FBXO7 Immunoreactivity in α-Synuclein–Containing Inclusions in Parkinson Disease and Multiple System Atrophy

Tianna Zhao, PhD, Lies-Anne Severijnen, Marcel van der Weiden, Ping Pin Zheng, PhD, Ben A. Oostra, PhD, Renate K. Hukema, PhD, Rob Willemsen, PhD, Johan M. Kros, MD, PhD, and Vincenzo Bonifati, MD, PhD

INTRODUCTION

Idiopathic Parkinson disease (PD) is one of the most common neurodegenerative disorders, affecting 1% to 2% of the population older than 60 years (1). The cardinal clinical features of the disease are resting tremor, bradykinesia, muscular rigidity, and postural instability (2, 3). The pathogenesis of PD remains poorly understood, and, as a consequence, no current treatment is available that can stop or even slow the disease progression. From the neuropathologic standpoint, PD is mainly characterized by the selective loss of dopaminergic neurons in the substantia nigra and by the presence of Lewy bodies (LBs) or Lewy neurites (LNIs) in the surviving neurons (4). Aggregated forms of the α-synuclein protein are the main component of these inclusions (5, 6). Furthermore, α-synuclein is also the major component of the glial cytoplasmic inclusions (GCIs), the pathologic hallmark of multiple system atrophy (MSA). The term “α-synucleinopathy” is currently adopted to indicate a spectrum of neurodegenerative diseases characterized by pathologic aggregation of α-synuclein (7).

There is growing evidence that genetic risk factors contribute to the etiology of PD (8). Mutations in several genes have been identified as the cause of human parkinsonism, inherited as autosomal dominant or autosomal recessive trait (9). Mutations in the F-box only protein 7 (FBXO7) gene cause PARK15, a rare autosomal recessive form of juvenile parkinsonism (10, 11). The brain pathology of PARK15 has not been explored so far, and whether α-synuclein–positive inclusions are present in these patients remains unknown. Intriguingly, the PARK15 patients display good response to levodopa, and they experience severe loss of dopaminergic nigrostriatal terminals, as shown by neuroimaging in vivo (11). These features resemble those of the patients with the classical late-onset form of PD. Understanding the pathogenesis of PARK15 might, therefore, illuminate the mechanisms of the selective dopaminergic neuronal degeneration, which could also be important for PD patients.

Two protein isoforms, of mostly unknown function, are expressed from the FBXO7 gene (12). The pattern of expression of these proteins in the human brain remains poorly characterized. Furthermore, the expression of the FBXO7 proteins in the brains of patients with late-onset PD or related neurodegenerative diseases has not been explored. Here, we...
studied the expression of the FBXO7 proteins in the normal human brain and in different late-onset neurodegenerative diseases, including the α-synucleinopathies PD and MSA and the tauopathies Alzheimer disease (AD) and progressive supranuclear palsy (PSP).

**MATERIALS AND METHODS**

Brain tissues from patients affected by idiopathic PD (n = 13), MSA (n = 5), AD (n = 5), PSP (n = 5), and age-matched controls (n = 9) were obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. The patient clinical and neuropathologic data are presented in Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A449. The study procedures were approved by the local ethical authorities. All materials were collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the Netherlands Brain Bank.

**Cell Culture and Western Blotting**

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco modified Eagle medium according to standard protocols. F-box only protein 7 knockdown in HEK 293T cells was obtained using FBXO7 shRNA (No. TRCN 0000004339; Sigma-Aldrich, St. Louis, MO). A nontargeting shRNA (shNT, SHC002; Sigma-Aldrich) was used as control.

Western blotting in cultured cells or in brain tissue was performed as previously described (12). Briefly, 40 μg of protein were loaded onto 6% to 12% Criterion XT 4.0 gel (Bio-Rad, Veenendaal, The Netherlands) and transferred to nitrocellulose membranes. The membrane was then incubated with FBXO7 purified mouse polyclonal antibody (B01P, 1:500; Abnova, Heidelberg, Germany) against the full-length FBXO7 longer isoform (isoform 1, 522 amino acids) or FBXO7 mouse monoclonal antibody (E8, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) against a C-terminal FBXO7 peptide (amino acids 427–461, present in both FBXO7 isoforms) and mouse monoclonal against β-actin (1:2000; Sigma-Aldrich). After washing with PBS with Tween 20, the membranes were incubated in the dark for 1 hour with anti-mouse secondary antibody and scanned using the Odyssey Infrared Imager (Li-COR Biosciences, Lincoln, NE).

**Immunohistochemistry and Immunocytochemistry**

Five-micrometer-thick sections were prepared from formalin-fixed, paraffin-embedded brain tissue. The endogenous peroxidase activity was blocked using 0.6% H2O2 and 0.125% NaNO3 in PBS. The sections were then incubated overnight at 4°C with different primary antibodies against FBXO7 (B01P, 1:100; or E8, 1:50), α-synuclein (1:400; Chemicon, Temecula, CA), or phosphorylated Tau (AT8, 1:500; Innogenetics, Ghent, Belgium) and followed with BrightVision horseradish peroxidase–linked secondary antibody (1:1; Immunologic, Duiven, The Netherlands) incubation for 1 hour at room temperature. After washing with PBS, the immunoreactivity was visualized by freshly prepared Liquid DAB Substrate Chromogen solution (DAKO, Glostrup, Denmark). Last, the sections were dehydrated through graded ethanol, cleared with xylene, and mounted with Entellan medium (Electron Microscopy Sciences, Hatfield, PA). To detect brainstem-type LBs, sections from substantia nigra and locus coeruleus were subjected to standard hematoxylin and eosin staining, and immunostaining was then performed on serial sections. For immunocytochemistry, HEK 293T cells were seeded on slides, permeabilized, incubated with the specific antibody, and then processed for DAB immunostaining.

**Double Immunofluorescence**

Double staining of the FBXO7 and α-synuclein proteins was performed on sections of the regions of substantia nigra and locus coeruleus. To block nonspecific antibody binding sites, the dehydrated sections were preincubated with 10% donkey and goat serum for 10 minutes. Sections were then incubated with primary mouse antibodies against FBXO7 (B01P, 1:25; or E8, 1:20) and rabbit α-synuclein (1:200) overnight at 4°C. Negative controls were generated by omission of primary antibodies. For double staining of FBXO7 and Tau, rabbit primary antibody against Tau (1:1000; DAKO) was used.

All primary antibodies and serum were diluted in PBS containing 1% (wt/vol) bovine serum albumin. After washing with PBS with Tween 20, sections were then incubated for 1 hour with Cy3-conjugated anti-mouse secondary antibody (1:200; Jackson ImmunoResearch Europe, Suffolk, UK) and Alexa 488–conjugated anti-rabbit secondary antibody (1:200; Invitrogen, Carlsbad, CA). To quench autofluorescence of brain tissue, the sections were treated with 0.1% Sudan Black B (Sigma-Aldrich) in the dark. The slides were then covered by coverslip and mounted with DAKO mounting medium. Fluorescence images were collected using a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) and analyzed with the Leica confocal software. The number of FBXO7-positive inclusions was expressed as the percentage of the α-synuclein– or tau-positive inclusions counted in the same sections.

**RESULTS**

**Specificity of the FBXO7 Antibodies**

Two FBXO7 protein isoforms of 522 and 443 amino acids, only differing at their N-terminus (also referred to as isoform 1 and isoform 2), are encoded by the human FBXO7 gene as a result of the usage of alternatively spliced 5′-exons (transcripts ENSP00000266087 and ENSP00000371490) (12). Two anti-FBXO7 antibodies were used in this study: a mouse polyclonal (B01P; Abnova) raised against the full-length human FBXO7 longer isoform and a mouse monoclonal antibody (E8; Santa Cruz Biotechnology) raised against a C-terminal FBXO7 peptide present in both the FBXO7 isoforms. To assess the specificity of these antibodies, we generated FBXO7 knockdown (FBXO7-KD) cells using shRNA targeting a sequence present in both the FBXO7 transcripts. Cells treated with a nontargeting shRNA (shNT) served as a

© 2013 American Association of Neuropathologists, Inc.
negative control. Two normal FBXO7 isoforms of the expected molecular weight were detected by Western blotting in the control (shNT) cells, whereas the expression of both proteins was markedly depleted in the FBXO7-KD cells (Fig. 1A; Supplemental Digital Content 2, http://links.lww.com/NEN/A450). Furthermore, FBXO7 immunoreactivity was detected using immunocytochemistry in control cells but not in the FBXO7-KD cells (Fig. 1B), further supporting the contention that these antibodies specifically detect the endogenous human FBXO7 proteins.

**FBXO7 Is Widely Expressed in Normal and PD Patient Brains**

To characterize the expression of the FBXO7 proteins in normal human brain and in PD, we studied different brain regions from 9 normal brain donors (controls) and 13 PD patients (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A449). The 2 endogenous FBXO7 protein isoforms were weakly but clearly detected by Western blotting in normal brain tissue (Supplemental Digital Content 3, http://links.lww.com/NEN/A451). F-box only protein 7 immunoreactivity in brain tissues was widely detected throughout the brain regions analyzed (Fig. 2); strong expression was detected in the neocortex (all areas), putamen, and cerebellum, whereas more moderate levels were seen in the hippocampus (including the dentate gyrus), substantia nigra, and locus coeruleus. Strong FBXO7 expression was observed in the pyramidal cells of the cerebral cortex (black arrows); weaker immunoreactivity is present in the hippocampal neurons (arrowhead), the Purkinje neurons (white arrow), the granular cells of the cerebellum, and the nigral neurons. Scale bar = 30 μm.

**FBXO7 Immunoreactivity in the α-Synuclein–Positive Inclusions of PD and MSA**

To investigate whether the FBXO7 proteins are present in the pathologic inclusions that characterize PD, we studied the FBXO7 immunoreactivity of LBs using immunohistochemistry.

---

**FIGURE 1.** Specificity of the polyclonal anti-F-box only protein 7 (FBXO7) antibody. (A) Western blotting using the (B01P) polyclonal anti-FBXO7 antibody yields 2 bands of the predicted molecular weight, corresponding to the 2 endogenous isoforms of FBXO7 proteins in nontransfected HEK 293T cells and in cells transfected with nontargeting shRNA (shNT). The intensity of these bands is markedly decreased after treatment with FBXO7-targeting shRNA (knockdown). Beta-actin is used as loading control. (B) Immunocytochemistry using the same polyclonal FBXO7 antibody reveals strong immunoreactivity in HEK 293T cells transfected with nontargeting shRNA (shNT). The immunoreactivity is drastically reduced after treatment of cells with FBXO7-targeting shRNA (knockdown). Scale bar = 30 μm.

**FIGURE 2.** Expression of F-box only protein 7 (FBXO7) proteins in the normal human brain. FBXO7 immunoreactivity is widely detected in different regions using the (B01P) polyclonal anti-FBXO7 antibody; shown are frontal cortex (A), parietal cortex (B), occipital cortex (C), dentate gyrus (D), cerebellum (E), and substantia nigra (F). Strong immunoreactivity is seen in the pyramidal neurons in the cerebral cortex (black arrows); weaker immunoreactivity is present in the hippocampal neurons (arrowhead), the Purkinje neurons (white arrow), the granular cells of the cerebellum, and the nigral neurons. Scale bar = 30 μm.
We focused on the classic brainstem-type LBs because they are necessary for the pathologic diagnosis of PD; they were present in all the 13 PD brains (Fig. 3). We detected FBXO7 immunoreactivity in the majority of the LBs in the substantia nigra and in the locus coeruleus (Fig. 3). Using either of the anti-FBXO7 antibodies yielded similar results, supporting the specificity of the staining. Using the polyclonal (B01P) FBXO7 antibody more frequently yielded a ringlike pattern of immunoreactivity in the periphery of LBs and less frequently a diffuse pattern of immunoreactivity in the whole inclusions. Using the monoclonal (E8) FBXO7 antibody, the diffuse pattern of immunoreactivity in the whole inclusions was more prevalent.

The main component of LBs and LNs is the α-synuclein protein (7). Therefore, we performed double immunofluorescence and confocal microscopy using anti-α-synuclein and β-FBXO7 antibodies. Colocalization of α-synuclein and FBXO7 was found in LBs and LNs using either of the anti-FBXO7 antibodies (Fig. 4). In particular, approximately 53% of the LBs (n = 90) displayed a peripheral ringlike pattern of immunoreactivity in the periphery of LBs and less frequently a diffuse pattern of immunoreactivity in the whole inclusions. Using the monoclonal (E8) FBXO7 antibody, the diffuse pattern of immunoreactivity in the whole inclusions was more prevalent.

The main component of LBs and LNs is the α-synuclein protein (7). Therefore, we performed double immunofluorescence and confocal microscopy using anti-α-synuclein and β-FBXO7 antibodies. Colocalization of α-synuclein and FBXO7 was found in LBs and LNs using either of the anti-FBXO7 antibodies (Fig. 4). In particular, approximately 53% of the LBs (n = 90) displayed a peripheral ringlike pattern of immunoreactivity in the periphery of LBs and less frequently a diffuse pattern of immunoreactivity in the whole inclusions. Using the monoclonal (E8) FBXO7 antibody, the diffuse pattern of immunoreactivity in the whole inclusions was more prevalent.

The main component of LBs and LNs is the α-synuclein protein (7). Therefore, we performed double immunofluorescence and confocal microscopy using anti-α-synuclein and β-FBXO7 antibodies. Colocalization of α-synuclein and FBXO7 was found in LBs and LNs using either of the anti-FBXO7 antibodies (Fig. 4). In particular, approximately 53% of the LBs (n = 90) displayed a peripheral ringlike pattern of immunoreactivity in the periphery of LBs and less frequently a diffuse pattern of immunoreactivity in the whole inclusions. Using the monoclonal (E8) FBXO7 antibody, the diffuse pattern of immunoreactivity in the whole inclusions was more prevalent.

To determine whether the FBXO7 proteins are also present in the α-synuclein–containing GCIs that are characteristic of MSA, we studied 5 brains from MSA patients (Fig. 5). There was a diffuse regular pattern of FBXO7 immunoreactivity in 78% of α-synuclein–positive GCIs (n = 156) (Fig. 5). The 2 anti-FBXO7 antibodies yielded similar results.

**FXB07 Immunoreactivity Is Rarely Detected in Tau-Positive Inclusions**

To determine whether the FBXO7 proteins are also present in the inclusions containing the microtubule-associated protein tau, we studied 5 brains from AD patients and 5 from PSP patients (Fig. 6; Supplemental Digital Content 4, http://links.lww.com/NEN/A452). F-box only protein 7 immunoreactivity was not detected in the majority (74%) of the neurofibrillary tangles (n = 27) in both AD and PSP brains. Moreover, the remaining inclusions (26%) only displayed weak FBXO7 immunoreactivity. The 2 anti-FBXO7 antibodies yielded similar results.

**DISCUSSION**

PARK15 is a Mendelian form of parkinsonism caused by recessive mutations in the FBXO7 gene (10, 11). We previously described the depletion of the FBXO7 protein in the brainstem in both AD and PSP brains. Moreover, the remaining inclusions (26%) only displayed weak FBXO7 immunoreactivity. The 2 anti-FBXO7 antibodies yielded similar results.
PARK15 patients, indicating that the loss of the normal FBXO7 function(s) underlies the pathogenesis of this disease (12). We have recently also generated the first vertebrate model of PARK15 by knocking down the FBXO7 expression in zebrafish (13). Here we show that the FBXO7 immunoreactivity is present in different regions of the normal human brain, and that this pattern is not altered in the brains of PD patients. Furthermore, we report for the first time that FBXO7 immunoreactivity is a shared feature of the majority of the α-synuclein-positive inclusions characteristic of PD and of MSA but not of the tau-positive inclusions in AD and PSP.

We performed a systematic investigation of a higher number of brain areas using brain tissue from 9 normal donors and 13 PD donors. Possible interindividual variability in FBXO7 immunoreactivity was, therefore, minimized.

**FIGURE 4.** Colocalization of F-box only protein 7 (FBXO7) and α-synuclein in Lewy bodies (LBs) and Lewy neurites (LNs). Double immunofluorescence reveals the colocalization of FBXO7 and α-synuclein immunoreactivity either in the periphery of LBs (A) or as a more diffuse pattern of colocalization (B, D). The colocalization of FBXO7 and α-synuclein immunoreactivity is also detected in LNs (C, E). The polyclonal (B01P) anti-FBXO7 antibody is used in (A) to (C), the monoclonal (E8) anti-FBXO7 antibody is used in (D) and (E). Scale bar = 5 μm.

**FIGURE 5.** Colocalization of FBXO7 and α-synuclein in glial cytoplasmic inclusions (GCIs). Typical GCIs of multiple system atrophy brains are strongly immunoreactive for α-synuclein (cerebellum shown in [A]). Weaker immunoreactivity for FBXO7 is also present in these inclusions, using the polyclonal (B01P) anti-FBXO7 antibody (B). Double immunofluorescence reveals the colocalization of FBXO7 and α-synuclein immunoreactivity in GCIs in the cerebellum (C, F), substantia nigra (D), and caudate putamen (E). The polyclonal (B01P) anti-FBXO7 antibody is used in (A) to (E); monoclonal (E8) anti-FBXO7 antibody is used in (F). Scale bars = (A, B) 50 μm; (C–F) 5 μm.
Brain tissue from patients with disease-causing \textit{FBXO7} mutations has not yet become available for autopsy study. Thus, the brain pathology of PARK15 remains unexplored. In particular, it is currently not known whether LBs are present in this disease. Here, we identified abundant \textit{FBXO7} immunoreactivity in most of the \(\alpha\)-synuclein–containing inclusions in the patients with the common form of PD and also in those from the MSA patients using 2 anti-\textit{FBXO7} antibodies. Furthermore, the striking difference in \textit{FBXO7} immunoreactivity between the \(\alpha\)-synuclein–containing and the tau-containing inclusions strongly supports the contention that the \textit{FBXO7} deposition is a specific feature of the synucleinopathies. This, in turn, suggests a role for the \textit{FBXO7} proteins in the pathogenesis of these diseases.

Using both antibodies, the \textit{FBXO7} immunoreactivity displayed a variable pattern of peripheral or diffuse staining of the LBs. Moreover, the antibodies yielded slightly different frequencies of these staining patterns. Intriguingly, immunoreactivity for the proteins encoded by other PD-causing genes, such as \textit{LRRK2} and \textit{PTEN-induced putative kinase 1 (PINK1)}, has also been detected, sometimes in the periphery of LBs or other times in the whole inclusions (14–16). These different patterns could be due to different epitope accessibility in different parts of these inclusions, to different stages of their biogenesis, as well as to different epitope specificity of the different antibodies. Taken together, our data suggest a possible important role for the \textit{FBXO7} proteins in the formation of these inclusions and in the pathogenesis of the common forms of synucleinopathies such as PD and MSA.

\section*{ACKNOWLEDGMENTS}

The authors thank Diana Nijholt (Erasmus MC, Rotterdam) for help with immunostaining and Tom de Vries-Lentsch (Erasmus MC, Rotterdam) for artwork.

\section*{REFERENCES}

2. Fahn S. Description of Parkinson’s disease as a clinical syndrome. Ann NY Acad Sci 2003;991:1–14

\section*{FIGURE 6.} F-box only protein 7 (\textit{FBXO7}) and Tau double immunofluorescence in the brains of Alzheimer disease (AD) and progressive supranuclear palsy (PSP) patients. Typical tau-positive pathology is shown using an anti–phosphorylated Tau antibody in the frontal cortex of AD (A) and in the caudate nucleus of PSP patients (B). Double immunofluorescence using the polyclonal (B01P) anti-FBXO7 and the anti-Tau antibody shows absent (C, E) or weak (D, F) colocalization of \textit{FBXO7} immunoreactivity in neurofibrillary tangles in AD (C, D) and PSP (E, F) patient brains. Scale bars = (A, B) 50 \(\mu\)m; (C–F) 5 \(\mu\)m.

In keeping with our previous preliminary report on \textit{FBXO7} localization in the normal human brain (12), \textit{FBXO7} immunoreactivity was widely expressed in all the regions analyzed. Moreover, there was ubiquitous distribution of the \textit{FBXO7} protein in all major neuronal groups throughout the brain, including dopaminergic neurons, pyramidal neurons, stellate neurons, and granule and Purkinje neurons. Furthermore, no major differences in \textit{FBXO7} immunoreactivity were observed between control and PD brains.