Research paper

Quantitative susceptibility mapping depicts severe myelin deficit and iron deposition in a transgenic model of multiple system atrophy

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ABSTRACT

Despite internationally established diagnostic criteria, multiple system atrophy (MSA) is frequently misdiagnosed, particularly at disease onset. While neuropathological changes such as demyelination and iron deposition are typically detected in MSA, these structural hallmarks were so far only demonstrated post-mortem. Here, we examine whether myelin deficit observed in a transgenic murine model of MSA can be visualized and quantified in vivo using specific magnetic resonance imaging (MRI) approaches. Reduced myelin content was measured histologically in prototypical white matter as well as mixed grey-white matter regions i.e. corpus callosum, anterior commissure, and striatum of transgenic mice overexpressing human α-synuclein under the control of the myelin basic protein promoter (MBP29-α-syn mice). Correspondingly, in vivo quantitative susceptibility mapping (QSM) showed a strongly reduced susceptibility contrast in white matter regions and T2*-weighted MR imaging revealed a significantly reduced grey-white matter contrast in MBP29-α-syn mice. In addition, morphological analysis suggested a pronounced, white matter-specific deposition of iron in MBP29-α-syn mice. Importantly, in vivo MRI results were matched by comprehensive structural characterization of myelin, iron, and axonal directionality. Taken together, our results provide strong evidence that QSM is a very sensitive tool measuring changes in myelin density in conjunction with iron deposition in MBP29-α-syn mice. This multimodal neuroimaging approach may pave the way towards a novel non-invasive technique to detect crucial neuropathological changes specifically associated with MSA.

1. Introduction

The atypical parkinsonian disorder multiple system atrophy (MSA) is an adult-onset neurodegenerative disease characterized by rapid progression and poor levodopa responsiveness (Wenning et al., 2013; Krismer and Wenning, 2017). Patients present distinct symptoms such as Parkinsonism, cerebellar ataxia, pyramidal symptoms, and severe autonomic dysfunction. Based on the predominant degeneration of striatonigral or olivopontocerebellar motor system, MSA is classified as a parkinsonian (MSA-P) or a cerebellar (MSA-C) subtype, respectively (Köllensperger et al., 2008; Köllensperger et al., 2010). Due to the clinical overlap with sporadic Parkinson’s disease or other atypical parkinsonian disorders, MSA is frequently misdiagnosed, in particular at the onset of symptoms. An accuracy of clinical diagnosis has been calculated between 62% - 86% precision in autopsy-confirmed patients (Hughes et al., 2002; Osaki et al., 2002; Koga et al., 2015; Koga and Dickson, 2018). This highlights the urgent need for improved diagnostic strategies, facilitating precise diagnosis of MSA in early stages of the disease.

Neuropathological hallmark of MSA is the accumulation of α-synuclein (α-syn) within oligodendrocytes, referred to as glial cytoplasmic inclusions (GCI) leading to oligodendrogial dysfunction and progressive demyelination (Papp et al., 1989; Waxman and Giasson, 2009; Ettle et al., 2016). Further important pathological changes observed in MSA are axonal and neuronal degeneration as well as neuroinflammation. Some of these neurodegenerative changes are associated with different types of parkinsonian disorders, however, pronounced demyelination is solely observed in MSA (Matsuo et al.,...
1998; Hunot and Hirsch, 2003; Fanciulli and Wenning, 2015; Hoffmann et al., 2019). Furthermore, an altered iron metabolism with abundant iron accumulation within the basal ganglia has been detected in MSA patients (Dexter et al., 1991; Berg and Hochstrasser, 2006). Therefore, both demyelination and iron accumulation may be particularly suitable hallmarks to diagnose MSA. However, a validated approach to quantify both in vivo is missing so far. Recent advantages in high field magnetic resonance imaging (MRI) facilitated novel approaches to assess white matter (wm) distribution and density as well as iron accumulation (Liu et al., 2011; Langkammer et al., 2012). Phase maps of gradient echo MRI exhibit high tissue contrast induced by magnetic susceptibility. These phase maps are processed in quantitative susceptibility mapping (QSM), which approximates the spatial distribution of tissue magnetic susceptibility using a series of complex post-processing algorithms (Deistung et al., 2017). Even though, the source of the QSM contrast is complex and needs further investigation, different tissue components such as iron (Haacke et al., 2005; Shmueli et al., 2009; Yao et al., 2009), calcium (Schweser et al., 2010; Straub et al., 2017), deoxyhaemoglobin (Marques et al., 2009; Lee et al., 2010), and microstructural orientation (He and Yablonskiy, 2009; Bender and Klose, 2010) are known to contribute to the susceptibility contrast. Furthermore, recent studies indicated that magnetic susceptibility is considerably affected by changes of myelin density (Zhong et al., 2011; Lodygensky et al., 2012; Argyridis et al., 2014). Intriguingly, the strong susceptibility contrast between grey matter (gm) and wm is significantly reduced in a murine model of acute toxic demyelination (Lee et al., 2012; Wang et al., 2019). Moreover, this contrast almost vanished in shiverer mice showing low myelination due to an autosomal recessive mutation in the myelin basic protein (MBP) gene (Liu et al., 2011; Lodygensky et al., 2012). Overall, these findings implicate that QSM may be a sensitive and specific imaging approach to quantify myelin and iron in vivo.

In order to assess underlying neuropathological mechanisms of MSA, Shults et al. generated a transgenic mouse line mimicking important aspects of MSA (Shults et al., 2005). Transgenic mice over-express human α-syn under the control of the oligodendrocyte-specific myelin basic protein promoter (line 29, MBP29-hα-syn mice) and develop a severe motor phenotype (2–3 month) before dying prematurely after 4–6 month of age (Shults et al., 2005; Hoffmann et al., 2019). Given the profound demyelination observed in post-mortem analysis of MSA and this corresponding model (Shults et al., 2005; Yazawa et al., 2005; Etelle et al., 2016), we hypothesized that the myelin deficit in the MBP29-hα-syn mouse model of MSA may be detected and quantified using QSM and T2-weighted MR imaging. In order to evaluate myelination and iron accumulation in MBP29-hα-syn mice, we performed in vivo imaging at two distinct time points (P21, P56), representing a presymptomatic as well as an advanced disease stage. In addition, in vivo imaging was corroborated by combining ex vivo MRI measurements and matching comprehensive histological characterization of myelin, iron, and axonal directionality in prototypical wm regions.

2. Material and methods

2.1. Animals and experimental design

Transgenic MBP29-hα-syn mice were compared to age-matched non-transgenic littermates. The model was initially described by Shults et al. in 2005 and shows a high expression of human α-syn in oligodendrocytes (Shults et al., 2005). All mice were bred on a B6D2F1 background and kept under standard animal housing conditions with a 12-h dark/light cycle and free access to food and water. Transgenic (n = 10; f = 6; m = 4) and control (n = 7; f = 3; m = 4) animals underwent longitudinal MRI measurements (Suppl. Fig. 1 B) at the postnatal age of 21 days (P21) and 56 days (P56). According to the European guidelines for the humane treatment of animals (ref. # 55.2-2532-2-450), mice were sacrificed at P56 under anesthesia and transcardially perfused using precooled 0.9% sodium chloride solution. Subsequent to fixation in 4% paraformaldehyde (PFA), the brains of 10 mice (controls n = 4, MBP29-hα-syn n = 6) were additionally scanned ex vivo. Afterwards, all brains were dehydrated in 30% sucrose for at least 72 h. For histological analysis, hemispheres were cut coronally into 40 µm thick sections and stored in cryoprotect solution at 4 °C until further histological characterization.

2.2. MRI scanning

All measurements were performed on a preclinical 7 T MRI scanner (ClinScan 70/30, Bruker, Ettlingen, Germany) using a dedicated mouse brain coil. For in vivo imaging, animals were anaesthetized using 5% isoflurane and maintained at 1.5% isoflurane. Respiration was monitored by breath sensors and kept constant throughout the entire imaging procedure. To reduce movement artifacts, mice were secured using custom-built 3D lateral head fixation bars, as previously described by Schweser et al. (Schweser et al., 2018). Body temperature was stabilized using a heating circulator bath (Thermo Fisher Scientific, Waltham, USA). Prior to ex vivo scanning, brains were washed in phosphate buffered saline (PBS) for 1–2 h in order to minimize signal interference due to PFA treatment (Dusek et al., 2019). Afterwards, all specimens were placed separately inside a conical tube (width: 15 mm, length: 118 mm; Sarstedt, Nümbrecht, Germany) filled with Fomblin® Y (perfluoropolyether, Sigma, St. Louis, USA). In order to prevent specimen movement during the MRI scan, the end of a Pasteur pipette was inserted into the tube. The longitudinal axis of every conical tube was aligned parallel to the magnetic field B0. In vivo susceptibility maps were calculated using a three dimensional (3D) gradient echo sequence (GRE) with a field-of-view (FOV) = (25.6 × 20.8 × 19.2) mm3, 0.1 mm3 isotropic voxel size, echo time (TE) = 6.7/13.3/20.0 ms, repetition time (TR) = 29 ms, bandwidth (BW) = 260 Hz/pixel, flip angle = 11°, and 3 averages. The total acquisition time (TA) was 45:36 min:sec. For T2-weighted imaging, a 2D turbo spin echo (TSE) sequence was used with FOV = (21 × 21 × 15) mm3, voxel size = (0.08 × 0.08 × 0.5) mm3, TE = 40 ms, TR = 5272 ms, BW = 130 Hz/pixel and TA = 6.26 min:sec. Ex vivo measurements were performed applying the identical 3D-GRE sequence as in vivo, but with adapted sequence parameters: FOV = (25.6 × 9.6 × 14.4) mm3, isotropic voxel size = 0.1 mm3, TE = 8.4/16.4/24.4/32.4 ms, TR = 43 ms, BW = 150 Hz/pixel, flip angle = 15°, 6 averages and TA = 46:44 min:sec. T2-weighted imaging was conducted using the identical TSE sequence and parameters as in vivo, except for a smaller number of slices, resulting in a FOV = (21 × 21 × 10) mm3, TR = 3.690 ms and TA = 4:30 min:sec.

2.3. Image processing and analysis

To reconstruct susceptibility maps, the phase maps of each echo time were processed using a Laplacian-based phase unwrapping algorithm (Schoefield and Zhu, 2003; Li et al., 2014) in order to remove phase wraps. A brain mask was semi-automatically calculated using morphological operations in Matlab (MathWorks, Natick, USA). Subsequently, the quality of each mask was visually assessed and, if necessary, manually adjusted. To eliminate background fields, V-SHARP was used (Wu et al., 2012). Finally, the susceptibility map was obtained using STAR-QSM (Wei et al., 2015). An echo-time-averaged susceptibility map was created by the squared echo time and the squared signal magnitude as a weight (Chen et al., 2018). All susceptibility values are depicted in parts per billion (ppb). Imaging of one transgenic animal showed major technical artifacts and was excluded from further analysis.

To quantify myelination deficits in MBP29-hα-syn mice, signal intensity ratio was calculated in T2-weighted MRI images and susceptibility was measured using QSM. Two representative wm regions (corpus callosum (CC) and anterior commissure (AC)) as well as the
striatum (ST) as a mixed grey-white matter structure were chosen as regions of interest (ROI). Additionally, corresponding gm regions (cortex, CTX) adjacent to each ROI were used as a reference. ROIs were segmented manually and separately for P21, P56, and ex vivo images using the MITK Software (Medical Imaging Interaction Toolkit, 2018.04.2, DKFZ, Heidelberg, Germany). The susceptibility value of each ROI was calculated relative to adjacent gm (wm - gm), while T2-weighted metrics are displayed as signal intensity ratio (wm / gm).

2.4. Histochemical staining

2.4.1. Heidenhain Woelcke

For all histological analyses, tissue was stained in parallel to ensure optimal comparability. For Heidenhain Woelcke myelin staining, free floating sections were processed using a modified version of the previously described protocol by Hutchins and Weber (Hutchins and Weber, 1983). Prior to staining, tissue was incubated in 2.5% iron alum (ferric ammonium sulfate) for 1 h and afterwards briefly rinsed in distilled water. Subsequently, sections were transferred into hematoxylin staining solution (1% hematoxylin, 20% lithium carbonate) for 1 h before excess staining was removed by washing in distilled water. In a final step, sections were mounted on glass slides using Neo-mount® (Merck Millipore, Darmstadt, Germany).

2.4.2. DAB intensified Perl’s Prussian blue staining

Sections were mounted on glass slides and dried for at least 5 days at 30 °C. The staining was performed using a modified version of the protocol by Nguyen-Legros and colleagues (Nguyen-Legros et al., 1980). At first, slides were incubated in a freshly prepared 10% potassium ferrocyanide solution for 5 min and subsequently transferred into the Pears solution (7.3, 10% potassium ferrocyanide and 10% HCl) for 20 min. Afterwards, sections were briefly rinsed in distilled water and the Pears reaction was intensified using a 3.3′ dianisobenzidine (DAB) solution (40 mg DAB in 100 ml PBS and 80 µl H2O2 (30%) for 17 min. Prior to embedding, slides were thoroughly washed in PBS for several times. Due to insufficient perfusion, one control mouse showed major staining artifacts and was excluded from further analysis.

2.5. Immunostaining

For immunofluorescent stainings, tissue was pretreated using citrate buffer (30 min, 80 °C) for antigen retrieval. Blocking was performed for 1 h using TBS supplemented with 3% donkey serum and 0.3% Triton X-100. Subsequently, sections were incubated over night at 4 °C with the respective primary antibodies: non-phosphorylated mouse-anti-neurofilament heavy chain (NFH; 1:1000; BioLegend, San Diego, USA), rat-anti-human α-syn (1:200; Enzo Life Sciences, Lörrach, Germany), and rabbit-anti-Olig2 (1:500; Millipore, Burlington, USA). Secondary antibodies were species-specific and labeled with Alexa-488 or Alexa-568 (both 1:1000, Life Technologies, Carlsbad, USA), respectively. DAPI was used as a nuclear marker (1:10000, Sigma, St. Louis, USA). Sections were mounted on glass slides using ProLong™ Gold Antifade Mountant (Invitrogen, Thermo Fisher Scientific, Waltham, USA).

In order to analyze expression of iron within oligodendrocytes, sections were stained with a DAB intensified Perl’s Prussian blue method as indicated above, and afterwards labeled with rabbit-anti-Olig2 (1:500; Millipore, Burlington, USA). Therefore, tissue was pretreated using citrate buffer (30 min, 80 °C) for antigen retrieval. Sections were incubated with 0.6% H2O2 in Tris-buffered saline (TBS) for 1 h to repress endogenous peroxidase activity. Blocking was performed for 2 h using TBS supplemented with 3% donkey serum and 0.3% Triton X-100. Subsequently, sections were incubated over night at 4 °C with the primary antibody. Chromogenic immunodetection was achieved using anti-rabbit alkaline phosphatase kit and blue alkaline phosphatase substrate kit (Vector laboratories, Burlingame, USA).

2.6. Microscopy and quantification

To characterize myelin levels and iron deposition in MBP29-α-syn mice, imaging was performed using a Zeiss Imager M2 microscope. Images were acquired with a 10-fold magnification and equal exposure time using the Stereo Investigator Software (Stereo Investigator 10.04, MicroBrightField, Williston, USA). Fiji was used to calculate the optical density for distinct brain regions (Suppl. Fig. 1 A; CTX, CC, AC, ST) in three coronal sections (Bregma 0.97 mm–0.37 mm) per animal. Visualization of axonal directionality and α-syn imaging was assessed using a ZEISS LSM 780 confocal scanning laser microscope (63× PL APO oil objective).

2.7. Statistics

Datasets were analyzed using SPSS (IBM SPSS Statistics V.21, New York, USA). Graphs are presented as mean ± standard deviation (SD). SD is calculated from the ROI-averaged values. A Shapiro-Wilks test was applied to all datasets to assess normal distribution. Group comparisons of normally distributed values were performed using a t-test. A Mann-Whitney-U test was applied if the assumption of normal distribution was not given. A two-way analysis of variance (ANOVA) was used to assess the effect of group and age as well as the interaction of both factors on susceptibility and signal intensity, as well as the effects of group and directionality on susceptibility. Correlation between susceptibility and optical densities was analyzed using the non-parametric Spearman correlation. To assess the effect of iron deposition on susceptibility, we performed partial correlation of susceptibility and myelin optical density, controlling for iron optical density using zero-order correlations and bootstrapping. To assess sensitivity and specificity of QSM and T2-weighted MRI, we performed a ROC analysis. P values ≤0.05 were considered statistically significant (**P ≤0.05, ***P ≤.01, ****P ≤.001 and ****P ≤ .0001).

3. Results

3.1. Pronounced myelin deficit in white matter regions of MBP29-α-syn transgenic mice

Since MSA pathology is characterized by profound demyelination, we asked whether myelin pathology is present in different wm regions of MBP29-α-syn mice. In order to assess the regional level of myelin, we analyzed coronal brain sections of 8-week old transgenic (n = 10) and control (n = 7) mice. Histological myelin staining of Heidenhain Woelcke (Fig. 1 A, B) revealed a severe myelin deficit in the forebrain of MBP29-α-syn mice. In detail, optical density in MBP29-α-syn mice was significantly decreased by 52% in the CC and the AC. The reduction in the wm within the ST was decreased to 62% (Suppl. Fig. 1 C).

We identified a pronounced human α-syn expression within oligodendrocytes in wm regions of MBP29-α-syn mice, whereas, only moderate levels of human α-syn were detected in the adjacent gm (Suppl. Fig. 2). α-Syn expression was absent in non-transgenic controls (data not shown). Expression of human α-syn is predominantly observed in the cytoplasm of oligodendrocytes, forming a characteristic “half-moon” shape (Suppl. Fig. 2 C). However, single cells within the CC and the AC showed a nuclear α-syn expression as well (Suppl. Fig. 2 A, B; yellow).

3.2. Loss of susceptibility contrast in MBP29-α-syn mice

To examine whether QSM is able to reflect the myelin level and the degree of myelin deficit over time, longitudinal imaging was performed at two defined time points. Mice were imaged at P21 representing a pre-symptomatic stage, and at P56 already showing motor deficits reflecting a more advanced stage of disease (Shults et al., 2005). In contrast to controls, MBP29-α-syn animals exhibit a very prominent
loss of susceptibility contrast among wm regions (Fig. 2 A, D). We compared MBP29-hsa-syn and control mice for both time points, separately. At the pre-symptomatic stage (P21; Fig. 2 B), an increase of susceptibility contrast was measured in MBP29-hsa-syn mice (n = 10) compared to controls (n = 7) by 23% (controls: −8.61 ± 1.57 ppb, MBP29: −6.62 ± 1.67 ppb) and 17% (controls: −21.98 ± 3.52 ppb, MBP29: −18.17 ± 5.45 ppb) in the CC and the AC of MBP29-hsa-syn mice, respectively. However, significance level was not reached in the AC region. Notably, QSM measurement at P56 depicted a pronounced loss of diamagnetic susceptibility in MBP29-hsa-syn mice (Fig. 2 E). Particularly, a significant increase of susceptibility by 48% (controls: −19.89 ± 2.05 ppb, MBP29: −10.42 ± 1.82 ppb) in the CC and by 38% (controls: −31.41 ± 0.99 ppb, MBP29: −19.72 ± 1.02 ppb) in the AC was observed in MBP29-hsa-syn mice compared to controls. Development of magnetic susceptibility relative to adjacent gm over time is shown in Fig. 2 C and F. Overall, an increased susceptibility contrast was observed from P21 to P56 in the selected wm regions (CC; Fig. 2 C and AC; Fig. 2 F) for both groups. Development of susceptibility over time was significantly influenced by group- and time- as well as by the interaction of both variables in both regions (CC: F = 23.01, p ≤ .0001; AC: F = 9.86, p = .004). Thus, MBP29-hsa-syn mice develop a significantly lower increase in susceptibility compared to controls. In order to improve the signal-to-noise ratio (SNR), in vivo QSM scans were complemented by ex vivo imaging. Subsequent to tissue collection and following fixation, we performed ex vivo QSM measurements of MBP29-hsa-syn mice (n = 6) and controls (n = 4). Matching the in vivo measurements, ex vivo susceptibility maps highlight reduced susceptibility contrast between wm and gm in MBP29-hsa-syn mice at P56 (Fig. 2 G). Overall, magnetic susceptibility of the wm regions is increased by 78% in the CC, 64% in the AC and 72% in the ST. Susceptibility values differ from −24.32 ± 4.83 ppb in control to −5.45 ± 0.59 ppb in transgenic mice for the CC, from −31.53 ± 5.15 ppb to −11.25 ± 1.42 ppb in the AC, and from 1.83 ± 2.11 ppb to 6.63 ± 1.47 ppb in the ST (Fig. 2 H). ROC analysis and youden indices (Fig. 3 A) revealed that using a cut off of −14.04 ppb in CC and of −23.63 ppb in AC provides 100% sensitivity and specificity to discriminate between MBP29-hsa-syn mice and controls (AUC = 1.0, P ≤ .001). The ST (AUC = 0.76) did not reach a significant level.

3.3. T2-weighted assessment of myelination reveals reduced grey-white matter contrast in MBP29-hsa-syn mice

In order to corroborate the findings observed using QSM measurements, T2-weighted images were acquired parallel to the susceptibility maps. Identical to the QSM analysis, MBP29-hsa-syn and control mice were scanned in vivo at a pre-symptomatic (P21) and an advanced stage (P56). Representative T2-weighted images of control mice show well-defined wm regions, whereas grey-white matter contrast appears nearly vanished in MBP29-hsa-syn mice at P21 (Fig. 4 A) and P56 (Fig. 4 D). In detail, quantification revealed a significant decline of grey-white matter contrast in MBP29-hsa-syn (n = 10) compared to control mice (n = 7) in both ROIs, at P21 (Fig. 4 B) and at P56 (Fig. 4 E). Precisely, the signal intensity was reduced by 26% in the CC and by 24% in the AC at P21, while we observed a reduction of 34% in the CC and of 35% in the AC at P56. Signal intensity in both groups at two different time points relative to CTX is shown in Fig. 4 C and F. While both variables either time or group significantly altered the signal-intensity-ratio, the interaction of both variables did not reach significance (CC: F = 0.009, p ≤ .925 AC: F = 1.92, p = .18). (CC; Fig. 4 C and AC; Fig. 4 F). Overall, T2-weighted imaging showed a significantly lower variation between MBP29-hsa-syn and control mice in comparison to QSM (Fig. 5). ROC analysis and youden indices (Fig. 3 B) showed that using a cut off of 0.7 in the CC and the AC provides 100% sensitivity and specificity to discriminate between MBP29-hsa-syn mice and controls (AUC = 1.0, P ≤ .001). The ST (AUC = 0.78) provides 78% sensitivity and 86% specificity, however, not reaching significance level.

3.4. Intraregional heterogeneity of magnetic susceptibility in white matter tracts due to axonal directionality

Interestingly, not only a difference in susceptibility between regionally different wm tract was observed, but also a high variation of susceptibility within the identical wm tract was detected. To assess this intraregional heterogeneity, the AC region was divided into the olfactory limb (oAC; black arrows) and the corpus (cAC; white arrows) as illustrated in Fig. 6 A and C. For in vivo QSM analysis, a significant gain of susceptibility in MBP29-hsa-syn mice was found in both subregions of
the AC (Fig. 6 B). Overall, we calculated an increase of 37% (controls: \(-31.64 \pm 1.08\) ppb, MBP29: \(-20.07 \pm 1.17\) ppb) and 44% (controls: \(-12.47 \pm 1.93\) ppb, MBP29: \(-7.02 \pm 0.98\) ppb) in the oAC and cAC of MBP29-hα-syn mice, respectively. For ex vivo analysis, susceptibility values of the MBP29-hα-syn mice were considerably higher compared to the controls as well (Fig. 6 D). Accordingly, an increase of 64% (controls: \(-31.53 \pm 5.15\) ppb, MBP29: \(-11.25 \pm 1.42\) ppb) was measured in the oAC, while an increase of 78% (controls: \(-21.81 \pm 1.14\) ppb, MBP29: \(-4.9 \pm 1.92\) ppb) was detected in the cAC. Furthermore, we observed an extensive variability of susceptibility within the AC region when comparing oAC and cAC of the identical animal, albeit, the difference appeared less pronounced in the in vivo study (Fig. 6 B). Since this intraregional variability was not observed in the AC (suppl. Fig. 3) we assumed that the magnetic susceptibility contrast of the CC (C) and the AC (F) relative to adjacent grey matter over time. (G) Representative ex vivo susceptibility maps exhibited a superior quality compared to the in vivo study. (H) MBP29-hα-syn mice (n = 5) showed significantly altered magnetic susceptibility in all regions of interest (CC, AC and striatum (ST)) compared to control mice (n = 4). QSM values are expressed in parts per billion (ppb). Data represent mean ± SD; B, E, H: t-test; C, F: two-way ANOVA; **P ≤ .01; ***P ≤ .0001.

3.5. Distinct intracerebral iron deposition in MBP29-hα-syn mice

Since iron deposition was observed in post-mortem analysis of MSA patients, we asked whether iron pathology is present in MBP29-hα-syn mice (DEXTER et al., 1991; Jellinger, 2003; Visanji et al., 2013). Indeed, DAB intensified Perl's Prussian Blue (PPB) staining revealed a strong increase in color intensity in MBP29-hα-syn mice (Fig. 7 B) compared to controls (Fig. 7 A), which was spatially restricted to wm regions. In addition to wm associated extracellular iron deposition, accumulation of iron was confirmed immunohistochemically within oligodendrocytes (Suppl. Fig. 4). Statistical analysis of PPB showed a significant increase of optical density in the CC (31%), the AC (24%), and the STwm (50%) (Fig. 7 C). However, analysis of the STc (8%) representing a mixed grey-white matter region, did not reach significance (p = .313).

3.6. Susceptibility contrast correlates with myelin and iron

Even though the origin of tissue contrast in QSM is rather complex and still under debate, numerous studies suggested a proportional contribution of myelin and iron (Liu et al., 2011; Langkammer et al., 2012). Since both are differentially present in MBP29-hα-syn and control mice, we asked to which extent iron accumulation and/or
myelin loss contribute to the detected susceptibility contrast. Consequently, the optical densitometry of myelin and iron stainings was correlated with the corresponding susceptibility values and analyzed separately for the CC, AC, and ST (Fig. 8). Note that for the analysis of the ST, the STt values were used, as QSM resolution does not allow to distinguish between wm striae and surrounding gm. Overall, we observed a negative correlation between myelin and susceptibility values in the CC ($r_{SP} = -0.779; \ p = .0004$) and AC ($r_{SP} = -0.715; \ p = .0026$), indicating an effect of reduced myelin density on magnetic susceptibility. A positive correlation between iron and susceptibility in the CC ($r_{SP} = 0.734; \ p = .0023$) and AC ($r_{SP} = 0.693; \ p = .0054$) suggests a direct correlation between iron load and susceptibility.

Nevertheless, analysis of the ST did neither reach significance for the correlation with myelin nor with iron. Given the high degree of correlation between susceptibility of myelin or iron we aimed to further separate both to analyze their contribution towards the QSM contrast. In order to control for the effect of iron, we applied a partial correlation between myelin and susceptibility, revealing a correlation for the CC ($-0.755, \ P < .0001$) and ST ($-0.590, \ P < .034$), while the correlation disappears in the AC after controlling for iron density. Taken together, this suggests that myelin plays a more important role in susceptibility contrast in comparison to iron deposition.

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Fig. 3. ROC analysis of QSM and T2-weighted imaging. (A) Magnetic susceptibility measured in the corpus callosum (CC; □) and anterior commissure (AC; Δ) in vivo at the age of 56 days distinguishes MBP29-ha-syn (n = 10) from control mice (n = 7; AUC = 1, $P \leq .001$). ROC curve of the striatum (ST; o) did not reach significance (AUC = 0.76; $P = .079$). (B) T2-weighted MR imaging in the CC and AC in vivo at P56 dissociates MBP29-ha-syn (n = 10) from control mice (n = 7; AUC = 1, $P \leq .001$). ROC curve of ST did not reach significance (AUC = 0.78; $P = .064$).

Fig. 4. T2-weighted analysis of MBP29-ha-syn mice. Representative T2-weighted images of the identical MBP29-ha-syn and control mice (Bregma 0.13 mm) at the age of 21 (P21; A) and 56 days (P56; D). Signal intensity (int.) ratio of the corpus callosum (CC) and the anterior commissure (AC) relative to the cortex (B, E). Compared to controls (n = 7), MBP29-ha-syn mice (n = 10) show significantly reduced grey-white matter contrast already at P21 (B) as well as at P56 (E). Signal intensity ratio of the CC (C) and the AC (F) relative to the cortex over time. Data represent mean ± SD; B, E: t-test; C, F: two-way ANOVA; ****$P \leq .0001$. 

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4. Discussion

Demyelination is a well described neuropathological hallmark of MSA and its respective models, in particular the MBP29-hα-syn mice. Despite recent advances in neuroimaging, the visualization of myelin deficit in vivo still remains difficult. Here, we demonstrate that QSM is a very sensitive tool both for myelin and iron imaging in vivo. In detail, we describe a profound myelin deficit in MBP29-hα-syn mice being paralleled by widespread iron accumulation, spatially restricted to wm regions. We were able to visualize and quantify these neuropathological changes in vivo and ex vivo using QSM and T2-weighted MR imaging. In fact, QSM imaging of the CC and AC allowed us to distinguish MSA mice from controls with a sensitivity and specificity of 100%. In addition, a significant correlation between magnetic susceptibility and optical densitometry of myelin and iron was observed within selected wm regions. Furthermore, we provide evidence that QSM is complementary to routine T2-weighted MR imaging regarding the detection of myelin deficit and iron accumulation.

In a first step, we analyzed α-syn expression and myelination in 8-week old MBP29-hα-syn and control mice. Consistent with previous studies, we detected abundant expression of human α-syn in the CC and AC, whereas, only moderate levels were observed in the ST and CTX of MBP29-hα-syn mice (Shults et al., 2005). Myelin intensity was severely reduced in MBP29-hα-syn mice compared to controls. Only a minor variability of about 8% was observed between the CC, the AC, and the STwm of the identical group, indicating that in general wm regions of the forebrain were affected equally in terms of myelin loss. Since recent studies in human neonates as well as shiverer mice identified myelin as the dominating source of diamagnetism and susceptibility contrast in wm (Liu et al., 2011; Zhong et al., 2011), we imaged MBP29-hα-syn and control mice at two different time points, a pre-symptomatic (P21)
and an advanced (P56) disease stage, in order to assess the temporal pattern of myelin density. Overall, both groups showed a trend towards increasing susceptibility over time indicating the presence of continuously progressing myelination between P21 and P56. However, the increase of susceptibility among the control group was by far higher than in the transgenic group implying an impaired formation or accelerated turnover of myelin in MBP29-hα-syn mice during postnatal development. The murine formation of myelin sheaths starts around P14, followed by the most rapid phase of myelination between P14 and P45, and continues at a lower rate until P240 (Sturrock, 1980; Vincze...
et al., 2008). Thus, the temporal pattern of susceptibility contrast most likely reflects the physiological generation of myelin in wm regions of the forebrain. Image quality between scans obtained at P21 and P56 was variable probably resulting from the difference in myelin density at the distinct time points. Myelin was identified to be an important source of magnetic susceptibility contrast, therefore, the image quality as well as signal-to-noise ratio may deteriorate in the absence of myelin (Liu et al., 2011). Confirming previous studies we observed a reduced susceptibility contrast and image quality at P21, reflected by the onset of myelination associated with a rather low myelin density (Lodygensky et al., 2012; Argyridis et al., 2014).

In line with recent studies, we observed high regional variability with increased susceptibility values within the AC region, suggesting an earlier and more dense myelination of this region compared to the CC (Lodygensky et al., 2012; Argyridis et al., 2014). Importantly, we detected an increase of susceptibility in vivo and ex vivo in the CC, the AC, and the ST in MBP29-ha-syn mice compared to the controls resulting in a reduced contrast between wm and surrounding gm. A similar observation was previously made in murine models characterized by low myelin content. Shiverer mice show reduced levels of myelination due to mutant MBP, while cuprizone fed mice present an acute toxic demyelination after feeding of the copper chelator (Liu et al., 2011; Wang et al., 2019). Taken together, this strongly supports the notion that the increased magnetic susceptibility reflects indeed the myelin deficit in MBP29-ha-syn mice. In addition to the regional variation of susceptibility between different wm tracts, we observed extensive intraregional susceptibility heterogeneity within the AC region, where the oAC consistently showed more negative values and a higher susceptibility contrast compared to the CAC. In contrast, we neither detected differences in myelin optical density between both subregions, nor susceptibility heterogeneity in the CC. Since axons within the oAC are oriented parallel towards B0, while they run perpendicular in the CAC, the findings of the present study are consistent with the assumption that QSM is influenced by axonal directionality (He and Yablonskiy, 2009; Wharton and Bowtell, 2012; Wharton and Bowtell, 2015). In line with Lancione and colleagues, we observed a more pronounced diamagnetic susceptibility in fiber bundles comprising axons oriented perpendicular relative to B0, compared to a parallel orientation (Lancione et al., 2017).

In terms of diagnostic accuracy in MSA, routine T2-weighted MRI reached only a moderate sensitivity or specificity (Schrag et al., 2000; Savoiardo, 2003; Lee et al., 2004). Using T2*-weighted MRI, we observed a pronounced and significant reduction of grey-white matter contrast in MBP29-ha-syn mice at both time points. In line with a recent study using the cuprizone model, we demonstrate that QSM yields higher discrimination between MBP29-ha-syn mice and controls compared to routine T2*-weighted MRI (Wang et al., 2019).

Even though our results indicate that myelin is the predominant source of grey-white matter contrast in QSM, it has to be considered that iron deposition is also a potential covariable to alter susceptibility contrast (Langkammer et al., 2012; Zheng et al., 2013; Sun et al., 2015). Iron is widely known for its pivotal role in brain development, since the formation of myelin is severely impaired in the absence of iron (Yu et al., 1986; Todorch et al., 2009). Nevertheless, increased iron accumulation ultimately leads to oxidative stress and is therefore closely linked to a variety of neurodegenerative processes (Zecza et al., 2004; Gaeta and Hider, 2005). Using an iron regulatory protein 2 knockout approach, LaVau et al. created a mouse model developing extensive iron deposition, predominantly in wm regions. These mice show a severe motor phenotype similar to parkinsonian syndromes including bradykinesia and tremor (LaVau et al., 2001). In our study, we detected increased iron levels in wm regions i.e. CC, AC, and STwm of MBP29-ha-syn mice. Moreover, these regions show considerably more α-syn expression than the gm or mixed gm and wm regions in which iron load was unchanged compared to controls. Interplay of α-syn and iron is further highlighted by previous findings showing a ferrireductase activity of α-syn, while α-syn expression is simultaneously regulated by iron-mediated translational control (Davies et al., 2011; Rogers et al., 2011; Brown, 2013). Taking together, this suggests a tight spatial and functional association between myelin, iron and α-syn.

Since both iron and myelin affect magnetic susceptibility contrast (Liu et al., 2011; Langkammer et al., 2012; Argyridis et al., 2014; Sun et al., 2015), we aimed to further dissociate both variables. Therefore, we correlated susceptibility values with the optical densitometry of myelin and iron. Both tissue variables showed a high correlation with magnetic susceptibility in the CC and AC. Interestingly, this correlation between susceptibility and myelin remained when applying a partial correlation to eliminate iron as a confounder, further underlining the strong influence of myelin towards magnetic susceptibility. In addition, this finding is corroborated by recent QSM based studies analyzing demyelination models without iron accumulation (Connor et al., 1993; Liu et al., 2011; Lodygensky et al., 2012; Wang et al., 2019). Myelin density was assessed in shiverer and cuprizone fed mice via susceptibility mapping and showed significantly increased magnetic susceptibility of wm regions most likely due to reduced myelin density. However, no correlation between imaging and histological data was generated in both models (Liu et al., 2011; Lodygensky et al., 2012; Wang et al., 2019). Previous studies revealed a strong correlation of myelin and magnetic susceptibility in wm regions of C57BL/6 mice and wistar rats (Lodygensky et al., 2012; Argyridis et al., 2014). A correlation of iron and magnetic susceptibility was demonstrated in the basal ganglia of patients with multiple sclerosis (MS) and healthy controls (Langkammer et al., 2012; Zheng et al., 2013; Sun et al., 2015). To our knowledge a simultaneous and significant correlation of both, iron and myelin vs. susceptibility was so far solely reported by Hametner et al. who analyzed healthy human subjects (Hametner et al., 2018). Nevertheless, a relationship between iron and susceptibility was solely detected in gm regions, while the correlation in wm regions did not reach significance (Argyridis et al., 2014). Furthermore, Lodygensky et al. demonstrated an unaltered signal phase, and therewith likely an unaltered magnetic susceptibility, after iron extraction using an iron chelating agent (Lodygensky et al., 2012). Overall, these studies imply that iron deposition is likely to contribute to susceptibility contrast, but may play a less crucial role in wm regions, where susceptibility contrast is rather related to diamagnetic myelin. In contrast, we observed a pronounced deficit of myelin combined with progressive iron accumulation in wm of MBP29-ha-syn mice. Both processes similarly affect QSM, since myelin shows a negative magnetic susceptibility due to its diamagnetic properties, while susceptibility is increased both by myelin loss and accumulation of paramagnetic iron (Liu et al., 2011; Haacke et al., 2015). However, the determination regarding their precise proportional contribution to susceptibility contrast will require quantitative biochemical analysis of iron and myelin content of the respective regions.

QSM has recently been employed in different studies to assess white matter irregularities in the cuprizone mouse model as a preclinical model of MS (Lee et al., 2012; Wang et al., 2019). MS is a chronic primarily autoimmune-mediated, neurological disease, neuropathologically characterized by multifocal formation of circumscribed demyelinating lesions (Meinl et al., 1993; Haider et al., 2016). While MS patients present with a rather early onset of symptoms at 30 years of age and different courses of disease, MSA is referred to as late onset, but rapid progressive disorder, characterized by diffuse inflammation and demyelination (Wenning et al., 2013; Etile et al., 2016; Krismer and Wenning, 2017; Louapre et al., 2017; Oh et al., 2018; Hoffmann et al., 2019). Intriguingly, even α-syn immunoreactivity, representing the neuropathological hallmark of MSA, was detected in MS lesions associated with inflammatory activity (Papp et al., 1989; Lu et al., 2009). Despite the neuropathological overlap, there are some crucial differences between both disorders. While the inflammatory component of MSA is characterized by upregulation and activation of microglia in the
wm, the immune-response in MS is predominantly T-cell mediated (Meinl et al., 1993; von Essen et al., 2018; Hoffmann et al., 2019). MS pathology is further defined by widespread oligodendroglial loss, in contrast post-mortem analysis of MSA patients and MBP29-hy-syn mice showed unchanged or even increased numbers of oligodendrocytes, respectively (Rodriguez et al., 2014; Salvesen et al., 2015; Nykjaer et al., 2017). Additionally, a disturbed iron homeostasis has been observed in both disorders. Two studies detected increased iron accumulation in the substantia nigra, the pons, and the putamen in MSA (Dexter et al., 1991; Visanji et al., 2013). In contrast, decreased iron levels in the wm accompanied by elevated iron in the basal ganglia were measured in MS (Hametner et al., 2013; Stankiewicz et al., 2014; Bergsland et al., 2017). QSM has been used to assess iron accumulation in MS and may be a complementary method to conventional T2*-weighted imaging for monitoring disease progression or interventional response in patients with MS (Barkhof and Thomas, 2018).

In conclusion, we demonstrate that QSM is an excellent non-invasive marker for probing two hallmarks of MSA: demyelination and increased iron deposition in wm. Both processes correlate well and positively with an increased magnetic susceptibility. Translating these findings into the clinic may help overcoming the current challenge of a reliable early differential diagnosis of parkinsonin syndromes such as MS and sporadic PD.

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Declaration of Competing Interest

None.

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