α-Synuclein Immunoreactivity Is Present in Axonal Swellings in Neuroaxonal Dystrophy and Acute Traumatic Brain Injury

KATHY L. NEWELL, MD, PHILIP BOYER, MD, PhD, ESTRELLA GOMEZ-TORTOSA, MD, PhD, WENDY HOBBS, BA, E. TESSA HEDLEY-WHYTE, MD, JEAN PAUL VONSATTEL, MD, AND BRADLEY T. HYMAN, MD, PhD

Abstract. The primary neuroaxonal dystrophies (NAD), which include infantile NAD and Hallervorden-Spatz syndrome (HSS), are characterized by dystrophic terminal axons and axonal swellings. Lewy bodies have been found in some cases. In Parkinson disease (PD) and dementia with Lewy bodies (DLB), Lewy bodies and neurites display prominent α-synuclein immunoreactivity. We examined 2 cases of HSS and 4 cases of infantile NAD with α-synuclein immunohistochemistry to test the hypothesis that these disorders with similar morphological findings might share a biochemical phenotype. Furthermore, we compared them to 8 cases of secondary or physiologic NAD of various causes and 2 cases of recent traumatic head injury. α-Synuclein positive neuronal cytoplasmic inclusions, including Lewy bodies, and neurites were numerous in 1 HSS and 1 infantile NAD case. In addition, axonal spheroids were immunostained in all 6 cases of primary NAD. 5 cases of secondary NAD, and 2 cases of recent head injury. Axonal spheroids were faintly stained in the 3 physiologic NAD cases. α-Synuclein positive axonal swellings may suggest a mechanism, such as axonal injury, leading to the neuronal cytoplasmic accumulation of α-synuclein in NAD and other disorders.

Key Words: α-synuclein; Axonal spheroids; Axonal swellings; Diffuse axonal injury; Hallervorden-Spatz syndrome; Lewy body; Neuroaxonal dystrophy.

INTRODUCTION

α-Synuclein immunoreactivity has been detected in the neuritic component of amyloid plaques in Alzheimer disease (AD) and Lewy body variant of AD (1), Lewy bodies, pale bodies, neurites, neuripil threads, and rare gli in Parkinson disease (PD) and dementia with Lewy bodies (DLB) (2–6), as well as in Lewy bodies or neuronal cytoplasmic inclusions in other disorders, including familial AD (7) and Hallervorden-Spatz syndrome (HSS) (6, 8). In addition, glial and neuronal cytoplasmic inclusions, neuronal intranuclear inclusions, some glial nuclei, neuripil threads, and swollen neuronal processes in sporadic multiple system atrophy are α-synuclein immunoreactive (6, 9–12).

Neuroaxonal dystrophies (NAD), defined histologically by the presence of dystrophic axonal swellings, can be classified as primary, secondary, or physiologic (normal) (13, 14). Primary NAD consists of such rare disorders as 1) infantile NAD, also called Seitelberger’s disease, 2) late infantile, juvenile, and adult NAD, 3) HSS, and 4) neuroaxonal leukodystrophy, which may include Nasu-Hakola disease (13–17). Little is known about the pathogenesis of these conditions, although a gene for HSS has been mapped to chromosome 20 (18). Secondary NAD is seen in degenerative diseases, such as PD and Wilson’s disease, certain metabolic disorders, chronic illness, vitamin E deficiency, human T-cell leukemia virus-1 infection, and leukencephalopathy associated with methotrexate therapy. Physiologic or normal NAD is a common finding in the aging central nervous system and occurs more often in the gracile than cuneate nuclei, the pars reticulata of the substantia nigra, and the inner segment of the globus pallidus.

In 2 recent cases of primary NAD, 1 HSS and the 1 infantile NAD, numerous Lewy bodies were identified by hematoxylin and eosin (H&E) staining. Neurofibrillary tangles, Lewy bodies, or both have been documented in association with a number of primary NAD cases, in addition to axonal swellings (17,19–27). We studied these 2 brains and 4 additional brains of primary NAD immunohistochemically with α-synuclein to see whether this sensitive marker for Lewy bodies and pale bodies in PD and DLB would uncover additional inclusions not observed with H&E staining and prove to be a common marker in primary NAD. In addition to numerous neuronal cytoplasmic inclusions, some corresponding to Lewy bodies, in multiple brain areas, we also observed the axonal swellings characteristic of primary NAD to be strongly immunoreactive for α-synuclein. This finding prompted the study of additional diseases with axonal swellings to confirm if similar morphological structures in conditions other than primary NAD are also α-synuclein immunoreactive.

Herein we make the observation that α-synuclein immunoreactivity is present in axonal swellings in a wide variety of settings, including primary neuroaxonal dystrophy (NAD), secondary NAD, including leukencephalopathy associated with methotrexate therapy, PD, DLB,
TABLE I

Demographic Data

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>NP</th>
<th>NAD type</th>
<th>Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.5</td>
<td>M*</td>
<td>NAD, inf</td>
<td>P</td>
<td>4.5 yr</td>
</tr>
<tr>
<td>2</td>
<td>6.5</td>
<td>F*</td>
<td>NAD, inf</td>
<td>P</td>
<td>5.5 yr</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>F</td>
<td>NAD, inf</td>
<td>P</td>
<td>14 yr</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>F</td>
<td>NAD, inf</td>
<td>P</td>
<td>19 yr</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>F</td>
<td>HSS</td>
<td>P</td>
<td>20 yr</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>F</td>
<td>HSS</td>
<td>P</td>
<td>41 yr</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>M</td>
<td>PD/d</td>
<td>S</td>
<td>12 yr</td>
</tr>
<tr>
<td>8</td>
<td>82</td>
<td>M</td>
<td>DLB</td>
<td>S</td>
<td>12 yr</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>M</td>
<td>MTX</td>
<td>S</td>
<td>1-7 m</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>M</td>
<td>NPC</td>
<td>S</td>
<td>7 yr</td>
</tr>
<tr>
<td>11</td>
<td>67</td>
<td>M</td>
<td></td>
<td>S</td>
<td>3 yr</td>
</tr>
<tr>
<td>12</td>
<td>90</td>
<td>F</td>
<td></td>
<td>Phy</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>86</td>
<td>M</td>
<td></td>
<td>Phy</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>73</td>
<td>M</td>
<td></td>
<td>Phy</td>
<td>NA</td>
</tr>
<tr>
<td>15</td>
<td>21</td>
<td>M</td>
<td>DA1</td>
<td></td>
<td>3.5 d</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>F</td>
<td>DA1</td>
<td></td>
<td>3 d</td>
</tr>
<tr>
<td>17</td>
<td>50</td>
<td>F</td>
<td>OPC/A/SN</td>
<td>NA</td>
<td>U</td>
</tr>
<tr>
<td>18</td>
<td>74</td>
<td>F</td>
<td>OPC/A</td>
<td>NA</td>
<td>U</td>
</tr>
</tbody>
</table>

Key: Age, age in years; NP, neuropathologic diagnosis; NAD, neuroaxonal dystrophy; Course, estimated duration of illness; M, male; *, siblings; inf, infantile type; P, primary; yr, years; F, female; HSS, Hallervorden-Spatz syndrome; PD/d, Parkinson’s disease plus dementia; S, secondary; DLB, dementia with Lewy bodies; MTX, leukoencephalopathy associated with methotrexate therapy; m, months; NPC, Niemann-Pick disease, type C; DP, dementia pigmentosa; C, elderly control subject without clinical neurological disease; Phy, physiological NAD associated with aging; NA, not applicable; DA1, diffuse axonal injury; d, days; OPC/A, olivopontocerebellar atrophy; SN, striatogniral degeneration; U, unknown.

Niemann-Pick disease type C, and dementia pigmentosa, physiologic NAD associated with aging, and cerebral trauma.

MATERIALS AND METHODS

Case material consisted of the following: 1) 6 cases of primary NAD, including 2 HSS brains and 4 infantile NAD brains; 2) 5 cases of secondary NAD, including 1 case each of PD with dementia and DLB, a case of methotrexate-associated leukoencephalopathy, a brain with features of Niemann-Pick disease type C, and 1 case of dementia pigmentosa; 3) 3 examples of physiologic NAD; and 4) 2 cases of recent head trauma with diffuse axonal injury (DA1). Two cases of multiple system atrophy (MSA), both diagnosed as olivopontocerebellar atrophy with 1 also showing features of striatogniral degeneration, were included (Table 1). With the exception of 2 cases that came from the Harvard Brain Tissue Resource Center at McLean Hospital, all other material was obtained from the files of the Massachusetts General Hospital and the Massachusetts Alzheimer Disease Research Center.

Common areas examined from 4 (cases 3-6, Table 1) of the 6 primary NAD brains included midbrain, pons with locus ceruleus, medulla with inferior olive, amygdala, hippocampal formation with adjacent temporal cortex, frontal cortex (including Brodmann area 4), parietal cortex (not available from 1 case), and occipital cortex. In addition, from cases 4 and 5 (Table 1), insular cortex, globus pallidus, and putamen were available for study; of these 2 cases, cingulate cortex was available only on case 5. From the other 2 primary NAD brains (cases 1 and 2, Table 1), a more limited number of areas were available; case 1: pons, parietal cortex, a section of unspecified cerebral cortex, and putamen, and from case 2: midbrain, pons, medulla, insular cortex, a section of unspecified cerebral cortex, globus pallidus, and putamen were examined.

Brain sections studied from the secondary NAD cases were midbrain with substantia nigra (PD with dementia and DLB), anterior cortical callosus (Niemann-Pick disease type C), medulla (methotrexate-associated leukoencephalopathy), and spinal cord (dementia pigmentosa). Sections of lower cervicomedullary junction with nucleus gracilis and nucleus cuneatus were obtained from the physiologic NAD cases. Corpus callosum or internal capsule, anterior and posterior limbs were selected in the head trauma cases. Sections of globus pallidus and pons were studied from the MSA cases.

Immunohistochemistry was performed on 7-micrometer-thick paraffin sections from multiple brain areas using monoclonal antisera to α-synuclein (H5C, gift of Dr. David Clayton; 1:7500 or 1:5000) counterstained with hematoxylin (3). In addition, adjacent sections from selected blocks were stained with polyclonal ubiquitin (DAKO, 1:300) or monoclonal glial fibrillary acidic protein (DAKO, 1:240) counterstained with hematoxylin to determine if the α-synuclein-positive structures were also identified with these antibodies. Detection was via the avidin-biotin-complex method (VECTASTAIN, Vector Labs) using diaminobenzidine as the chromogen. Sections from some of the older archival cases were microwaved in citrate buffer or distilled water for 4.5 minutes at 70°C power following deparaffinization to enhance detection. Negative controls, with the primary antibody omitted, were uniformly blank.

To establish whether α-synuclein (monoclonal) and ubiquitin (polyclonal) colocalized in axonal swellings, fluorescence-tagged secondary antibodies (y3-anti-mouse, 1:200, and bodipy-anti-rabbit, 1:100) were used on a subset of sections. Double immunofluorescence was detected using a Bio-Rad MRC 1024 Confocal microscope (3).

In the 2 primary NAD cases with detectable Lewy bodies by H&E staining, sections from frontal, temporal, parietal, cingulate, and entorhinal cortices, and 3 levels of brainstem were assessed with α-synuclein to quantitate cytoplasmic inclusions. In each cortical region (frontal, temporal, parietal, cingulate) from 1 gyrus crest to sulcus, 5 areas spanning the cortical thickness from pial surface to gray-white junction (25× objective, Zeiss microscope) spaced throughout the cytoarchitectural field were assessed for neurons bearing α-synuclein positive inclusions using a 10 mm² counting reticle. No attempt was made to select fields with high numbers of α-synuclein positive bodies. In the brainstem, 5 fields in the substantia nigra, pars compacta, locus ceruleus, and dorsal vagal nucleus were similarly assessed. Perinuclear cytoplasmic inclusions were counted. The number of neurons bearing such inclusions was expressed per mm² (Table 2).

RESULTS

Cytoplasmic Inclusions in Primary NAD Cases

Two of the 6 primary NAD brains, 1 from a 20-year-old woman with infantile NAD (case 4, Table 1) and the
TABLE 2
Quantitation of Perinuclear Cytoplasmic α-Synuclein Immunoreactive Inclusions in Two Cases of Primary NAD (number of inclusion-bearing neurons/mm²)

<table>
<thead>
<tr>
<th>Age</th>
<th>S</th>
<th>NP</th>
<th>Fr</th>
<th>T</th>
<th>P</th>
<th>C</th>
<th>EC</th>
<th>SN</th>
<th>LC</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>F</td>
<td>NAD-i</td>
<td>16.1</td>
<td>10.2</td>
<td>21.6</td>
<td>NA</td>
<td>12.4</td>
<td>32.2</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>HSS</td>
<td>7.5</td>
<td>5.2</td>
<td>13.2</td>
<td>13.2</td>
<td>7.8</td>
<td>35.0</td>
<td>1.4</td>
<td>0</td>
</tr>
</tbody>
</table>

Key: Age, age in years; S, sex; NP, neuropathological diagnosis; Fr, frontal cortex (including Brodmann area 4); T, temporal cortex (Brodmann area 20); P, parietal cortex (Brodmann area 7b); C, cingulate gyrus (Brodmann area 24); EC, entorhinal cortex (Brodmann area 28); SN, substantia nigra, pars compacta; LC, locus ceruleus; V, dorsal nucleus of the vagus; F, female; NAD-i, neuroaxonal dystrophy, infantile type; NA, not available; HSS, Hallervorden-Spatz syndrome.

![Fig. 1](http://jnen.oxfordjournals.org/)

α-Synuclein positive cytoplasmic inclusions were seen in, but not limited to neurons of the substantia nigra pars compacta, locus ceruleus, inferior olivary nucleus, cingulate cortex, substantia innominata, amygdala, and claustrum. Cortical staining was robust especially in frontal (primary motor and adjacent cortex), temporal, and parietal cortex. Occipital cortex contained fewer inclusions, and visual association cortex was relatively spared. Within the hippocampus, there were numerous positive cytoplasmic inclusions in CA2-4, with essentially no inclusions seen in CA1 adjacent to CA2. In both cases, but especially the infantile NAD patient, this region of CA1 contained many ghost neurofibrillary tangles with severe neuronal loss. In the prosubiculum, the overlap region bordering CA1 and subiculum, frequent inclusions were noted. The dentate gyrus granule cells contained small, α-synuclein immunoreactive cytoplasmic bodies in the infantile NAD brain (case 4, Table 1), some of which were morphologically similar to Pick bodies (Fig. 1).

The neuronal α-synuclein positive inclusions in these 2 cases displayed multiple morphologies. They ranged from round to ovoid bodies with intensely stained outer rings and lighter-staining centers to homogeneously-staining inclusions. Some neurons had multiple inclusions (2 or 3) while the majority contained a solitary inclusion. In many cases, only a small dot-like structure was seen. Other neurons displayed diffuse staining of the perinuclear cytoplasm instead of discrete inclusions.

Neuropil Threads, Dystrophic Neurites, and Axonal Swellings in Primary NAD

α-Synuclein positive neuropil threads and dystrophic neurites were seen throughout cases 4 and 5 (Table 1), especially in the areas containing neurons with abundant perinuclear inclusions but not in the other 4 cases. Hippocampal CA2–3 neurites stained positively in both cases. In addition, there were many α-synuclein positive axonal swellings in all 6 primary NAD brains (cases 1–6, Table 1). The staining patterns ranged from spheroids displaying diffuse α-synuclein-positivity, to those containing a positively-stained central core or dot-like structure surrounded by a pale zone, to others consisting of a synuclein-positive rim, or halo, with a pale inner region, very
similar to the staining pattern often seen in Lewy bodies (Fig. 2).

Axonal Swellings Are α-Synuclein Positive in Head Trauma and Other Conditions

We also tested the hypothesis that axonal swellings, regardless of underlying etiology, may be immunopositive for α-synuclein. α-Synuclein immunoreactive axonal swellings were detected in brains from PD with dementia and DLB cases (cases 7 and 8, Table 1), methotrexate-associated leukoencephalopathy (case 9, Table 1), storage disorder with features of Niemann-Pick disease type C (case 10, Table 1), and in the spinal cord from a case of dementia pugilistica (case 11, Table 1). In 2 cases of head trauma with diffuse axonal injury (cases 15 and 16, Table 1), the axonal swellings also stained with α-synuclein. The majority of the axonal swellings in the nucleus gracilis from 3 elderly individuals (cases 12–14, Table 1) were palely stained with α-synuclein, although some of the axonal swellings were more darkly stained (Fig. 2). The axonal swellings in these multiple settings did not react with an irrelevant primary antibody (e.g., glial fibrillary acidic protein).

Comparison of Ubiquitin and α-Synuclein Labelling of Axonal Swellings

Double-labeling experiments with α-synuclein and ubiquitin revealed colocalization of both markers in some but not all of the axonal swellings in primary NAD and diffuse axonal injury. α-Synuclein stained more axonal swellings than ubiquitin as judged by both immunofluorescence and comparison of adjacent stained sections with light microscopy.

DISCUSSION

We found α-synuclein in axonal swellings in 6 primary NAD brains, 5 secondary NAD brains, 2 brains with post-traumatic diffuse axonal injury, and, to a lesser degree, 3 aged brains with physiological NAD, thereby indicating that α-synuclein immunoreactivity, while not specific to the primary axonal dystrophies, is common to axonal swellings. α-Synuclein, a 140-amino acid protein expressed throughout the central nervous system is normally present in both cell bodies and presynaptic nerve terminals. Mutations in the α-synuclein gene have been implicated in the pathogenesis of some forms of familial PD, for instance autosomal dominant familial PD (28). α-Synuclein has also been localized to Lewy bodies and
neurites in DLB (2–5, 10). However, α-synuclein has a broader expression in neurodegeneration as indicated by the observation that α-synuclein is found not only in inclusions in multiple system atrophy (6, 9–12), but also in the neuronal inclusions and axonal swellings in primary NAD and other instances of axonal injury.

Dystrophic terminal axons, also referred to as spheroids, range from 20 to 120 μm in diameter, and can be seen with H&E and silver impregnation stains. Axonal swellings secondary to axonal injury in a variety of settings frequently contain ubiquitin and several axonally transported proteins, demonstrated by immunoreactivity for neurofilament and β-amyloid precursor protein (29–32).

α-Synuclein seems to be a marker for axonal injury both in acute axonal injury and in neurodegenerative disease, such as primary NAD. From the time course of the injury of the DAI/head trauma cases (Table 1), α-synuclein can be identified in injured axons at least as early as 72 hours after traumatic insult. Whether the intensity of this staining pattern declines with time following injury, as reported for ubiquitin, neurofilament, and β-amyloid precursor protein (33), remains to be determined.

Our observations highlight some features in common between primary NAD and PD and/or DLB. In some cases of primary NAD, as well as PD/DLB brains, α-synuclein accumulates in the cytoplasmic perinuclear region. In 2 such cases of primary NAD, numerous eosinophilic intraneuronal inclusions resembled Lewy bodies both morphologically as well as immunocytochemically with α-synuclein. The distribution of neurons containing α-synuclein positive cytoplasmic inclusions in NAD showed overlap with PD/DLB, yet there was far more extensive brain involvement in the 2 primary NAD cases, and the number of such inclusions per area greatly exceeded that seen in PD or DBL (34). In both primary NAD cases, neuropathological criteria for DLB, neocortical type (35) would have been met. Although the genetic basis and pathophysiology of the primary neuroaxonal dystrophies are still for the most part unknown, these data raise the question of whether there might be common underlying factors for primary NAD and PD or DLB.

In summary, our observations extend the range of conditions in which α-synuclein accumulates. We found α-synuclein in neuronal cytoplasmic deposits or inclusions (Lewy bodies, pale bodies, and NAD-inclusions), neurites, axonal swellings, or spheroids, and in oligodendroglia (glial cytoplasmic inclusions). The multiple cellular locations of this protein as well as the diverse conditions in which it has been recognized imply a broader role in neurodegenerative processes than initially suspected based on Lewy body staining and genetic linkage with familial PD.

ACKNOWLEDGMENTS

We are indebted to the late Dr. E. P. Richardson Jr for sharing his enthusiasm for neuropathology and his patience in teaching us so generously what he knew about neurodegenerative disorders. We have appreciated the opportunity to study brains submitted to the Harvard Brain Tissue Resource Center (MH/NS 31862) and the Massachusetts Alzheimer Disease Research Center (AG8534). Supported by the MGH/MIT Parkinson's Disease Research Center, PONS 38372. We thank Drs. David Clayton and Julia George, University of Illinois, for their generous gift of antibody H3C.

Dr. Newell is the E. P. Richardson Jr Research Fellow in Neuropathology.

REFERENCES


Received July 16, 1999
Revised received September 20, 1999
Accepted September 20, 1999