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Short Communication

Widespread Nitration of Pathological Inclusions in Neurodegenerative Synucleinopathies

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Reactive nitrogen species may play a mechanistic role in neurodegenerative diseases by posttranslationally altering normal brain proteins. In support of this hypothesis, we demonstrate that an anti-3-nitrotyrosine polyclonal antibody stains all of the major hallmark lesions of synucleinopathies including Lewy bodies, Lewy neurites and neuraxonal spheroids in dementia with Lewy bodies, the Lewy body variant of Alzheimer's disease, and neurodegeneration with brain iron accumulation type 1, as well as glial and neuronal cytoplasmic inclusions in multiple system atrophy. This antibody predominantly recognized nitrated α-synuclein when compared to other *in vitro* nitrated constituents of these pathological lesions, such as neurofilament subunits and microtubules. Collectively, these findings imply that α-synuclein is nitrated in pathological lesions. The widespread presence of nitrated α-synuclein in diverse intracellular inclusions suggests that oxidation/nitration is involved in the onset and/or progression of neurodegenerative diseases. *(Am J Pathol 2000, 157:1439–1445)*

Oxidative injury has been implicated in the pathogenesis of numerous neurodegenerative diseases including Alzheimer's disease,[1] Parkinson's disease,[2,3] dementia with Lewy bodies (DLB),[4] amyotrophic lateral sclerosis,[5] and Huntington's disease.[6] Oxidative injury occurs when an imbalance is created by the production of reactive species that escape or overwhelm the compensatory antioxidant capacity of a cell. Both reactive oxygen and nitrogen species are produced *in vivo* and may act synergistically to form nitrating agents that can modify proteins as well as lipids and thiol and aldehyde moieties in other biomolecules.[7,8] More specifically, tyrosine residues or free tyrosine can be modified by peroxynitrite, a compound generated by the reaction of superoxide radical and nitric oxide, to generate 3-nitrotyrosine (3-NT). The formation of the peroxynitrite-CO_2 adduct or the presence of other catalysts (redox active metal, metalloproteins) increases the reactivity of peroxynitrite.[9,10] Further, in the presence of myeloperoxidase or eosinophil peroxidase, hydrogen peroxide can oxidize nitrite to another biologically active nitrating agent,[11,12] which also generates 3-NT. Nitrated tyrosine residues have been detected in Lewy bodies (LBs) of Parkinson's disease brains[13] and in neurofibrillary tangles of Alzheimer's disease brains,[14,15] but no studies have examined these or additional hallmark lesions of other neurodegenerative disorders and the molecular target(s) of nitration in these lesions have yet to be identified.

α-Synuclein (α-syn) is a 140-amino acid long highly conserved protein that is abundant in neurons, particularly in presynaptic terminals.[16,17] Two mutations in the α-syn gene have been shown to be pathogenic for familial Parkinson's disease in rare kindreds,[18–20] and it has been demonstrated that α-syn is the major component of LBs and Lewy neurites (LNs) in Parkinson's disease, DLB, and the LB variant of Alzheimer's disease (LBVAD).[21–27] More recently, α-syn has been recognized to be a major component of the glial (GCIs) and neuronal cytoplasmic inclusions in multiple system atrophy (MSA) brains[28–34] as well as of the LB-like inclusions, neuraxonal spheroids, and LNs in neurodegeneration with brain iron accumulation type 1 (NBIA1; previously known as Hallervorden-Spatz dis-

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ease).[33,35,36] Thus, neurodegenerative disorders characterized neuropathologically by α-syn lesions now are referred to as synucleinopathies.

Here, we report that the majority of α-syn inclusions in DLB, LBVAD, MSA, and NBIA1 contain nitrated proteins. Further, we also demonstrate that α-syn, nitrated *in vitro*, is recognized by the rabbit anti-3-NT polyclonal antibody (3-NT pAb) in Western blots thereby implicating α-syn as a plausible target for nitrative modification in these inclusions. Thus, we infer from these data that oxidative/nitrative modifications of α-syn are involved in mechanisms underlying neurodegenerative synucleinopathies.

Materials and Methods

In Vitro *Nitration and Western Blot Analysis*

To assess the relative specificity of the 3-NT pAb for proteins previously detected in synucleinopathy lesions, we performed Western blot analyses with this antibody on purified proteins after *in vitro* nitration. Recombinant human α-syn was expressed and purified from bacteria as previously described.[37] Recombinant mouse low molecular weight neurofilament (NF) protein (NFL) were expressed in *Escherichia coli* BL21 (DE3) using a mouse NFL cDNA cloned into the pET-23d expression vector (Novagen, Inc. Madison, WI) after which transformed bacteria were selected and maintained in Luria-Bertani medium (10 g/ml bacto-tryptone, 5 g/ml bacto-yeast extract, 10 g/ml NaCl) or Terrific Broth (12 g/ml bacto-tryptone, 24 g/ml bacto-yeast extract, 0.4% gycerol, 17 mmol/L KH_2PO_4, 72 mmol/L K_2PO_4) containing 100 μg/ml ampicillin. Bacteria were grown to an OD_{600} of 0.6 and the expression of the recombinant protein was induced with 0.5 mmol/L of isopropyl-β-D-thiogalactopyranoside for 2 hours. To recover bacterially expressed NFL, cells were pelleted, resuspended into lysis buffer (25% sucrose, 1 mmol/L ethylenediaminetetraacetic acid, 50 mmol/L Tris, pH 8.0, 2 mg/ml lysozyme, and a cocktail of protease inhibitors) and incubated on ice for 30 minutes. Ten mmol/L of $MgCl_2$, 1 mmol/L $MnCl_2$, 10 μg/ml DNase 1 and 10 μg/ml RNase A were added to the homogenate, which was incubated on ice for another 30 minutes. Two ml of detergent buffer (0.2 mol/L NaCl, 1% deoxycholic acid, 1% Nonidet P-40, 20 mmol/L Tris, pH 7.5, 2 mmol/L ethylenediaminetetraacetic acid) per ml of lysis buffer were added and, after vigorous mixing, the insoluble material was sedimented at 5,000 × *g* for 30 minutes. The supernatant was discarded and the pellet was repeatedly washed with buffer containing 0.5% Triton and 1 mmol/L ethylenediaminetetraacetic acid to generate a highly compact pellet which was resuspended in 8 mol/L urea, 1% β-mercaptoethanol, 10 mmol/L $NaPO_4$, pH 7.0, for subsequent purification of NFL using hydroxylapatite (Bio-Rad Laboratories, Richmond, CA).[38] Medium (NFM) and high (NFH) molecular weight NF proteins were purified from bovine spinal cords as previously described.[39] Tubulin proteins recovered from phosphocellulose purified bovine microtubules were purchased from Cytoskeleton, Inc., Denver, CO. All of these proteins were nitrated with a 10-fold molar excess of peroxynitrite as previously described,[40] and then the relative specificity of the 3-NT pAb for each of these proteins was assessed by Western blot methods reported earlier.[33,37,40]

Table 1. Case Demographics

Case no.	Age	Sex	Postmortem interval	Diagnosis
1	55	M	7	MSA
2	67	F	5	MSA
3	60	F	10.5	MSA
4	64	F	17	MSA
5	72	F	14	MSA
6	43	M	16.5	MSA
7	79	M	16	MSA
8	58	M	20.5	DLB
9	83	F	14.5	DLB
10	76	M	13.5	DLB
11	74	M	15.5	DLB
13	63	F	22	DLB
14	79	M	12	DLB
12	65	M	9.5	LBVAD
15	73	M	12	LBVAD
16	79	M	20.5	LBVAD
17	82	F	8	LBVAD
18	77	M	10.5	LBVAD
19	29	M	6	NBIA1

Tissue Collection and Processing

The harvesting, fixation, and further processing of the brain tissue specimens were conducted as previously described.[22,41] Briefly, tissue blocks were removed at autopsy from the cingulate cortex and mesencephalon of six DLB and five LBVAD brains, the cerebellar white matter from seven MSA brains, and the insular cortex and globus pallidus from one NBIA1 brain (see Table 1). The diagnostic assessment of all cases was performed in concordance with published guidelines.[42–44] Samples from these brains were fixed by immersion in 70% ethanol with 150 mmol/L NaCl for 24 to 36 hours, and paraffin embedded according to a previously described schedule.[45] These blocks were then cut into multiple, near serial, 6-μm sections for immunohistochemical staining.

Antibodies and Immunohistochemistry

The presence of 3-NT modified substrates in human tissue was detected by immunohistochemistry using the 3-NT pAb (generously provided by Joseph S. Beckman; previously characterized by Beckman et al[46] and Ye et al[47]), and the avidin-biotin complex (ABC) system (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA) with the chromagen 3,3′-diaminobenzidine as previously described.[48] Briefly, sections were heated at 60°C for 60 minutes, deparaffinized and hydrated through graded ethanols, rinsed in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.1, and endogenous peroxidases were neutralized with 5% H_2O_2 in methanol for 20 minutes. Sections were then washed and blocked in PBS containing 10% goat serum and 1% bovine serum albumin for 30 minutes at 37°C. 3-NT pAb was diluted 1:400 in PBS

containing 10% goat serum and 1% bovine serum albumin, and incubated at 37°C for 90 minutes. Slides were washed and incubated with biotinylated goat anti-rabbit antibody at 37°C for 40 minutes. Slides were washed and incubated with ABC solution at 37°C for 30 minutes. Bound antibody complexes were visualized with 3,3′-diaminobenzidine followed by a brief wash in distilled water. The sections were then lightly counterstained with hematoxylin, dehydrated, and coverslipped. Negative controls for the 3-NT pAb included preabsorption of this antibody with a 10-fold excess of purified 3-NT and preincubation of tissue sections with 100 mmol/L dithionite as described previously.[48]

Quantitation of GCIs and LBs

Consecutive 6-μm sections were taken from each of the MSA (cerebellum) or DLB/LBVAD (cingulate cortex) brains and immunostained with either the 3-NT pAb as described above, or a mouse anti-α-syn-specific monoclonal antibody, Syn 202,[49] to count the total number of GCIs or LBs in a given field. Immunostaining with the Syn 202 was performed as described previously.[45,50] Briefly, the sections were deparaffinized, hydrated through graded ethanols, treated with 5% H_2O_2 in methanol, and blocked in 2% donor horse serum in 0.1 mol/L Tris (Tris/donor horse serum) for 5 minutes. Primary incubation was performed with Syn 202 diluted 1:1,500 in Tris/DHS overnight at 4°C. After washing, sections were sequentially incubated with biotinylated secondary antibodies for 1 hour and avidin-horseradish peroxidase complex (Vectastain Standard ABC kit; Vector Laboratories) for 1 hour. Bound antibody complexes were visualized using 3,3′-diaminobenzidine.

For GCI quantification, five adjacent medium power ($\times$200) photomicrographs were taken from the MSA cerebellar white-matter tissue sections stained with either the 3-NT pAb or Syn 202 and the total number of GCIs in all five photomicrographs was obtained. For LB quantification, the total number of LBs recognized by each antibody within entire tissue sections of cingulate cortex from DLB and LBVAD cases were counted for each antibody. The percentage of LBs or GCIs labeled with the 3-NT pAb was determined as a ratio of 3-NT pAb inclusion counts over Syn 202 inclusion counts.

Results

Immunohistochemical Localization of 3-NT in Neurodegenerative Brain Lesions

In all of the DLB and LBVAD brain sections (see Table 1 for a summary of cases studied), the 3-NT pAb robustly stained cortical and nigral LBs, as well as many LNs (Figure 1, a–c). In the substantia nigra pars compacta, the core and peripheral halo of classical LBs were labeled by this antibody (Figure 1c), whereas robust 3-NT immunoreactivity was observed throughout many GCIs in the cerebellar white matter in all MSA brains examined here (Figure 1, d–f), and these 3-NT-positive GCIs were seen in satellite, interfascicular, and perivascular oligodendrocytes.

In the NBIA1 brain, the 3-NT pAb immunolabeled LB-like inclusions (Figure 1, g and h) and neuraxonal spheroids (Figure 1i) of the globus pallidus. As in LBs of DLB and LBVAD brains, 3-NT immunoreactivity was seen in the core and halo regions of LB-like inclusions of the NBIA1 brain (Figure 1g), and white-matter GCIs also were extensively immunoreactive for 3-NT (Figure 1j). The specificity of this 3-NT pAb for 3-NT residues was confirmed using control sections treated with dithionite (which reduces 3-NT to 3-aminotyrosine), and by preabsorbing the 3-NT pAb with 3-NT before immunohistochemistry because both control experiments completely abolished the labeling of all pathological inclusions by this antibody (data not shown).

Quantitation of 3-NT Immunoreactive LBs and GCIs

The percentage of α-syn-positive cortical LBs that were also 3-NT immunoreactive ranged from 61 to 100% (mean, 76.2%) in DLB brains and from 57 to 80% (mean, 67.8%) in LBVAD brains (Table 2). Presumably because of section-to-section variation in the number of LBS, one DLB brain had slightly more 3-NT-immunolabeled LBs than those labeled by antibodies to α-syn in adjacent sections, and this case (identified by an asterisk in Table 2) was assigned a grade of 100%. Further, the percentage of α-syn-positive GCIs in the MSA brains that were 3-NT immunoreactive ranged from 56 to 76% (mean, 65.4%). Finally, there was no apparent relationship between the percentage of 3-NT-positive LBs or GCIs and the severity of the neuropathology, as reflected by the abundance of these α-syn inclusions.

Western Blot Analysis of Nitrated Proteins Recognized by the 3-NT pAb

α-Syn is the major component of LBs and GCIs, however other proteins such as NF subunits[41,51,52] and tubulins[53,54] are prominent constituents of LBs and GCIs, respectively. Because it is plausible that the 3-NT pAb could recognize a variety of nitrated proteins, Western blot analysis was used to compare the relative specificity of this antibody for these proteins after *in vitro* nitration. Human α-syn, bovine microtubule-derived tubulins, as well as mouse NFL and bovine NFM and NFH were exposed under identical conditions to the same quantity of peroxynitrite in the presence of CO_2. As shown in Figure 2, the immunoreactivity for *in vitro*-nitrated α-syn was significantly more intense than that for *in vitro*-nitrated tubulins or NF proteins. This dramatic difference may be because of more extensive nitration of α-syn, or greater affinity of this 3-NT pAb for nitrated α-syn which is known to be nitrated *in vitro* at all four of its tyrosine residues, primarily because of the random coil conformation that this protein assumes in solution.[40] Indeed, other proteins may assume alternative secondary structures

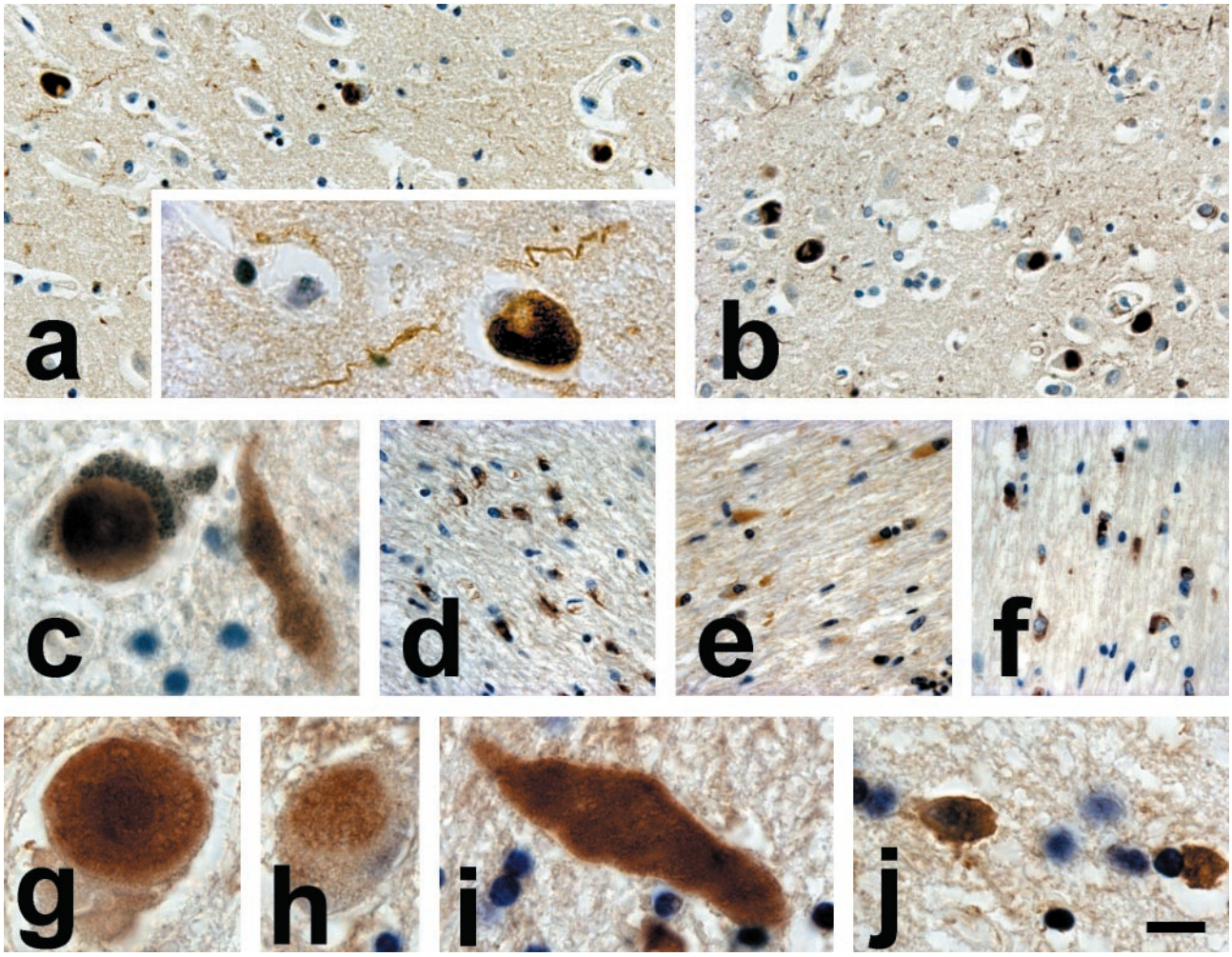

Figure 1. Immunostaining with the 3-NT pAb in sections of DLB, LBVAD, MSA, and NBIA1 brains. **a** and **b:** 3-NT immunostaining of α-syn lesions in the cingulate cortex of DLB and LBVAD brains, respectively. The **insert** in **a** shows a high-power magnification of a typical 3-NT-positive LB and LN from the same section. **c:** Intense 3-NT immunostaining throughout a classical LB in a pigmented substantia nigra pars compacta neuron of an LBVAD brain. **d–f:** Robust 3-NT immunostaining is seen in multiple GCIs throughout cerebellar white matter of three different MSA brains. In the NBIA1 brain, intense 3-NT immunoreactivity is seen in LB-like inclusions of the globus pallidus (**g** and **h**), as well as in neuraxonal spheroids (**i**), and GCIs from the insular cortex (**j**). Scale bars, 30 μm (**a**, **b**, **d–f**)and 10 μm (**inset** in **a**, **c**, **g–j**).

that confer a selective susceptibility of their tyrosine residues to nitration, as in NFL.[55] Notably, the higher molecular mass species of α-syn seen in Figure 2 probably reflect peroxynitrite-induced *o-o′*-dityrosine cross-link formation because α-syn has been show to be modified by this reaction more readily than other proteins.[40]

Discussion

Although evidence that oxidative and nitrative modifications occur in several different neurodegenerative diseases continues to accumulate, it remains unclear if these modifications play a mechanistic role in brain degeneration, or if they deleteriously affect neurons and glia in these disorders. Similarly, although many signature lesions of a large number of distinct neurodegenerative diseases are composed of abnormal aggregates of different brain proteins,[56] it remains incompletely understood how they compromise the function and viability of neurons and glia. Because oxidative and nitrative injury

Table 2. Percentage of 3-NT-Positive Inclusions

Case no.	Diagnosis	Percentage
1	MSA	76
2	MSA	57
3	MSA	60
4	MSA	56
5	MSA	63
6	MSA	70
7	MSA	76
8	DLB	63
9	DLB	61
10	DLB	NA
11	DLB	100*
12	DLB	90
13	DLB	67
14	LBVAD	79
15	LBVAD	60
16	LBVAD	57
17	LBVAD	80
18	LBVAD	63

Asterisk indicates a DLB brain that had slightly more 3-NT-immunolabeled LBs than those labeled by antibodies to α-syn in adjacent sections.

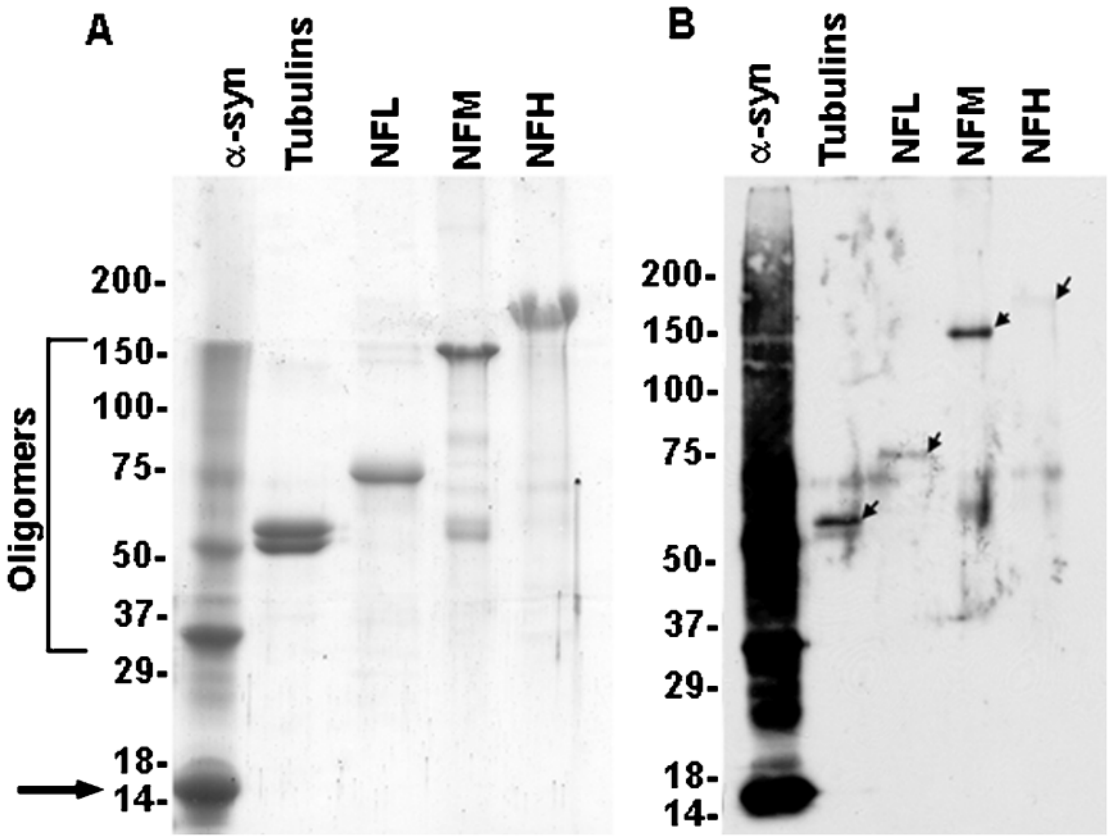

Figure 2. Western blot analysis of 3-NT pAb antibody. **A:** Coomassie-stained gel of *in vitro* nitrated α-syn, tubulin, NFL, NFM, and NFH. Five μg of each protein were resolved by electrophoresis on separate lanes of a discontinuous 6% to 10% to 12% sodium dodecyl sulfate-polyacrylamide gel. The **arrow** on the **left** denotes monomeric α-syn. **B:** In Western blots the 3-NT pAb variably recognizes each of the nitrated proteins but none are as intensely immunoreactive as nitrated α-syn. Note the robust recognition of monomeric α-syn as well as higher oligomeric α-syn species by this antibody. The **arrows** in **B** identify the far less intensely labeled immunobands for each of the other proteins. Fifty ng of each protein were resolved by electrophoresis on a discontinuous 6% to 10% to 12% sodium dodecyl sulfate-polyacrylamide gel. After electrophoretic transfer to nitrocellulose membrane, the blot was blocked with 5% skimmed milk, sequentially incubated with the 3-NT pAb and anti-rabbit-horseradish peroxidase-conjugated antibody and developed with enhanced luminol reagents (Dupont-New England Nuclear). The position of the molecular weight markers are indicated on the **left**.

may play a mechanistic role in the pathogenesis of one or more of these signature lesions, the detection of 3-NT immunoreactivity in some of these pathological protein aggregates by a limited number of published studies implies that components of these lesions have been modified by nitrative and oxidative injury.[13–15] Thus, to extend previous reports of the presence of 3-NT-modified residues in studies that focused exclusively on Alzheimer's disease neurofibrillary tangles and LBs in Parkinson's disease brains,[13–15] we conducted the studies described here which showed that protein nitration is not limited to the LBs of Parkinson's disease. Indeed, we demonstrated that this also occurs in LBs of DLB and LBVAD brains, as well as in NBIA1 LB-like inclusions. The presence of 3-NT immunoreactivity also was demonstrated in all other α-syn rich neuropathological hallmarks of MSA, NBIA1, DLB, or LBVAD (ie, LNs, GCIs, neuronal cytoplasmic inclusions, and neuraxonal spheroids) and our quantitative analysis showed that the majority of these inclusions contain 3-NT modified protein.

Taken together with previous observations, our studies suggest that α-syn is nitrated in the α-syn lesions detected here by the 3-NT pAb. The abundance of α-syn in LBs and GCIs and the recent observation that α-syn is an excellent substrate for nitration[40] provide compelling support for this view. Further, because we also showed that nitrated α-syn was far more intensely labeled by the 3-NT pAb than any of the other nitrated proteins examined here by Western blot, it is highly likely that α-syn is the major 3-NT-modified protein in the lesions we studied. Nonetheless, additional studies are needed to confirm this using complementary methods and additional synucleinopathy brains.

Because the majority (57 to 100%) of the inclusions counted here, (ie, LBs and GCIs), contained 3-NT immunoreactivity, it is tempting to speculate that nitrative or oxidative damage may cause aggregation of proteins to form these inclusions. However, not all inclusions were labeled by the 3-NT pAb and this may be because of the fact that the generation of 3-NT by nitration is enzymatically reversible, and denitration of 3-NT may occur throughout an extended period of time if normal cellular reductive capacities are re-established. Thus, the generation of additional antibodies that specifically recognize the more stable *o-o′*-dityrosine modification induced by nitration may demonstrate the presence of nitrative injury in all of these α-syn inclusions.

The effects of α-syn nitration are undetermined, but it is possible that nitration may render α-syn more resistant to proteolysis or alter other properties of this synaptic protein thereby playing a mechanistic role in the formation of α-syn lesions as well as in the onset/progression of synucleinopathies. Nitrating species also may contribute to the pathogenesis of α-syn lesions by oxidizing tyrosine residues to form *o-o′*-dityrosine resulting in the covalent cross-linking of α-syn and the formation of stable α-syn polymers.[40] Indeed, *o-o′*-dityrosine formation may be more damaging than the 3-NT modification because this alteration may be reversed enzymatically whereas *o-o′*-dityrosine cross-linking is more stable and it may retard or prevent the removal of abnormal protein aggregates.[57,58]

These uncertainties not withstanding, based on the data described here, together with evidence that *o-o′*-dityrosine cross-linking of α-syn leads to the formation of stable α-syn polymers,[40] we suggest that loss of neuronal or glial oxidative protective mechanisms may have deleterious effects on the normal functions or metabolism of α-syn and thereby contribute to the onset/progression of neurodegenerative synucleinopathies. Thus, further studies of the role of oxidative and nitrative injury in mechanisms underlying these and other neurodegenerative disorders may lead to the identification of therapeutic targets for the prevention or reversal of these diseases.

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