

α -Synuclein-induced myelination deficit defines a novel interventional target for multiple system atrophy

Benjamin Eittle¹ · Bilal E. Kerman^{2,8} · Elvira Valera³ · Clarissa Gillmann⁴ · Johannes C. M. Schlachetzki^{1,9} · Simone Reiprich⁵ · Christian Büttner⁶ · Arif B. Ekici⁶ · André Reis⁶ · Michael Wegner⁵ · Tobias Bäuerle⁴ · Markus J. Riemenschneider⁷ · Eliezer Masliah³ · Fred H. Gage² · Jürgen Winkler¹

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Abstract Multiple system atrophy (MSA) is a rare atypical parkinsonian disorder characterized by a rapidly progressing clinical course and at present without any efficient therapy. Neuropathologically, myelin loss and neurodegeneration are associated with α -synuclein accumulation in oligodendrocytes, but underlying pathomechanisms are poorly understood. Here, we analyzed the impact of oligodendrocytic α -synuclein on the formation of myelin sheaths to define a potential interventional target for MSA. Post-mortem analyses of MSA patients and controls were performed to quantify myelin and oligodendrocyte numbers. As pre-clinical models, we used transgenic MSA mice, a myelinating stem cell-derived oligodendrocyte-neuron co-culture, and primary oligodendrocytes to determine functional consequences of oligodendrocytic α -synuclein overexpression on myelination. We detected myelin loss accompanied by preserved or even increased numbers of oligodendrocytes in

post-mortem MSA brains or transgenic mouse forebrains, respectively, indicating an oligodendrocytic dysfunction in myelin formation. Corroborating this observation, overexpression of α -synuclein in primary and stem cell-derived oligodendrocytes severely impaired myelin formation, defining a novel α -synuclein-linked pathomechanism in MSA. We used the pro-myelinating activity of the muscarinic acetylcholine receptor antagonist benztropine to analyze the reversibility of the myelination deficit. Transcriptome profiling of primary pre-myelinating oligodendrocytes demonstrated that benztropine readjusts myelination-related processes such as cholesterol and membrane biogenesis, being compromised by oligodendrocytic α -synuclein. Additionally, benztropine restored the α -synuclein-induced myelination deficit of stem cell-derived oligodendrocytes. Strikingly, benztropine also ameliorated the myelin deficit in transgenic MSA mice, resulting in a prevention of neuronal cell loss. In conclusion, this study defines the α -synuclein-induced myelination deficit as a novel and crucial pathomechanism in

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✉ Jürgen Winkler
juergen.winkler@uk-erlangen.de

- ¹ Department of Molecular Neurology, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Schwabachanlage 6, 91054 Erlangen, Germany
- ² Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA, USA
- ³ Department of Neurosciences, University of California San Diego, La Jolla, CA, USA
- ⁴ Institute of Radiology, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany

- ⁵ Institute of Biochemistry, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
- ⁶ Institute of Human Genetics, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
- ⁷ Department of Neuropathology, Regensburg University Hospital, Regensburg, Germany
- ⁸ Present Address: Research Center for Regenerative and Restorative Medicine, Istanbul Medipol University, Istanbul, Turkey
- ⁹ Present Address: Department of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA, USA

MSA. Importantly, the reversible nature of this oligodendrocytic dysfunction opens a novel avenue for an intervention in MSA.

Keywords Multiple system atrophy · Oligodendrocytes · Oligodendrocyte progenitor cells · Myelin · α -Synuclein

Introduction

The atypical parkinsonian disorder multiple system atrophy (MSA) is a sporadic, rare, and age-related neurodegenerative disease with rapid progression. From onset of symptoms, the median survival is less than 10 years [32]. Importantly, there are currently no efficient symptomatic or disease-modifying therapies available [13]. MSA patients present with a heterogeneous combination of autonomic dysfunction, parkinsonism, cerebellar ataxia, and pyramidal features as a consequence of pronounced neurodegenerative changes. The predominance of degeneration within the striatonigral or olivopontocerebellar system classifies MSA in a parkinsonian (MSA-P) and a cerebellar (MSA-C) subtype [16]. In addition to widespread axonal and neuronal loss, major neuropathological features of MSA are myelin loss, micro- and astrogliosis [16, 35, 38]. Although MSA etiology is poorly understood, convergent lines of evidence indicate that oligodendrocytes, the myelinating cells of the central nervous system, may be causatively involved in MSA pathogenesis [15, 16, 47].

Myelin sheaths are generated throughout life by pre-existing or newly formed oligodendrocytes derived from adult oligodendrocyte progenitor cells (OPCs) [54]. Active myelination in the adult brain mediates a continuous myelin turnover [53] and ensures myelin remodeling required for learning processes [37], but also contributes to myelin repair upon demyelination under pathological conditions [22, 39]. Remarkably, proper remyelination is essentially required to prevent demyelination-associated axonal and neuronal degeneration [23]. In contrast, myelin loss without sufficient remyelination leads to a breakdown of oligodendrocyte-derived metabolic supply at the myelin-axon interface, which consequently results in axonal dysfunction and neuronal loss [21, 46, 51]. Hence and although not yet been considered, targeting myelin regeneration may represent a promising approach to slow or even halt disease progression in MSA.

The key neuropathological feature of MSA is the accumulation of α -synuclein in glial cytoplasmic inclusions within oligodendrocytes, which classifies MSA as synucleinopathy [15]. According to the current consensus guidelines [19], post-mortem detection of abundant α -synuclein-positive glial cytoplasmic inclusions is

required for definite MSA diagnosis. The association of genetic variants in the SNCA gene coding for α -synuclein points toward a crucial role of α -synuclein during MSA pathogenesis [43]. Furthermore, most recent studies have shown increased α -synuclein mRNA levels in MSA patients [4, 11], suggesting that pathological α -synuclein accumulation in MSA is the result of excessive α -synuclein gene expression. In line, transgenic mice overexpressing α -synuclein in oligodendrocytes recapitulate the most important neuropathological features of MSA, including myelin loss, neurodegeneration, and microgliosis [24, 45, 52]. Intriguingly, the level of α -synuclein overexpression corresponds to the severity of functional and neurodegenerative features [45]. In fact, transgenic mice with highest oligodendrocytic α -synuclein overexpression die prematurely at 4–6 months of age, resembling the rapidly progressing clinical course of MSA patients [45].

In addition to its pathological overexpression in oligodendrocytes of MSA patients, α -synuclein is physiologically expressed in murine and human OPCs and downregulated upon maturation toward pre-myelinating oligodendrocytes [8, 40]. In this context, we have recently shown that sustained α -synuclein expression in primary rat OPCs delays myelin gene expression [14].

Considering the increasing evidence for a physiological role of α -synuclein in oligodendrocyte maturation, we hypothesized that α -synuclein accumulation within oligodendrocytes impairs myelin maintenance and repair, potentially explaining myelin loss in early stages of MSA. While we detected profound myelin loss in the presence of widespread α -synuclein pathology in the putamen of MSA patients, oligodendrocyte numbers were surprisingly preserved. Corroborating this observation, overexpression of human α -synuclein in mice, in mouse embryonic stem cell-derived oligodendrocytes co-cultured with cortical neurons, and in primary rat oligodendrocytes severely impaired myelin formation. Intriguingly, the deficit in myelin formation linked to α -synuclein accumulation was restored in vitro by the muscarinic acetylcholine receptor antagonist benztropine, which has recently been identified from a high-throughput screen as a potent pro-myelinating small molecule [7]. In addition, benztropine attenuated the myelin deficit in transgenic mice overexpressing α -synuclein in oligodendrocytes, resulting in the prevention of neuronal loss, whereas microglial cell density was not altered.

Our data provide compelling evidence for α -synuclein-induced myelination deficit without oligodendrocyte loss in MSA. Given that the pro-myelinating intervention using benztropine was able to overcome the α -synuclein-induced oligodendrocytic dysfunction in vitro and in vivo, our study

provides a possible translation for a novel treatment of MSA.

Methods

Human post-mortem samples

Human post-mortem samples used in this study were obtained from the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam (open access: <http://www.brainbank.nl>). MSA was clinically and neuropathologically diagnosed according to current consensus guidelines [19]. Tissue blocks were cut into 5 μm sections and processed using standard protocols for hematoxylin and Luxol Fast Blue/Periodic Acid Schiff. α -Synuclein-positive inclusions were detected using a rabbit anti-human α -synuclein antibody (clone 5G4; Analytik Jena AG; #847-0102004001). Luxol Fast Blue intensity was quantified by a blinded researcher using ImageJ as mean gray value in at least 10 outlined striae crossing the putamen. Oligodendrocytic cell density was quantified by a blinded researcher using ImageJ in at least five outlined striae.

Animals and treatment

Mice overexpressing human α -synuclein under the control of a murine myelin basic protein (*Mbp*) promoter (high-expressing line 29; on a BDF1 background) were previously generated [45]. In contrast to moderately expressing lines (e.g., line 1), strongest α -synuclein overexpression in the forebrain of line 29 results in severe neurological deficits, such as tremor, ataxia, and pronounced seizure activity, and premature death at 4–6 months of age [45]. Mice were maintained under standard animal housing conditions with a 12-h-day/night cycle and free access to food and water. Mice (mixed gender) received daily intraperitoneal injections of vehicle or benzotropine mesylate (Alfa Aesar; in saline; 2 mg/kg) starting at the age of 8 weeks for 30 consecutive days. All experiments were carried out in accordance with the guidelines of the National Institutes of Health (NIH) in regard to the care and use of animals for experimental procedures. Mice were transcardially perfused with PBS and brains were collected. Brains were post-fixed in 4 % paraformaldehyde (in PBS) overnight and transferred into 30 % sucrose (in PBS).

Magnetic resonance imaging of mouse brains

Left hemispheres ($n = 3$ per group, females) were scanned on a pre-clinical ultra-high field magnetic resonance imaging system (7 Tesla ClinScan 70/30, Bruker). The imaging protocol consisted of a 3D T2-weighted turbo spin

echo sequence for morphological images and a 3D multi-echo gradient echo sequence was acquired to calculate voxel-based maps of T2* (Syngo software, Siemens). On T2-weighted images, the corpus callosum was segmented using Chimaera's segmentation tool (Chimaera). As previously described [27], T2* relaxation times were determined to quantify myelin. By transferring the segmentation mask to the T2*-map, T2* relaxation time of the corpus callosum was calculated.

Histology and electron microscopy

Left hemispheres ($n = 5$ per group, males) were cut on a sliding microtome into 40 μm coronal sections. For Luxol Fast Blue staining, one section per animal (at Bregma +1) was used to group all samples on a single glass slide, ensuring optimal comparability. Sections were stained in 0.1 % Luxol Fast Blue solution and destained using 0.05 % lithium carbonate and 70 % ethanol until white matter became apparent dark blue. Images were taken with equal exposure time using StereoInvestigator software (MicroBright-Field) on an Imager.M2 microscope (Zeiss). ImageJ was used to calculate the mean gray value of inverted images. Immunofluorescent analyses were performed as described previously [36] using following primary antibodies: rabbit anti-Olig2 (Millipore; AB9619; 1:500), goat anti-platelet-derived growth factor receptor- α (*Pdgfra*) (R&D Systems; AF1062; 1:250), mouse anti-glutathione-S-transferase- π (*Gst π*) (BD; 610718; 1:100), mouse anti-NeuN (Millipore; MAB377; 1:200), rabbit anti-Iba1 (WAKO; 019-19741; 1:1000), and rat anti-human α -synuclein (15G7, ENZO; ALX-804-258; 1:200). For quantification of cell density, 3 coronal sections per animal were analyzed. Three images per section were taken using ApoTome technology (Zeiss). The corpus callosum and the motor cortex (at Bregma -1 to $+1$) were outlined and quantified using ImageJ by a blinded researcher.

Ultrastructural analysis of myelin was performed as previously described [33]. Briefly, vibratome sections ($n = 6$) were post-fixed in 1 % glutaraldehyde, treated with osmium tetroxide, embedded in epon araldite and sectioned with an ultramicrotome (Leica). Electron micrographs were randomly obtained from three grids at a magnification of 5000 \times and 20,000 \times using a Zeiss OM 10 electron microscope. Myelinated axons and myelin layers were quantified in the corpus callosum in areas of similar axon size.

Lentiviral vectors

Third-generation lentiviral vectors coding for human α -synuclein and/or an internal ribosomal entry site (IRES) followed by the enhanced green fluorescent protein (Gfp) sequence were generated as previously described [14]. For

transducing myelinating co-cultures, the identical *Mbp* promoter [20] as used for transgene expression in MBP29 mice was cloned into the lentiviral vector using the restriction enzymes *Clal* and *AgeI*, resulting in the α -synuclein expression vector MBP-SYN-IRES-GFP (MBP-SYN-IG) and the control vector MBP-IRES-GFP (MBP-IG). For primary oligodendrocyte monocultures, an elongation factor 1- α promoter was used to constitutively drive transgene expression (α -synuclein expression vector: EF1a-SYN-IG; control vector: EF1a-IG) [14]. Lentiviral titers were determined by biological titration in HEK293T cells using flow cytometry.

Myelinating co-culture

The protocol to obtain a myelinating stem cell-derived co-culture has recently been described [25]. In this study, the functionality of stem cell-derived oligodendrocytes in terms of myelination has been confirmed by demonstrating the formation of physiological myelin on an ultrastructural level. Briefly, E14Tg2a mouse embryonic stem cells were differentiated into cortical neurons [17]. To obtain mature oligodendrocytes, neural progenitor cells were derived from mouse D3 embryonic stem cells as described elsewhere [34] and underwent a two-step differentiation/maturation protocol [25]. OPCs were differentiated from neural progenitor cells using differentiation medium (DMEM/F12-GlutaMax, N2, B27, 10 ng/ml insulin-like growth factor-1, 10 ng/ml Pdgf-aa) for 7 days before switching to maturation medium for another 7 days [DMEM/F12-GlutaMax, N2, B27, 10 ng/ml ciliary neurotrophic factor, 5 ng/ml neurotrophin-3, 40 ng/ml triiodothyronine (T3)]. Medium change was conducted every other day.

Twelve hours prior to starting the co-culture, oligodendrocytes were lentivirally transduced using MBP-SYN-IG or MBP-IG. Oligodendrocytes were detached using accutase and seeded on cortical neurons at a density of 50,000 per well. Additionally, oligodendrocytes were plated in poly-ornithine and laminin-coated 8-well chamber slides to control for specificity of lentiviral transduction. Co-cultures were maintained for 14 days in neuron medium (1:1 DDM and Neurobasal/B27, 20 ng/ml glial cell-derived neurotrophic factor, 500 μ g/ml 3'5'-cyclic adenosine monophosphate, 0.2 μ M ascorbic acid) supplemented with T3 at 40 ng/ml. Benzotropine was added at 1 μ M to assess its effects on myelination. Medium was changed every other day.

Immunocytochemistry

Myelinating co-cultures and D3 embryonic stem cell-derived oligodendrocytes were stained as recently

described [25]. The following primary antibodies were used in combination for overnight incubation: chicken anti-Gfp (Aves Labs, GFP-1020; 1:250), rat anti-Mbp (AbD Serotec; MCA409S; 1:50), mouse anti-beta-III-tubulin (Tuj1, Covance, MMS-435P; 1:1000), rat anti-human α -synuclein (15G7, ENZO; ALX-804-258; 1:200), rabbit anti-Pdgfra (Santa Cruz; sc-338; 1:400), rabbit anti-cleaved Caspase 3 (Asp175; Cell Signaling; #9661; 1:500). For O4 staining, the primary antibody (mouse anti-O4; MAB1326; R&D Systems; 1:40) was added to the supernatant 30 min prior to fixation and permeabilization. Images were acquired on a laser scanning microscope LSM710 (Zeiss) using ZEN black software.

Quantification of myelination in vitro

Myelination in the oligodendrocyte-neuron co-culture was semi-automatically quantified using the Computer-assisted Evaluation of Myelin formation (CEM) tool [25]. Myelination was assessed by analyzing individual oligodendrocytes. Upon lentiviral transduction, Gfp/Mbp-positive oligodendrocytes were randomly selected from at least 8 different wells per experiment and condition by a blinded researcher. Images were processed using ImageJ (generation of binary images; calculation of Mbp-positive, Tuj1-positive, and Mbp/Tuj1-positive pixels; pictures with less than 200,000 Tuj1 pixels were excluded) and MATLAB (MathWorks; cell body removal). Myelin pixels (Mbp/Tuj1-positive) relative to total Mbp-positive pixels were calculated and considered as myelination (in percentage) of an individual oligodendrocyte.

Primary OPC culture

Primary rat OPCs were isolated, maintained, and transduced as previously described [14]. Briefly, mixed glial cultures were obtained from P0 to P2 neonatal Wistar rats and kept in DMEM supplemented with 10 % FCS. After 14 days in vitro, OPCs were separated by overnight orbital shaking at 37 °C and plated on poly-ornithine coated plates or coverslips. OPCs were allowed to recover by culturing in SATO medium [6] supplemented with Pdgf-aa and fibroblast growth factor-2 at 10 ng/ml. Twenty-four hours after seeding, OPCs were lentivirally transduced at a multiplicity of infection of 2. Maturation was initiated 48 h after seeding by withdrawal of growth factors. To assess the effects of benzotropine on OPC maturation, benzotropine mesylate (Alfa Aesar) dissolved in deionized water was added at the initiation of maturation and compared to vehicle control. Medium was completely replaced after 2 days of maturation. Oligodendrocyte maturity and the transcriptome were analyzed after 4 days.

Western blot

Western blot analysis was performed according to standard protocols. The NuPage[®] gel electrophoresis system (Invitrogen) was used according to the manufacturer's protocol. Primary antibodies: rat anti-Mbp (Abd Serotec; MCA409S; 1:500) and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (Gapdh; Millipore; MAB374; 1:100,000).

Quantitative PCR (qPCR)

Total RNA of primary oligodendrocytes was extracted using the RNeasy mini kit according to the manufacturer's instructions (Qiagen). CDNA was generated using GoScript[™] Reverse Transcription System (Promega). qPCRs were performed on a Light Cycler 480 (Roche) using the Sso Fast EvaGreen Supermix (Biorad). Primers: *Mbp* (5'-ACTACGGCTCCCTGCCCCAG-3', 5'-GGGATGGAGGGGGTGTACGAGG-3'; detecting all five *Mbp* transcript variants in *Rattus norvegicus*), *Gapdh* (5'-CACAGTCAAGGCTGAGAATGGGAAG-3', 5'-GTGGTTCACACCCATCACAAACATG-3').

Transcriptome analysis

Before and during library preparation, RNA quality was ascertained using a 2100 Bioanalyzer system (Agilent Technologies). Barcoded strand-specific whole transcriptome sequencing libraries were prepared from 100 ng of DNase digested total RNA using Ovation Human FFPE RNA-Seq System (NuGEN) according to the manufacturer's instructions. Rat rRNA and tRNA were depleted using custom-designed oligonucleotides in strand selection. Pooled libraries were sequenced on a HiSeq 2500 platform (Illumina) generating on average 86 million 101 bp single-end reads.

After alignment against the *Rattus norvegicus* reference genome Rnor5.0 using STAR v.2.4.0i [9] absolute read counts for all Ensembl genes (version 75) were determined with HTSeq count v.0.6.1 [3]. Differential expression analysis accounting for the paired design with respect to primary cell cultures was performed using the DESeq 2 package v.1.6.3 [30]. Gene ontology (GO) term enrichment of differentially expressed genes against background of expressed genes after independent filtering was performed using the Gene Ontology Enrichment Analysis and Visualization (GORilla) tool [12]. *P* values were corrected using the false discovery rate method.

Statistics

Data were analyzed using GraphPad Prism[®]. Graphs are presented as mean \pm standard error of mean. One-way

analysis of variation (ANOVA) with post hoc Dunnett's multiple comparison test was used when comparing to control. Two-tailed, unpaired student's *t* test was performed for comparing two groups. *P* values <0.05 were considered significant.

Results

Myelin loss despite preserved oligodendrocyte numbers in MSA and in mice overexpressing α -synuclein in oligodendrocytes

While myelin loss has previously been reported as pathological feature present already in early stages of MSA [35, 38, 47], underlying causes for this loss have not yet been elucidated. To assess whether demyelination is a result of oligodendrocyte degeneration or rather oligodendrocytic dysfunction in terms of myelin formation, we quantified myelin and the density of oligodendrocytic cells in post-mortem tissue of MSA patients. Selection criteria for MSA patients were the parkinsonian phenotype (predominance of striatonigral degeneration, MSA-P) based on clinical history and the abundant presence of α -synuclein-positive inclusions within the putamen (Fig. 1; for details of the cohort see Suppl. Table T1). A combined Luxol Fast Blue/Periodic Acid Schiff staining was used to visualize myelin (Fig. 1a). Oligodendrocytes were identified by morphology in hematoxylin-stained sections according to Salvesen and colleagues [41] (Fig. 1a, b). While myelin was reduced by 30 ± 5 % in MSA patients relative to age- and gender-matched controls, no significant change in the density of oligodendrocytes was detected within fiber tracts crossing the putamen (Fig. 1c). To confirm this dichotomy, we compared myelin with oligodendrocyte density in mice overexpressing α -synuclein in oligodendrocytes under the control of a murine *Mbp* promoter (high-expressing line 29; hereafter referred to as MBP29). In this well-established pre-clinical MSA mouse model, we analyzed the corpus callosum, which represents a prototypical and—in contrast to the striatum—pure white matter region, showing most abundant α -synuclein expression in the forebrain and allowing a comprehensive quantification of myelin and oligodendrocytes (Suppl. Fig. S1a, b). Furthermore, mature oligodendrocytes strongly expressed α -synuclein in MBP29 mice, whereas OPCs did not show α -synuclein immunoreactivity (Suppl. Fig. S1c). In similarity to MSA patients, we observed a reduction of myelin by 44 ± 5 % accompanied by an 1.7-fold increased oligodendrocyte density in MBP29 mice compared to non-transgenic littermates (Fig. 2).

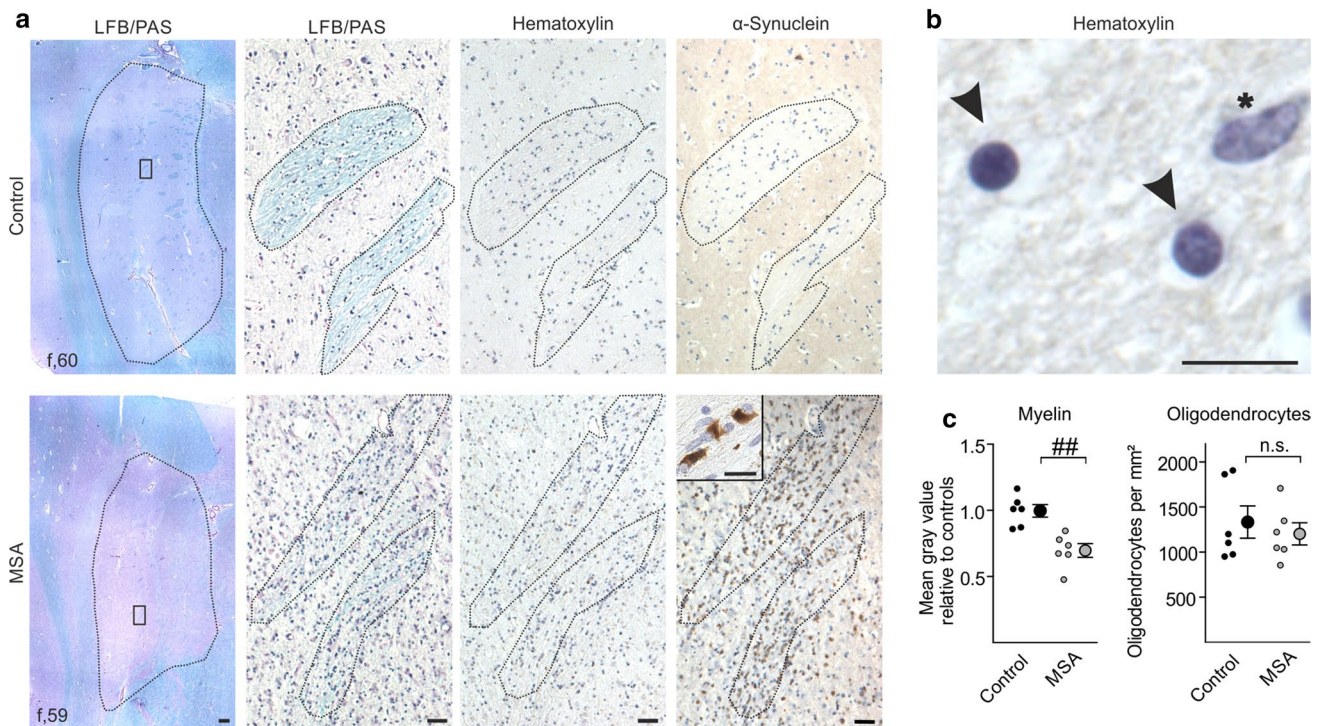


Fig. 1 Putaminal myelin loss in multiple system atrophy (MSA) without alterations in oligodendrocyte density. **a** Representative Luxol Fast Blue/Periodic Acid Schiff (LFB/PAS) stained images (left) depict the putamen (encircled) of a control (upper panel) and a MSA patient (lower panel). The putamen of the MSA patient showed a reduced Luxol Fast Blue staining intensity reflecting severe loss of myelin. Scale bar 500 μ m. Boxed areas within the left images are magnified showing LFB/PAS-, hematoxylin-, and α -synuclein-stained striatae. Note abundant α -synuclein-positive inclusions in the putamen

of the MSA patient (magnified as insert). Scale bars 50 μ m, insert 10 μ m. **b** Oligodendrocytes (arrowheads; round-shaped, dense chromatin) were distinguished by morphology from astrocytes (asterisk; larger and more elongated in size, loose chromatin) in the hematoxylin-stained sections. Scale bar 10 μ m. **c** While myelin was significantly reduced in MSA patients (gray dots) compared to controls (black dots), oligodendrocyte density was unaltered ($n = 6$). Data are shown as mean \pm standard error of mean. *T* test: ## $p < 0.01$, n.s. (not significant) $p > 0.05$. *f* female

Impaired myelin formation upon oligodendrocytic α -synuclein overexpression in an oligodendrocyte-neuron co-culture

The intriguing dichotomy of a profound myelin deficit despite preserved or even increased oligodendrocyte numbers in MSA patients (Fig. 1) and MBP29 mice (Fig. 2), respectively, suggests that an oligodendrocytic dysfunction may underlie myelin loss in MSA. Thus, we next asked whether oligodendrocytic α -synuclein accumulation impacts the process of myelin formation. To analyze functional consequences of α -synuclein on myelination, we took advantage of a recently described approach to assess myelin sheath formation in vitro by co-culturing mouse embryonic stem cell-derived oligodendrocytes and cortical neurons [25] (Fig. 3a). We adapted the protocol to quantify myelination of individual oligodendrocytes. Withdrawal of the pro-myelinating thyroid hormone T3 from the culture medium significantly decreased myelin formation, demonstrating that this method is suitable to quantify myelination of individual oligodendrocytes (Suppl. Fig. S2). To study

the effect of intraoligodendrocytic α -synuclein on myelin formation, lentiviral vectors with the *Mbp* promoter as a regulatory element were generated (α -synuclein expression vector: MBP-SYN-IG; control vector: MBP-IG), yielding a high proportion of α -synuclein-overexpressing oligodendrocytes (Fig. 3b). The usage of the *Mbp* promoter resulted in α -synuclein and/or Gfp expression in mature oligodendrocytes, but not in OPCs (Fig. 3c). Upon transduction, there was no difference in the numbers of apoptotic, activated Caspase 3-positive oligodendrocytes between α -synuclein-overexpressing and control oligodendrocytes (Suppl. Fig. S3). A comprehensive and computer-assisted quantification on a single cell level revealed that α -synuclein overexpression significantly reduced myelin sheath formation by $21 \pm 3\%$ compared to oligodendrocytes transduced with the control vector lacking the α -synuclein-coding sequence (Fig. 3d, e). Importantly, Tuj1- and *Mbp*-positive pixels were not altered upon α -synuclein overexpression (Suppl. Fig. S4), indicating that there was no effect of *Mbp* promoter-driven α -synuclein overexpression on neuronal and oligodendrocytic survival in the co-culture.

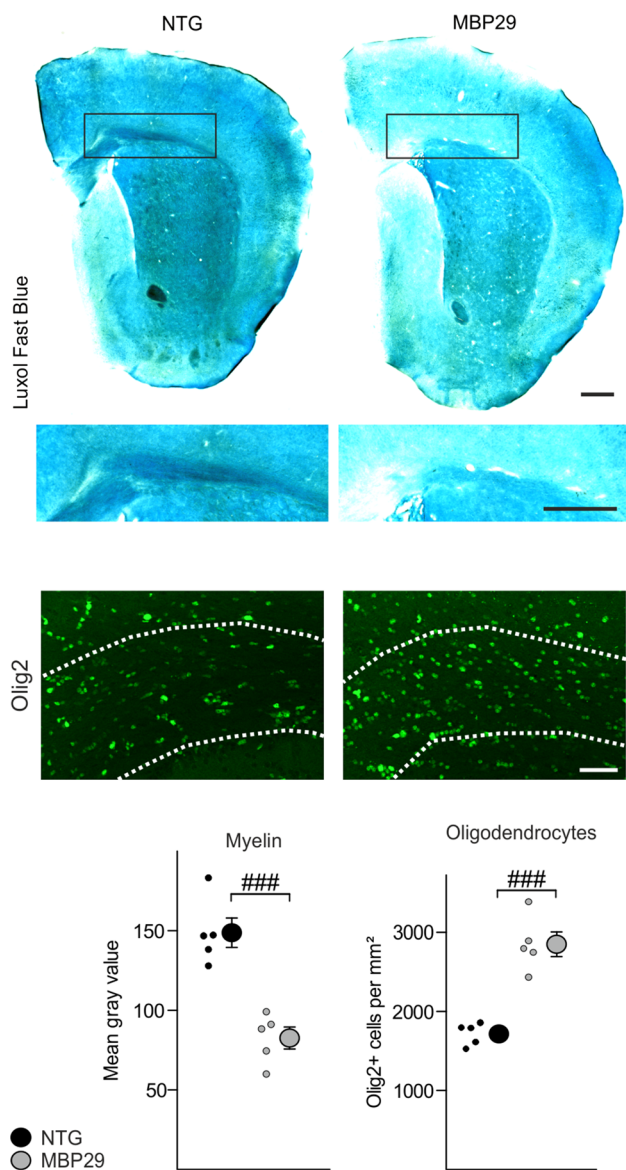


Fig. 2 Severe myelin deficit despite increased oligodendrocyte density in mice overexpressing human α -synuclein under a myelin basic protein-promoter (MBP29). MBP29 mice (3 months of age) showed a profound myelin deficit (illustrated by images of a Luxol Fast Blue staining and quantified within the corpus callosum; scale bars 500 μ m). Density of callosal oligodendrocytes (identified by Olig2; scale bar: 50 μ m) was increased by 1.7 fold. Data are shown as mean \pm standard error of mean. *T* test ($n = 5$): ### $p < 0.001$. NTG non-transgenic mice

Restored myelination by benzotropine in vitro

Next, we asked whether the interference of α -synuclein with myelin formation is reversible and thus, might represent a novel interventional target for MSA. For this purpose, we chose benzotropine, which has recently been described as a potent small molecule to enhance myelination [7]. First, we validated the effect of benzotropine on

myelin gene expression in non-modified primary OPC monoculture. Compared to vehicle, benzotropine dose dependently increased *Mbp* protein in primary OPCs (Fig. 4a). Gene expression analysis revealed an effective dose in the range of 0.1–1.0 μ M, peaking at 0.5 μ M with an increase in *Mbp* expression by 2.4 ± 0.2 fold (Fig. 4b). Immunofluorescence against *Mbp* confirmed that benzotropine at its most effective dose (0.5 μ M) strongly increased the number of *Mbp*-positive cells (Fig. 4c). We next evaluated the effect of benzotropine on *Mbp* expression in primary oligodendrocytes overexpressing human α -synuclein. For this purpose, primary oligodendrocytes were lentivirally transduced with a constitutively active α -synuclein expression vector (EF1a-SYN-IG) or the control vector lacking the α -synuclein-coding sequence (EF1a-IG). As previously described [14], α -synuclein overexpression significantly reduced *Mbp* protein (Fig. 4d) and transcription (Fig. 4e) compared to control condition. Addition of benzotropine increased *Mbp* expression in α -synuclein-overexpressing oligodendrocytes, restoring it to the level of control cells. Based on morphology, vehicle-treated α -synuclein-overexpressing oligodendrocytes were bi- or tripolar indicative for an immature phenotype, whereas benzotropine induced the formation of multiple and highly arborized branches in α -synuclein-overexpressing oligodendrocytes reflecting an advanced maturation stage comparable to control cells (Fig. 4f).

To globally analyze the effects of α -synuclein overexpression and benzotropine treatment on the myelinogenic capacity of oligodendrocytes, transcriptome profiles of vehicle- or benzotropine-treated primary OPCs transduced with the α -synuclein expression vector (EF1a-SYN-IG) or the respective control vector (EF1a-IG) were determined by RNA sequencing (Fig. 5). The genes, which were differentially regulated by both α -synuclein and benzotropine compared to respective controls, were subjected to a GO analysis (Fig. 5a, b). Enriched GO terms were implicated in myelin formation (GO:0042552 myelination, GO:0007272 ensheathment of neurons, GO:0008366 axon ensheathment), membranogenesis (GO:0006695 cholesterol biosynthetic process, GO:0044283 small molecule biosynthetic process, GO:0061024 membrane organization), and the maintenance of oligodendrocyte immaturity (GO:0050793 regulation of developmental process, GO:0051093 negative regulation of developmental process, GO:0021782 glial cell development). Intriguingly, transcripts related to myelin formation and membranogenesis were significantly underrepresented in α -synuclein-overexpressing cells and likewise upregulated by benzotropine (e.g., *Mbp*, *Plp1*, *Hmgcr*, and *Ank3*) (Fig. 5c). In contrast, transcripts involved in maintaining the immaturity of OPCs were significantly enriched upon α -synuclein overexpression and accordingly downregulated by benzotropine treatment (e.g., *Nkx2.2*, *Sox9*, and *Tcf7l1*).

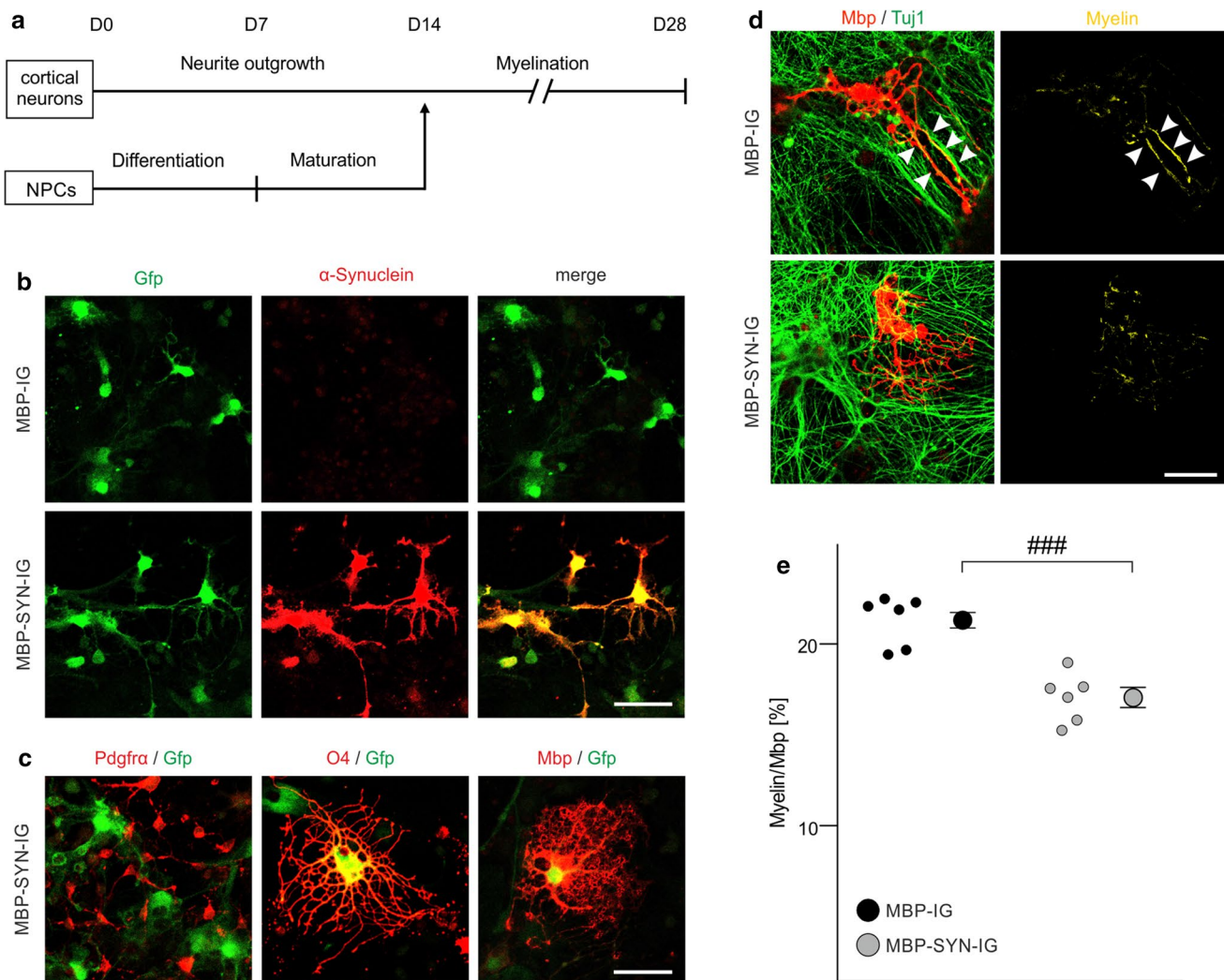


Fig. 3 Impaired myelination of α -synuclein-overexpressing mouse embryonic stem cell-derived oligodendrocytes. **a** The experimental paradigm to analyze myelination of individual oligodendrocytes in the mouse embryonic stem cell-derived oligodendrocyte-neuron co-culture is schematically illustrated according to [25]. **b** Stem cell-derived oligodendrocytes expressed human α -synuclein (when transduced with MBP-SYN-IG expression vector only; lower panel) and green fluorescent protein (Gfp) (also when transduced with the MBP-IG control vector; upper panel). Expression of α -synuclein overlapped with GFP expression in MBP-SYN-IG transduced oligodendrocytes. Scale bar 20 μ m. **c** Co-labeling with stage-specific oligodendrocytic markers revealed the specificity of myelin basic protein (*Mbp*) promoter-driven transgene expression for mature oligodendrocytes (positive for the early maturation marker O4 and *Mbp* as a marker for terminal maturation), whereas transgene expression was not detectable in platelet-derived growth factor receptor- α

(*Pdgfra*)-positive oligodendrocyte progenitor cells. Scale bar 20 μ m. **d** Representative images of control (transduced with MBP-IG prior to starting the co-culture) and α -synuclein-overexpressing (transduced with MBP-SYN-IG) *Mbp*-positive oligodendrocytes co-cultured with beta-III-tubulin (*Tuj1*)-positive cortical neurons are depicted. Myelination (yellow; arrowheads) was regularly detected under control condition, whereas α -synuclein overexpression impaired myelin formation. Scale bar 20 μ m. **e** Myelination of individual oligodendrocytes (altogether: MBP-IG: 235 cells, MBP-SYN-IG: 215 cells) in the stem cell-derived co-culture was quantified ($n = 6$) by calculating the ratio of *Mbp*/*Tuj1*-positive pixels over total *Mbp*-positive pixels and is shown as percentage. α -Synuclein-overexpressing oligodendrocytes (gray dots) formed significantly less myelin than controls (black dots). Data are shown as mean \pm standard error of mean. *T* test: ### $p < 0.001$

To extend the findings from primary oligodendrocyte monocultures, we next analyzed whether bentsropine is also able to restore the myelination deficit of α -synuclein-overexpressing stem cell-derived oligodendrocytes in co-culture with cortical neurons. First, the myelinogenic effect

of bentsropine was validated in non-modified stem cell-derived oligodendrocytes (Suppl. Fig. S5). Strikingly, bentsropine treatment also increased myelin sheath formation of α -synuclein-overexpressing oligodendrocytes by $29 \pm 4\%$ to the level of control cells (Fig. 6).

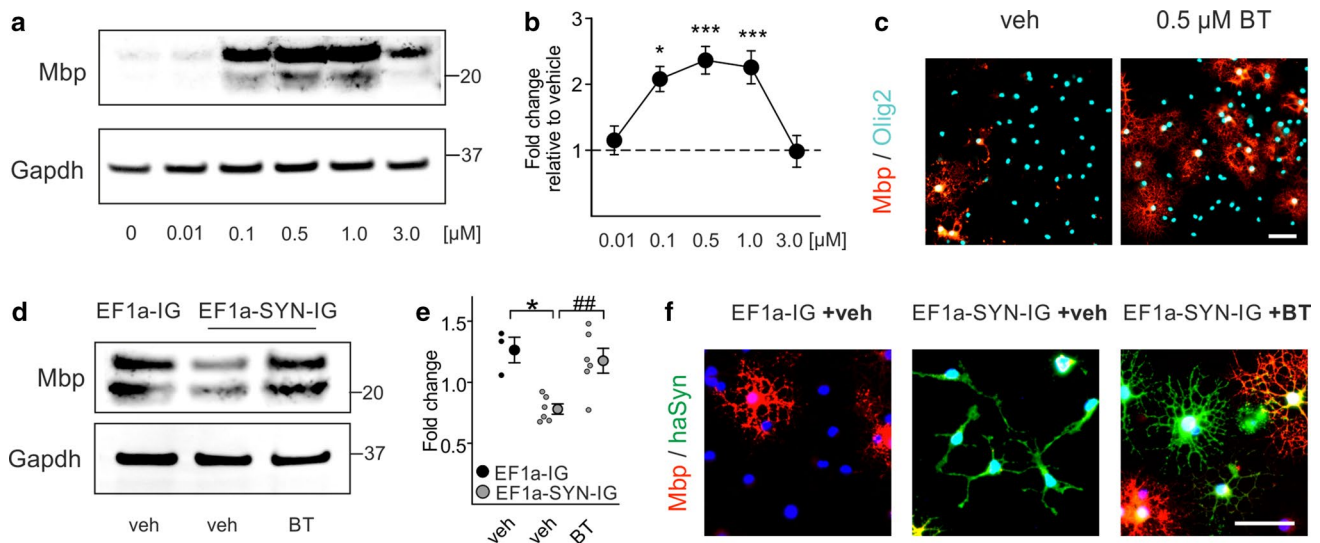


Fig. 4 Benztropine restores myelin basic protein (Mbp) expression in α -synuclein-overexpressing primary oligodendrocyte progenitor cells (OPCs). **a** The dose-dependent effect of benzotropine on Mbp expression in primary OPCs is illustrated using Western blot. Gapdh signal served as control. **b** Benztropine significantly increased Mbp gene expression in a range of 0.1–1.0 μ M as quantified by real-time PCR ($n = 3$ –6). **c** Immunocytochemistry for Mbp and Olig2 illustrates the effect of 0.5 μ M benzotropine (BT) on OPC maturation in comparison to vehicle- (veh) treated cells. Scale bar: 20 μ m. **d** The restoration of Mbp expression in α -synuclein-overexpressing cells (EF1a-SYN-IG

+veh) upon treatment with 0.5 μ M benzotropine (EF1a-SYN-IG +BT) to control levels (EF1a-IG +veh) is demonstrated by Western blot. Gapdh signal served as control. **e** Benztropine restored Mbp gene expression in α -synuclein-overexpressing OPCs as quantified by real-time PCR ($n = 3$ –6). **f** α -Synuclein-overexpressing cells exhibited the typical morphology of OPCs (bi-/tripolarity), whereas treatment with benzotropine induced Mbp expression and formation of multiple processes as signs of advanced maturity comparable to control condition. Scale bar 20 μ m. Data are shown as mean \pm standard error of mean. ANOVA: * $p < 0.05$, *** $p < 0.001$. *T* test: ## $p < 0.01$

Attenuated myelin deficit in MBP29 mice upon benzotropine administration

After observing the restoration of the α -synuclein-induced myelination deficit by benzotropine in vitro (Figs. 4, 5, 6), we next administered benzotropine at 2 mg/kg to MBP29 mice to assess whether benzotropine is also able to enhance myelination in the presence of oligodendrocytic α -synuclein accumulation in vivo. T2-weighted magnetic resonance imaging revealed a reduced gray-white matter contrast in vehicle-treated MBP29 mice compared to non-transgenic littermates, which corresponds to a myelin reduction (Fig. 7a). A significant gain of contrast was observed in benzotropine-treated MBP29 mice. For quantification, T2*-weighted magnetic resonance imaging was performed because of its previously demonstrated sensitivity in detecting myelin loss in murine brains [27]. Compared to controls, vehicle-treated MBP29 mice showed a significant increase by 19 ± 3 % in the T2* relaxation time within the corpus callosum, confirming the myelin deficit (Fig. 7b). Treatment with benzotropine significantly reduced the callosal T2* relaxation time in MBP29 mice by 9 ± 1 %, thus restoring the myelin deficit by almost 50 %. Matching this imaging approach, benzotropine treatment significantly increased the mean Luxol Fast Blue signal intensity in the

corpus callosum of MBP29 mice, ameliorating the myelin deficit by more than 50 % (Figs. 2, 7c, d). Results were further confirmed by ultrastructural analysis of myelin in the corpus callosum using electron microscopy (Fig. 8). In vehicle- and benzotropine-treated non-transgenic mice, myelin showed a highly organized multilaminar structure. In contrast, myelin in vehicle-treated MBP29 mice appeared disorganized and substantially reduced. Both density of myelinated axons and myelin layers per axon were significantly reduced. In benzotropine-treated MBP29, numbers of myelinated axons and myelin layers per axon were comparable to non-transgenic controls.

Taken together, these data demonstrate that the pro-myelinating activity of benzotropine attenuates the severe myelin deficit upon α -synuclein overexpression in MBP29 mice.

Unchanged oligodendrocyte dynamics in MBP29 mice upon benzotropine administration

We next examined whether benzotropine impacts the dynamics of oligodendrocytic populations in MBP29 mice. To this end, we histologically identified distinct oligodendrocytic subpopulations within the corpus callosum of vehicle- and benzotropine-treated MBP29 mice and non-transgenic littermates using maturation stage-specific markers (Fig. 9a, b): OPCs

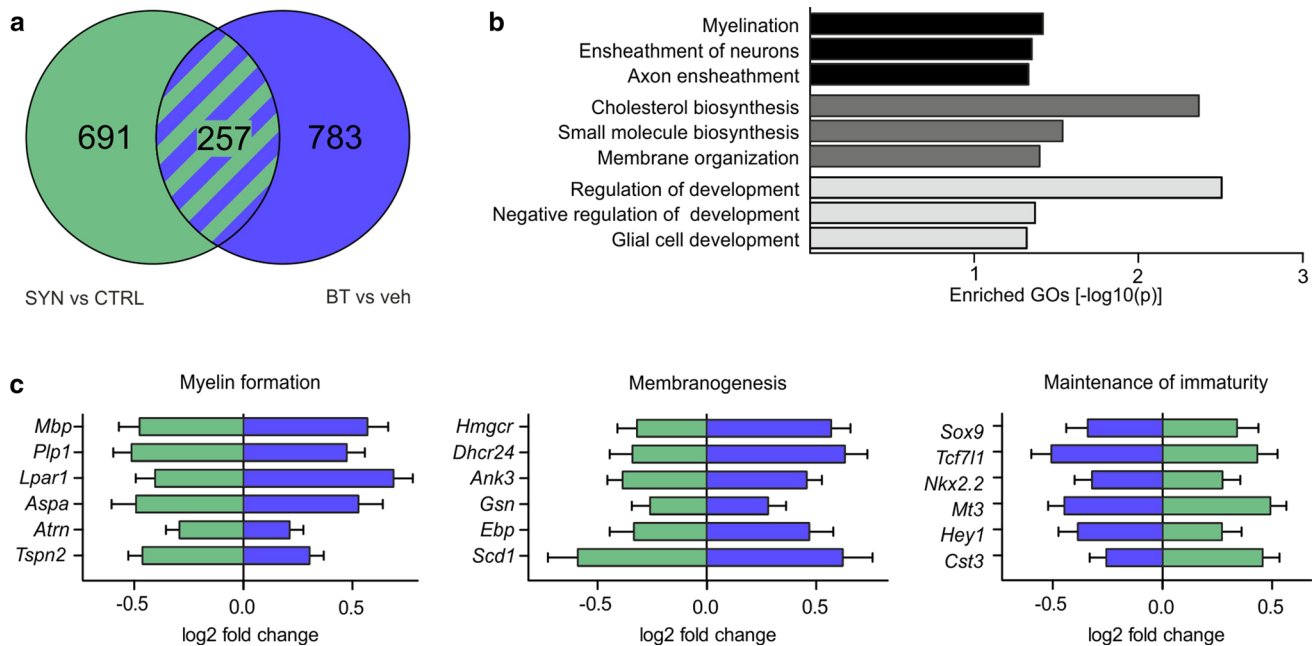


Fig. 5 Restored myelinogenic capacity of α -synuclein-overexpressing primary rat oligodendrocyte progenitor cells (OPCs) by benzotropine. **a** A Venn diagram depicts differentially regulated genes revealed by RNA sequencing of primary rat OPCs ($n = 3$). *Green circle*: α -synuclein-overexpressing (SYN) versus control cells (CTRL). *Blue circle*: benzotropine (BT) versus vehicle (veh)-treated α -synuclein-overexpressing cells. **b** Genes differentially regulated by both α -synuclein overexpression and benzotropine treatment classified into gene ontologies (GOs) implicated among others in myelin formation (*black*), membranogenesis (*dark gray*), and maintenance of oligodendrocyte immaturity (*light gray*). **c** Expression dynamics of representative genes identified by GO term enrichment are

listed. Benzotropine (*blue*) oppositely regulated genes differentially expressed upon α -synuclein overexpression (*green*). Myelin formation: *Mbp* myelin basic protein, *Plp1* proteolipid protein 1, *Lpar1* lysophosphatidic acid receptor 1, *Aspa* aspartoacylase, *Atrn* attractin, *Tspn2* tetraspanin 2; Membranogenesis: *Hmgcr* 3-hydroxy-3-methylglutaryl-CoA reductase, *Dhcr24* 24-dehydrocholesterol reductase, *Ank3* ankyrin 3, *Gsn* gelsolin, *Ebp* emopamil binding protein (sterol isomerase), *Scd1* stearyl-CoA desaturase-1; Maintenance of immaturity: *Sox9* sex determining region Y-box 9, *Tcf7l1* transcription factor 7-like 1, *Nkx2.2* Nk2 homeobox 2, *Mt3* metallothionein 3, *Hey1* hes-related family bHLH transcription factor with YRPW motif 1, *Cst3* cystatin C

(*Pdgfra*/*Olig2*-positive) were distinguished from mature oligodendrocytes (*Gst π* /*Olig2*-positive), whereas a minority of *Olig2*-positive cells co-labeled for neither *Pdgfra* nor *Gst π* (*Olig2* only). *Mbp* promoter-driven α -synuclein overexpression in MBP29 mice resulted in a 1.5- to 2.2-fold increased density of all distinct subpopulations compared to non-transgenic control animals (Fig. 9c). In contrast to α -synuclein overexpression, it is remarkable that benzotropine did not affect oligodendrocyte density. Given that the proportional distribution of oligodendrocytic subpopulations was unchanged in all groups (Suppl. Fig. S6), these findings suggest that treatment with benzotropine directly acts on myelin formation, but does not increase OPC maturation in MBP29 mice.

Prevention of motor cortical neuronal loss without affecting microgliosis in MBP29 mice upon benzotropine treatment

In addition to the severe loss of myelin, MBP29 mice recapitulate crucial neurodegenerative and inflammatory features of MSA [45]. Thus, we next examined whether

benzotropine treatment had an impact on neuronal and microglial cell densities in MBP29 mice. To this end, we first quantified the density of NeuN-positive neurons within the motor cortex (Fig. 10). While vehicle-treated MBP29 mice showed a moderate (14 %), but significant reduction in neuronal cell density, benzotropine treatment prevented neuronal loss in MBP29 animals.

We next assessed the effect of benzotropine on motor cortical and callosal microglial cells in MBP29 mice by determining the density of Iba1-positive microglia (Suppl. Fig. S7). Microgliosis was apparent in the corpus callosum of vehicle-treated MBP29 mice (2.5-fold increase in Iba1-positive cells), whereas the motor cortex showed no apparent signs of microgliosis when compared to control mice (1.1-fold increase). Benzotropine treatment neither affected callosal nor motor cortical Iba1 cell density in MBP29 mice.

Taken together, these findings suggest that benzotropine treatment has a beneficial effect on survival of motor cortical neurons, but does not alter microglial cell densities in MBP29 mice.

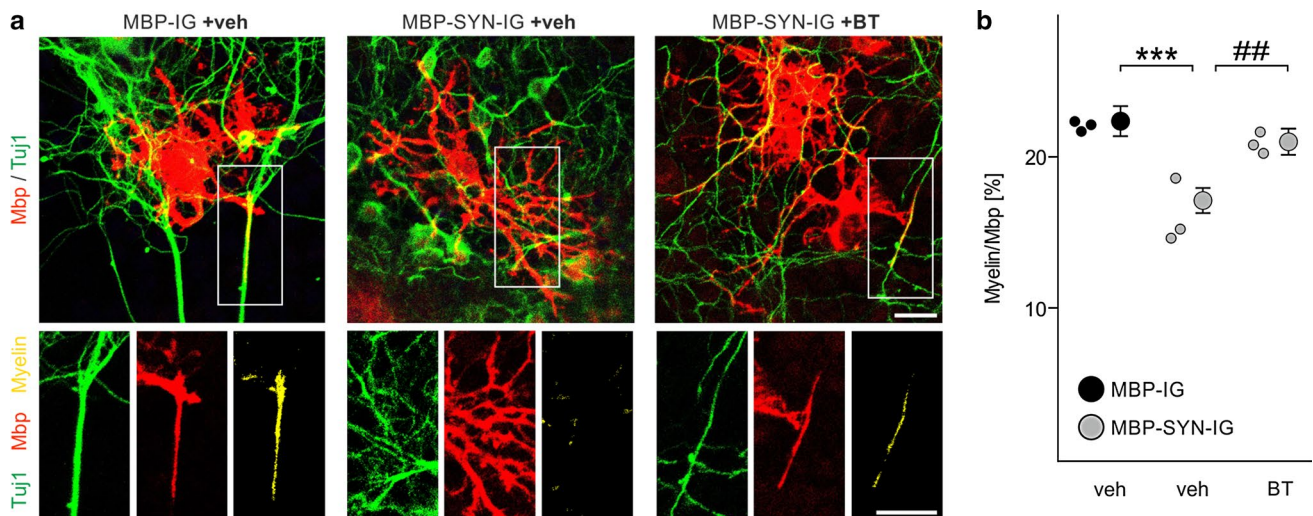


Fig. 6 Restored myelination deficit of α -synuclein-overexpressing mouse embryonic stem cell-derived oligodendrocytes by benzotropine. **a** Representative images show that overexpression of α -synuclein (transduced with MBP-SYN-IG prior to starting the co-culture) impaired myelin formation of stem cell-derived oligodendrocytes (stained for myelin basic protein, Mbp) co-cultured with cortical neurons (beta-III-tubulin, Tuj1). Addition of benzotropine enhanced myelin formation of α -synuclein-overexpressing cells [comparable to vehicle (veh)-treated control cells, which were transduced with

MBP-IG]. Mbp-positive pixels co-localizing with Tuj1-positive pixels were considered as myelin (shown in yellow). Scale bar 20 μ m. **b** Myelination of individual oligodendrocytes (in total 90–110 cells per condition) was quantified ($n = 3$). α -Synuclein-overexpressing oligodendrocytes (gray dots) in the presence of vehicle (veh) formed significantly less myelin than controls (black dots, veh). Benzotropine (BT) restored the myelination deficit of α -synuclein-overexpressing oligodendrocytes. Data are shown as mean \pm standard error of mean. ANOVA: *** $p < 0.001$. *T* test: ## $p < 0.01$

Discussion

Cellular pathomechanisms that underlie profound myelin loss and neurodegeneration in MSA are poorly understood. In this regard, our study provides compelling evidence for impaired myelin formation in MSA, which is directly associated with the oligodendrocytic accumulation of α -synuclein and not accompanied by loss of oligodendrocytes. Considering that oligodendrocytic α -synuclein accumulation is the primary event during MSA pathogenesis, the deficit in myelin formation may be an early and crucial pathomechanism during disease progression. Given that efficient interventional strategies for MSA do not exist and by demonstrating that the interference of α -synuclein with myelin formation is reversible, our *in vitro* and *in vivo* data imply pro-myelinating strategies as a novel approach for an urgently needed therapy in MSA.

Using histological analyses of MSA post-mortem tissue, we showed that myelin loss is a widespread pathological feature in MSA. Loss of myelin, downregulation of myelin proteins [1, 35, 38, 47], and widespread lipid alterations [5, 11] have previously been reported in post-mortem brains of MSA patients, supporting our finding of a severe myelin deficit in MSA. Despite strong evidence for myelin degeneration in MSA, the underlying pathomechanisms of this myelin loss are not well understood. In the MSA cohort analyzed in the present study, the putamen

exhibited extensive oligodendrocytic α -synuclein pathology within white matter tracts, suggesting that oligodendrocytic α -synuclein accumulation underlies loss of myelin. This is supported by the fact that a substantial myelin deficit was also present upon transgenic overexpression of α -synuclein in oligodendrocytes of MBP29 mice. In line, a similar myelin reduction has also been observed in transgenic mice with α -synuclein overexpression under alternative myelin gene promoters, namely the proteolipid protein and the 2',3'-cyclic-nucleotide 3'-phosphodiesterase promoter [48, 52]. Notably, structural alterations, including nuclear shrinkage, cytosolic re-localization of myelin proteins, and oligodendrocytic swelling, were also observed in oligodendrocytes without α -synuclein accumulation and possibly contribute to myelin loss in MSA [47, 49]. Moreover, we selectively used post-mortem material of MSA patients with striatonigral degeneration, limiting the interpretation of the present study to the parkinsonian variant of MSA (MSA-P).

Myelin loss in MSA patients and in transgenic MSA mice may be linked to either oligodendrocyte loss or impaired ability of oligodendrocytes to form new myelin sheaths. The observation of an unchanged or even increased oligodendrocyte density in human MSA post-mortem tissue and MBP29 mice suggests that rather a dysfunction of oligodendrocytes in terms of myelin sheath formation than pronounced loss of oligodendrocytes

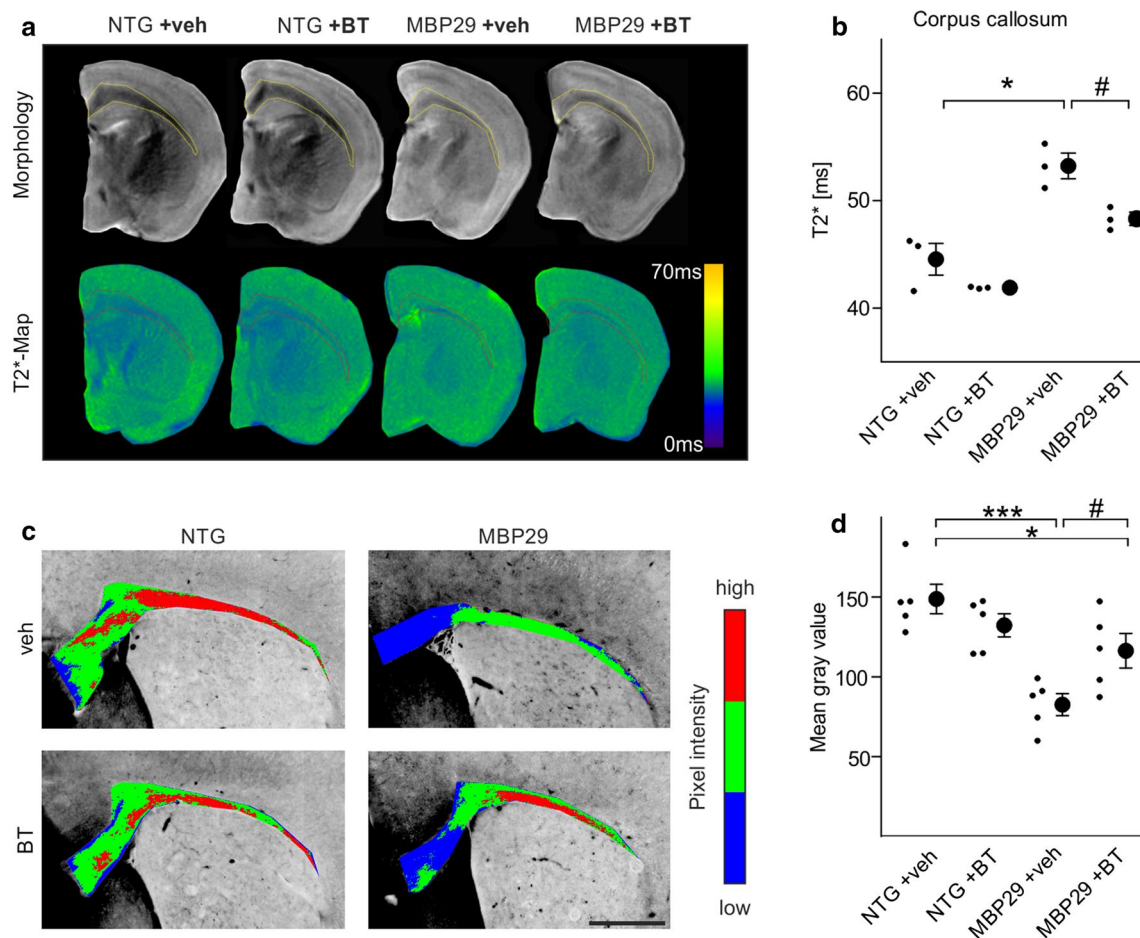


Fig. 7 Amelioration of the myelin deficit in mice with oligodendrocytic overexpression of α -synuclein (MBP29) upon benztrapine treatment. **a** Callosal myelin levels of non-transgenic (NTG) and MBP29 mice treated with vehicle (veh) or benztrapine (BT, 2 mg/kg) were analyzed using magnetic resonance imaging. Upper panel illustrates morphology as determined by T2-weighted magnetic resonance imaging, whereas lower panel depicts T2*-weighted magnetic resonance imaging (shown as *color-coded maps*), which was used for myelin quantification. **b** MBP29 mice ($n = 3$) showed a reduction in myelin levels detected by increased T2*-relaxation times within

the corpus callosum. Benztrapine attenuated the myelin deficit in MBP29 confirmed by a reduction of T2*. **c** Representative pictures of a Luxol Fast Blue staining (color-coded for signal intensity: *blue* low, *green* intermediate, *red* high intensity), confirming the effects of α -synuclein overexpression and benztrapine treatment on myelination. *Scale bar* 500 μ m. **d** MBP29 mice ($n = 5$) exhibited a reduction in Luxol Fast Blue intensity, which was attenuated, but not completely restored by benztrapine. Data are shown as mean \pm standard error of mean. ANOVA: * $p < 0.05$, *** $p < 0.001$. *T* test: # $p < 0.05$

underlies myelin loss in MSA. A recent stereological analysis, focusing on basal ganglia, has indicated that oligodendrocytes are by far more preserved than neurons in MSA [41]. In line, we and others have observed increased numbers of OPCs in the striatum and the cerebellum of MSA patients, supporting the notion of preserved oligodendrocytic cells in MSA [2, 36]. Similarly, we have shown that mice moderately overexpressing α -synuclein driven by the *Mbp* promoter (line 1) exhibit an increased number of OPCs accompanied by myelin loss [36, 45]. Given that the *Mbp* promoter did not drive α -synuclein overexpression in OPCs, the observed increase in OPC density of MBP29 mice indicates enhanced OPC proliferation to compensate the myelin deficit.

Supporting these observations in MSA post-mortem and transgenic mouse forebrains, our *in vitro* data provide further evidence, that oligodendrocytic α -synuclein impairs myelin formation: α -synuclein-overexpressing oligodendrocytes derived from mouse embryonic stem cells exhibited a reduced capacity to form myelin sheaths around axons of cortical neurons. Moreover, transcriptome profiles of primary rat OPC monocultures showed that α -synuclein overexpression in maturing oligodendrocytes impaired expression of gene families essentially involved in myelin sheath formation. The temporal dynamic of α -synuclein expression in cultured murine and human OPCs during maturation toward pre-myelinating oligodendrocytes suggests that α -synuclein is physiologically restricted to

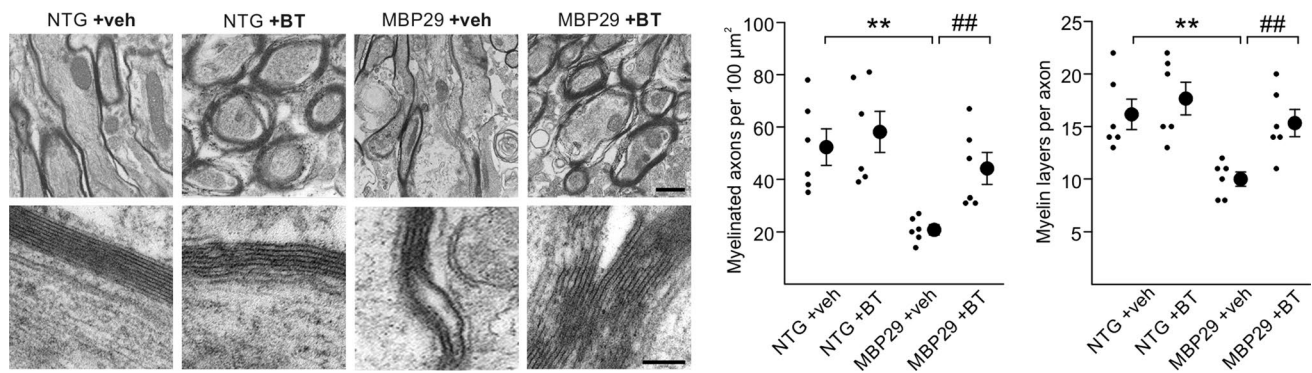


Fig. 8 Structural restoration of myelin by bntropine in mice over-expressing α -synuclein in oligodendrocytes (MBP29). Representative electron micrographs of the corpus callosum at low (*upper panel*, scale bar 1 μ m) and high (*lower panel*, scale bar 0.2 μ m) magnification depict myelin sheaths of non-transgenic (NTG) and MBP29 mice treated with either vehicle (veh) or bntropine (BT). In vehicle-

treated MBP29 mice only, myelin appeared strongly reduced and disorganized. Quantification of myelinated axons and myelin layers per axon revealed a significant myelin deficit in vehicle-treated MBP29 mice, which was restored by bntropine treatment to control level. Data are shown as mean \pm standard error of mean. ANOVA ($n = 6$): ** $p < 0.01$. *T* test: ## $p < 0.01$

immature oligodendrocytes [8, 40]. In addition, we have previously demonstrated that the sustained presence of intracellular α -synuclein delays the upregulation of myelin gene expression in monocultures of primary rat oligodendrocytes [14], further supporting that α -synuclein expression needs to be downregulated for timed and physiological oligodendrocyte maturation and myelination. Thus, our data further strengthen the view on α -synuclein as an inhibitor of oligodendrocyte maturation and myelination and suggest that pathologic oligodendrocytic α -synuclein accumulation in MSA impairs myelin formation.

In recent years, the view on myelin dynamics in the adult brain has fundamentally changed. The fact, that myelin is physiologically formed in the adult murine and human central nervous system throughout life, has only lately been recognized [18, 37, 42, 53, 54]. In mice, adaptive myelination in the adult forebrain is mediated mostly by newly formed oligodendrocytes derived from OPCs, occurs upon neuronal activity, and is essentially required for motor skill learning [18, 37]. In line, motor training has also induced a remodeling of white matter structures in humans [42]. Importantly, a continuous physiological myelin turnover during adulthood, which is mostly mediated by preexisting oligodendrocytes, has been detected in the human corpus callosum by determining the integration of nuclear bomb test-derived (14)C [53]. Therefore, impaired myelination in the presence of α -synuclein may result not only in compromised remyelination, but also in reduced myelin turnover. In this context, it is important to note that disturbances in myelin homeostasis result in axonal and neuronal degeneration [51]. The impaired capacity to form myelin sheaths may thus represent a fundamental and early pathological mechanism in MSA, favoring myelin loss and ultimately triggering widespread axonal dysfunction and neuronal loss.

To restore oligodendrocyte functionality, we used the small molecule bntropine, which has recently been identified by a high-throughput screen to accelerate OPC maturation and directly promote myelin formation, thereby ameliorating functional deficits in pre-clinical mouse models of multiple sclerosis [7]. We chose this small molecule to target α -synuclein-induced effects on oligodendrocyte function because (i) it is clinically used for the treatment of tremor in patients diagnosed with sporadic Parkinson's disease and thus promises a fast translation into clinics; and (ii) bntropine has a dual mode of action as it promotes myelin gene expression in pre-myelinating oligodendrocytes and directly enhances myelin formation in mature oligodendrocytes [7]. Both processes are essentially required for myelination and are compromised by α -synuclein overexpression. While the effect of bntropine on myelin gene expression has been attributed to its antagonistic activity at the muscarinic acetylcholine receptors M1 and M3 [7], the mechanism by which bntropine induces myelin sheath formation is not yet known. Our observation, that bntropine increased myelination without changing the density of OPCs and mature oligodendrocytes in MBP29 mice, favors rather a direct action of bntropine on myelin sheath formation in this model. Importantly, bntropine even ameliorated the myelination deficit in MBP29 mice by approximately 50 %, although used at a low dose (2 mg/kg in comparison to the most efficient dose of 10 mg/kg according to Deshmukh and colleagues [7]). Higher dosages (5 and 10 mg/kg) were not tolerated due to the severe phenotype of MBP29 mice with a premature death at 4–6 months of age [45]. Moreover, our experiments using the stem cell-derived oligodendrocyte-neuron co-culture also point toward a direct pro-myelinating effect of bntropine. Our transcriptome analysis suggests that bntropine readjusts

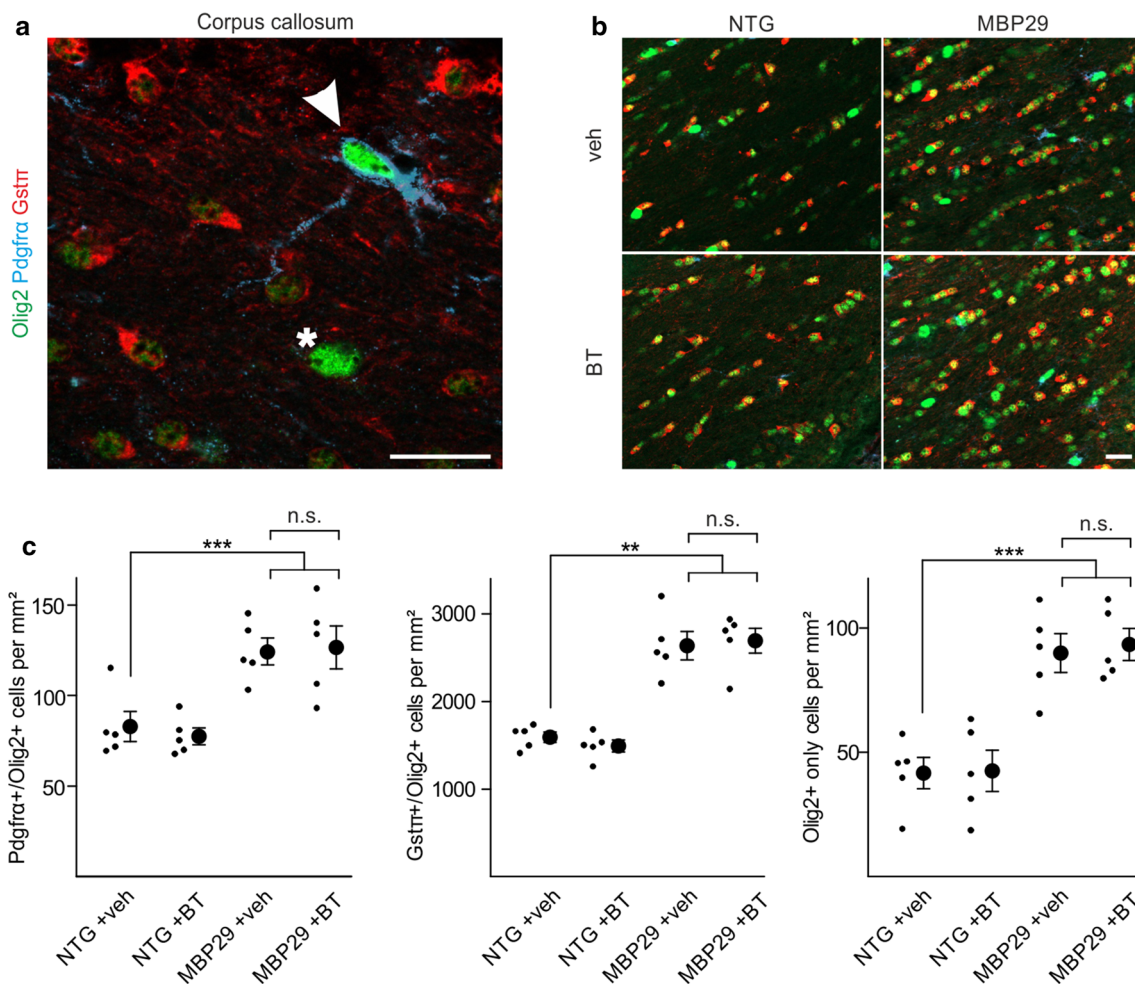


Fig. 9 No alteration of oligodendrocyte density by benzotropine. **a** Oligodendrocytic cells within the corpus callosum were identified using stage-specific markers [entire population: Olig2; oligodendrocyte progenitor cells: platelet-derived growth factor receptor- α (Pdgfra)/Olig2^{strong} (arrowhead), mature oligodendrocytes: glutathione-*s*-transferase- π (Gstr)/Olig2^{weak}, as well as cells staining for Olig2^{strong} only (asterisk)]. Scale bar 20 μ m. **b** A profound increase of oligodendrocytic cells within the corpus callosum of mice

overexpressing α -synuclein in oligodendrocytes (MBP29) compared to non-transgenic controls (NTG) was observed. Scale bar 20 μ m. **c** While MBP29 mice exhibited an almost twofold increased density of the entire spectrum of oligodendrocytic subpopulations, benzotropine (BT) had no effect on oligodendrocytic cell density as compared to vehicle (veh)-treatment. Data are shown as mean \pm standard error of mean. ANOVA ($n = 5$): ** $p < 0.01$, *** $p < 0.001$. *T* test: not significant (n.s.) $p > 0.05$

disturbed lipid metabolism in oligodendrocytes with α -synuclein accumulation, thereby enhancing myelin formation. While not in the focus of the present work, future studies need to dissect the complex pharmacology and define molecular targets underlying the pro-myelinating activity of benzotropine.

Benzotropine treatment also prevented motor cortical neuronal cell loss in MBP29 mice, suggesting that myelin regeneration supports neuronal survival. Although we cannot exclude a direct effect of benzotropine on neuronal cells, the preservation of neurons might be attributed to oligodendrocyte-derived factors and their importance for axonal maintenance and neuronal survival [29]. In line, protection of cortical neurons was also achieved upon

immunotherapeutic prevention of myelin loss in transgenic mice overexpressing α -synuclein in oligodendrocytes [33]. In this study, oligodendrocytic α -synuclein pathology was reduced via specific antibodies against α -synuclein. Importantly, benzotropine injections did not alter microglial cell numbers in MBP29 mice, supporting that neuronal preservation is not linked to decreased neuroinflammation. In line, Deshmukh and colleagues have demonstrated that benzotropine has no immunomodulatory effect in experimental mouse models for multiple sclerosis [7].

The surprising dichotomy of a substantial myelin deficit despite a preserved oligodendrocyte density opens novel avenues for restorative interventional strategies in MSA. Up to now, experimental and clinical trials for disease-modifying

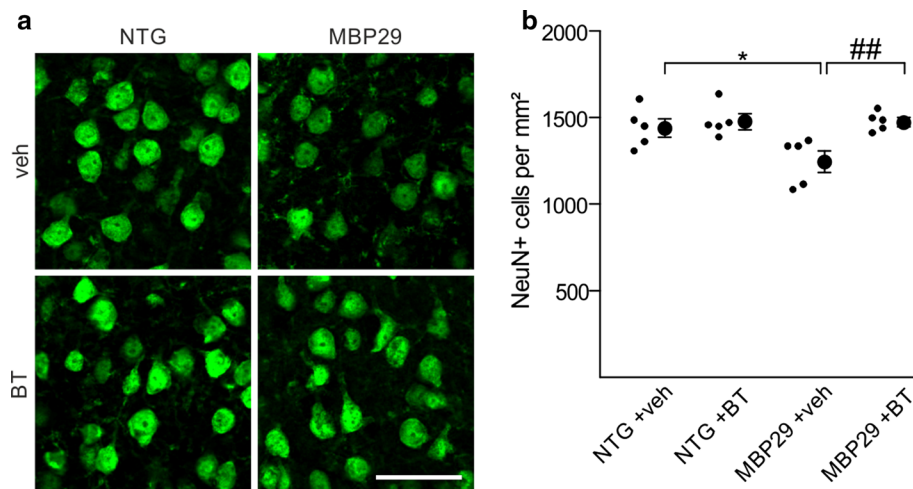


Fig. 10 Prevention of motor cortical neuronal cell loss in mice overexpressing α -synuclein in oligodendrocytes by benzotropine. **a** Representative images depict NeuN-positive neuronal cells within the motor cortex of non-transgenic controls (NTG) and α -synuclein transgenic mice (MBP29) treated with vehicle (veh) or benzotropine (BT). Scale bar 20 μ m. **b** Densities of motor cortical neurons are

shown ($n = 5$). In vehicle-treated MBP29 mice, a significant reduction in neuronal cell density was observed. Benzotropine treatment prevented the loss of cortical neurons in MBP29 mice. Data are shown as mean \pm standard error of mean. ANOVA: * $p < 0.05$. *T* test: ## $p < 0.01$

therapies in MSA have focused on direct protection of neurons (e.g., riluzole [44]), prevention or clearance of α -synuclein aggregates (e.g., rifampicin [31]), reduction of neuroinflammation (e.g., minocycline [10]), and neurorestorative approaches (e.g., systemic mesenchymal stem cell delivery [28]). However, all of these strategies showed only very moderate—if any—clinical efficacy (for review see [26]). We propose the myelination deficit of dysfunctional oligodendrocytes as a novel and promising interventional target for MSA because of two reasons: firstly, α -synuclein accumulation within oligodendrocytes is considered as the primary and pathognomonic event during MSA pathogenesis [50]; and secondly, we showed that oligodendrocytes are preserved in MSA and—albeit being dysfunctional—their ability to form myelin was not irreversibly lost in the presence of α -synuclein. Consequently, restoring the capacity to form new myelin may provide a new basis for a therapeutic strategy even in early stages of MSA. It should be emphasized that—although we observed prevention of neuronal cell loss in our model—we cannot state whether a pro-myelinating monotherapy will lead to a functional recovery in the context of MSA, given that the usage of the most severe pre-clinical mouse model for MSA, the MBP29 mouse line, did not allow a comprehensive behavioral assessment. Thus, additional pre-clinical studies have to explore whether a myelinogenic monotherapy or a synergistic combination of a pro-myelinating intervention with strategies targeted against other pathological hallmarks of MSA, including neuronal degeneration and neuroinflammation, may be the basis for treating MSA patients.

To conclude, our findings shed new light into α -synuclein-linked pathomechanisms in MSA. Intriguingly, the observed reversibility of the α -synuclein-induced

myelination deficit in primary rat OPCs, in mouse embryonic stem cell-derived oligodendrocytes, and in MBP29 mice identified a new direction to treat MSA.

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Compliance with ethical standards

Human brain samples used in this study were obtained from the NBB and have been collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB. All animal procedures were conducted with approval of the animal care and use committees of the University of California San Diego, the Friedrich-Alexander-Universität Erlangen-Nürnberg, and the state of Bavaria.

Conflict of interest The authors declare that they have no conflict of interest.

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