Neuroserpin Mutation S52R Causes Neuroserpin Accumulation in Neurons and Is Associated with Progressive Myoclonus Epilepsy

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The protein neuroserpin has recently been found to be involved in the pathogenesis of a neurodegenerative disease (1, 2). This protein, a serine protease inhibitor mainly expressed in the central nervous system, is a regulatory element of extracellular proteolytic events and inhibits the activity of a tissue plasminogen activator (tPA) (3, 4). The gene encoding neuroserpin is mapped to chromosome 3q26 and its open reading frame encodes 410 amino acids (5, 6). This gene is referred to as PII2 and Neuroserpin; throughout this paper we will use the latter. Recent studies have shown that genetic mutations in the Neuroserpin gene are associated with the deposition of neuroserpin in neurons and the formation of intracytoplasmic bodies containing the mutant protein (1, 2, 7).

The first genetic mutation found in the Neuroserpin gene causes a proline for serine substitution at residue 49 (S49P) and is associated with a newly recognized form of hereditary presenile dementia (1, 2). A second mutation in the Neuroserpin gene, which causes an arginine for serine substitution at residue 52 (S52R), has been found in 2 families, 1 from Oregon and 1 from Indiana (1, 7). The phenotypes associated with the S49P and S52R mutations differ considerably; in fact, while presenile dementia appears as the main clinical feature of the S49P mutation, we have found that the neurodegenerative disease caused by the S52R mutation is characterized clinically by generalized seizures, myoclonus, and progressive dementia, i.e. progressive myoclonus epilepsy.

In order to begin understanding the basis of the neurological dysfunction seen in this group of diseases and to highlight differences in the phenotypes caused by Neuroserpin mutations, we studied the neuropathological phenotype associated with the Neuroserpin S52R mutation. We describe for the first time this neuropathological phenotype by giving an account of the distribution of the lesions and providing an analysis of the intracellular pathological changes as they relate to neuroserpin accumulation.

MATERIALS AND METHODS

Pedigree and Clinical Evaluation of the Proband

The family of the proband, identified here as “family J,” has been in Indiana for several generations. We were able to reconstruct a pedigree consisting of 46 members over 7 generations. Figure 1 shows an abbreviated pedigree of this family. The proband (subject III-1) first presented neurological signs affecting activities of daily living at 24 yr of age. Neurological,
psychiatric, and neuropsychological examinations were obtained throughout the course of the disease, including 9 electroencephalograms (EEGs), 1 auditory brainstem response (ABR), 2 electromyograms (EMGs), 1 somatosensory evoked potentials (SEPs), 2 brain computed tomographic (CT) scans, and 3 brain magnetic resonance images (MRIs). A slit-lamp microscope examination was carried out in order to rule out the diagnosis of Wilson disease.

Biopsy Studies: At 3 separate times during the disease course, tissue biopsies (2 skin and 1 liver) were done to exclude a diagnosis of a metabolic disease. Tissue was processed for histological and electron microscopic studies according established protocols.

DNA Extraction and Genetic Analysis: A molecular genetic analysis was originally carried out in order to rule out the possibility of a mutation in the Prion Protein (PRNP) gene. Informed consent from the next of kin was obtained for DNA studies. DNA from the proband was extracted from peripheral leukocytes and the PRNP gene was sequenced (8, 9).

At the time of the proband’s death, a definite clinical diagnosis had not been made.

Autopsy and Anatomic Pathology

An autopsy was carried out 2 h after death and included the examination of the organ systems, eyes, central nervous system, dorsal root ganglia, and a segment of the sciatic nerve.

Tissue samples of the pituitary, lung, heart, liver, kidney, pancreas, gall bladder, spleen, small and large intestines, esophagus, testes, lymph nodes, vertebrae, and aorta as well as the thyroid, parathyroid, adrenal, and prostate glands were fixed with 10% formalin. Additional samples of the spleen, heart, liver, and muscle were frozen and stored at $-70^\circ$C. Special chemical analyses (7).

Neurohistology:

The fresh brain was hemisected along the sagittal plane; the left cerebral hemisphere, the left half of the brainstem and of the cerebellum were fixed with 10% formalin. The right cerebral hemisphere, the right half of the brainstem and of the cerebellum were sliced and stored at $-70^\circ$C for genetic and biochemical analyses (7).

Following formalin fixation the tissue samples were dehydrated in graded alcohols, cleared in xylene, embedded in paraffin and 8-µm-thick sections were cut with a Leica rotary microtome. In addition, coronal slices were also processed for histology using a similar method and cut for whole mount on a Leica-Jung Polycut microtome. The sections were then stained with H&E, the Heidenhain-Woelcke method for myelin, Bodian method for fibrils, PAS, and PAS after diastase as well as Congo red and thioflavin S for amyloid, and alcin blue for mucopolysaccharides. Samples of formalin fixed tissue, frozen at $-20^\circ$C, were cut in a cryostat and sections were stained with Sudan black B and oil red O.

Immunohistochemistry:

Polyclonal antibodies raised against human neuroserpin (1:2,000) (4, 10), glial fibrillary acidic protein (GFAP) (Dako, Carpinteria, CA) (1:100), a-B crystallin (Vector Laboratories, Burlingame, CA) (1:750), a synthetic peptide corresponding to residues 119–137 of human a-synuclein (1:300), ubiquitin (Dako) (1:1,000), the low molecular neurofilament protein (68 kD) (Chemicon, Temecula, CA) (1:100), and the medium molecular weight neurofilament protein (145 kD) (Chemicon) (1:100) were used, as were monoclonal antibodies against the amyloid B protein (AB) [10D5 (Elan Corporation, San Francisco, CA) (1:100)], the phosphorylated and nonphosphorylated high-molecular weight neurofilament subunit [SMI 31 and SMI 32, (Sternberger Monoclonal, Luther ville, MD) (1:2,000)] (11), tissue-plasminogen activator (Chemicon) (1:500), microtubule associated protein 2 (MAP-2) [SMI 31 and SMI 32, (Sternberger Monoclonals) (1:2,000)] (12) and tubulin (Chemicon) (1:200). Phosphorylation-dependent and phosphorylation-independent anti tau monoclonal antibody [AT8 (Polymedco, Cortlandt Manor, NY) (1:400) and Alz 50 (donated by P. Davis) (1:20)] were used; the former recognizes phosphorylated Ser202/Thr205 (13) and the latter preferentially recognizes a conformation of assembled tau protein (14).

Double immunolabeling studies, using anti-neuroserpin and anti-high molecular weight neurofilament antibodies, were carried out to determine the location of neuroserpin accumulation, and specifically to establish if they were in the neuronal perikarya, cell processes, or both. Polyclonal antibodies raised against neuroserpin were detected using avidin-biotin with goat anti-rabbit immunoglobulin as secondary antibody, and horseradish peroxidase-conjugated streptavidin visualized with chromogen diaminobenzidine or tetramethylbenzidine. Monoclonal antibody SMI 32 was revealed by avidin-biotin using goat anti-mouse immunoglobulins as secondary antibody and streptavidin conjugated with alkaline phosphatase.

Fig. 1. Pedigree of “family J.” The 3 subjects affected by progressive myoclonus epilepsy (II-1, III-1, and III-2) are shown by the symbols $\bigcirc$ and $\bullet$. The proband is indicated by $\bigcirc$. Deceased members are indicated by $\bigcirc$. The fresh brain was hemisected along the sagittal plane; the left cerebral hemisphere, the left half of the brainstem and of the cerebellum were fixed with 10% formalin. The right cerebral hemisphere, the right half of the brainstem and of the cerebellum were sliced and stored at $-70^\circ$C for genetic and biochemical analyses (7).
At age 27 seizures reappeared; they were myoclonic, complex partial, and tonic-clonic seizures. In spite of aggressive treatment with phenytoin, carbamazepine, valproic acid, and clonazepam, his seizures were difficult to control and there were several episodes of status epilepticus.

A neurological examination at age 29 revealed slow speech, diplopia, vertical and horizontal directional nystagmus, dysarthria, and myoclonus in the extremities. The tendon reflexes of the extremities were increased, except for the Achilles tendon reflex, which could not be elicited. Sensory examination showed hypoalgesia in a glove and stocking distribution. A neuro-opthalmologic examination was negative for both a cherry red spot in the fundus ocular and a Kayser-Fleischer ring in the cornea. Routine laboratory tests were normal.

*Neuropsychology:* The patient was tested 5 times over a 10-yr interval. He was first examined in the context of a disability evaluation at age 28. The proband had stopped working and his daily activities consisted of keeping house, going to church, and caring for his 4-yr-old son. He was described as somewhat depressed. Intelligence was measured in the borderline defective range on Wechsler Adult Intelligence Scale-Revised (WAIS-R, Full Scale IQ = 73, Verbal IQ = 78, Performance IQ = 68) (16). This represented a drop of approximately 30 points (or 2 standard deviations) from estimated premorbid levels based on educational and occupational attainment. The second examination occurred about 13 months later and revealed significant interval decline in intelligence which was now in the mildly defective range (WAIS-R, Full Scale IQ = 65, Verbal IQ = 69, Performance IQ = 65). The most significant deficits were in working memory, visuoconstructual ability, and fine manual dexterity. The patient’s short-term memory was relatively intact (Wechsler Memory Scale, Logical Memory, Story A = 8.5/24 units recalled immediately and 7/24 units recalled after 30-min delay; Visual Reproduction 8/14 units recalled immediately and 6/14 after a 30-min delay). The third assessment took place at age 34, when the patient was a resident in a nursing home, and consisted of the Mini-Mental State Examination (MMSE) (17). His score was 24/30 indicating mild cognitive impairment with more trouble in attention (0/5 on serial subtraction) than memory (2/3 on delayed recall of objects). The fourth examination occurred when the patient was 37-yr-old and consisted of the Consortium to Establish a Registry for Alzheimer Disease (CERAD) neuropsychological battery (18). His MMSE score was fairly stable at 23/30. The most significant deficits on this battery occurred on tests of constructional ability (Constructional Praxis = 1st percentile) and sequential tracking (Trail Making Test Part A = unable to complete); short-term memory was within broad normal limits (3/3 objects recalled after a delay; fully oriented to time and place;

**Electron Microscopy:** Tissue obtained from the frontal and temporal cortices, the hippocampus and the liver were fixed with 4% formaldehyde and post-fixed with 1% osmium tetroxide, dehydrated in graded alcohols, cleared in propylene oxide, and embedded in Epon. One-μm-thick sections were stained with toluidine blue. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and scanned with an electron microscope.

**DNA Extraction from Brain Tissue and Genetic Analysis**

DNA was extracted from the brain of the proband using the method previously described (15). The DNA was analyzed by direct sequencing of exons 2–9 of *Neuroserpin*. Amplification and sequencing were done using primers previously reported (1).

**RESULTS**

**Family History**

The pedigree is shown in Figure 1 with the proband listed as subject III-1. Only limited medical information was available on most family members. Subject II-1, the proband’s mother, presented seizures at 25 yr of age and was diagnosed as having progressive myoclonus epilepsy. She died at 37 yr of age following third degree burns. Subject III-2, the proband’s brother, is currently alive and has been diagnosed as having progressive myoclonus epilepsy. Genetic testing on subject II-2 has revealed the presence of the S52R mutation in the *Neuroserpin* gene (7).

Prior to subject II-1, only 2 subjects were known to have had a neuropsychiatric disorder. These were a paternal uncle and a sibling of subject I-2. The paternal uncle is reported to have been institutionalized in a mental hospital and diagnosed as having epilepsy, psychosis, and cerebral arteriosclerosis, and to have died at age 57. The sibling died at age 64 after a 14-yr history of a dementing illness diagnosed clinically as Alzheimer disease and confirmed neuropathologically after death. Subjects I-1 and I-2 died at 66 and 89 yr of age, respectively and the cause of their deaths is unknown. Subject II-2 is alive and well at age 60.

**Clinical History of the Proband**

**Subject III-1:** Prior to age 24, the proband, a Caucasian male, had been healthy and had conducted an uneventful life working as an architectural drafter. According to information obtained from family members, he had frequent episodes of somnambulism since adolescence and was often found profusely sweating following these episodes. At 24-yr of age, he had an episode of generalized seizures during the night and periodically there after continued to have action myoclonus. His performance at work deteriorated and he had difficulties with memory, occasionally being unable to write his own name.
CERAD Word List Learning sum recall = 27th percentile, delayed recall = 25th percentile. The fifth examination was done 18 months later, at age 38, again using the CERAD neuropsychological battery. The patient’s MMSE score had dropped to 10/30 with marked decline in visuoconstructional ability and expressive language (Animal Fluency Test < 1st percentile; Boston Naming Test < 1st percentile) and milder decline in verbal short-term memory (Word List Learning sum recall = 1st percentile).

In summary, at the age of 28, 3 yr after the onset of the illness, the patient had already experienced disabling intellectual loss consistent with mild dementia. There was disturbance to his fund of knowledge but even more marked impairment in novel problem solving. Subsequent examinations revealed progressive deterioration with impairments in visuoconstructional ability more prominent than those in language and short-term memory. By age 38, there was severe generalized impairment consistent with advanced dementia.

Electrophysiology: EEGs, obtained at regular intervals, showed similar results throughout the course of the proband's illness; frequent spikes and spike and wave complexes were particularly prominent in the central and temporal regions bilaterally. An ABR examination, carried out when the patient was 29 yr of age, suggested a disturbance in the right pontomedullary junction. At the same time, a SEP examination showed normal response to median and tibial nerve stimulation and an EMG showed mildly decreased motor conduction velocity (MCV) and compound muscle action potentials (CMAP) of the left peroneal nerve. Another EMG, obtained at age 37, showed severely decreased MCV and CMAP of the right peroneal and deep tibial nerve. The possibility of a severe peripheral neuropathy was entertained; however, no further studies were carried out.

Skin and Liver Biopsies: A skin biopsy and a liver biopsy were obtained when the patient was 28 and 29 yr of age, respectively; a repeated skin biopsy was obtained when the patient was 37. The pathologic examination revealed neither Lafora bodies nor cellular deposits consistent with any storage or metabolic disease.

Neuroradiology: At 32 yr of age, an MRI showed no abnormal findings; while at age 35, mild cerebellar atrophy was noted. At age 37, cerebellar atrophy was evident on CT and MRI scans.

Generalized seizures and myoclonus continued to persist despite pharmacological treatment. At the age of 30, he was slow in mental processing and calculations, but he was still oriented to time and place; his walk was impaired due to severe myoclonus. He was placed in a nursing home at age 32. As the disease progressed, myoclonus of the face and extremities worsened as well as cerebellar ataxia. He died from aspiration pneumonia at age 43.

Autopsy and System Pathology

The general autopsy revealed a normocephalic, well-developed male. On gross examination, patchy consolidation of the lower lobes of the lungs, mild atheromatous changes of the thoracic aorta, and petechial hemorrhages of the mucosa of the urinary bladder were seen.

Light Microscopy: The lungs showed an organized aspiration pneumonia and an acute bronchopneumonia. The spleen showed a reduction of the white pulp and the urinary bladder showed an acute cystitis. Tissue samples of the peripheral organs were otherwise histologically unremarkable and the cytological examination did not reveal pathologic intracellular inclusions. Immunohistochemical analysis of skeletal muscles, lung, liver, spleen, and pancreas as well as pituitary, adrenal and thyroid glands, using antibodies against neuroserpin, did not reveal immunopositivity. An examination of the striate muscle revealed mild type II fiber atrophy.

Electron Microscopy: Parenchymal cells of the liver did not show intracytoplasmic bodies.

Neuropathology

Gross Neuropathology: The fresh brain weighed 980 g and showed diffuse atrophy, which was most evident at the level of the frontal lobes. There was no atherosclerosis in the major cerebral arteries. On coronal sections, the caudate nucleus and cerebellum were mildly atrophic and the brainstem appeared reduced in volume.

Neurohistology: Neuronal loss was moderate in the frontal, cingulate, temporal, parietal, insular, and occipital cortices and the most affected were layers III, IV, V, and VI. Gliosis was present throughout the cortical layers. Mild neuronal loss and gliosis were also seen in the entorhinal and transentorhinal cortices, hippocampus, amygdala, basal ganglia, and thalamus. Small vessels with mineralized walls were noted in the globus pallidus. Mild gliosis was present in the white matter of the centrum semiovale. In the cerebellum, a mild to moderate loss of Purkinje cells was noted; many of these cells had an eosinophilic cytoplasm consistent with agonal hypoxia. The Golgi epithelial cells appeared more numerous than in control cerebellum and a mild hypertrophy of the Bergmann glia was seen. Furthermore, a mild gliosis was noted in the cerebellar white matter and dentate nucleus. The substantia nigra and locus coeruleus showed moderate neuronal loss and gliosis. Amyloid deposits and neurofibrillary pathology were not seen in thioflavin S and Congo red preparations.

The most striking cytological finding was the presence of intraneuronal eosinophilic homogeneous bodies in the neuronal perikaryon and the neuropil in most gray matter areas of the brain and spinal cord (Figs. 2–6). Within a single neuronal perikaryon, 1 or more bodies were present; in some instances, they were so numerous and
Fig. 2. A section of the proband’s frontal cortex (cingulate gyrus) immunolabeled with antibody to human neuroserpin. Layers II to VI contain numerous NBs of different sizes. Magnification: ×90.
crowded together that they resembled a cluster of grapes. When a major portion of the perikaryon was occupied by 1 or more intracytoplasmic bodies, the nucleus was eccentric and often deformed. The diameter of these bodies ranged from 1.5 to 25 μm with the smaller more likely to be in a cluster.

In addition to being strongly eosinophilic, these bodies were PAS-positive, with and without diastase treatment (Fig. 3A, B). In Bodian preparations, large bodies were darkly stained in the core and lightly stained in the outer part, while smaller bodies were diffusely pale throughout; in Heidenhain-Woelcke preparations, the bodies were darkly stained throughout, regardless of their size (Fig. 3C–E).

Antibodies against neuroserpin strongly labeled bodies of all sizes (Figs. 2, 4–6). The largest bodies were strongly immunopositive at the periphery with the inner part being less immunoreactive (Fig. 4). In some instances, a clear, crescent shaped space was seen on the side of the body with the distal surface of the space being neuroserpin-immunopositive. Using single and double immunohistochemistry, it was possible to establish the localization of the bodies in neuronal cell processes (Figs. 4–6). Double immunohistochemistry was particularly useful when antibodies against both non-phosphorylated high molecular weight neurofilament protein and neuroserpin were used in combination (Fig. 5). Intracytoplasmic inclusions of various sizes were seen in processes identified as dendrites (Figs. 4–6). These findings were particularly frequent in neurons adjacent to the thalamic fasciculus, substantia nigra, and the anterior horn cells (Figs. 4, 5, 6A–C). In some instances, a body was seen at a considerable distance from the perikaryon and its diameter was much wider than that of the dendrite (Figs. 4, 6A). In other instances, a body was in the proximity of the origin of the dendrites, while in other cases they appeared as string of beads (Figs. 4, 5A, B, 6C).

Immunopositivity, using anti-neuroserpin antibodies, suggested that the intracytoplasmic bodies in the patient of the Indiana family were composed of the protein neuroserpin. Indeed, these data were later confirmed by
biochemical studies (7). Therefore, these intracellular deposits will be referred to as neuroserpin bodies (NBs) in the rest of the manuscript.

Round NBs measuring 2 to 5 μm were observed in the neuropil and it was not possible to determine the cell type with which they were associated; however, in some instances they were seen adjacent to small cells, with round or oval nucleus, resembling glial cells.

NBs appeared more numerous in sections immunolabeled using neuroserpin antibodies than in those stained with H&E or PAS. NBs did not immunoreact with antibodies against GFAP, α-B crystallin, α-synuclein, neurofilament proteins, amyloid β protein, tissue-plasminogen activator, MAP-2, tubulin, tau, and ubiquitin.

NBs were numerous in the frontal, cingulate, temporal, parietal, insular and occipital cortices (Figs. 2, 7). In the neocortex, NBs were numerous throughout the cortical layers, with the exception of layer I and II. NBs were not seen in layer I and rarely were they found in layer II. In addition, they could occasionally be seen in the subcortical white matter. NBs were seen in most regions of the gray matter, including basal ganglia, thalamus, amygdala, hippocampus, subiculum, entorhinal cortex, substantia innominata, colliculus, periaqueductal gray, substantia nigra, red nucleus, oculomotor nerve nucleus, locus coeruleus, dorsal nucleus of raphe, pontine nucleus, hypoglossal nerve nucleus and dentate nucleus of the cerebellum. The substantia nigra was the area of the central nervous system most affected with NBs; in fact, they were consistently seen in neurons and cell processes of pigmented cells (Figs. 3, 4). In the spinal cord, they were seen in the nucleus of Clark, intermediolateral column.
Fig. 7. The topographical distribution of NBs is shown at several levels of the cerebral hemisphere (A–C), cerebellum (D), brainstem (E–G), and spinal cord (H). ●, frequent; △, moderate; ○, sparse.

nucleus, posterior column and posterior funiculus and anterior horn cells (Figs. 6C). No NBs were seen in the retina.

As neuroserpin immunoreactivity was seen in association with the round bodies, in some instances, neuronal perikarya and processes were diffusely immunopositive (Figs. 4, 6A, B). The latter pattern was observed in neurons of the substantia nigra and the thalamic fasciculus (Figs. 4, 6A, B).

In addition to central nervous system neurons, NBs were consistently found in the dorsal root ganglion cells (Fig. 6D). A significant observation was the moderate loss of these neurons; in the place of a degenerated neuron a cluster of satellite cells was found. The topographic distribution of NBs is shown in Figure 7 and summarized in the Table.

Electron Microscopy: In toluidine blue-stained sections, NBs appeared homogeneously blue with a lighter ring in the outer part (Fig. 3F).

Low magnification electron micrographs revealed numerous osmiophilic NBs within neuronal perikarya or neuronal processes. NBs were consistently round, oval, or with a slightly irregular contour and their diameter ranged from 1 to 31 μm (Figs. 8–10). In high magnification electron micrographs, the NBs appeared to be composed of a fine granular material (Fig. 10). Frequently, perikarya that contained only 1 body showed an electron-dense cytoplasm and a deformed nucleus, which was

Fig. 6. Sections of the proband’s substantia nigra (A), thalamic fasciculus (B), anterior horn of the spinal cord (C), and dorsal root ganglion (D) immunolabeled with antibody to human neuroserpin. A single NB in a cell process (A), several NBs in perikarya and cell processes (arrows) (B, C) and the dorsal root ganglion neuron (D) are seen. In addition, diffuse immunopositivity is seen in cell processes (A, B) and in the neuronal perikaryon (B). Magnification: A, B, ×960; C, D ×600.
shaped, electron lucent space that was limited by a unit membrane (Figs. 8, 9A). These were surrounded by an irregularly shaped, electron lucent space filled with normal organelles; however, neurons had a round or oval nucleus and electron-chromatin near the nuclear membrane. In some instances, structure with electron-dense nucleoplasm and clumps of no longer round, but it appeared as a narrow, elongated structure with electron-dense nucleoplasm and clumps of chromatin near the nuclear membrane. In some instances, neurons had a round or oval nucleus and an electron-lucent cytoplasm filled with normal organelles; however, a portion of the cytoplasm was occupied by 1 or 2 NBs (Figs. 8, 9A). These were surrounded by an irregularly shaped, electron lucent space that was limited by a unit membrane, which was most likely the rough endoplasmic reticulum (Fig. 10). The space between this membrane and the outer surface of the NB contained different amounts of floccular material. In these neurons, dilated spaces surrounded by endoplasmic reticulum membranes were also seen that contained floccular material only. In some instances, more than 1 body was surrounded by the electron lucent space containing the floccular material. Often, the electron lucent spaces had a crescent shape on 1 side of the NB. In electron micrographs that included the entire perikaryon and the NB, the electron dense material surrounded by the electron lucent space formed a compartment that appeared to be separated from the rest of the cytoplasm (Figs. 8, 9).

NBs were also found in neuropil elements (Fig. 9B). In several instances, it was not possible to identify the nature of the cell process; however, the body was always granular, electron dense, and surrounded by an electron lucent space. The latter was limited by a unit membrane. Some NBs appeared as round, granular structures that contained 1 or 2 smaller round and more electron-dense structures (Fig. 9B). In other instances, the granular electron dense body appeared as though it had incorporated multiple lipofuscin grains within its boundaries. Some NBs could be clearly identified within dendrites that were cut in cross sections (Fig. 9B). The membranes surrounding the NB were often well preserved and showed ribosomes that were adherent to the outer layer of the membrane. Some dendrites contained granular material that was diffuse throughout the cell process without the presence of dense osmiophilic round NBs.

**Molecular Genetics**

The DNA sequence of exons 3–9 was normal in the proband. Sequence of exon 2 showed a single nucleotide (A to C) substitution in 1 allele. This causes an amino acid change in codon 52 from a serine to an arginine (S52R).

**DISCUSSION**

Three subjects across 2 generations of an Indiana family have been diagnosed with progressive myoclonus epilepsy and we have found the Neuroserpin S52R mutation in 2 of them. Neuropathologic studies in 1 of these patients revealed that neuroserpin accumulates in most gray matter regions of the central nervous system and in neurons of the dorsal root ganglia. Immunohistochemical and neurocytological studies showed that in most instances, neuroserpin accumulates as round, compact deposits in neuronal perikarya and cell processes, forming bodies that are well defined from the rest of the cytoplasm by the membranes of the endoplasmic reticulum. Less frequently, neuroserpin immunoreactivity was detected diffusely throughout the neuronal cytoplasm without the formation of well-demarcated bodies.

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<thead>
<tr>
<th>Anatomical region</th>
<th>Semiquantitative assessment of NBs</th>
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<td>Cerebrum</td>
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<td>Frontal cortex</td>
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<td>Cingulate cortex</td>
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Semiquantitative analysis of neuroserpin bodies, 1+ = sparse, 2+ = moderate, 3+ = frequent.
Fig. 8. Electron micrographs showing neurons containing NBs. Note that the space surrounding the NBs contains floccular amorphous material and is circumscribed by a unit membrane. Magnification: A, ×7,000; B, ×6,300.
Mutations in the *Neuroserpin* gene have been found in 3 families, including the 1 currently reported. These mutations cause either a proline for serine substitution at residue 49 or an arginine for serine substitution at residue 52; the former was previously reported in a New York family and the latter is reported here in an Indiana family and previously in an Oregon family (1, 2).

The disorders caused by *Neuroserpin* mutations are autosomal dominant and the clinical phenotype associated with each of the 2 *Neuroserpin* mutations differs for the
age at onset of the neurological signs and in the type and evolution of symptoms. The age at onset of the neurological signs in the Indiana family is as early as 24 or 25 yr of age, similar to that seen in patients of the Oregon family where the onset is as early as age 18 (1, 2, 19). In contrast, in patients carrying the S49P mutation, the age at onset of the neurological signs occurs as early as age 39 and as late as age 50 (1, 2). It is noteworthy that the neurological syndrome in the patients of the 3 families shows important phenotypic differences. The individuals with the S49P mutation do not have seizures, but suffer from presenile dementia (1, 2). In contrast, in both the Indiana and Oregon families, subjects with the S52R mutation have seizures and dementia (19, 20). Moreover, the patients from the Indiana family meet the criteria for a diagnosis of progressive myoclonus epilepsy (21, 22).

Several significant observations were made in the course of our studies. The neuropathologic data show that neuroserpin accumulation is associated with differing degrees of brain atrophy. In fact, the brain of the patient of the Indiana family with the S52R weighed 980 g, while in patients with the S49P mutation, its weight ranged from 1,400 to 1,530 g (2). It appears that the severity of neuroserpin accumulation may also vary as we compare the Indiana family with the New York family. In fact, NBs are located mostly in layers III and V of the cortex in the latter, while in the former the deposits are abundant in layers III, IV, V, and VI and only occasionally in layer II and in the subcortical white matter. Further studies may clarify these neuropathologic differences.

Except for a cortical biopsy study in a patient with the S52R Neuroserpin mutation from the Oregon family, no other neuropathologic data have been reported on patients with this mutation. In the proband of the Indiana family, NBs are present throughout most gray matter regions and are most numerous in the neocortex and substantia nigra. The severity of neuroserpin deposition in the cortex of the proband seems to be greater than that seen in the cortical biopsy of the Oregon patient. It should be emphasized that while the biopsy was carried out on an individual who had symptoms for only 3 yr, in the patient of the Indiana family, the disease had occurred for 19 yr.

The severe cortical pathology correlates well with the severe deterioration in cognitive function, documented by repeated neuropsychological testing. In addition, the severe neuroserpin deposition in the substantia nigra may be the cause of the extrapyramidal signs, which developed as the disease progressed. It is significant to note that the spinal cord and the dorsal root ganglia were also affected, a finding that may be correlated with the sensory abnormalities detected clinically. The distribution of NBs involves practically all cortical layers of both association and projection cortices as well as multiple subcortical neuronal populations; therefore, a specific clinicopathologic correlation relevant to the myoclonus cannot be made at this time without the results of a detailed intra vitam neurophysiologic analysis. It is of interest, however, that the cerebellar cortex and the dentate nucleus are practically free of NBs.

In diseases caused by Neuroserpin mutations, the distribution of NBs may reflect the regional expression of this protein. In fact, in the human brain, neuroserpin is expressed in the neocortex, putamen and spinal cord, but weakly in the cerebellum (4, 6). Neuroserpin was originally extracted from dorsal root ganglion neurons of chicken embryos (23). In addition to the central nervous system, neuroserpin is expressed in the pancreas and adrenal gland (4, 24); however, our studies have not revealed neuroserpin bodies in any internal organs.

In the proband of the Indiana family, we found that the NBs are present in the perikaryon and cell processes in the central nervous system. Within cell processes, they can be seen in dendrites; however, we can speculate that some may also be present in axons. These observations may be relevant to the function of the protein in nerve cells. At this time, we cannot establish whether neuroserpin accumulates in glial cells, even though we have occasionally seen neuroserpin-immunopositive structures very close to glial cells nuclei.

The immunohistochemical results, using antibodies to human neuroserpin, show strong immunoreactivity of the NBs and are consistent with biochemical data showing that the main constituent of these bodies is neuroserpin (7). Biochemical studies of cortical tissue from the proband have shown that NBs can be isolated in a relatively pure fraction and contain a major protein with a mass of ca. 50 kD. Amino acid sequence of this protein identified it as neuroserpin with a substitution of arginine for serine at residue 52, numbering according to Schrimpf et al, which includes the signal peptide (6). Further analysis will be needed to determine the changes in molecular structure occurring as the result of this genetic mutation.

Our electron microscopic data show that the accumulation of neuroserpin occurs in association with cellular compartments that can be identified as dilated cisterns of the rough endoplasmic reticulum. The NB, per se, is not membrane-bound, but it appears to be contained within a space limited by the membranes of the endoplasmic reticulum. The modalities of neuroserpin accumulation in compact homogenous bodies needs to be further investigated in cell models. However, on the basis of the biochemical studies on the patient from the Indiana family, NBs do not appear to be the result of neuroserpin polymerization (7).

The disease caused by the S52R mutation in the Indiana family may have some analogies with Lafora disease. In both conditions, progressive myoclonus epilepsy is the main clinical feature. However, the age of onset of Lafora disease is most often between 10 and 18 yr of age (25, 26). The gene responsible for that condition has been
localized on chromosome 6q and the disease is autosomal recessive, however, the enzyme defect in Lafora disease is still unknown (27). The Lafora bodies are mostly composed of polyglucosans (28). Their morphological characteristics are only superficially similar to NBs. Both Lafora and NBs are strongly PAS-positive and they are approximately in the same size range, i.e. 1 to 30 μm. Both may be present in perikarya or cell processes. However, electron microscopic examination reveals substantial differences between Lafora and NBs. Lafora bodies are nonmembrane-bound and are composed of glycogen-like granules interspersed with fine filaments measuring 8 to 12 nm in diameter, while NBs are composed of fine granular material and are found within dilated cisterns of the rough endoplasmic reticulum. Furthermore, the distribution of these inclusions in the central nervous system differs, particularly at the level of the cerebellum where Lafora bodies may be numerous and neuroserpin only rarely seen. Lafora bodies may be present in glial cells; however, this is not confirmed for NBs. Lafora bodies may be found in capillary endothelial cells of the brain, in the retina, skeletal and cardiac muscle, liver and in cells of the sweat ducts of the skin; on the contrary, we found NBs only in the central nervous system and dorsal root ganglia but not in any of the other organs.

Four separate reports (29–32) have described 4 unrelated patients affected by progressive myoclonus epilepsy associate with intraneuronal deposits referred to as “atypical inclusion bodies” that have histological, histochemical, and electron microscopic characteristics similar to those seen in diseases caused by neuroserpin accumulation. The disease in these cases did not appear to be familial (29–32). These individuals, who had similar signs, suffered from seizures, myoclonus, extrapyramidal signs, and mental deterioration or dementia. No pathologic changes were found outside the nervous system in the patients studied at autopsy. Interestingly, Ota et al reported that the small inclusions were often surrounded by the rough endoplasmic reticulum (31). Based on the reported studies, it is likely that type II myoclonus bodies are composed of neuroserpin. Genetic analysis of patients with type II myoclonus bodies may clarify the nature of these cytopathologic lesions.

In conclusion, we describe for the first time the association of progressive myoclonus epilepsy with the deposition of neuroserpin in the central nervous system. We emphasize the phenotypic differences within this recently recognized group of neurodegenerative diseases and highlighted the pathologic differences between progressive myoclonus epilepsy caused by neuroserpin accumulation and Lafora disease. The main findings are as follows: 1) neuroserpin accumulates in both the brain and the spinal cord; 2) outside the central nervous system, neuroserpin accumulates in the dorsal root ganglion neurons; 3) neuroserpin accumulates intracellularly within the cisterns of the rough endoplasmic reticulum; and 4) no intracellular accumulation of neuroserpin is found in other organs.

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