Published in final edited form as:

Neurobiol Aging. 2014 October ; 35(10): 2357–2368. doi:10.1016/j.neurobiolaging.2014.02.028.

α -Synuclein impairs oligodendrocyte progenitor maturation in multiple system atrophy

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1. Introduction

Multiple system atrophy (MSA) is an age-related neurodegenerative synucleinopathy characterized by fast progression and severe disability (Wenning and Colosimo, 2010). Distinct MSA subtypes predominantly present besides parkinsonism (MSA-P) or cerebellar ataxia (MSA-C) with autonomic failure and poor levodopa responsiveness. The MSA related neuropathological hallmark are glial cytoplasmic inclusions (GCI) in oligodendrocytes frequently observed in the cortex, the corpus callosum, and the striatum (Papp, et al., 1989) correlating with severe neurodegenerative changes such as axonal degeneration, severe myelin loss, and gliosis (Ahmed, et al., 2012b, Ozawa, et al., 2004). The most prominent constituent of GCIs is alpha-synuclein (α -syn), a presynaptic protein involved in vesicular and synaptic transport of neurons, but its origin and function in oligodendrocytes still remain elusive (Nemani, et al., 2010, Tofaris and Spillantini, 2007). More recently, studies indicate that extracellular α -syn may be taken up by neurons, astrocytes, microglia, and oligodendroglia (Hansen, et al., 2011, Kisos, et al., 2012, Kordower, et al., 2011, Lee, et al., 2008, Lee, et al., 2010). Importantly, numerous studies

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The authors declare no competing financial interest.

Disclosure Statement: There are no actual or potential conflicts of interest.

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suggest that synucleinopathies interfere with neuronal plasticity in the human adult central nervous system (CNS) such as neurogenic regions, like the hippocampus and the subventricular zone/olfactory bulb (Marxreiter, et al., 2012, Winner, et al., 2011). However, much less is known about the influence of α -syn on glial plasticity.

Oligodendrogenesis occurs during development and is maintained throughout adulthood (Dawson, et al., 2000, Miller, 2002). An oligodendroglial precursor cell (OPC) population is present throughout the grey and white matter (Richardson, et al., 2011) and comprise approximately 5 % of all cells within the adult CNS. These cells are characterized by expression of the chondroitin sulfate proteoglycan NG2 (Trotter, et al., 2010) and coexpress platelet derived growth factor receptor α (PDGFR α ; Rivers, et al., 2008). Upon further differentiation to mature oligodendrocytes, PDGFR α expression is continuously downregulated, paralleled by increased expression of mature oligodendrocytic proteins, e.g. proteolipid protein (PLP) and myelin basic protein (MBP; Fancy, et al., 2011), recapitulating developmental signaling pathways during adult oligodendrogenesis and remyelination. However, very little is known on the effect of α -syn on oligodendrogenesis and remyelination under pathological conditions. As MSA is characterized by widespread myelin loss, we hypothesized that α -syn interferes with adult oligodendrogenesis preventing OPCs from remyelination and therefore contributing to MSA pathology. Indeed, in *post-mortem* analyses we detected an increase in the number of PDGFR α ⁺ OPC in the striatum of MSA patients suggesting a maturation deficit of these cells. Supporting our hypothesis, mice overexpressing human wild-type α -syn under the control of the MBP promoter exhibit increased striatal OPC numbers while numbers of mature oligodendrocytes remain unaffected. A delayed expression pattern of stage-specific transcription factors accompanied by downregulation of MBP expression in differentiated CG4 cells (central glia 4, a permanent oligodendroglial cell line) stably expressing human wild-type α -syn also point towards an interference of intracellular α -syn with maturation of OPCs. Taken together, our data suggest that α -syn impairs maturation of OPCs thereby contributing to MSA pathology.

2. Material and Methods

2.1. Human specimen

A total of 12 human brains (n = 6 MSA-P cases; n = 6 controls) were analyzed for the present study (Table 1). Autopsies were performed within 24 h *post mortem*. Brains were fixed with 4 % paraformaldehyde and dissected according standard procedure as described previously (Winner, et al., 2012). Basal ganglia were processed in 40 μ m sections on a vibratome for subsequent immunohistochemical analysis.

2.2. Animals and experimental design

Transgenic (tg) mice overexpressing human wild type α -syn under the control of the MBP promoter were compared to age matched littermate controls. The generation of mice was previously described (Shults, et al., 2005); MBP expresser line 1 was used for the present study. Animals were housed in a 12 h light/12 h dark cycle and had free access to nutrients. Body weight was monitored daily. Experiments for this study were performed for two animal groups with mixed gender, aged either 5 or 9 months at the time of perfusion (n =

5-8). To detect dividing cells, 5'-bromo-2'-deoxyuridine (BrdU; 50 mg/kg) was intraperitoneally injected twice a day for three consecutive days. Three weeks later, animals were perfused transcardially with 4 % paraformaldehyde (PFA; Sigma) in 100 mM phosphate buffer (PB), pH 7.4, brains were dissected and postfixed in 4 % PFA in 100mM PB for 48 hours. Following NIH guidelines for the humane treatment of animals, mice were sacrificed under anesthesia. For longer storage, brains were transferred into a 30 % sucrose/PBS solution. Brains were cut into 40 μ m sections on a sliding microtome (Leica), sections were stored in cryoprotectant (ethylene glycol, glycerol and PB, pH 7.4, 1:1:2 by volume) at -20 °C until further processing.

2.3. Staining procedures

Primary antibodies—Stainings on tissue were performed with the following primary antibodies: rat monoclonal anti-BrdU (1:500; AbD Serotec), mouse monoclonal anti-neuron-specific nuclear protein (NeuN; 1:100; Chemicon), rat monoclonal anti-human α -synuclein 15G7 (1:10; Axxora GmbH), rabbit anti-PDGFR α (c-20; 1:100; Santa Cruz Biotechnology), mouse monoclonal anti-glutathione-S-transferase π (GST π ; 1:500 for fluorescent stainings, 1:1000 for DAB-stainings; BD Transduction Laboratories), rabbit polyclonal anti-galactocerebroside (GalC; 1:100; AB 142; Chemicon), and mouse monoclonal anti α -synuclein 211 for human samples (1:250; MA1-12874, Thermo Scientific). For immunocytochemistry monoclonal mouse anti-MBP (1:250; MCA184S; AbD Serotec), rabbit anti-PDGFR α (c-20; 1:100; Santa Cruz Biotechnology), rat monoclonal anti-human α -synuclein 15G7 (1:250; Axxora GmbH), and rabbit monoclonal anti-GFP (E385; 1:500; Abcam) were used and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:10.000; Sigma).

Secondary antibodies—For immunohistochemistry, donkey anti-mouse and anti-rat biotinylated secondary antibodies were used 1:1000, as well as the avidin-biotin-peroxidase complex, 1:100 (Vectastain Elite, Vector Laboratories).

For immunofluorescence, donkey-anti-mouse antibodies were used coupled to Alexa 568 or Cy5 (both 1:1000), donkey-anti-rat coupled to Alexa 488 (1:1000) or Rhodamine Red-X (1:500), and donkey-anti-rabbit coupled to Alexa 488, Alexa 568, Cy5 or biotin (all 1:1000). Alexa-coupled antibodies were purchased from Invitrogen, Life Technologies, Cy5-, Rhodamine Red-X- and biotin-coupled antibodies from Dianova, respectively.

Immunohistochemistry—Free-floating sections were incubated in 0.6 % H₂O₂ in Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 min. Afterwards blocking was performed in TBS/0.25% Triton-X100/3% normal donkey serum for 30 min followed by incubation with primary antibodies in blocking solution over night at 4 °C. Biotinylated secondary antibody incubation was performed for 1 h in blocking solution. After rinsing in TBS, avidin-biotin-peroxidase complex was added for 1 h, followed by peroxidase detection for 10 min (25 mg/ml diaminobenzidine, 0.01% H₂O₂, 0.04 % NiCl in TBS). The following denaturation procedure was performed for BrdU staining prior to the primary antibody: 30 min incubation in 2 M HCl at 37 °C; and 10 min rinse in 0.1 M boric

acid, pH 8.5. Sections were mounted on glass slides, submerged in NeoClear and coverslipped with NeoMount (both from Merck).

Immunofluorescence—Free-floating sections were treated to denature DNA as described above. Consecutively, a combination of primary antibodies was applied in TBS-donkey serum for 24 h at 4°C. Rinsing in TBS was followed by secondary antibody incubation for 1 h in darkness. After final washing steps, sections were mounted on glass slides and coverslipped in Mowiol (Carl Roth). The tyramide signal amplification kit (TSA™ Biotin System; Perkin Elmer) was used for PDGFR α stainings: incubation of 4 μ l biotinylated tyramide reagent per ml amplification diluent for 10 min at room temperature was performed after addition of avidin-biotin-peroxidase complex. Fluorescently labeled streptavidin (1:250; Alexa 488, invitrogen, Life Technologies) was consecutively incubated for 30 min at room temperature.

Immunocytochemistry—Cells were fixed with PFA for 10 min, and stained as described for immunofluorescence.

2.4. Counting procedures

All counting procedures were performed on 40 μ m thick coronal sections. Regions were analyzed at the following coordinates: Interaural 4.98 mm to 4.06 mm and Bregma from 1.18 mm to 0.26 mm (according to Franklin & Paxinos, The mouse brain in stereotaxic coordinates, 3rd Edition, 2008). Three different regions of the forebrain were analysed: the motor cortex M2 together with the cingulate cortex Cg1 and Cg2 (termed “cortex” throughout this study, Fig. 2), the corpus callosum, and the striatum. BrdU⁺ cells were exhaustively counted in all regions. For double labeled cells at least 20 BrdU⁺ cells in the striatum and the cortex were analysed for their coexpression of PDGFR α using a confocal microscope (LSM 780, Zeiss). Scanning was performed through all confocal planes. Analysis of GST π ⁺ cells was performed using a systematic unified random counting procedure similar to the optical dissector (Gundersen, et al., 1988) with a semi-automatic stereology system (Stereoinvestigator, MicroBright-Field) using Zeiss objectives (Plan-APOCHROMAT 40 $\times$ /0,95 Korr). Positive cells touching the uppermost focal plane (exclusion plane) or the lateral exclusion boundaries of a counting frame were not counted. Every sixth section in the defined regions was analyzed. For the cortex the following parameters were used: counting frame size: 50 μ m $\times$ 50 μ m; sampling grid size: 250 μ m $\times$ 250 μ m. For the striatum the counting frame measured 75 μ m $\times$ 75 μ m and the sampling grid size 350 μ m $\times$ 350 μ m. The GST π ⁺ cells of the corpus callosum were counted exhaustively caudal to the predefined cortex. The area was measured using Stereoinvestigator software and GST π ⁺ cells are given per area.

For GalC expression, brightness measurements of 5 images within the predefined corpus callosum region were taken of each individual animal (Plan-APOCHROMAT 40 $\times$ /0,95 Korr). The corpus callosum was outlined manually and luminescence information was collected (both using Stereoinvestigator software).

2.5. Plasmid construction

The pT2/HB vector was used for the Sleeping Beauty transposon-based expression vector generation (Addgene, plasmid # 26557). To insert the sequence of human wild-type α -syn under the CMV promoter into the pT2/HB plasmid, we expanded the original multiple cloning site within the backbone vector pT2/HB. The following oligonucleotides were used: MluI/BstBI blunt f: TTTCTAGAACGCGTTTCGAAGCGGCCGCTATA and MluI/BstBI blunt r: TATAGCGGCCGCTTCGAAACGCGTTCTAGAAA to insert the restriction sites for MluI and BstBI in between the restriction sites of XbaI and NotI. 20 pmol of each oligonucleotide were annealed at 95 °C for 2 min before cutting them with XbaI and NotI (both NEB). The backbone vector pT2/HB was identically restricted, purified (Wizard SV Gel & PCR Clean Up System; Promega), and treated with Shrimp Alkaline Phosphatase (SAP; Promega) to avoid relegation. Restricted vector and oligonucleotides were ligated using T4 ligase (Promega). This new plasmid called pT2/HBMB was transformed, and sequencing revealed the predicted order of the new multiple cloning site. The constructs for human wild-type α -syn containing a Neomycin resistance gene for selection purposes (Klucken, et al., 2012) and pT2/HBMB was cut with BstBI and MluI, fragments with the expected size were cut and purified from an agarose gel, SAP treated, and ligated with T4 ligase. Control digestions and sequencing were consistent with the proposed insertion of the fragment leading to the new vector pT2-WTS.

2.6. Cell Culture

The rodent oligodendroglial cell line CG4 was cultured under mycoplasma free conditions (regularly tested with MycoAlert™ Mycoplasma Detection kit, Lonza). Cells were cultured in poly-L-ornithine coated flasks and grown in DMEM (PAN-Biotech) containing 4.5 g/l glucose and stable glutamine supplemented with N2 (Gibco), biotin (10 ng/ml), basic fibroblast growth factor (bFGF; 10 ng/ml), and platelet derived growth factor (PDGF; 10 ng/ml). CG4_wt- α -syn were cultured in the presence of 200 μ g/ml geneticin. Differentiation of CG4, CG4_venus, and CG4_wt- α -syn cells was achieved by withdrawal of growth factors and addition of triiodothyronine (T3; 30 ng/ml; Sigma-Aldrich) and low dose of fetal calf serum (FCS; 0,5%) for 6 days. In order to induce differentiation, BDNF (50 ng/ μ l; PeproTech) was added at day 1 of differentiation or daily during the 6 days differentiation paradigm.

2.7. Stable transfection of CG4 cells

To generate a CG4 cell line stably expressing human wild-type α -syn or the fluorescent control protein Venus the plasmids pT2-WTS or pT2-Venus (obtained from Dr. S. Iszvak, Max Delbrück Center for Molecular Medicine, Berlin, Germany), respectively, were cotransfected with the transposase containing plasmid pCMV(CAT)T7-SB100X using Attractene Transfection reagent (Qiagen). CG4_wt- α -syn cells were selected using geneticin. CG4_venus cells were sorted twice by fluorescence-activated cell sorting reaching a purity of more than 95%.

2.8. RNA extraction and quantification by real time-PCR

Total RNA was extracted from MBP tg and non-tg mice (n = 3 per group) using the RNeasy Lipid tissue kit (Qiagen) according to manufacturer's instructions. RNA from CG4 cell lines was isolated using RNeasy Mini Kit (Qiagen). RNA quantification was determined using Nanodrop (PqLab). For cDNA synthesis, 1 µg total RNA was reverse transcribed using GoScript™ Reverse Transcription System (Promega). Real time-PCR experiments were performed using the LightCycler480 (Roche). Amplification was performed on a cDNA amount equivalent to 62,5 ng total RNA with SsoFast EvaGreen Supermix (BioRad) containing dNTPs, MgCl₂, Taq DNA polymerase, and forward and reverse primers. The PCR cycling parameters was initiated with 95 °C for 44 s, and 45 cycles were run with primer specific conditions given in table 2. After each cycle fluorescence was measured at 73 °C for 3 s. The amount of studied cDNA in each sample was calculated relatively by the 2^{-Cp} method and expressed using mouse 18S rRNA as an internal control.

2.9. Western Blot analysis

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) cell lysates of CG4, CG4_venus, and CG4_wt-α-syn cells (n = 3 each) were extracted using RIPA buffer and 15 µg of proteins were loaded on a 4-12 % Novex® NuPAGE Bis-Tris-Mini gels (Invitrogen) and afterwards transferred on a polyvinylidene fluoride membrane (Immobilon PVDF-FL, 0.45 µm; Millipore). Membranes were blocked (PBS containing 0.1 % Tween-20 and 1 % BSA) for 1 h at RT and incubated in primary antibodies diluted in 0.1 % sodium azide, 0.1 % Tween 20, and 1 % BSA in PBS overnight at 4°C: monoclonal rat anti-MBP (1:200; MCA409S; AbD Serotec), monoclonal mouse anti-GAPDH (1:100.000; clone GB-69; Sigma), and monoclonal anti-CNPase (1:1000; clone 11-5B; Millipore) were detected with respective secondary antibodies diluted in blocking buffer (1 h at RT): donkey anti-mouse 488 (1:3000), donkey anti-rat 488 (1:3000), and donkey anti-mouse 647 (1:1000; all Invitrogen). Fluorescent signals were captured using a Fusion FX7 detection system (PqLab). Results were quantified using Bio1D software (Vilber Lourmat) and normalized to GAPDH.

2.10. Statistical analysis

Experimental groups were compared using Student's t-test (Prism 5; GraphPad Software). Data are presented as mean ± SEM. Asterisks indicate the following significance level: ***: p < 0.001; **: 0.01 < p < 0.05; *: 0.001 < p < 0.01; not significant (ns): p > 0.05.

3. Results

3.1. Numbers of OPCs are increased in the striatum of MSA-P patients

The presence of α-syn within OPCs *in vivo* was not yet demonstrated, although the uptake of α-syn by OPCs was recently shown *in vitro* (Kisos, et al., 2012). Therefore, we first asked whether α-syn is present in PDGFRα⁺ OPCs of MSA-patients. Indeed, a small fraction of striatal OPCs (5 - 8%) in MSA-P cases showed α-syn immunoreactivity which was absent in control subjects (Fig. 1A). Furthermore, we quantified PDGFRα⁺ cells within this region and determined a significant increase in OPCs in MSA-P patients compared to controls (Fig. 1B). Interestingly, numbers of PDGFRα⁺ OPCs increase with age in both MSA-P patients

and controls (Fig. 1C). Moreover, by performing an univariate analysis of co-variation (ANCOVA), the diagnosis contributes significantly to the observed difference in PDGFR α ⁺ cell number (F-value = 23.0, $p < 0.01$) whereas the age does not (F = 2.9, $p > 0.05$).

3.2. Mature oligodendrocytes in MBP tg mice are dysfunctional

Increased striatal OPC numbers in MSA-P patients prompted us to investigate this cell population in the MSA mouse model expressing human wild-type α -syn under the control of the MBP promoter (Shults, et al., 2005). First, we asked whether mature oligodendrocytes in the MBP tg mice are dysfunctional. To confirm α -syn expression oligodendrocytes, we adopted a coronal section of the mouse brain (Franklin and Paxinos, 2008) to delineate the cortex, the corpus callosum, and the striatum (Fig. 2A). The right panel of figure 2A depicts α -syn expression of a representative coronal section in an MBP tg animal. In all regions analyzed, numerous cell bodies and processes stained for α -syn. Interestingly, α -syn is predominantly present within the cytoplasm of GST π ⁺ mature oligodendrocytes. The expression of α -syn is sometimes more intensely accumulated in close proximity of both cellular poles (see arrows in Fig. 2B and arrowheads in Fig. 2C). Within the corpus callosum (Fig. 2C in between dashed lines) numerous cells show cytoplasmic α -syn⁺ expression sparing the nucleus, whereas in the cortex and the striatum α -syn expression is present in both, the nucleus and the cytoplasm. The white matter regions within the striatum are well defined by their pronounced α -syn⁺ processes (dashed ellipse in Fig. 2D). Almost all of the α -syn⁺ cells co-express GST π (Fig. 2B''-D'') representing mature oligodendrocytes in all regions analyzed (see asterisks and merged images in Fig. 2B''-D''). GST π is specifically expressed in mature oligodendrocytes where it catalyzes detoxifying reactions to reduce reactive oxygen species (Hayes and Pulford, 1995, Tamura, et al., 2007, Tansey and Cammer, 1991). As the loss of myelin in white matter regions is one of the most important characteristics in *post-mortem* tissue of MSA patients and its models, we evaluated the number and functionality of mature oligodendrocytes in the MBP tg animals. Therefore, we quantified GST π ⁺ cells and examined expression intensity of GalC, a major component of myelin, in the corpus callosum (Fig. 3). Whereas the number of GST π ⁺ oligodendrocytes in the corpus callosum is not altered (Fig. 3A-C), GalC expression is significantly reduced in the corpus callosum of MBP animals compared to controls (Fig. 3F). Furthermore, myelin is disposed in patchy structures less present in MBP tg animals (Fig. 3D, E). These myelin abnormalities paralleled by persisting numbers of mature oligodendrocytes suggest a functional deficit of oligodendrocytes in MBP tg animals.

3.3. Numbers of new born OPCs are increased in MBP tg mice

The presence of dysfunctional mature oligodendrocytes suggests that they may not be replaced by maturing OPCs. We hypothesized that this failure is due to an α -syn mediated deficit of OPC maturation. Thus, we asked whether the number of surviving new born OPCs is altered in different cerebral regions of MBP tg mice with a high α -syn load, i.e. the corpus callosum, the striatum, and the cortex. Using BrdU injections with consecutive immunohistochemical analyses three weeks later, we labeled recently divided and surviving cells (Fig. 4). The number of BrdU⁺ cells in 5 month old animals was not significantly different neither in the cortex and striatum (Fig. 4A) nor in the corpus callosum (data not shown) between MBP tg and control animals. However, at the age of 9 month, we observed

significantly more newly generated BrdU⁺ cells in the cortex and the striatum of tg animals compared to controls (Fig. 4B). BrdU⁺ cells were frequently observed as pairs in the cortex and the striatum (Fig. 4). No significant changes were observed in the corpus callosum (data not shown).

Approximately 50 % of the BrdU⁺ cells in the striatum and the cortex are coexpressing the immature oligodendroglial marker PDGFR α (Fig. 5A'''). Again, numerous of double positive cells appear in pairs suggesting to be derived from one precursor cell (Fig. 5A-A'''). We did not find a significant proportional difference in BrdU⁺/PDGFR α ⁺ cells between MBP tg and control animals (Fig. 5B, D). Next, we calculated the number of newly generated immature oligodendrocytes (BrdU $\times$ % PDGFR α) analogous to standard approaches used for calculation of neurogenesis (Kohl, et al., 2012) and observed a significantly increased number of PDGFR α ⁺ OPCs in the striatum of MBP tg animals (Fig. 5E), but not in the cortex (Fig. 5C). BrdU colabeling was not observed with GST π ⁺ mature oligodendrocytes (arrowhead in Fig. 5A''). To evaluate whether the increased numbers of new born OPCs in the striatum also result in more mature oligodendrocytes within this region, we quantified striatal GST π ⁺ cells. We did not find a significant difference between tg and control animals (Fig. 5H) suggesting an impaired maturation of newly generated oligodendrocytes progenitors.

3.4. Intracellular α -syn impairs OPC maturation in vitro

To validate and dissect the interference of α -syn with OPC maturation we generated the oligodendroglial cell line CG4 stably expressing human wild-type α -syn or the yellow fluorescent protein (YFP) derivative Venus. The cytoarchitecture of undifferentiated and differentiated CG4, CG4_venus, and CG4_wt- α -syn cells is presented using PDGFR α and MBP (Fig. 6A). It is important to note, that human wild-type α -syn is expressed in CG4_wt- α -syn cells, only. In a higher magnification, CG4 and CG4_venus cells show much more delicate branches after differentiation compared to the shorter and thickened branches observed in CG4_wt- α -syn cells (Fig. 6A, lower row). The number of MBP⁺ cells, a late stage oligodendrocyte specific differentiation marker, was markedly reduced in CG4_wt- α -syn cells compared to CG4_venus (Fig. 6B), but not between CG4 and CG4_venus cells (data not shown, $p = 0.98$).

Furthermore, protein levels of MBP are significantly reduced in CG4_wt- α -syn cells compared to CG4_venus (Fig. 6C, D). However, the reduction in the expression of CNPase does not reach significance indicating that the potential to obtain a mature oligodendrocytic phenotype may be maintained (Fig. 6C, D). No significant differences were observed between CG4 and CG4_venus cells for protein levels of CNPase ($p = 0.71$) and MBP ($p = 0.53$), excluding an effect of transgenesis itself. Matching the observation of fewer differentiated MBP⁺ cells present in the immunocytochemistry staining of CG4_wt- α -syn cells (Fig. 6B) and their reduced MBP protein levels (Fig. 6D), we also detected significantly reduced levels of MBP mRNA (Fig. 6E). To further unravel the effect α -syn exerts on transcription factors regulating oligodendroglial maturation, we analyzed transcription factors upstream of MBP. Hairy enhancer of split-5 (Hes5) as a repressor of myelin gene activation was significantly increased in CG4_wt- α -syn cells compared to

control cells (Fig. 6E). Transcription factor-4 (Tcf4) marks oligodendroglial cells in an intermediate stage of maturation and remains unaffected by the presence of human wild-type α -syn in CG4 cells (Fig. 6E). Furthermore, the myelin-gene regulatory factor (MYRF) is significantly reduced in CG4_wt- α -syn cells (Fig. 6E). No significant differences in mRNA levels were observed between the CG4 cell line and the control cell line CG4_venus for all genes analyzed (MBP: $p = 0.07$; Hes5: $p = 0.18$; Tcf4: $p = 0.75$; MYRF: $p = 0.17$). Taken together, the stage specific transcriptional profile indicates an impaired maturation of CG4_wt- α -syn cells.

3.5. BDNF partially rescues OPC maturation

Since reduced neurotrophic factor levels are observed in MSA and their models (Ubhi, et al., 2012, Ubhi, et al., 2010), we determined BDNF mRNA levels to gain more insight in possible changes of trophic factors involved in MBP tg mice. BDNF mRNA is significantly reduced in the striatum of MBP tg animals (Fig. 7A). Matching this *in vivo* finding α -syn leads to significantly reduced BDNF mRNA-levels in CG4_wt- α -syn cells compared to CG4_venus cells (Fig. 7B). Again, transgenesis itself had no effect on BDNF expression level ($p = 0.59$).

Thus, in order to rescue impaired maturation of oligodendrocytes induced by human wild-type α -syn we applied BDNF *in vitro*. Albeit not observing a pro-differentiating effect for CG4_venus cells, the elevated level of Hes5 mRNA induced by wild-type α -syn (Fig. 6E) was significantly reduced by single or daily administration of BDNF in CG4_wt- α -syn cells (Fig. 7C). The mRNA levels of MYRF and MBP, however, were unaffected by BDNF in both CG4_venus and CG4_wt- α -syn cells (Fig. 7C) suggesting that BDNF may act on early stages of oligodendrocytic maturation of CG4_wt- α -syn cells, but lacks the potential to induce myelin gene expression.

4. Discussion

The effect of α -syn on the generation and maturation of oligodendroglia is not well understood in diseases such as MSA, an atypical, rapidly progressing parkinsonian disorder characterized by intraoligodendroglial α -syn bearing GCIs. In a *post mortem* analysis of MSA-P patients we detected a significant increase in PDGFR α ⁺ OPCs within the striatum. To further elucidate the effects of α -syn on oligodendrogenesis, we used MBP:wt- α -syn mice as a model for MSA showing numerous α -syn⁺ oligodendrocytes with GCIs and myelin loss within the corpus callosum. Yet, no loss of mature oligodendrocytes was observed within this region or the adjacent striatum. However, the number of newly generated cells (BrdU⁺) was increased in the striatum and the cortex of aged MBP tg animals (9 month) predominantly characterized as PDGFR α ⁺ OPCs. In the striatum, we found significantly elevated levels of newborn PDGFR α ⁺ OPCs paralleled by decreased striatal BDNF levels. These findings indicate a regional specific increased number of immature OPCs, and more importantly an impaired oligodendrogenesis in MSA. To better understand the underlying molecular mechanisms, we stably overexpressed human wild-type α -syn in the oligodendroglial cell line CG4. The maturation of CG4_wt- α -syn⁺ cells was halted in conjunction with increased levels of the oligodendroglial differentiation repressor Hes5 and severely reduced levels of the pro-myelinating factor MYRF as well as

the major myelin protein MBP. In order to promote differentiation in CG4_wt- α -syn cells, administration of BDNF was able to downregulate increased levels of Hes5, however, without inducing terminal maturation.

Tg mice expressing human wild-type α -syn under the control of the MBP promoter feature characteristic pathological α -syn associated hallmarks of MSA: GCIs. α -Syn⁺ immunofluorescence is frequently present within GST π ⁺ oligodendroglial cells confirming previous observations that α -syn⁺ cells are of oligodendroglial origin expressing also GalC (Shults, et al., 2005). Interestingly, the interfascicular GST π ⁺ mature oligodendrocytes are not altered by the expression of α -syn in white matter regions within the corpus callosum or the striatum, despite of the severe myelin loss observed within MSA patients and the MBP tg model (Ahmed, et al., 2012b, Shults, et al., 2005).

Various animal models mimic the clinical and neuropathological phenotype of MSA expressing human wild type α -syn under different mature glial promoters such as the proteolipid (Kahle, et al., 2002) or the 2, 3 -cyclic nucleotide 3-phosphodiesterase (CNP) promoter (Yazawa, et al., 2005) showing GCIs with a loss of oligodendrocytes and neurons in the spinal cord. The MSA model expressing human wild type α -syn under the promoter for myelin basic protein, however, showed a pronounced loss of dopaminergic fibers within the basal ganglia and of neocortical dendrites associated with a severe motor deficit (Shults, et al., 2005). Additionally, oligodendroglial α -syn accumulation was observed in the neocortex, basal ganglia, corpus callosum, cerebellum, and brain stem accompanied by astrogliosis and loss of myelin reproducing important neuropathological and functional aspects of MSA. Similar to the present study, the density of CNPase⁺ cells, a mature type of oligodendrocytes, was also not altered in the striatum of PLP tg α -syn mice (Stefanova, et al., 2005). In humans, no changes between control and MSA patients were reported for the mature oligodendrocytic PLP mRNA levels (Ozawa, et al., 2001). However, comprehensive analyses for mature oligodendrocytes are still missing in MSA. Recently, an increased density of NG2⁺ OPCs was detected in human cerebellar white matter regions in MSA (Ahmed, et al., 2012a), which is also in line with our observation of increased PDGFR α ⁺ OPCs in the striatum of MSA-P patients. However, these findings have to be confirmed in larger, better age-matched cohorts. In conjunction with these findings, we hypothesize that human wild-type α -syn interferes with the maturation of oligodendrocytes during adult oligodendrogenesis.

Furthermore, GCIs are observed throughout gray and white matter regions (Ahmed, et al., 2012b) without a distinct regional pattern for these inclusions in both MSA subtypes (Dickson, et al., 1999). Although others failed to detect α -syn in OPCs of MSA patients (Ahmed, et al., 2012a), α -syn was present in immature PDGFR α ⁺ OPCs within the striatum of MSA-P patients. This discrepancy might be attributable to the specificity of antibodies used for human α -syn, differences in tissue processing, and our selected patient cohort of MSA-P patients only. Furthermore, in contrast to our co-labeling approach using PDGFR α - and α -syn antibodies, Ahmed and colleagues used co-labeling with NG2. The presence of α -syn within OPCs might repress NG2 expression explaining the observed discrepancy between both studies. Additionally, *post mortem* studies indicate a correlation of GCI load with neuronal degeneration (Ozawa, et al., 2004, Papp and Lantos, 1994) for striatonigral,

olivopontocerebellar, and motor cortical areas. In MBP tg mice, dendritic and axonal alterations were severe in regions with an increased load of GCIs, e.g. corpus callosum, cortex, basal ganglia, and brainstem (Shults, et al., 2005).

Transcriptional networks regulating developmental oligodendrogenesis have extensively been examined (Emery, 2010, Nicolay, et al., 2007, Wegner, 2008). A recapitulation of developmental signaling pathways controls adult oligodendrogenesis and remyelination (Arnett, et al., 2004, Fancy, et al., 2009, John, et al., 2002, Patel and Klein, 2011). To study the impact of intracellular α -syn on oligodendrocytic differentiation, we used a permanent oligodendrocytic cell line expressing human wild-type α -syn. Consecutively, we analyzed stage-specific transcription factors encompassing early, late, and intermediate oligodendrocytic phenotypes.

Hes5 is an effector of the Notch pathway, well known to maintain OPCs and to repress oligodendrocytic differentiation (Liu, et al., 2006, Wang, et al., 1998). Moreover, Notch1 receptors and the downstream effector Hes5 are expressed in OPCs (Wang, et al., 1998). In the present *in vitro* MSA model, we observed significantly increased mRNA levels of Hes5 in CG4_wt- α -syn cells after 6 days of differentiation. Tcf4, an important mediator of the wingless/int (Wnt)/ β -catenin pathway, is intermediately expressed during oligodendrocytic differentiation (Ye, et al., 2009). The mRNA levels of this intermediate marker were not altered in CG4_wt- α -syn cells. Recently, MYRF, strongly expressed in mature oligodendrocytes throughout the CNS, is considered as central activator for myelination since loss of MYRF prevents expression of a broad range of myelin genes (Emery, et al., 2009). After differentiation, significantly reduced levels of MYRF mRNA were detected in CG4_wt- α -syn cells. Additionally, mRNA levels of the target gene of MYRF, MBP, were dramatically reduced, paralleled by decreased MBP protein levels. This expression pattern suggests that CG4_wt- α -syn are arrested in an earlier stage of differentiation again supporting our notion that α -syn interferes with the maturation of oligodendrocytes. Yet, CNPase, an earlier marker of oligodendrocyte differentiation is not affected, indicating that cells may still have the potential to further obtain a mature oligodendroglial phenotype. Thus, accumulation of α -syn in OPCs results in downregulation of myelin associated genes possibly explaining the reduced remyelination in MSA (Wakabayashi and Takahashi, 2006).

Impaired oligodendrocytic differentiation was also observed in multiple sclerosis (MS) as another primary oligodendropathy (Kuhlmann, et al., 2008). The Notch1 ligand Jagged1 is highly expressed in non-myelinating lesions of MS, however, it is almost absent in remyelinated lesions. Thus, Notch1 signaling may interfere with further maturation of oligodendroglial precursors in chronic lesions of MS (John, et al., 2002). Additionally, Fancy and co-workers reported an upregulation of Tcf4 expression in oligodendrocytes precursors in MS lesions arguing for its importance during adult oligodendrogenesis (Fancy, et al., 2009).

The dual function of oligodendrocytes has been recently highlighted for white and grey matter regions (Jones, 2012). On one hand, mature oligodendrocytes form myelin to sheath axons thus facilitating rapid saltatory conduction, on the other hand, provide metabolic and trophic support to axons. BDNF is one neurotrophic factor promoting oligodendrocytic

differentiation and myelination (Du, et al., 2006a, Du, et al., 2003, Du, et al., 2006b, Xiao, et al., 2010). However, due to the cell cycle duration of NG2⁺ OPCs (Simon, et al., 2011), the present time period between BrdU labeling and phenotyping may be too short in order to observe newly generated mature GST π ⁺ cells.

As BDNF levels are reduced both *in vivo* and *in vitro*, we tested to promote differentiation in CG4_wt- α -syn cells by addition of BDNF either using a single or a daily dosing regimen. Its pro-myelinating effect was previously demonstrated in contused rat spinal cord, where an increased number of re-myelinated axons was observed upon BDNF injections (Lang, et al., 2008, McTigue, et al., 1998). Furthermore, fluoxetine, a selective serotonin reuptake inhibitor, ameliorated motor deficits in the MBP tg mice, and improved neurodegenerative pathology in the basal ganglia, neocortex and hippocampus in a BDNF dependant manner (Ubhi, et al., 2012). Although we did not observe a differentiation enhancing effect of BDNF on CG4_venus cells, BDNF is able to downregulate Hes5 in CG4_wt- α -syn cells, however without upregulation of MYRF and MBP. Thus, the sole administration of BDNF is not sufficient to overcome the α -syn induced impaired maturation in CG4_wt- α -syn cells.

In summary, we observed an impaired maturation of OPCs as a potential novel pathological mechanism involved in the course of MSA present in *post-mortem* tissue as well as in its *in vivo* and *in vitro* models. Increased PDGFR α ⁺ cells in the striatum of patients, reduced levels of myelin in the corpus callosum with unchanged numbers of mature oligodendrocytes, altered transcription factor profile as well as decreased MBP protein levels in CG4_wt- α -syn cells supported by reduced pro-differentiating and pro-myelinating BDNF levels point towards the interference of α -syn with oligodendrocyte progenitor maturation. Future studies may help further dissecting the underlying molecular mechanisms leading to better understanding of the rapidly progressing MSA pathogenesis. In particular, our study suggests that mediation of oligodendrocyte differentiation with consecutive remyelination may be a promising target for novel therapeutic approaches to interfere with the progression in MSA.

Acknowledgments

This study was supported by the Bavarian State Ministry of Sciences, Research, and the Arts, ForNeuroCell (V.M., J.W.; Erlangen, Germany), the German Ministry for Education and Science (BMBF grant 01GN0979), the Adalbert-Raps-Stiftung, "Parkinson and Nutrition", (01/2010-12/2013), the Interdisciplinary Center for Clinical Research (IZKF N3 and TP E9; The Role of glial cells in synucleinopathies_Wegner/Winkler). The authors would like to thank Drs. Z. Kohl, N. Ben Abdallah, and J. Klucken for scientific discussions.

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Figure 1. Increased numbers of striatal oligodendrocyte progenitor cells in MSA-P patients PDGFR α ⁺ OPCs in the striatum of MSA-P patients (upper panel) show accumulation of α -syn⁺ GCIs, whereas in control tissue (lower panel) no α -syn⁺ GCIs were detected (A). Quantification of PDGFR α ⁺ cells revealed a significant increase of total striatal OPCs in MSA-P (n = 6) compared to control patients (n = 6; B; p = 0.0215). The numbers of striatal PDGFR α ⁺ OPCs correlate in an age-dependent manner for MSA-P and control. Note the increased number of OPCs in MSA-P compared to controls (C). Scale bar: 20 μ m. Data represent mean $\pm$ SEM. Statistical analysis was performed using student's t-test. *: p < 0.05.

Figure 2. Regional expression pattern of α -syn in MBP tg animals

Coronal section of a 9 month old MBP tg animal adopted from Franklin & Paxinos is depicted as a topographical map (A); coordinates: Interaural: 4.98 mm, Bregma: 1.18 mm (Franklin and Paxinos, 2008). Regions analyzed within this study are marked and include the cortex (motor cortex M2, cingulate cortex Cg1 and Cg2), the corpus callosum (cc), and the striatum (caudate putamen, CPu). The right panel depicts an overview of human wild type α -syn expression in MBP tg mice. Regions marked with a rectangle in A termed B-D are shown at a higher magnification (B-D). Z-stacks were recorded and a mean intensity projection of each stack is depicted. α -syn is expressed in numerous cortical cells as well as in processes proceeding column-like within the cortex (B). In the corpus callosum, α -syn staining is predominantly observed in the cytoplasm of cells. Fibers are stained parallel to the direction of the corpus callosum (C). In the striatum, α -syn is present in cell bodies, both

within and in between striae (framed by a dashed line). A punctual α -syn expression is observed within the striae resembling orthogonally transected fibers (D). Accumulation of α -syn⁺ GCIs is observed within one (arrow in B) or two cellular poles (arrowhead in C). GST π staining is depicted in B''-D''. * exemplarily indicates α -syn⁺/GST π ⁺ cells. Merged pictures show colabeling of α -syn⁺ cells (red) with GST π ⁺ cells (green; B'''-D''') indicating α -syn accumulations within mature oligodendrocytes. Scale bars: 500 μ m (A), 20 μ m (B-D).

Figure 3. Myelin loss in MBP tg animals with preserved numbers of mature oligodendrocytes
 DAB-staining depicts GST π^+ mature oligodendrocytes in the corpus callosum of 9 months old control (A; n = 5) and MBP tg animals (B; n = 6). Cells were stereologically quantified revealing no differences between both groups (C; p = 0.36). Myelin is visualized using GalC fluorescent staining in the corpus callosum of control (D) and MBP tg animals (E). Intensity was significantly decreased in MBP tg animals measured as brightness per area (F; p = 0.03). Five images were analyzed per animal. Note the patchy GalC⁺ myelin structures in control animals that are less abundant in MBP tg animals. Data represent mean $\pm$ SEM. Scale bars: 50 μ m. Statistical analyses were performed using student's t-test. *: p < 0.05.

Figure 4. Age dependant survival of newly generated cells in the cortex and striatum of MBP tg animals

BrdU-DAB labeled cells of control and MBP tg animals at 9 month of age are depicted for both regions. The quantification of BrdU⁺ cells at 5 months (striatum $p = 0.96$; cortex $p = 0.96$) and at 9 months (striatum $p = 0.03$; cortex $p = 0.02$; $n = 5 - 8$) is given. A significant increase of BrdU⁺ cells is observed in the cortex and the striatum at 9 month of age in MBP tg animals. Note the pairs of BrdU⁺ cells in all regions analyzed (see also higher magnification). Data represent mean $\pm$ SEM. Scale bar: 50 μ m. Statistical analyses were performed using student's t-test. *: $p < 0.05$.

Figure 5. Increased newborn striatal OPCs in MBP tg animals

Mean intensity projection of a merged confocal image depicts double positive cells for BrdU (red; A) and PDGFRα (green, arrows; A') that do not colocalize with GSTπ (white, arrowhead; A'''). Quantification of BrdU cells colabeling for PDGFRα revealed no significant proportional differences in the cortex (B; $p = 0.4468$) or the striatum (D; $p = 0.2173$) of MBP animals ($n = 6$) compared to controls ($n = 5$). Newly generated immature oligodendrocytes (BrdU * %PDGFRα) of MBP tg animals were significantly increased in the striatum (E; $p = 0.0240$), but not in the cortex (C; $p = 0.1606$). In contrast, quantification of total GSTπ+ cells (H; $p = 0.4719$) in the striatum revealed no alterations between control (F) and MBP tg (G) animals. Data represent mean ± SEM. Scale bars: 10 μm (A); 50 μm (F, G). Statistical analyses were performed using student's t-test. *: $p < 0.05$.

Figure 6. Differentiation delay in CG4_wt-α-syn cells

The morphology of undifferentiated CG4, CG4_venus, and CG4_wt-α-syn cells is depicted using PDGFRα (red; upper row; scale bar: 20 μm) expression with dapi stained nuclei. α-syn (green) is solely present in the CG4_wt-α-syn cells. MBP expression (red; lower two rows) is observed after 6 days of differentiation (scale bar second row: 50 μm; third row: 20 μm). Note the typical branched and multiprocessed morphology of CG4 and CG4_venus (YFP in green; lower row, first and second panel) in contrast to shorter and thickened branches in α-syn expressing CG4s (α-syn in green; lower row, third and fourth panel). Quantification of MBP⁺ cells (n = 3) revealed significant less MBP⁺ oligodendrocytes in CG4_wt-α-syn cells compared to CG4_venus control cells (B; p = 0.0020). Decreased levels of MBP expression were confirmed using Western Blot (C; densitometry shown in D; p = 0.0045) and real time-PCR (E; p < 0.0001). Note that CNPase protein expression was unaffected (C, D; p = 0.1018). Real time-PCR analysis of stage specific oligodendrocytic transcription factors in CG4_wt-α-syn cell revealed significantly increased levels of Hes5 mRNA (p = 0.0157), unchanged levels of Tcf4 (p = 0.3563), and significantly reduced levels of MYRF (p = 0.0052) compared to CG4_venus. Changes in protein (normalized to GAPDH expression; n = 3) and mRNA levels (normalized to 18S rRNA expression; n = 5) of

CG4_wt- α -syn cells are presented as fold change relative to CG4_venus. Data represent mean $\pm$ SEM. Statistical analyses were performed using student's t-test. *: p $\leq$ 0.05, **: p $\leq$ 0.01, ***: p $\leq$ 0.001.

Figure 7. BDNF partially restores transcriptional delay in CG4_wt-α-syn cells

BDNF mRNA levels are significantly decreased both in the striatum of MBP tg animals (A; $n = 3$) and CG4_wt-α-syn cells prior differentiation (B; $n = 5$). To induce differentiation, BDNF (50 ng/μl; single dose prior differentiation (light grey) or six times during differentiation (dark grey)) was administered to CG4_venus or CG4_wt-α-syn cells, respectively. Real time-PCR analysis shows that BDNF administration reverses the upregulation of Hes5 expression in CG4_wt-α-syn cells without altering Hes5 expression in CG4_venus cells. Note unaltered expression levels of MYRF and MBP after BDNF administration for CG4_venus and CG4_wt-α-syn cells. (C; $n = 3$; single dose BDNF [CG4_venus]: Hes5 $p = 0.2816$, MYRF $p = 0.2282$, MBP $p = 0.4194$; single dose BDNF [CG4_wt-α-syn]: Hes5 $p = 0.0172$, MYRF $p = 0.5503$, MBP $p = 0.8113$; daily BDNF [CG4_venus]: Hes5 $p = 0.8485$, MYRF $p = 0.1488$, MBP $p = 0.2682$; daily BDNF [CG4_wt-α-syn]: Hes5 $p = 0.0181$, MYRF $p = 0.1554$, MBP $p = 0.4088$). Changes in mRNA levels (normalized to 18S rRNA expression) are presented as fold change relative to control signals. Data represent mean $\pm$ SEM. Statistical analyses were performed using student's t-test. *: $p < 0.05$, ***: $p < 0.001$.

Figure 8. Delayed oligodendrocytic differentiation induced by α -syn

Temporal transcription factor network during adult oligodendrocyte differentiation is depicted for CG4 cells. Alterations in transcription factors and surface markers induced by human wild-type α -syn are presented for CG4/CG4_venus (upper panel) and CG4_wt- α -syn cells (lower panel). Hes5 is strongly upregulated in α -syn expressing cells, whereas the promyelinating factor MYRF is significantly downregulated. MBP is severely reduced in CG4_wt- α -syn cells on mRNA and protein level. OPC oligodendrocyte precursor cell, OL oligodendrocyte, Hes5 hairy enhancer of split-5, Tcf4 transcription factor 4, MYRF myelin-gene regulatory factor, MBP myelin basic protein.

Table 1**Summary of clinicopathological characteristics of patients**

PMT: post mortem time. MMSE: mini-mental state examination. M: male. F: female. Statistical analysis was performed using student's t-test.

Diagnosis	n	Age [yrs]	PMT [h]	Gender [M/F]	Duration [yrs]	MMSE	Brain weight [g]
MSA-P	4	66.2 ± 3.2 *	13.3 ± 2.3	3/1	7.8 ± 4.9	23.6 ± 0.9 **	1425 ± 75.7
Control	4	80.8 ± 4.9	12.0 ± 1.5	1/3	NA	28.5 ± 0.7	1262 ± 83.9

* p = 0.0313;

** p = 0.0018.

Table 2
Primer pairs used for mRNA analysis

Forward and reverse primers used for real time-PCR analysis are listed. BDNF: brain derived neurotrophic factor; Hes5: hairy enhancer of split-5; MBP: myelin basic protein; MYRF: myelin-gene regulatory factor; Tcf4: transcription factor 4

	Forward Primer	Reverse Primer
BDNF	TCATACTTCGGTTGCATGAAGG	AGACCTCTCGAACCTGCCC
Hes5	GCGTCGGGACCGCATCAACA	GCGGCGAAGGCTTTGCTGTG
MBP	ACTACGGCTCCCTGCCCCAG	GGGATGGAGGGGGTGTACGAGG
MYRF	GGTGGTGGACGAGACCGAAGC	GCTCAGATGGTGGAGCCCCG
Tcf4	GCCCACCGTGAAGATGGCGT	GGCCTGGTGGCATCCCTCTGT
18S rRNA	GTGATGGGGATCGGGGATTG	GGCGGTGTGTACAAAGGGCAG