Neurobiology

p25 α Relocalizes in Oligodendroglia from Myelin to Cytoplasmic Inclusions in Multiple System Atrophy

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p25 α is an oligodendroglial protein that can induce aggregation of α -synuclein and accumulates in oligodendroglial cell bodies containing fibrillized α -synuclein in the neurodegenerative disease multiple system atrophy (MSA). We demonstrate biochemically that p25 α is a constituent of myelin and a high-affinity ligand for myelin basic protein (MBP), and *in situ* immunohistochemistry revealed that MBP and p25 α colocalize in myelin in normal human brains. Analysis of MSA cases reveals dramatic changes in p25 α and MBP throughout the course of the disease. *In situ* immunohistochemistry revealed a cellular redistribution of p25 α immunoreactivity from the myelin to the oligodendroglial cell soma, with no overall change in p25 α protein concentration using immunoblotting. Concomitantly, an ~80% reduction in the concentration of full-length MBP protein was revealed by immunoblotting along with the presence of immunoreactivity for MBP degradation products in oligodendroglia. The oligodendroglial cell bodies in MSA displayed an enlargement along with the relocalization of p25 α , and this was enhanced after the deposition of α -synuclein in the glial cytoplasmic inclusions. Overall, the data indicate that changes in the cellular interactions between MBP and p25 α occur early in MSA and contribute to abnormalities in myelin and subsequent α -synuclein aggregation and the ensuing neuronal degeneration that characterizes this disease. (Am J Pathol 2007, 171:1291–1303; DOI: 10.2353/ajpath.2007.070201)

The myelin sheaths surrounding nerve axons represent a specialized structure produced by oligodendroglia in the central nervous system. The myelin sheath not only facilitates the propagation of action potentials along axons but also supplies trophic support for axons and participates in communication between the axons and oligodendroglia.¹ The significances of these functions are highlighted by the devastating impact of diseases that primarily target oligodendroglia, eg, multiple sclerosis with profound demyelination and multiple system atrophy (MSA) that develop oligodendroglial cytoplasmic inclusions containing aggregates of the protein α -synuclein. Myelin basic protein (MBP) comprises 30% of the total protein in myelin, making it the second major constituent after proteolipid protein.² Genetic mutations reducing functional MBP cause substantial neurological symptoms, as demonstrated in shiverer mice and shaker rats.^{3,4}

p25 α is an oligodendroglial-specific phosphoprotein^{5,6} also designated tubulin polymerization-promoting protein because of its microtubule-binding activity.⁷ Its expression initiates at the time of myelination along with MBP, and p25 α can be used as a marker of myelinating oligodendroglia.⁸ Furthermore, the expression of p25 α and MBP in myelinating oligodendroglia in the rat brain appears at the same time.^{8,9} Its normal function is unclear, but accumulating evidence points

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to a role related to the microtubular system as it copurifies with kinases directed to the microtubule-binding protein tau and promotes the bundling of microtubules.^{6,7} p25 α binds tubulin according to a 1:2 stoichiometric model,¹⁰ and expression of a fusion protein between human p25 α and enhanced green fluorescent protein in transfected cells leads to a colocalization of the fusion protein with microtubules during specific cell-cycle stages.¹¹

α -Synuclein is normally a neuron-specific protein that is localized in nerve terminals. However, its aggregation and relocation to cellular inclusions in a group of neurodegenerative diseases forms the basis for designating these disorders α -synucleinopathies.¹² In addition, α -synuclein deposition can also occur in a large variety of central nervous system conditions, including Alzheimer's disease, Gaucher's disease, Niemann-Pick type C1 disease, gangliosidoses, and more recently multiple sclerosis.^{13–18} MSA represents a special disease among the α -synucleinopathies, which primarily are dominated by diseases with intraneuronal Lewy body inclusions such as Parkinson's disease and Lewy body dementias. In MSA, the principal depositing of α -synuclein takes place in oligodendroglial cell bodies as glial cytoplasmic inclusions (GCIs),¹⁹ although accumulation of α -synuclein in neurons also occurs.^{20,21} MSA is an aggressive parkinsonian syndrome displaying a widespread development of GCIs and degeneration of both oligodendroglia and neurons, accounting for its symptoms that range from autonomic dysfunctions, parkinsonism, and cerebellar signs. The tissue load of aggregated α -synuclein is high in comparison to Parkinson's disease and Lewy body dementia and is best appreciated by immunoblotting of denatured brain extracts.¹⁹ GCI genesis in MSA remains unclear because oligodendroglial α -synuclein mRNA expression has not been demonstrated *in vivo*, although oligodendroglia are able to express α -synuclein in culture.²² Transgenic oligodendroglial-specific expression of human α -synuclein causes a neurodegenerative phenotype in mice that resembles MSA and clearly demonstrates that neuronal degeneration develops subsequent to an α -synuclein-mediated dysfunction of oligodendroglia.^{23,24}

We have previously shown that p25 α is a potent stimulator of α -synuclein aggregation and demonstrated in MSA that p25 α colocalizes with oligodendroglial α -synuclein-immunopositive GCIs.²⁵ To investigate the role of p25 α in oligodendroglia, and MSA in particular, this study analyzes the normal association of p25 α to myelin, its binding to MBP, and the changes that occur with these components in relation to MSA. We find that p25 α is a ligand of MBP and colocalizes with MBP in brainstem myelinated fiber tracts. The colocalization with MBP is lost in MSA where p25 α appears to relocate within the oligodendroglia to accumulate in the cell body, enlarging this structure before colocalization with α -synuclein. Along with the relocation of p25 α from myelin, there is a reduction in the level of MBP and an increase in degraded MBP that also becomes relocated to the oligodendroglial cell bodies. The results suggest a sequence of events in which early pathogenic signals impede the normal cellular function of

p25 α in myelin resulting in a decreased stability of MBP and a build-up of degraded MBP and p25 α in the expanding cell bodies that favor subsequent deposition and fibrillization of α -synuclein leading to neurodegeneration.

Materials and Methods

All chemicals were of analytical grade. Bovine MBP was purchased from Sigma, St. Louis, MO. Recombinant human p25 α was expressed in *Escherichia coli* and purified as described previously.²⁵ Complete ethylenediamine tetraacetic acid (EDTA)-free mini protease inhibitors were purchased from Roche (Indianapolis, IN) and PEFA Bloc SC was from Pentapharm AG (Basel, Switzerland).

Surface Plasmon Resonance (Biacore)

Kinetic analysis was performed on a Biacore 3000 instrument (Biacore, Uppsala, Sweden) equipped with CM5 sensor chips maintained at 25°C. A continuous flow of CaHBS buffer (150 mmol/L NaCl, 1 mmol/L EGTA, 1.5 mmol/L CaCl₂, 0.01% P-20, and 10 mmol/L HEPES, pH 7.4) was passed over the sensor surface at a rate of 5 μ l/minute. The carboxylated dextran matrix of the sensor chip flow cells was activated by injection of 60 μ l of 0.2 mol/L *N*-ethyl-*N*-(3-dimethylaminopropyl) carbodiimide and 0.05 mol/L *N*-hydroxysuccinimide. A solution of recombinant human p25 α (80 μ l, 100 μ g/ml in CaHBS buffer) was then injected at a flow rate of 5 μ l/minute. Remaining binding sites were blocked by injection of 35 μ l of 1 mol/L ethanolamine, pH 8.5, at the same flow rate. The surface plasmon resonance signal from immobilized p25 α generated 4489 Biacore response units equivalent to 190 fmol/mm². MBP samples (40 μ l, in CaHBS buffer) at concentrations of 2 to 100 nmol/L were injected through the flow cell at 5 μ l/minute. Binding was expressed in relative response units, ie, the difference in response between the immobilized protein flow cell and a corresponding control flow cell (activated and blocked, but without any protein). Regeneration of the sensor chip after each cycle of analysis was performed by injecting 20 μ l of 200 mmol/L Na₂CO₃, pH 11.0. The kinetic parameter was obtained using BIAevaluation 4.1 (Biacore).

Fluorescence Titration

Eight hundred μ l of a 2 μ mol/L MBP solution was added to a 1.7-ml quartz cuvette and p25 α was added in 10- to 50- μ l steps from an 8 μ mol/L stock solution and mixed. After the addition of each aliquot, the solution was allowed to equilibrate for 30 seconds before measuring fluorescence intensity F_{obs} (excitation at 295 nm, emission at 310 nm, excitation and emission slit widths 10 nm on a LS55 Luminescence Spectrometer; Perkin Elmer, Boston, MA). Intensities of the p25 α and MBP stock solutions were recorded separately in the same cuvette before the titration experiment.

The fluorescence emission intensity F_{exp} expected from the titrated solution in the absence of interactions between MBP and p25 α was calculated as follows:

$$F_{\text{exp}} = I_0^{\text{MBP}} * \frac{V_{\text{MBP}}}{V_{\text{MBP}} + V_{\text{p25}}} + I_0^{\text{p25}} * \frac{V_{\text{p25}}}{V_{\text{MBP}} + V_{\text{p25}}}$$

I_0^{p25} and I_0^{MBP} are the individual fluorescence intensities of the MBP and p25 α stock solutions, whereas V_{MBP} and V_{p25} are the volumes of the MBP solution (fixed, 800 μl) and added p25 α solution (0 to 400 μl). The difference in the expected and the observed (measured) intensities, $F_{\text{exp}} - F_{\text{obs}}$, was plotted versus the ratio [p25 α]:[MBP].

Circular Dichroism (CD)

Far-UV CD studies were performed on a Jasco J-715 spectropolarimeter (Jasco Spectroscopic, Malmö, Sweden) with a Jasco PTC-348W temperature control unit, essentially as described previously¹⁰ with protein concentrations of 14 $\mu\text{mol/L}$ MBP and 17 $\mu\text{mol/L}$ p25 α .

Porcine Brain Tissue Processing

Adult porcine brains were obtained from an Aarhus slaughter house. The cerebellum and medulla oblongata were removed, and the brains were frozen in liquid nitrogen and stored at -80°C . Two separate protocols were used to extract different fractions for different experimental procedures.

Porcine Protocol 1 for Affinity Chromatography and Mass Spectrometry

All steps were performed at 4°C . Brain tissue was homogenized in a solution of 0.32 mol/L sucrose, 50 mmol/L NaF, 20 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 1 mmol/L vanadate, 2 mmol/L EDTA, 2 mmol/L PEFA Bloc SC, and 10 mmol/L Tris-HCl, pH 7.4. The homogenate was centrifuged at $1000 \times g$ for 15 minutes, and the cleared supernatant was centrifuged ($145,000 \times g$, 3 hours). The resulting supernatant was filtered through a 0.45- μm filter (soluble fraction). Protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Porcine Protocol 2 for Myelin Isolation

Myelin was isolated from porcine brain according to a previously published protocol.²⁶ All steps were performed at 4°C . In brief, porcine brain tissue was homogenized in 20 vol (w/v) 0.32 mol/L sucrose and layered over 0.85% sucrose, followed by centrifugation ($75,000 \times g$, 30 minutes). The crude myelin fraction was collected at the interphase of the sucrose layers and resuspended in water and centrifuged ($75,000 \times g$, 15 minutes). The supernatant was discarded, and the myelin pellet was osmotically shocked by resuspension in water followed by centrifugation ($12,000 \times g$, 10 minutes). This step was repeated. The resulting myelin pellet was resuspended in 0.32 mol/L sucrose, layered over 0.85 mol/L sucrose, and

centrifuged ($75,000 \times g$, 30 minutes). The purified myelin was collected at the interphase of the two sucrose layers. The extraction of myelin involved the resuspension of myelin (200 μg) in phosphate-buffered saline (PBS; pH 7.2), 1% Triton X-100, 0.1 mol/L Na_2CO_3 , pH 11.5, 1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 1 mol/L NaCl, and incubated for 30 minutes at 37°C . The myelin samples were centrifuged ($13,500 \times g$, 10 minutes), and both the pellets (P) and supernatants (S) were used. Protein concentrations were determined using the BCA method.

Human Brain Tissue Processing

Human brain tissue was obtained from the Australian Brain Donor Programs and the South Australia Brain Bank. For all cases (16 MSA) and controls,¹² consent for brain autopsy was given and brain removal for research studies approved by the Human Ethics Committees of the institutions involved. The causes of death for the MSA cases were pneumonia ($n = 8$), cardiorespiratory arrest ($n = 6$), multiorgan failure, and drowning. The causes of death for the controls were cancer ($n = 4$), cardiorespiratory arrest ($n = 5$), renal failure ($n = 2$), and postoperative complications. Two separate protocols were used: a protocol to extract different fractions for analysis of protein amount and a standard protocol for *in situ* protein localization.

Human Protocol 1 for Analysis of Protein Amount

One g of white matter under the superior precentral gyrus (region that does not contribute to clinical presentation and is without cell loss but with GCIs²⁷) was sampled from frozen brain hemispheres of four MSA cases (three categorized according to Jellinger and colleagues²⁸ as MSA-parkinsonian and one as MSA-cerebellar) and four age-matched controls that had been stored for <2 years at -80°C . Brain proteins were extracted using a previously described protocol.²⁹ In brief, tissue was homogenized in a Tris-sucrose homogenization buffer (0.32 mol/L sucrose, 20 mmol/L Tris-Cl, and 5 mmol/L EDTA, pH 7.4), in the presence of a protease inhibitor cocktail (Complete, EDTA-free; Roche). This was followed by sonication (2×10 seconds) and centrifugation ($23,500 \times g$, 10 minutes, 4°C), and the resulting supernatant was stored as the soluble particulate fraction. Pellets were resuspended and sonicated (2×10 seconds) in a SDS-urea solubilization buffer (6 mol/L urea, 1% SDS, and 5 mmol/L EDTA) (1:5 w/v) and centrifuged ($23,500 \times g$, 10 minutes, room temperature) to obtain the SDS-extractable insoluble particulate fraction. Protein concentrations were determined using the BCA method.

Human Protocol 2 for *in Situ* Tissue Localization of Proteins

Fifteen percent buffered formalin-fixed samples of the pons (region that contributes to a MSA-cerebellar pre-

sensation²⁸) were taken from transversely cut slices of either whole or midsagittally dissected brainstems of 12 MSA cases (eight categorized as MSA-parkinsonian and four as MSA-cerebellar²⁸) and eight age-matched controls. Tissue samples were paraffin-embedded, cut at 5 μ m on a microtome, and mounted on 3-aminopropyltriethoxysilane (TESPA)-coated slides for immunohistochemical staining.

Gel Electrophoresis and Western Blotting

Brain protein samples were added with SDS-loading buffer (4% SDS, 40% glycerol, 50 mmol/L Tris-HCl, pH 6.8) supplemented with 20 mmol/L DTE for reducing SDS-polyacrylamide gel electrophoresis (PAGE) and heated for 3 minutes at 95°C. Proteins were separated by 10 to 16% (w/v) gradient SDS-PAGE. For Western blotting, SDS-PAGE gels were electroblotted onto nitrocellulose or polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK; and Bio-Rad, Hercules, CA). Membranes were blocked in 5% skimmed milk (dissolved in 50 mmol/L NaCl, 0.05% Tween 20, and 20 mmol/L Tris-HCl, pH 7.4), followed by incubation with several primary and secondary antibodies depending on the experiments required and tissues used. To detect p25 α , polyclonal rabbit anti-p25 α 1 antibody raised against recombinant human p25 α was used as described previously.²⁵ Monoclonal rat anti-MBP IgG (ab7349; Abcam, Cambridge, UK) or rabbit anti-MBP IgG (18-0444, 1:2000; Zymed Laboratories, South San Francisco, CA) was used to detect MBP. For standardization and internal controls, monoclonal mouse anti-BiP/Grp78 IgG (610978, 1:500; BD Biosciences, Franklin Lakes, NJ) and monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) IgG (4300, 1:4000; Ambion, Austin, TX) were used. Detection was done with enhanced chemiluminescence (Amersham Biosciences; and NEL104; Perkin Elmer, Boston, MA) using secondary antibodies conjugated to horseradish peroxidase (1:1000: DAKO, Glostrup, Denmark; or 1:5000: Sigma; 1:4000: Bio-Rad).

Affinity Chromatography and Mass Spectrometry

Three different columns for chromatography were developed using either recombinant human p25 α , anti-p25 α 1 IgG, or nonimmune IgG immobilized to CNBr-activated Sepharose 4B according to the manufacturer's instructions (Amersham Biosciences). Polyclonal rabbit anti-p25 α 1 antibody was raised against recombinant human p25 α as described previously,²⁵ and rabbit nonimmune IgG was prepared by subjecting preimmune serum to protein A chromatography. All following procedures were performed at 4°C. Soluble cytosol fraction from porcine protocol 1 (see above) was applied to each of the columns. The columns were equilibrated with 90 mmol/L NaCl, 50 mmol/L NaF, 20 mmol/L Na₄P₂O₇, 1 mmol/L vanadate, and 10 mmol/L NaH₂PO₄, pH 7.4. The columns were eluted with 0.1 mol/L glycine, pH 2.5, and 1-ml

Figure 1. MBP is a p25 α -binding protein. **A–C:** Interacting partners for p25 α were investigated by affinity chromatography. Porcine brain cytosol was subjected to affinity chromatography using columns having immobilized either recombinant human p25 α (**A**), anti-p25 α IgG (**B**), or nonimmune IgG (**C**). The columns were eluted with acid, and equal volumes of the fractions were analyzed by reducing SDS-PAGE and silver staining. **Arrowhead** indicates pH shift. The protein bands indicated by **arrows** and marked by numbers were subjected to tryptic digestion and mass spectrometric peptide mapping for protein identification. 1, α - and β -tubulin; 2, p25 α ; and 3, MBP. **D:** The interaction between porcine p25 α and MBP was verified by immunoprecipitation. Porcine brain cytosol was immunoprecipitated (IP) with anti-p25 α 1 IgG or anti-MBP IgG. The precipitates were subjected to reducing SDS-PAGE followed by immunoblotting (IB) with antibodies against p25 α and MBP. The molecular size markers on the left apply to **A–D**.

fractions were collected. The protein elution profiles were determined by gel electrophoresis and silver staining.

Protein bands indicated in Figure 1 were excised from silver-stained gels and subjected to tryptic digestion.³⁰ The resulting peptides were analyzed using a Voyager STR MALDI instrument operating in reflector mode (Applied Biosystems, Foster City, CA). The peptide mass list was analyzed using the PeakEraser program³¹ before analysis on the Mascot search engine (Matrix Science, London, UK) for a positive identification in the National Center for Biotechnology Information nonredundant protein database. The spectral data correlated with the MBP sequence using the GPMW software.³²

Immunoprecipitation

The soluble cytosol fraction from porcine protocol 1 was subjected to immunoprecipitation by adding 10 μ g of anti-p25 α 1 IgG (50 μ l) or 50 μ l of anti-MBP IgG, followed by incubation for 16 hours at 4°C. Polyclonal rabbit anti-p25 α 1 antibody was raised against recombinant human p25 α as described previously,²⁵ and monoclonal rat anti-MBP IgG (ab7349; Abcam) was used. Hereafter, 25 μ l of protein A-Sepharose (Amersham Biosciences) was added, followed by a 1-hour incubation at 4°C to immobilize the antibody. The beads were washed in an ice-cold buffer (0.5% Triton X-100 and PBS, pH 7.2), and SDS-loading buffer was added. The samples were subjected to gel electrophoresis and Western blotting.

Immunohistochemistry

Double-fluorescence immunohistochemical labeling to assess myelin changes was performed using previously established methods, including antigen retrieval²⁰ with antibodies against either rabbit anti-MBP IgG (18-0444, 1:3000; Zymed Laboratories) or mouse anti-MBP IgG (MCA184S, 1:250; Serotec, Oxford, UK) or 2'3'-cyclic

nucleotide 3'-phosphodiesterase (CNPase) (C5922, 1:400; Sigma) and p25 α (1:50, as previously described^{20,25}) or neurofilament (NFM) (814342, 1:100; Boehringer Mannheim Biochemica, Roche, Indianapolis, IN) or mouse anti-peptide β III-tubulin (1:1000; Promega, Madison, WI). Single peroxidase immunohistochemistry was also performed using these antibodies, an antibody to degraded MBP (AB5864, 1:2000; Chemicon International, Temecula, CA) and appropriate biotinylated secondary antibodies and tertiary peroxidase complexes (Vector Laboratories, Burlingame, CA). Paraffin sections were dewaxed through xylene and graded ethanol to water for 3 minutes each (2 $\times$ xylene, 2 $\times$ 100% ethanol, 1 $\times$ 95% ethanol, and 1 $\times$ 75% ethanol) and incubated in 0.1 mol/L citrate buffer (sodium citrate, citric acid, pH 6.0) for 3 to 15 minutes at 70 to 100% power in the microwave. Slides were then cooled, washed in distilled water and 0.1 mol/L Tris buffer placed in a solution of 3% H₂O₂ and 50% ethanol for 40 minutes at room temperature and then placed in 20% normal horse serum solution for 1 hour at room temperature. For double labeling, primary antibodies were then incubated overnight at 4°C and visualized with a cocktail of anti-rabbit secondary antibody conjugated to Alexa 568 and anti-mouse secondary antibody conjugated to Alexa 488 (Molecular Probes, Invitrogen, Carlsbad, CA). Slides were then coverslipped with the Vectashield mounting medium (Vector Laboratories). To test the specificity for all immunohistochemical reactions, and to ensure noncross-reactivity of secondary fluorescent probes, a section without primary antibodies was included for each staining procedure as a negative control. In addition, a cocktail of the secondary antibodies were applied to sections with only one primary antibody incubated on each section. Labeled sections were evaluated on a confocal microscope analysis system (TCS SP inverted microscope, Leica TCS software; Leica, Wetzlar, Germany) or an Olympus BX51 fluorescence microscope (Tokyo, Japan) fitted with specific filter systems and a computerized image analysis system (SPOT camera, Image Pro Plus software).

For triple labeling, a combined method of double-fluorescence labeling and peroxidase visualization was used. Pretreatment differed to include 99% formic acid for 3 minutes to ensure maximal α -synuclein binding.^{33,34} p25 α was visualized using standard peroxidase immunohistochemistry.^{20,35} Briefly, slides were incubated with the anti-p25 α 1 IgG (1:50) overnight at 4°C and then incubated with a rabbit biotinylated secondary antibody (1:200; Vector Laboratories) for 30 minutes at 37°C. The tertiary complex involved the incubation with an avidin-biotin-peroxidase tertiary complex (1:500, Vectastain; Vector Laboratories) for 30 minutes at room temperature. Visualization was achieved by incubation with 0.7% H₂O₂, 0.15% diaminobenzidine tetrahydrochloride (Sigma) for 15 minutes at room temperature, followed by several 0.1-mol/L Tris washes. This was then followed by α -synuclein immunofluorescence. Briefly, sections were incubated with anti- α -synuclein IgG (1:200; BD Biosciences, San Jose, CA) for 1 hour at 37°C. After 0.1-mol/L Tris washes, sections were incubated with the secondary antibody (anti-mouse conjugated to Alexa 568; Molecular Probes,

Invitrogen) for 2 hours at room temperature. Thioflavine S staining was then used to determine the protein conformation as it detects β -pleated sheets of protein.³⁶ Briefly, slides were placed in a 1% thioflavine S (Sigma) solution for 5 minutes at room temperature, differentiated with 70% ethanol, and then coverslipped with Vectashield. To ensure specificity of the immunohistochemical technique, a positive (section containing Lewy bodies) and negative (see above) control were included in each experiment. Labeled sections were evaluated and photographed using an Olympus BX51 fluorescence microscope fitted with specific filter systems and a computerized image analysis system (SPOT camera, Image Pro Plus software).

Evaluation of Tissue Changes in MSA

All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL), and a *P* value <0.05 was considered as significant.

Assessment of Protein Levels

For all control and MSA samples, the relative amounts of MBP, p25 α , and GAPDH on Western immunoblots were analyzed using image analysis software (Quantity One 1D-Analysis software, version 4.6; Bio-Rad). GAPDH (30 to 40 kd) label intensity was used for standardization on each blot. Protein bands were identified using a computer mouse for each sample, the average band intensity extracted and standardized against the intensity of the GAPDH protein band for that case in that blot. Density values for the soluble particulate and SDS-extractable insoluble particulate fractions were added together to assess total protein levels, and changes in solubility identified using ratios. Differences between groups were assessed using Mann-Whitney *U*-tests and correlations with clinical indices determined using Spearman rank tests.

Assessment of Protein Association with Myelin

For each control and MSA case, double-labeled immunofluorescent sections using either p25 α , MBP, or CNPase antibodies were graded in five random sites within the pontine basis at $\times$ 200 magnification. Samples were photographed using standard digital camera settings, and the normal distribution of proteins was determined in controls. MSA-related changes in protein localization were graded as either no change from controls (0), mild loss (1), moderate loss (2), or severe loss (3) of immunofluorescence labeling of protein antigens, and any associations between protein loss and clinical indices was determined using the Spearman rank test (across all MSA cases) and Mann-Whitney *U*-test (for MSA subtypes).

Analysis of Oligodendroglia and GCI

For each case sections triple-labeled using p25 α immunoperoxidase, α -synuclein immunofluorescence, and thioflavine S fluorescence were analyzed using merged digital

images. Fibrillization was assessed using thioflavine S fluorescence and oligodendroglial cells categorized into four types according to their protein content and presence of fibrillized thioflavine S-positive inclusions: i) those containing nonfibrillar p25 α only, ii) those colocalizing α -synuclein and p25 α but not thioflavine S, iii) those colocalizing fibrillized α -synuclein and p25 α (thioflavine S-positive), or iv) those containing only fibrillar α -synuclein (thioflavine S-positive). High-magnification ($\times 1000$) of 15 to 20 random sample areas of 13,767 μm^2 in affected regions in each section were photographed using standard digital camera settings. Images were enlarged 2.6-fold and the density of GCIs determined and the size of oligodendroglial GCIs and entire soma in each image measured using point-counting by randomly overlaying a 121 $\times$ 175-mm grid on each image (each point represented 1.826 μm^2). To ensure adequate *in situ* visualization of α -synuclein using the fluorescent probe, assessment of the density and size of α -synuclein-immunoreactive oligodendroglia was also performed on single immunoperoxidase-labeled sections. For all analyses a total of 40 to 60 oligodendroglial cells were measured in each control and MSA case. *t*-Tests revealed no significant differences in the density or size of α -synuclein-immunoreactive oligodendroglia when measured within sections prepared using either the triple-labeling or single-labeling procedures ($P > 0.7$). Repeated measurements by the same or different investigators gave an average variance of less than 10% for all variables. Differences between groups in oligodendroglial density were evaluated using Mann-Whitney *U*-tests. Differences between groups in the distribution of GCI sizes or oligodendroglial cell body sizes were identified using χ^2 tests. Correlations with clinical indices were determined using Spearman rank tests.

Analysis of Axon Diameter, Fiber Diameter, and Calculation of g Ratios

Sections of the pons double labeled using MBP and NFM immunofluorescence were analyzed using merged digital images and Axiovision software (version 4.6; Carl Zeiss Imaging Solutions, Thornwood, NY). Only transversely sectioned pontine fiber tracts were assessed, and in MSA cases these fiber tracts were chosen as those depleted of p25 α immunoreactivity (see Results). For each axon, axon area and external fiber area were outlined separately, the diameter in μm calculated from the area. Forty to sixty-five axons per case were analyzed, and repeated measurements of 10 axons in three cases gave $<10\%$ variability. Differences between groups in g ratio and axon diameter were identified using Mann-Whitney *U*-tests.

Results

Identification of MBP as a p25 α -Binding Protein

The identification of proteins that bind to the oligodendroglial p25 α protein may shed light on its physiological

functions. Affinity chromatography was used to isolate putative p25 α -binding proteins in a crude cytosolic preparation of porcine brain, and reducing SDS-PAGE was used to resolve the eluted proteins (Figure 1). Subjecting crude porcine brain cytosol to affinity chromatography using a column with immobilized recombinant human p25 α permitted the isolation of protein complexes that interact directly with p25 α . By doing so, a range of proteins was isolated, among which a 50-kd protein and three proteins of 22, 20, and 18 kd were prominent (Figure 1A). Using a column with immobilized anti-p25 α 1 antibody allowed the purification of proteins that are parts of putative complexes with the endogenous porcine brain p25 α . This resulted in the isolation of a predominant protein of 27 kd, two prominent proteins of 50 kd and 20 kd and a few less abundant proteins (Figure 1B). Based on its electrophoretic migration, the 27-kd protein was expected to be p25 α , and this was corroborated by immunoblotting (data not shown). As a negative control, porcine brain cytosol was subjected to affinity chromatography on a column having nonimmune rabbit IgG immobilized and expected to be devoid of any specific protein binding. This procedure resulted in an elution profile absent of distinct protein bands but with faint smears ~ 25 , 50, and 60 kd and where the 50-kd smear was most intense (Figure 1C).

The prominent proteins, which are marked by arrows in Figure 1, A–C, were all chosen for identification by tryptic mass spectrometric peptide mapping (data not shown). The spectra identified the 27-kd band (indicated by “2” in Figure 1B) as the bovine homologue of human p25 α (accession no. O94811) because the porcine p25 α was not present in the database. The three 18- to 22-kd peptides (indicated by “3” in Figure 1, A and B) were identified as porcine MBP homologues (accession no. P81558) and the 50-kd protein (indicated by “1”) in all three Figure 1 panels as porcine α - and β -tubulin (accession nos. P02250 and P02254).

p25 α and MBP are both oligodendroglial proteins so their putative interaction was further investigated. The significance of the interaction was corroborated by co-immunoprecipitation of p25 α and MBP from porcine brain cytosol using antibodies raised against MBP and p25 α , respectively (Figure 1D). To demonstrate direct binding and determine the affinity of interaction between isolated p25 α and bovine MBP, surface plasmon resonance analysis was performed using a sensor chip coated with purified recombinant human p25 α . Figure 2A shows that purified MBP binds to p25 α in a reversible and concentration-dependent manner with an estimated K_d of 10 nmol/L demonstrating an interaction of high affinity. This interaction was further analyzed by tryptophan (Trp) fluorescence spectroscopy (Figure 2B) and far-UV CD spectroscopy (Figure 2D) to gain information on the stoichiometry of the interaction and putative structural alterations as a consequence thereof. Both p25 α and MBP have Trp residues. In the absence of any p25 α -MBP interaction, Trp fluorescence spectroscopy would reveal a spectrum of only the sum of the individual spectra of MBP and p25 α . However, even though there is no shift in the emission wavelength of the peak maximum, the p25 α :MBP complex had an

Figure 2. Biophysical analysis of the interactions between MBP and p25 α . **A:** Surface plasmon resonance analysis of the binding affinity of bovine MBP to immobilized recombinant human p25 α . Sensorgrams obtained using increasing concentrations of MBP are demonstrated. Analysis of the obtained sensorgrams estimated a k_d of 10 nmol/L. The ordinate shows arbitrary response units. **B:** Spectroscopic alterations induced by the p25 α :MBP complex formation as measured by Trp fluorescence spectroscopy. The Trp spectra of 2 μ mol/L MBP or p25 α were measured, and the mathematical sum of the individual spectra was calculated (denoted with the **closed circles**). The spectrum of the p25 α :MBP complex (2 μ mol/L) is denoted by the **open circles**. **C:** Titration of MBP with p25 α . The emission difference $F_{exp} - F_{obs}$ (where F_{exp} is the mathematical sum of the fluorescence of the two protein components and F_{obs} is measured fluorescence) is plotted versus the ratio of the concentrations of the two protein components. The difference between expected and measured fluorescence is a measure of the interaction between MBP and p25 α . The increase in fluorescence at low ratios indicates that all added p25 α forms a complex with MBP, whereas the plateau at higher ratios occurs when all MBP binding sites for p25 α are saturated and no more complexes are formed. The **straight lines** are drawn to guide the eye to these two regions. The ratio where the two regions meet (indicated by the crossing of the two lines) indicates the stoichiometry of the MBP:p25 α complex. **D:** Spectroscopic alterations induced in the p25 α :MBP complex as measured by far-UV CD spectroscopy. The spectrum of the p25 α :MBP complex is denoted by open circles; the mathematical sum of the two protein components is denoted with the **closed circles**. In **B** and **D**, spectra of free MBP and p25 α are omitted for clarity.

~25% reduced intensity compared with the sum of the individual proteins (Figure 2B).

The binding-induced reduction in fluorescence intensity allowed the investigation of the stoichiometry of the interaction between p25 α and MBP, as described previously for the binding of tubulin to p25 α .¹⁰ p25 α was titrated into a solution of MBP in small increments while measuring the fluorescence. The difference between the measured and the calculated fluorescence from each protein contribution ($F_{exp} - F_{obs}$) is an indication of the binding extent between p25 α and MBP. An absence of interaction would result in the difference being zero throughout the titration interval. A plot of $F_{exp} - F_{obs}$ versus the concentration ratio between p25 α and MBP ($[p25\alpha]/[MBP]$) shows a steep increase in $F_{exp} - F_{obs}$ at low $[p25\alpha]/[MBP]$ before reaching a constant level (Figure 2C). The data indicate that binding is saturated at a ratio of 0.75 molecule MBP per molecule p25 α ; thus the data can be interpreted as the formation of complexes of no more than a simple 1:1 binding model.

Structurally, p25 α is a highly flexible yet structured protein that displays structural changes on interaction with tubulin,^{7,10} whereas MBP is an intrinsically unstruc-

tured protein.² Because of Trp's general environmental sensitivity as mentioned above, fluorescence spectroscopy cannot distinguish between binding and conformational changes. In contrast, far-UV CD spectroscopy primarily monitors conformational changes. Far-UV CD measurements of a 1:1 mixture of the two protein components were performed. As shown in Figure 2D, the measured spectrum of the complex was significantly different from the calculated spectrum that represents the sum of the individual spectra of MBP and p25 α . This observation suggests that p25 α and MBP interact and that the interaction results in structural alterations in these proteins.

p25 α Is a Component of Myelin

The interaction of p25 α with the myelin-specific MBP prompted us to investigate whether p25 α is a constituent of myelin. Porcine brain was homogenized, and myelin was purified accordingly.²⁶ Figure 3A demonstrates that the concentration of MBP is increased as expected in the purified myelin as compared with the homogenate. For p25 α , there is a clear signal in the purified myelin, but its concentration is reduced as compared with the homogenate. This demonstrates that p25 α is present in myelin but not as a myelin-specific constituent like MBP. The tightness of the association of p25 α to myelin was compared with MBP by subjecting myelin to extraction under different stringencies (Figure 3B). We incubated myelin in

Figure 3. p25 α colocalizes with MBP in myelin extracts. **A:** Porcine brain homogenate (H) and purified myelin (M), each fraction (25 μ g), was analyzed by SDS-PAGE and Western blotting using antibodies raised against p25 α and MBP. p25 α is clearly present in purified myelin but at a concentration lower than that in the homogenate, which contrasts with the MBP that is enriched in the myelin fraction. **B:** The association of p25 α to myelin was investigated by subjecting the purified myelin to washing in buffers of different stringencies. Purified porcine myelin (200 μ g) was suspended in PBS, 1% Triton X-100, 1% SDS, 1 mol/L NaCl, and 0.1 mol/L Na₂CO₃, pH 11.5, and separated into pellet (P) and supernatant (S) fractions. The samples were analyzed by SDS-PAGE and immunoblotted with anti-p25 α 1 (P) and anti-MBP (M) antibodies. The data clearly indicate that p25 α is a component of myelin but is less tightly associated to the membrane fraction than MBP.

the presence of denaturing and nondenaturing detergents (1% SDS and 1% Triton X-100), high ionic strength (1 mol/L NaCl), and high pH (100 mmol/L Na₂CO₃, pH 11.5) and separated soluble and membrane fractions by centrifugation. As a control, the fractions were incubated in PBS, pH 7.2. The pellets and soluble fractions were analyzed by immunoblotting with antibodies against p25 α and MBP. In the control with PBS, a minor fraction of p25 α was found in the supernatant, which indicates a reversible interaction, whereas the MBP species remained entirely in the insoluble pellet fraction. Increasing the ionic strength did not increase the fraction of released p25 α compared with PBS whereas a small but reproducible release of MBP occurred. The pH 11.5 treatment released the majority of p25 α and more than half of the MBP from the pellet. Complete release of both proteins was obtained using the detergents Triton X-100 and SDS. These data indicate that p25 α is less tightly associated with the myelin as compared with MBP.

The association of p25 α to MBP and to myelin was verified *in situ* in control human brain tissue. Double-labeling immunofluorescence in controls revealed substantial colocalization of MBP and p25 α in the myelinated corticospinal fiber tracts, particularly surrounding the NFM-immunopositive axons (Figure 4). In addition, intense p25 α immunoreactivity was also present in the cytosol of oligodendroglial cell bodies (Figure 4D), as previously described.^{25,37}

Figure 4. p25 α and MBP localization in myelinated fiber bundles *in situ*. Representative confocal photomicrographs of immunofluorescently labeled, paraffin-embedded sections of the pontine basis in a control case. **A–C:** p25 α (Alexa 568, red; **A**) and MBP (Alexa 488, green; **B**) immunoreactivity colocalizes in the same axon bundles and structures as indicated (merged micrographs, **C**). Scale in **C** equivalent to **A** and **B**. **D** and **E:** Merged images of p25 α (Alexa 568, red; **D**) and MBP (Alexa 568, red; **E**) with NFM (Alexa 488, green) immunoreactivity, indicating that both proteins (p25 α and MBP) colocalize to the myelin sheaths surrounding the NFM-immunopositive axons in these myelinated fiber bundles. **D:** Note the intensely p25 α -immunopositive oligodendroglial cell body.

Figure 5. Western blots of human brain extracts (four MSA cases compared with controls). Brain protein extracts were analyzed by SDS-PAGE and immunoblotted for MBP, p25 α , and GAPDH in both the soluble particulate (**A**) and SDS-extractable insoluble particulate (**B**) tissue fractions. There was a significant reduction in the relative amount of MBP protein but not p25 α in the insoluble particulate fraction in MSA compared with controls (see Table 1). There was no significant redistribution of these proteins between soluble and insoluble fractions. Molecular size markers are indicated on the right.

A Reduction in MBP and Its Dissociation from p25 α in MSA

To confirm protein type and amount in human tissue, similar Western blot studies of MSA and control cases were performed. The major MBP and p25 α protein bands were consistently identified in all tissue fractions in all cases (Figure 5). Densitometric assessment of the SDS/urea-extractable insoluble particulate fraction revealed a reduction in relative MBP protein level in MSA with no increase in the soluble particulate fraction (Figure 5, Table 1). In contrast, there was no change in the relative amount of p25 α in either fraction (Figure 5, Table 1). This reduction in MBP protein was in accordance with an earlier study suggesting that myelin pathology was more widespread³⁸ and not readily appreciated by immunohistochemistry. Further examination of *in situ* protein localization revealed no change in the localization of MBP or CNPase, and no gross structural demyelination (Figure 6). However, in the MSA cases there was a patchy distribution of degraded MBP in myelinated fiber bundles and in some oligodendroglia and GCIs (Figure 6, E and F), as previously shown³⁹ and consistent with the reduction in the relative amount of full-length MBP protein in MSA (Figure 5). There was a redistribution of p25 α immunoreactivity in the myelin leaving patchy areas with reduced p25 α , and an enhancement of staining in the oligodendroglial cell bodies (Figure 6, B and D) in all MSA cases irrespective of their subtype. Semiquantitative assessment of the immunofluorescence labeling of protein antigens confirmed the mild to moderate patchy loss of p25 α immunoreactivity from myelinated fibers to cell bodies with no apparent correlation to disease duration (Table 1) or subtype, suggesting an early, nonprogressive change.

Table 1. Demographic Variables and Changes in Immunoreactivity within Myelinated Fiber Tracts

Group	n (M:F)	Mean age (years)*	Mean duration (years)*	Method used	MBP*	p25 α *	CNPase*
Control	3:1	71 $\pm$ 7	N/A	WB	54 $\pm$ 27 [†]	22 $\pm$ 10 [†]	
MSA	1:3	71 $\pm$ 6	4 $\pm$ 3	WB	17 $\pm$ 3 [†]	16 $\pm$ 3 [†]	
Control	4:4	73 $\pm$ 9	N/A	IHC	Normal	Normal	Normal
MSA	7:5	69 $\pm$ 8	4 $\pm$ 2	IHC	0 $\pm$ 0 [‡]	1.33 $\pm$ 0.49 [‡]	0 $\pm$ 0 [‡]

F, female; M, male; WB, Western blotting; IHC, immunohistochemistry; N/A, not applicable. The majority of MSA cases were the subtype MSA-Parkinsonian with one (male) MSA-cerebellar used for WB and four (two males) MSA-cerebellar used for IHC. There were no differences between MSA subtypes in demographics ($P > 0.10$).

*Mean $\pm$ SD. Significant differences from control values are indicated in italics (Mann-Whitney U -test, $P < 0.05$).

[†]Densitometric measurement standardized to the level of GAPDH immunoreactivity.

[‡]Measured using the semiquantitative scale: 0, no loss; 1, mild loss; 2, moderate loss; 3, severe loss.

Morphological Assessment of Oligodendroglial Changes in MSA

To establish and quantify the magnitude of change in the cellular relocation of p25 α from myelin in MSA, triple-labeling experiments were performed to identify α -synuclein-positive GCIs, p25 α protein, and thioflavine-S-positive fibrils (Figure 7), and the density and cell body size of oligodendroglia containing only p25 α was determined in affected regions compared with those containing α -synuclein-posi-

tive GCIs (Table 2). In this analysis, thioflavine-S-positive fibrillar inclusions measured in cross-section $45 \pm 17 \mu\text{m}^2$. In controls only small α -synuclein-negative p25 α -immunoreactive oligodendroglia were observed with an average cell size of $12.9 \pm 3.9 \mu\text{m}^2$ (Figure 7A, Table 2) and an average density of $4.0 \pm 0.9/\text{high-power field}$. In MSA, this α -synuclein-negative population was significantly reduced in density (average of $1.8 \pm 1.1/\text{field}$, $P = 0.002$) and had a significant enlargement of their cell size (approximately triple the size; Figure 7B, Table 2).

Assessment of oligodendroglia containing α -synuclein-immunoreactivity (not seen in controls) revealed a similar number ($1.7 \pm 1.2/\text{field}$) to those without α -synuclein immunoreactivity (see above), with the majority of these colocal-

Figure 6. Changes in the location of myelin-associated proteins in MSA. Representative merged confocal photomicrographs of immunofluorescently labeled, paraffin-embedded sections of the pontine basis in controls (**A** and **C**) and MSA (**B** and **D**) cases. **A** and **B**: p25 α (Alexa 568, red) and MBP (Alexa 488, green) significantly colocalize in control myelinated fiber tracts (**A**). In MSA, there is a reduction of p25 α but not MBP immunoreactivity in the myelinated fiber tracts (**B**). Scale in **B** is equivalent to **A**. **C** and **D**: Similar to MBP, p25 α (Alexa 568, red) and CNPase (Alexa 488, green) significantly colocalize in control myelinated fiber tracts (**C**), and there is a loss of p25 α but not CNPase in MSA (**D**). **D**: Enlarged p25 α -immunoreactive oligodendroglia remain in the MSA cases. Scale in **D** equivalent to **C**. **E** and **F**: Representative bright-field photomicrographs of degraded MBP immunoperoxidase staining in paraffin-embedded sections of the pontine basis from two MSA cases show abnormally enlarged oligodendroglia containing GCI and degenerating myelin. No degraded MBP was observed in tissue sections from controls (not shown). Scale in **F** equivalent to **E**.

Figure 7. Control and MSA oligodendroglia. Representative photomicrographs of paraffin-embedded sections of the pontine basis in a control (**A**) and MSA (**B–F**) case triple labeled for p25 α (bright-field immunoperoxidase), α -synuclein (Alexa 568, red immunofluorescence), and thioflavine-S (green histofluorescence; scale in **F** is equivalent to **A–E**). **A** and **B**: The majority of oligodendroglia sampled in both controls (**A**) and MSA cases (**B**) contained only p25 α immunoreactivity (α -synuclein- and thioflavine-S-negative, bright-field images shown). **A**: In controls, p25 α immunoreactivity was found surrounding the nucleus in a thin sheet of cytoplasm, as well as in the surrounding myelin. **B**: In MSA, there was a reduction of p25 α immunoreactivity in the surrounding myelin and an obvious enlargement of the oligodendroglia cytoplasm in the majority of this cell type. **C**: A small proportion of the MSA oligodendroglia sampled contained only fibrillar α -synuclein inclusions (thioflavine-S-positive, p25 α -negative, merged bright-field and red fluorescence shown). **D–F**: A significant proportion of MSA oligodendroglia colocalized p25 α (bright-field immunoperoxidase, **D**) and α -synuclein (Alexa 568 red immunofluorescence, **E**) immunoreactivity, and ~20% of this cell type contained fibrillized GCI (thioflavine-S green histofluorescence, **F**; asterisk indicates the same cell).

Table 2. Cell Size Measurements in μm^2 (Mean $\pm$ SD)

Group	<i>n</i>	p25 α only (%N, range)	p25 α + α -syn (%N, range)	p25 α + α -syn + thioflavine (%N, range)	α -syn only + thioflavine (%N, range)
Control	377	12.9 $\pm$ 3.9 (100%, 3.7 to 31)			
MSA	689	36 $\pm$ 18* (53%, 11 to 141)	72 $\pm$ 29 (27%, 22 to 181)	89 $\pm$ 34 (7%, 22 to 161)	44 $\pm$ 24 (13%, 13 to 120.5)

α -syn, α -synuclein.

*Significantly different distribution from controls using χ^2 test ($P < 0.000$) with 87% of MSA p25 α only oligodendroglia sampled >2 SDs from control mean value.

izing p25 α protein (average density of $1.2 \pm 0.9/\text{field}$). However, these double-labeled oligodendroglia had an even larger cell size compared with those without α -synuclein immunoreactivity (average of $\sim 80 \mu\text{m}^2$, Table 2) and were of a similar size to MSA oligodendroglia containing degraded MBP immunoreactivity ($P = 0.29$; Figure 6, E and F). These very large p25 α and α -synuclein-positive oligodendroglia could be divided into nonfibrillar ($\sim 80\%$) and thioflavine-S-positive fibrillar cells with the latter having the largest average size (Figure 7, D–F; Table 2). A small proportion of the oligodendroglia sampled contained no p25 α immunoreactivity but displayed α -synuclein- and thioflavine-S-positive fibrillar inclusions (Figure 7C; average density of $0.5 \pm 0.4/\text{field}$). These cells had a reduced average size compared with those containing both α -synuclein and p25 α immunoreactivity (Table 2) or those containing degraded MBP immunoreactivity ($P = 0.004$). In MSA, there was no correlation between the number of α -synuclein-positive and α -synuclein-negative oligodendroglia ($P = 0.27$), and the size of the GCIs did not differ within the different cell types detected and was not different from the oligodendroglia containing only fibrillar α -synuclein ($P = 0.77$). There was also no difference between the size of any cell type between the two MSA phenotypes included in the analyses ($P > 0.08$).

Associated Axonal Changes in MSA

To determine whether these changes in myelination had any affect on axons, the *in situ* location of axonal proteins and axonal size and myelination were assessed. Overall axon integrity appeared preserved as evaluated by NFM and β III-tubulin immunoreactivity in the corticospinal or pontocerebellar pathways in MSA compared with controls (Figure 8). This is despite the significant reduction in p25 α immunoreactivity in the myelin surrounding these axons (Figure 8) and the enlargement of the p25 α -immunoreactive oligodendroglia observed in MSA (Figure 8D). To determine whether the surrounding myelin itself was morphologically changed and could therefore affect axonal function, g ratios were calculated. There was a significant increase in the g ratio in MSA cases compared with controls (0.63 ± 0.08 in MSA versus 0.59 ± 0.10 in controls, $P = 0.004$), suggesting thinner myelin as identified using MBP immunofluorescence. Assessment of the axonal diameter for these cases identified a small but significant increase in diameter of NFM-immunoreactive axons in the MSA cases compared with controls ($2.6 \pm 0.4 \mu\text{m}$ in MSA versus $2.4 \pm 0.6 \mu\text{m}$ in controls, $P < 0.03$). This was attributable to a relative de-

myelination of smaller caliber axons in MSA rather than any overall enlargement in the MSA axons.

Correlations between Changes in MSA Oligodendroglia and the Time Course of Events

To gain insight into the time course of events in the MSA cases assessed, Spearman rank correlations were performed. There was a correlation between the size of these highly expanded p25 α - and α -synuclein-containing oligodendroglia and those containing only p25 α ($P = 0.03$) and those containing only α -synuclein immunoreactivity ($P = 0.001$). However, there was no correlation between the size of p25 α -only- and α -synuclein-only-immunoreactive MSA oligodendroglia. The relationship between the size of the expanded oligodendroglia containing only p25 α and those also containing α -synuclein across the cases evaluated suggests that such cell expansion may predispose to abnormal α -synuclein deposition. The relationship between the size of the oligodendroglia containing both p25 α and α -synuclein and the smaller number containing only fibrillized α -synuclein suggests further intracellular modifications targeting p25 α influence this pathology.

Correlating disease duration with density and average size data revealed a correlation with the cell size of p25 α - and α -synuclein-immunoreactive oligodendroglia (Spear-

Figure 8. Loss of p25 α without axonal degeneration in MSA. Representative merged photomicrographs of immunofluorescently labeled paraffin-embedded sections of pontine basis in controls (**A** and **C**) and MSA (**B** and **D**) cases. Scale in **D** equivalent for all photomicrographs. **A:** p25 α immunoreactivity (Alexa 568, red) concentrates in the myelin surrounding NFM-immunoreactive (Alexa 488, green) axons in controls. **B:** There is a significant loss of p25 α immunoreactivity in MSA myelin without the loss of NFM immunoreactive axons. **C** and **D:** Similar to NFM, β III-tubulin immunoreactivity (Alexa 488, green) concentrated in the axons in both controls and MSA. In areas of reduced p25 α immunoreactivity (Alexa 568, red) in MSA myelin, axonal β III-tubulin immunoreactivity remained preserved (**D**) and enlarged p25 α -immunoreactive oligodendroglia were obvious.

Figure 9. Correlations between the duration of MSA and oligodendroglia cell size. Spearman rank correlations in the MSA cases show that the average size of two oligodendroglia cell types positively correlated with increasing disease duration; there seems to be an increase in the size of both p25 α - and α -synuclein-colocalizing oligodendroglia ($P = 0.04$) and of those containing only fibrillized α -synuclein-immunoreactive GCI ($P = 0.009$) throughout the disease course. The average size of the enlarged p25 α -only-immunoreactive oligodendroglia does not appear to expand further with disease progression.

man rank, $P = 0.04$) as well as the cell size of those containing only fibrillized α -synuclein-immunoreactive GCI (Spearman rank, $P = 0.009$) but not those containing only p25 α immunoreactivity (Figure 9) or with any density measures. The lack of correlation between disease duration and the density measures suggests that tissue volume changes play a significant role in MSA, with remaining tissue primarily containing similar numbers of oligodendroglial elements. In addition, as α -synuclein-immunoreactive GCI size was similar across cases, there was no correlation with disease duration.

Overall, these data suggest that in MSA there is an early redistribution of p25 α from the myelin to the oligodendroglial cell soma with consequent cell expansion (around the time of symptom onset), which impacts on the normal MBP/p25 α interaction in myelin and accompanies the degradation of full-length MBP. This change in oligodendroglial proteins is associated with a reduction in smaller diameter myelinated axons within the corticospinal and pontocerebellar pathways. Throughout time, there is an increasing number of oligodendroglial cells that accumulate degraded MBP and α -synuclein in their cytoplasm causing further cell expansion and eventually α -synuclein fibrillization. In some cases the fibrillization appears to be followed by a loss of normal cytoplasmic p25 α and abnormally accumulated degraded MBP and is associated with a reduction in the overall size of the oligodendroglial cytoplasm to include mainly the fibrillized inclusion.

Discussion

Oligodendroglia are highly polarized cells that elaborate distinct domains such as the soma, and the more distant

compact and noncompact myelin, with distinct lipid and protein composition.⁴⁰ The complexity is demonstrated by single oligodendroglial cells that produce and maintain the myelin structures enwrapping several axons in the central nervous system.⁴¹ The composition of myelin is dominated by a limited number of molecular species whereby the protein MBP represents ~30% of the total protein.² The functional significance of MBP is highlighted by its association with demyelinating conditions,^{3,4} although the precise role of MBP in maintaining a functional myelin sheath remains unclear. However, the different expression of MBP isoforms and their extensive posttranslational modifications² indicates that it is subject to tightly controlled expression and signaling.

We demonstrate that the oligodendroglial protein p25 α is a novel component of myelin as revealed biochemically by its presence in purified myelin and immunohistochemically by its diffuse presence in MBP-immunopositive myelinated fiber tracts. This contrasts former analyses that have described p25 α as a protein specific for the oligodendroglial cell body,^{8,25,37} although the first demonstration of p25 α in rat brain did describe neuropil staining⁵ in accordance with our data. This discrepancy likely reflects the abundance of p25 α in the cell body that may have masked the appreciation of its association with myelin. We convincingly demonstrate the presence of p25 α in MBP-immunopositive myelin sheaths by its presence in these ring-like structures around the NFM- and β III-tubulin-immunopositive axons and by colocalization experiments using two independent antibodies. A functional role for p25 α in the myelin structure is supported by our biochemical demonstration of a complex between p25 α and MBP in porcine brain extracts and the direct high-affinity interaction between purified MBP and p25 α demonstrated by plasmon surface resonance analysis. GAPDH was recently identified as a p25 α -binding protein by p25 α affinity purification from bovine brain cytosol,⁴² and we do detect some weakly stained bands of ~35 kD eluting from our affinity chromatography columns from porcine brain cytosol that may represent GAPDH, but their presence is minor compared with tubulin, MBP, and the endogenous p25 α . The Trp spectroscopic analysis allowed an assessment of a stoichiometry of the complex that indicated a 1:1 p25 α :MBP complex, which differed from the 1:2 p25 α :tubulin complex demonstrated using a similar biophysical approach.¹⁰ As p25 α is less abundant than MBP, our data indicate that p25 α may serve a dynamic regulatory role rather than a structural role that facilitates interactions between MBP and tubulin or MBP-directed kinases such as glycogen synthase kinase, of which p25 α has been identified as an inhibitor.⁴³

We demonstrate that the normal presence of p25 α in myelin is altered in MSA as a patchy disappearance from the myelin along with an accumulation in oligodendrocytes with expanded cell bodies. The accumulation of p25 α in GCI-bearing oligodendroglia in MSA is well documented,^{25,37,44} but we describe for the first time that ~50% of the non- α -synuclein-expressing oligodendroglia in MSA-affected fiber tracts have an abnormal accumulation of p25 α , based on their strong immunoreactivity and approximately tripled size. Oligodendroglia possess

an advanced system for intracellular sorting and transport of constituents for the different subdomains in the myelin sheets. This is exemplified for the two major myelin proteins in which the proteolipid protein uses the endoplasmic reticulum-Golgi pathway for sorting of membrane proteins,⁴⁰ whereas MBP in myelin relies on the transport of its RNA, which becomes translated within the myelin structure.⁴⁵ The reduction in full-length MBP and relocation of p25 α from myelin, along with the accumulation of MBP degradation products in the oligodendroglial cell bodies, suggests that the cellular transport system maintaining MBP levels in myelin is perturbed. Gross myelin disruption is not a feature of MSA, although myelin changes have been previously noted by Papp and colleagues³⁸ using histochemical techniques and by Matsuo and colleagues³⁹ using degraded MBP immunohistochemistry. Our analyses of related axonal changes suggests that within the myelinated fiber bundles these changes are more targeted at oligodendroglia myelinating smaller rather than larger diameter axon fibers in MSA. Targeting of these axon fibers appears specific to MSA⁴⁶ and relates to increased ataxia.⁴⁷ However, it remains to be investigated whether the reduction in full-length MBP and its abnormal proteolysis in MSA (previously noted³⁹) result from an initial problem with MBP mRNA transport from the cell body destabilizing its protein associations in myelin or a direct loss of the stability of these associations because of a change in its interaction with p25 α in myelin or to some combination of these events.

α -Synuclein was present in ~50% of the MSA oligodendroglia sampled, which suggests the consistent overall oligodendroglial expansion and accumulation of p25 α preceded the α -synuclein accumulation. However, the significance of p25 α in the generation of the large amounts of aggregated α -synuclein present in MSA tissue¹⁹ is still unclear, despite p25 α possessing the potential to stimulate α -synuclein aggregation.²⁵ Our quantitative data suggest the earliest morphological change observed is the relocation of p25 α to the cell body and subsequent oligodendroglial soma expansion. There seems to be continued p25 α accumulation and somal expansion throughout time until after the accumulated α -synuclein has aggregated as revealed by the thioflavine S positivity of the GCIs. Hereafter a fraction of the GCI-containing cell bodies compact and lose their p25 α positivity. The presence of large amounts of p25 α in the GCI may explain the enigmatic lack of correlation between the number of GCIs in the tissue sections and the large amount of α -synuclein protein revealed by immunoblotting after extracting the tissue using denaturing buffers,¹⁹ as the abundant p25 α may have covered the α -synuclein epitopes in the GCI. How the physiological role of p25 α is disrupted to initiate these processes remains to be determined, but transgenic mouse models show that the accumulation of α -synuclein in oligodendroglia causes subsequent degeneration of both oligodendroglia and neurons.^{23,24} In these transgenic mouse models the expression of human α -synuclein in oligodendroglia leads to oligodendroglial pathology with aggregates of human α -synuclein and neuronal degeneration displaying aggregates of mouse α -synuclein.^{23,24} It will be important to understand further the earlier pathologi-

cal changes involving p25 α noted in the present study to be able to develop better models of the early pathogenic events and provide potentially preventative therapeutic strategies for this type of degeneration.

The abnormal localization of p25 α in neurons has also been described in MSA in both the absence and presence of abnormal α -synuclein deposition.²⁰ The extent of this abnormal neuronal localization of p25 α mirrors to some degree the abnormal accumulation of the neuron-specific α -synuclein deposits observed within MSA oligodendroglia, both concentrating in basal ganglia and cerebellar pathways.¹⁹ The present study does not address the mechanism(s) for these ectopic accumulations of either p25 α in neurons or α -synuclein in oligodendroglia. However, there is no evidence for an increase in mRNA expression of either of these proteins in MSA pons, putamen, or cerebellum,^{48,49} and our data show no increase in the relative protein amount of p25 α in MSA. The possibility of a direct transfer of these proteins in pathological brain tissue may be considered.

In conclusion, p25 α is a normal constituent of myelin and a high-affinity ligand for MBP. In MSA there is relocation of p25 α from the myelin to expanding oligodendroglial cell bodies in parallel with a reduction in full-length MBP and demyelination of smaller caliber axons in corticospinal and pontocerebellar pathways. The expansion of the oligodendroglial cell bodies continues in those cells that also accumulate α -synuclein and degraded MBP, with the subsequent development of the fibrillar form of α -synuclein. We have thus described novel early MSA-specific oligodendroglial changes affecting the p25 α protein before the development of the current disease-specific hallmark, the α -synuclein-containing GCIs.

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