Early Neurodegeneration in the Brain of a Child Without Functional PKR-like Endoplasmic Reticulum Kinase

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INTRODUCTION

Wolcott-Rallison syndrome (WRS) is the clinical manifestation of a lack of function of protein kinase R–like endoplasmic reticulum kinase (PERK). The syndrome was first described by Wolcott and Rallison (1) in 1972 in a series of 3 siblings. To date, approximately 60 cases have been described in the literature. They largely occur in geographic areas with high levels of consanguinity, such as the Middle East, north Africa, Pakistan, and Turkey. Wolcott-Rallison syndrome is an autosomal recessive condition with manifestations of infancy-onset diabetes mellitus, multiple epiphyseal dysplasia, osteopenia, microcephaly, mental retardation or developmental delay, and hepatic and renal dysfunction (2).

Histologic studies of the bone, pancreas, and liver—but not the brain—of patients with WRS have been performed. Bone tissue shows an irregular proliferation of chondrocytes, spongy bone, and ramified collagen fibers (3). Liver tissue outside phases of hepatic failure shows progressive fibrosis with mild steatosis and noninflammatory necrosis (2, 4). Pancreatic tissue shows severe reduction in acinar tissue and severe β cell deficit with evidence of necrosis (2). Similar findings have also been found in PERK−/− mice (5). Examination of neural function in mice with brain-specific deletion of PERK also shows impaired behavioral flexibility (6).

Wolcott-Rallison syndrome is generally caused by mutations in the EIF2AK3 gene, which encodes PERK (7); however, mutations in other genes related to PERK function, such as IER3IP, have also been implicated (8). Protein kinase R–like endoplasmic reticulum kinase is part of the unfolded protein response, which coordinates cellular responses to an accumulation of unfolded or misfolded proteins in the endoplasmic reticulum. Protein kinase R–like endoplasmic reticulum kinase phosphorylates 2 known downstream targets: EIF2A and Nrf2 (9). Phosphorylated EIF2A reduces the rate of translation of most proteins and selectively enhances ATF4 translation, which in turn promotes autophagy (10, 11) and affects mitochondrial protection (12). NRF2, meanwhile, acts to protect cells from oxidative stress (9, 13).

Protein kinase R–like endoplasmic reticulum kinase activity has recently attracted attention in the field of neurodegeneration (14–16). Phosphorylated (i.e., activated) PERK has been detected in the brains of patients with Alzheimer disease (17), Parkinson disease (18), and progressive supranuclear palsy (19), among others. A genomewide association study of progressive supranuclear palsy showed that EIF2AK3 is genetically associated with the disease (20). One of the mechanisms by which PERK is thought to play a role in neurodegeneration is through its function in regulating autophagy.

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Julius Bruch was funded by the Bavarian Research Foundation (Bayerische Forschungsstiftung). Günter U. Höglinger was funded by DFG (Deutsche Forschungsgemeinschaft; Grant No. HO2402/6-2).

The authors declare no conflicts of interest.

Abstract

We report the first detailed examination of the brain of a patient with Wolcott-Rallison syndrome. Wolcott-Rallison syndrome is an extremely rare clinical manifestation of a lack of protein kinase R–like endoplasmic reticulum kinase (PERK) function caused by mutations in the PERK gene EIF2AK3. Protein kinase R–like endoplasmic reticulum kinase is thought to play a significant pathogenetic role in several neurodegenerative diseases, including Alzheimer disease, other tauopathies, and Parkinson disease. The brain of a male patient aged 4 years 7 months showed pathologic and immunohistochemical evidence that the absence of PERK for several years is sufficient to induce early changes reminiscent of various neurodegenerative conditions. These include neurofibrillary tangles (as in progressive supranuclear palsy), FUS-immunopositive and p62-immunopositive neurons, and reactive glial changes. We also detected an increased amount of p62-positive puncta coimmunostaining for LC3 and ubiquitin, suggesting changes in autophagic flux. Studying a human brain with absent PERK function presents the opportunity to assess the long-term consequences of nonfunctioning of PERK in the presence of all of the compensatory mechanisms that are normally active in a living human, thereby confirming the importance of PERK for autophagy in the brain and for neurodegeneration.

Key Words: Alzheimer disease, Autophagy, FUS, p62, Parkinson disease, PERK, Tau, Wolcott-Rallison syndrome.
(10, 11, 21) and other cell protective mechanisms, such as oxidative stress defense (9). Autophagy is thought to be critical to the health of neurons because they cannot rely on mitosis to rid themselves of excess proteins and cellular material (22). In fact, stimulation of autophagy has been shown to mitigate pathology in models of Alzheimer disease, other tauopathies, and Parkinson disease (22–25).

The present study presents the first immunohistochemical examination of the brain of a patient with WRS. In light of recent developments in the role of PERK in neurodegeneration, we examined the brain of a 4-year-old boy with WRS for findings of neurodegenerative diseases.

**MATERIALS AND METHODS**

**Search for Tissue From Patients With WRS**

We started our search for WRS postmortem brain material by contacting all institutions that had published cases of the syndrome or had written about the syndrome in PubMed-listed literature. This step yielded samples of 1 patient from Lyon University Pediatric Hospital (Lyon, France) in which EIF2AK3 had previously been identified as the gene responsible for WRS. Next, we searched the Brain Net Europe database and contacted leading pediatric units across the world. We were unable to find brain material from additional WRS cases with confirmed EIF2AK3 mutations.

**Genotyping**

DNA was extracted from peripheral blood collected on EDTA. The diagnosis of WRS was confirmed by direct sequencing of EIF2AK3 on genomic DNA, as previously described (5). This showed a c.3009C→T substitution that is homozygous in the patient and heterozygous in the parents, resulting in a p.R903* nonsense mutation and a truncated protein.

**Postmortem Tissue**

Three blocks (2 blocks from the frontal cortex and 1 block from the cerebellum) of paraffin-embedded tissue from a single patient were obtained from the Department of Pathology and Neuropathology, Groupement Hospitalier Est (Bron, France). The parents had given full permission for the use of the material and medical records for research purposes, according to the Declaration of Helsinki. Equivalent blocks of paraffin-embedded tissue from 3 age-matched control cases were obtained from the Center for Neuropathology and Prion Research, University of Munich (Munich, Germany). These were anonymized routine biopsy cases. The use of the material was in accordance with the directives of the local ethics commission regarding the use of archive material for research purposes. Table 1 shows an overview of the cases described.

**Immunohistochemistry**

The paraffin blocks were cut on a microtome to a thickness of 5 μm. Tissue was deparaffinized and progressively rehydrated according to the Abcam protocol (www.abcam.com/protocols). Hematoxylin and eosin (Hoffmann-LaRoche, Basel, Switzerland) staining was performed according to the manufacturer’s guidelines. Antigen retrieval was performed by heating slides in 10 mmol/L sodium citrate (Sigma-Aldrich, St Louis, MO) buffer at 90°C for 20 minutes. Immunohistochemical staining was performed semiautomatically on a BenchMark IHC device (Ventana, Tucson, AZ [now Hoffmann-LaRoche]). The primary antibodies, concentrations, and incubation times used are shown in Table 2. iView DAB and ultraView DAB (Perkin-Elmer, Waltham, MA) were used as detection systems. Nuclear counterstaining was performed with hematoxylin (Hoffmann-LaRoche). Microscopy and imaging were performed on a Leica CTR 6000 microscope (Leica Microsystems, Wetzlar, Germany).

**Double-Label Immunofluorescence**

Tissue sections were cut, deparaffinized, and rehydrated, and antigens were retrieved, as described previously. The slides were blocked in 5% normal goat serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline (PBS) with 0.2% Tween (Sigma-Aldrich). Primary antibody was incubated in 2% normal goat serum in PBS with 0.2% Tween overnight at 4°C. The antibodies and concentrations used are listed in Table 2. The slides were washed 3 times for 5 minutes in PBS and incubated for 2 hours in 2% normal goat serum in PBS with 0.2% Tween at room temperature with the fluorescent secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (Life Technologies [now Thermo-Fisher Scientific, Carlsbad, CA]) and Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies). DAPI dihydrochloride (Thermo-Fisher Scientific) 300 nmol/L was added and incubated for 10 minutes. The slides were washed 3 times for 5 minutes in PBS and coverslipped with polyvinyl alcohol mounting medium with DABCO antifading mounting medium (Sigma-Aldrich). Microscopy and imaging were performed on a Leica TCS SP5 II laser confocal microscope (Leica Microsystems).

**RESULTS**

**Clinical Case History**

The patient (Case 2 in [26]) was a 4-year-old boy born to consanguineous Kosovar parents. He showed the first signs of diabetes mellitus at age 6 months, which were treated with
twice daily insulin injections. A clinical diagnosis of WRS was made only at age 4 years when he first presented to the Lyon University Pediatric Hospital together with his older sister, who was also affected.

At the time of diagnosis, the glycated hemoglobin level was 8.6%. Radiographs showed skeletal dysplasia in carpal bones and phalanges, capital femoral epiphyses, and vertebral bodies. There were no further abnormalities on ultrasound examination of organs or on liver and kidney function tests. No neurologic abnormality was noted.

Simon later, at age 4 years 7 months, the patient presented to the intensive care unit of the Lyon University Pediatric Hospital with sudden-onset coma, vomiting, hepatomegaly (7 cm), tachycardia (111 beats/minute), and mild pyrexia. Blood pressure dropped from 90/46 mm Hg initially to 53/27 mm Hg. There was coagulopathy (factor V, 14%; prothrombin time, 13 seconds; fibrinogen, 0.9 g/L). Liver function values were outside the reference range (ammonia, 859 K mol/L; lactate, 17.6 mmol/L; alanine aminotransferase, 8,000 IU/L; aspartate aminotransferase, 16,000 IU/L). Renal function was also compromised, with a potassium level of 6.6 mEq/L and a serum creatinine level of 270 K mol/L. Diagnoses of hepatic encephalopathy and renal failure were made. Treatment was supportive (with mechanical ventilation) until death occurred by vascular hypotension and ventricular arrhythmia.

Genetics

Genetic testing revealed the patient to be homozygous for the mutation R902stop in the EIF2AK3 gene. This mutation has been described before in 2 siblings of Kosovo-Albanian origin with similar clinical history (27). The mutation leads to truncation of much of the second active site (Fig. 1). This suggests that PERK in this patient is present as a truncated, functionally inactive fragment.

General Autopsy Findings

No external abnormality, except for delayed growth and reduced body weight (12 kg), was noted. The liver was enlarged (722 g), showing mildly expanded and inflamed portal table: 2. Primary Antibodies and Immunostaining Conditions

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PHF, paired helical filament; RT, room temperature.
tracts and hypereosinophilic or vacuolated hepatocytes. The pancreas was small (20 g) with hypereosinophilic exocrine tissue. Results of the full pathologic examination are described in the study by Collardeau-Frachon et al (26).

Because the brain was not the center of attention at the time of autopsy examination, only limited information was recorded. There was slight cerebral edema with early tonsillar herniation. Gyration was not abnormal, but sections had pale appearance. No vascular abnormality or focal lesion was observed. The cerebellum and brainstem had no macroscopic abnormality. No brain weight or size was recorded.

Histologic Findings

Hematoxylin and eosin staining of the frontal cortex and cerebellum showed reduced cell density in the molecular layer, compared with age-matched control cases (Fig. 2). The subpial region of the molecular layer was irregularly rarefied. No overt oligodendrocyte pathology was noted, and there was no evidence of proteinaceous inclusions in oligodendrocytes, such as coiled bodies. Luxol fast blue-periodic acid Schiff myelin staining did not show any abnormality of white matter tracts. On NeuN immunostaining, there was no visible abnormality of layering.

Immunostaining for glial fibrillary acidic protein revealed unusually prominent and corkscrew-like extensions of Bergmann glial cells in the cerebellum (Fig. 3B) that were not present in normal controls (Fig. 3A). Glial fibrillary acidic protein staining also showed focal clusters of activated astrocytes, especially in the subpial region of the cortex (Fig. 3D), which were much larger than those in control cases (Fig. 3C). These findings are consistent with long-term gliosis. To investigate this further, we stained for microglia with the Iba1 marker (Figs. 3E, F). The WRS brain showed higher density and some clustering of microglia compared with the 3 control brains. Staining for the activated microglia marker CR3/43 in the WRS brain, however, yielded negative results.

There were individual neurons with FUS-positive inclusions (Figs. 4A, B), which are associated with certain types of frontotemporal dementia (28). Other neurons stained positive for AT8 (Figs. 4C, D), an antibody against phosphorylated paired helical filament tau; AT8-positive tau is considered to be pathologic (29). The appearance was that of a globose neurofibrillary tangle, possibly suggesting the beginnings of tau pathology akin to that in progressive supranuclear palsy. These findings were absent in the 3 control cases and in cerebellar tissue.

**FIGURE 2.** Hematoxylin and eosin staining (10×) of sections of the frontal cortex and cerebellum. There was reduced cell density in the molecular layer of the cerebellum and in the outer gray matter layers of the frontal cortex in the WRS case compared with age-matched controls. Scale bar = 50 μm.
The WRS brain yielded negative immunostaining for α-synuclein, β-amyloid, and TDP-43 aggregates and for cleaved caspase 3 (positive control: lymphoma). Gallyas staining also did not reveal any abnormal changes (data not shown).

**Impairment of Autophagic Flux**

We looked further into the impact of absent PERK function in this brain on autophagy. We saw several cells that were positive for p62, a marker that can accumulate in cells with interrupted autophagic flux (Figs. 4E, F). These were not observed in the age-matched control brains.

Using double-label immunofluorescent laser confocal microscopy, we observed p62 in scattered puncta in a wide distribution in the WRS brain; some cells had pronounced density (Fig. 5A). Although there were fewer puncta that were positive for p62 than for LC3 overall, there was considerable overlap. As expected, there was also a high level of overlap of p62 with ubiquitin (Fig. 5B). The control brains showed virtually no such p62-positive puncta.

**DISCUSSION**

Examination of samples from the brain of a 4-year-old boy without PERK function presented a unique opportunity to

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**FIGURE 3.** Glial histology. (A) Glial fibrillar acidic protein immunostaining (63×) with hematoxylin counterstaining in a control brain shows only thin straight processes of Bergmann glial cells. (B) Glial fibrillar acidic protein immunostaining (63×) with hematoxylin counterstaining in the WRS brain shows thickened and corkscrew-like processes of Bergmann glial cells. (C) Glial fibrillar acidic protein immunostaining (63×) in a control brain shows a normal pattern of glial fibrillar acidic protein-positive astrocytes. (D) Glial fibrillar acidic protein immunostaining (63×) in the WRS brain shows a cluster of reactive enlarged astrocytes adjacent to the pia mater. (E) Iba1 immunostaining (4×) of the cerebellum of a control brain shows normal distribution of microglia. Scale bar = 350 μm. (F) Iba1 immunostaining (4×) of the cerebellum of the WRS case shows increased density and clustering of microglia. Scale bars = (A–D) 20 μm; (E, F) 350 μm.
learn about the effects of PERK dysfunction without the limitations of animal models and short-term cell culture models.

We found evidence that a lack of PERK function for several years is sufficient to induce changes reminiscent of the early stages of several neurodegenerative diseases (i.e., formation of globose AT8-positive neurofibrillary tangles typical of tauopathies and FUS-positive neurons occurring in some forms of frontotemporal dementia). There were generally only small numbers of cells affected by these immunohistochemical changes; however, these findings were completely absent

FIGURE 4. Immunohistochemical findings for the frontal cortex of the WRS brain. (A) FUS immunostaining shows an individual FUS-positive neuron. Original magnification: 63×. (B) FUS immunostaining shows 2 immunopositive neurons. The box indicates the field shown in (A). Original magnification: 10×. FUS-positive cells were generally farther apart. (C) AT8 tau immunostaining showing globose neurofibrillary tangles in the cytoplasm of a neuron. Original magnification: 63×. (D) AT8 tau immunostaining showing 2 immunopositive neurons, including the one shown in the box in (C). Original magnification: 10×. AT8 tau immunostaining showing 2 immunopositive neurons, including the one shown in the box in (C). Original magnification: 10×. Positive cells were generally farther apart. (E) p62 immunostaining showing an immunopositive cell. Original magnification: 63×. (F) p62 immunostaining showing 2 p62-positive cells. Original magnification: 10×. Immunopositive cells were generally farther apart. Box indicates the field shown in (E). Scale bars = (left column) 10 μm; (right column) 50 μm. Arrowheads point to immunopositive cells.
in the 3 age-matched control brains. In addition, there were also changes suggesting activation of astrocytes and Bergmann glial cells, and increased density and clustering of microglia. These may represent process similar to the fibrotic and inflammatory processes observed in other organs of patients with WRS.

We also observed changes suggestive of impaired autophagy. Although there is already an emerging body of knowledge on the role of PERK in regulation of autophagy, the case described allowed, for the first time, an assessment of the impact of absent PERK function for several years on autophagy in a human. In the process of marking misfolded proteins for macroautophagy, proteins get ubiquitinated and tagged with p62, which in turn binds to LC3 proteins on nascent autophagophores. Colocalization of p62, LC3, and ubiquitin in the WRS brain suggests that this process works well in the absence of PERK function. However, the increased amount of p62-positive puncta coimmunostaining for LC3 and ubiquitin and the existence of some cells staining very intensely for p62 suggest that, in numerous cells in the WRS brain, formed vesicles accumulate and do not proceed to lysosomal fusion and degradation. This corresponds to our knowledge that ATF4, the transcription factor acting downstream of PERK, induces a set of autophagy-related genes, including ATG5, ATG7, and ATG12. These genes are involved mainly in the completion and coating of autophagic vesicles. This observation is relevant because autophagy has been implicated in a large number of neurodegenerative conditions.

In conclusion, the present study shows, for the first time, evidence that the absence of PERK is sufficient to induce early changes reminiscent of various human neurodegenerative diseases and confirms the importance of PERK for autophagy in the pathogenesis of neurodegeneration.

ACKNOWLEDGMENTS

Ms Brigitte Kraft performed many of the immunohistochemical stainings. We thank Cécile Julien and Valérie Seneé (Inserm UMR-S958, Medical Faculty Paris 7, site Villemin, Paris, France) for their work in mutation analysis. We would like to acknowledge the many people we contacted in our
search for brain autopsy material from WRS cases who searched their databases and brain banks for us.

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