for age. The two healthy samples performed at normal levels by the MMSE and Clinical Neurological Scales (Table 1). As expected, the patients displayed substantial cognitive and neurological deficits, despite being examined early in the course of their disease. Within the patient sample, age was not significantly associated with the clinical neurological scale score, but MMSE scores declined with age ($r=-0.54$, $P<0.005$). As expected, the MMSE and clinical neurological scale scores were correlated ($r=-0.58$, $P<0.0001$), reflecting their joint sensitivity to severity of the disease. While the three samples were not matched for gender, no significant effects of gender were detected on any relevant variable. The complete set of imaging results is provided in the Supplementary Tables 1–3. Here we detail only the quantitative diffusion findings in the thalamus and basal ganglia. To assess the early diffusion changes due to the disease, we compared the fCJD patients (group S+) to the healthy, mutation-negative controls from the same families (Fig. 1A and Table 2). Diffusion was significantly reduced in specific areas of the thalamus (primarily the pulvinar, mediodorsal and ventrolateral nuclei), the putamen and the caudate nucleus.

The effects of the mutation, without overt clinical disease, were tested in the comparison of healthy mutation-carriers (C+) to non-carriers (C–). These results are depicted in Fig. 1B and detailed in Table 3. Diffusion was significantly and bilaterally reduced in the putamen and thalamus (primarily the mediodorsal, pulvinar and ventrolateral nuclei), as well as cerebellar and neocortical foci (Supplementary Table 2). The thalamic and putaminal locations of diffusion reduction were identical to the disease effects noted above.

Finally, diffusion changes due to disease onset were assessed by comparing the S+ patients to the C+ healthy subjects. These groups are matched on all variables, including the presence
of the E200K, but the patients had already developed overt disease of short duration. This comparison, thus, reveals the effects of disease onset (Fig. 1C and Table 4). Diffusion was reduced primarily in the putamen, caudate nucleus and thalamus (mediodorsal, ventrolateral and pulvinar).

To illustrate the overall pattern of results, we extracted two large significant clusters from the ‘C− > S+’ contrast above. These are the first and third clusters in Table 2 and Supplementary Table 1, with their peaks located in right putamen and left thalamus. The corresponding voxels were then outlined.
for all subjects in all three groups, and the plot of their mean apparent diffusion coefficient values is depicted in Fig. 2. In both structures, a small decline is evident from C− to C+, followed by a larger decline in the symptomatic S+ subjects.

**Discussion**

This is the first quantitative, voxel-level and whole-brain analysis of MRI changes in CJD. Our results confirm and extend previous reports of sporadic CJD, and document substantial similarity between the cerebral patterns of fCJD and sporadic CJD. In addition, this is the first study of early and pre-morbid brain abnormality in human prion diseases, and suggests intriguing hypotheses about the initial neuropathological processes associated with disease onset.

We report the main abnormalities in specific thalamic nuclei (pulvinar, mediodorsal and ventrolateral) and the putamen. Neuropathologically, striatal and thalamic degeneration, and sparing of the globus pallidus, are well established in CJD. Significant thalamic degeneration is commonly noted in the mediodorsal and ventrolateral thalamic nuclei (Masters and Richardson, 1978; Tschampa et al., 2002), and the thalamus is the major site of injury in FFI, another human prion disease (Macchi et al., 1997). The ventrolateral and mediodorsal also showed loss of inhibitory parvalbumin-expressing neurons in sporadic CJD (Tschampa et al., 2002), and severe neuronal loss was similarly reported in the mediodorsal, pulvinar and ventrolateral (Macchi et al., 1997). Specifically, in fCJD with the E200K mutation, case reports demonstrated pathological involvement of the pulvinar and mediodorsal, as well as caudate and putamen (Chapman et al., 1996; Taratuto et al., 2002). Hyperintense MRI appearance of the pulvinar, mediodorsal, caudate nucleus and putamen was already noted as characteristic of vCJD, the variant of CJD associated with exposure to BSE-contaminated beef (Collie et al., 2003). While few imaging studies noted thalamic abnormalities in sporadic CJD, a recent report demonstrated significant diffusion reductions by manual region of interest measurements of apparent diffusion coefficient (despite ambiguous DWI images) in the pulvinar and mediodorsal, as well as the caudate nucleus and putamen, in six sporadic CJD patients (Tschampa et al., 2003). Thus, our findings in early fCJD are fully consistent with the current literature on vCJD and sporadic CJD, and suggest that a thalamo-striatal network is centrally involved in several forms of human prion diseases, and that this network is affected early in the course of the disease.

Notably, we have also detected diffusion reductions in healthy subjects who carry the E200K mutation. Long incubation periods are characteristic of prion diseases. The infectious agent has been detected years before onset of clinical symptoms in sheep infected by Scrapie (Georgsson et al., 2008) and in BSE-infected cattle (Espinosa et al., 2007). In humans, incubation times of Kuru (cannibalistic form) and vCJD can be decades long, and vCJD infection can occur from blood transfusions obtained from donors up to three years before they developed symptoms (Brown, 2007). Thus, prion protein (PrPSc) accumulation occurs long before clinical onset in several variants of infectious CJD. In the case of our E200K subjects, born with the mutation that is assumed to predispose them to produce PrPSc, it appears that cerebral diffusion changes can be detected years before disease onset, similar to scrapie-infected mice (Broom et al., 2007). These diffusion reductions were detected in healthy E200K mutation carriers in the same brain areas noted in patients with the full-blown disease, namely the putamen and the same thalamic nuclei, but not the caudate nucleus. These abnormalities were obtained in comparison to healthy relatives (free from the mutation) from the same families. The two groups share environmental, culinary, and genetic traits (other than the specific mutation), as well as being matched for age. It is likely, therefore, that these brain abnormalities are due to the mutation itself and, presumably, the cerebral deposition of the prion protein and its degenerative properties. However, we cannot yet exclude the possibility that the abnormalities in mutation carriers could be developmental; we are testing this hypothesis by studying younger subjects.

![Figure 2](image-url) Mean volume averaged apparent diffusion coefficient (ADC) values of (A) Right Putamen at (-26, -4, 8) and (B) Left Thalamus at (-12, -14, 8) are plotted by diagnosis. The error bars represent standard deviations.
Finally, diffusion changes due to disease onset were assessed by comparing the S+ patients to the C+ healthy subjects. These groups are matched on all variables, including the presence of the E200K mutation, but the patients had already developed overt disease. Diffusion was reduced primarily in the putamen, caudate nucleus and thalamus (mediodorsal, ventralateral and pulvinar). This striatal-thalamic circuit is already affected in healthy mutation carriers (at the mean age of 57 years), and the pathology intensifies, but does not yet spread to other grey matter areas, at the time of disease onset. Disease onset, therefore, is associated with quantitative increase of pathology in the same areas, rather than further involvement of other areas. This pattern was also reported in the brain stem of BSE-affected cattle (Siso et al., 2007), where preclinical cases showed the neuropathology in the same areas as symptomatic animals, but to a lesser extent.

Remarkably, not only did we fail to detect diffusion reductions in additional areas upon disease onset, but cortical and cerebellar findings that were noted in healthy mutation carriers were absent in the patients. The pathological lesions of sporadic CJD-type MM1, which the E200K variant closely resembles, are typically found in cortex, striatum, cerebellum and thalamus (Parchi et al., 1999), and previous investigators have reported cortical abnormalities, using visual ratings of DWI scans (Young et al., 2005). There are several possible explanations for this apparent discrepancy. First, cortical—and possibly cerebellar—lesions may occur randomly in multiple small loci over a large cerebral region. While visual examination of individual MRI scans often reveals characteristic cortical diffusion reductions, they do not occur consistently in a particular gyrus. Therefore, when averaged across many patients in a statistical group comparison, this variable location may prevent significant findings. Second, this may be a statistical artifact of sample sizes. While the healthy samples in this study included 40 subjects, only 14 patients were analysed to date, and the smaller sample size may have limited the significance. However, when we tested this conjecture by manipulating significance thresholds in our analyses, we could not corroborate it. Third, DWI may demonstrate cortical and cerebellar lesions through T2 shine-through, without diffusion reductions. We believe the likely explanation is pathophysiologic. We have previously showed that cerebellar diffusion reductions cannot be detected even in CJD patients specifically selected for cerebellar symptomatology (Cohen et al., in press). In that study, we also documented significant cerebellar atrophy. Atrophy is associated with cerebral spinal fluid accumulation and elevated diffusion, and thus would mask the reduced diffusion caused by CJD-specific changes. In the current material, we have analysed apparent diffusion coefficient elevations and found them significant in cortex and cerebellum of patients, but not in the basal ganglia or thalamus of patients and not anywhere in the healthy mutation carriers (manuscript in preparation). Therefore, we can propose that the CJD-specific changes, probably associated with PrPSc accumulation and/or vacuolation (Haik et al., 2002) that cause apparent diffusion coefficient reductions, are the ones that occur very early in the course of the disease and in fact before clinical onset in our mutation carriers. These changes occur in all four sites (cerebellum, cortex, putamen and thalamus), and are detectible in the mutation carriers. However, with disease onset, there is atrophy of cortex and cerebellum in the patients, which raises apparent diffusion coefficient and masks the reductions, and thus they become invisible in these areas in patients. Because there is no appreciable atrophy of putamen and thalamus in early CJD, these changes become stronger with disease onset.

Despite great progress in understanding molecular aspects of prion diseases, their early effects in the brain have not been well documented. Here we report that the initial changes are detectible in a network consisting of the putamen and specific thalamic nuclei, the pulvinar, mediodorsal and ventrolateral. These structures all belong to a well-known extrapyramidal circuit, known to be disrupted in movement disorders, with major involvement in the initiation, sequencing and modulation of motor activity, as well as affective and cognitive aspects. Detailed anatomical mapping of the relevant cerebral areas is beyond the scope of this article, but can be summarized as follows: the ventral thalamic nuclei group, which includes the ventrolateral, is part of the motor subdivision of the thalamus, which receives afferents from the cerebellum and pallidum and projects to the motor cortex and supplementary motor area. The motor cortex, in turn, innervates the putamen, which sends efferent to the contralateral cerebellar cortex. The output nucleus of the cerebellum, the dentate nucleus, also projects to motor cortex (Middleton and Strick, 1994), and to the mediodorsal and ventrolateral, which in turn project to prefrontal Brodmann areas 9, 12 and 46; these areas, in turn, project heavily on pontine nuclei that provide access to the input stage of cerebellar processing (Middleton and Strick, 2001). The pulvinar and mediodorsal are connected to both associative cortex and limbic structures (Macchi et al., 1997). The putamen and caudate nucleus receive input from diverse cortical areas, including motor, sensory, prefrontal and limbic structures. Non-motor output of the basal ganglia targets at least three cortical areas via the thalamus (particularly the mediodorsal and ventrolateral)—the dorsolateral prefrontal cortex, lateral orbitofrontal cortex and the anterior cingulate. Thus, it is not surprising that basal ganglia dysfunction has substantial cognitive effects, or at least influence on prefrontal processing. The same cortical areas that are the targets of basal ganglia output also project to the basal ganglia input stage (caudate nucleus and putamen), thus forming multiple parallel closed loops with a large number of cortical areas (Middleton and Strick, 2002).

Anatomically, therefore, the rich interconnectivity of the putamen and thalamus can explain both the motor and cognitive aspects of CJD symptomatology. The putamen, mediodorsal, ventrolateral and pulvinar are critical parts of an extensive network that also includes several sensorimotor and associative neocortical areas, as well as the cerebellum. The globus pallidus pars externa also receives massive input from the putamen, but surprisingly is not affected in CJD, as evidenced by multiple neuropathological and imaging studies (Zemanick et al., 1991). How the abnormal PrPSc propagates across the brain is not known, but two reasonable hypotheses would be spatial proximity and neuronal pathways. Propagation along neuronal pathways has been shown in mice following intra-ocular inoculation (Fraser and Dickinson, 1985), and PrPSc was demonstrated in white matter tracts (Taraboulos et al., 1992). The bilaterality of diffusion disruption may also support the latter, since it suggests cross-hemispheric
propagation through commissural fibers. The globus pallidus is spatially contiguous with the putamen and thalamus, as well as richly connected to both, yet appears unaffected both by neuropathological examinations and our own findings. The reason for this surprisingly heterogeneous sensitivity to prion disease is unknown, and the present findings indicate the need for better mapping of PrP^Sc to specific synapses.

The same critical structures, the putamen, pulvinar, mediodorsal and ventrolateral were already affected in healthy subjects carrying the E200K mutation. These subjects are highly likely to develop the disease later in life, possibly shortly, since penetrance was reported to be close to 100% (Chapman et al., 1994). Disease onset commonly occurs near age 60 (Simon et al., 2000; Kovacs et al., 2005), and our subjects had a mean age of 57. Notably, disease onset appears associated with only about 10% further reduction in diffusivity in these same structures, without detectible propagation to additional cerebral areas (other than the caudate nucleus). Thus, we may offer the hypothesis that the E200K mutation causes gradual accumulation of PrP^Sc in the brains of these subjects throughout life, and clinical symptoms emerge when some threshold is crossed in the thalamic-putaminal network, perhaps consisting of disrupted synaptic pathways or neuronal loss. Aging certainly may play a role—prior evidence suggests that the E200K mutation does not automatically generate PrP^Sc; a further conversion process is necessary, and this may require age-dependent factors, consistent with the late onset age (Rosenmann et al., 2001). Alternatively, though less likely, significant damage to the caudate nucleus may be associated with clinical symptom onset. If the first hypothesis is valid, future studies tracking the diffusion abnormalities in younger healthy subjects should demonstrate that the lesions diminish as the subjects are examined further, chronologically, from disease onset. On the other hand, these diffusion abnormalities may not be the correlate of impending clinical disease onset, but could reflect stable developmental and maturational effects of the E200K mutation. We are not aware of any evidence, in humans or animal models, of aberrant brain development in prion mutation carriers, but we cannot rule it out. We propose, therefore, that our healthy mutation carriers expressed an identical pattern of brain abnormalities because they were temporally close to disease onset. We are studying younger subjects to test this hypothesis.

The major caveat in interpreting these results involves the nature of statistical parametric mapping. By definition, we report the average results in a group of subjects, following the inevitable step of spatial normalization, i.e. warping all brains into a standard anatomical space. Thus, heterogeneity is masked, and only common features are emphasized. Spatial normalization is known to be limited in its accuracy, and this problem is exacerbated in severely atrophic brains, as CJD patients tend to have. Therefore, the anatomical identification of abnormal loci should be interpreted with caution. Finally, generalizability to other forms of CJD cannot be guaranteed, although it is likely. Clinical similarity of the E200K fCJD to sporadic CJD was verified as ‘more classical’ (Kahana et al., 1991), presumably due to the genetic homogeneity, which was not fully appreciated at that time. Pathological appearance is also similar to the predominant sporadic CJD subtype, MM1 (Gambetti et al., 2003), as are the radiological abnormalities detectable by MRI (Fulbright et al., 2006).

In conclusion, the present results provide the first quantitative, prospective, voxel-based mapping of early CJD disease process in the human brain, in a genetically uniform, relatively large sample of well-characterized patients. We have documented a cerebral network, encompassing the putamen, caudate nucleus, mediodorsal, ventrolateral and pulvinar, which is affected in early fCJD. Reduced diffusion was also detected in all of these structures (apart from the caudate nucleus) in healthy pre-symptomatic mutation carriers. The globus pallidus was normal throughout. Future work will need to investigate the unique characteristics of these structures that confer their sensitivity to prion-associated damage, and the microscopic process responsible for this diffusion reduction.

Supplementary material
Supplementary material is available at Brain online.

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