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Postmortem and imaging based analyses reveal CNS decreased myelination in restless legs syndrome

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Abstract

Background—Restless legs syndrome (RLS) is a neurological disorder characterized by a strong urge to move the legs and has been linked in many studies with abnormally low brain iron. Iron deficiency is associated with hypomyelination in brains of animals. Therefore we hypothesized that a myelin deficit should be present in the brains of patients with RLS.

Methods—We performed western blot analysis on myelin isolated from RLS (n=11) and control (n=11) brain tissue obtained at autopsy for the expression of the integral myelin proteins, myelin basic protein (MBP), and proteolipid protein (PLP) and the oligodendrocyte specific enzyme 3'5'-cyclic nucleotide phosphohydrolase (CNPase). To expand the postmortem findings to *in vivo*, we analyzed the brains of RLS patients (n=23) and controls (n=23) using Voxel-based morphometry (VBM).

Results—The expression of MBP, PLP and CNPase in the myelin from RLS was decreased by approximately 25% (p<0.05) compared to controls. The amounts of transferrin (Tf) and H-ferritin (H-Frt) in the myelin fraction were also significantly decreased in RLS compared to controls. The imaging analysis revealed significant small decreases in white matter volume in RLS patients compared to controls in the corpus callosum, anterior cingulum and precentral gyrus.

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Conclusion—A decrease in myelin similar to that reported in animal models of iron deficiency was found in the brains of individuals with RLS. The evidence for less myelin and loss of myelin integrity in RLS brains, coupled with decreased ferritin and transferrin in the myelin fractions, is a compelling argument for brain iron insufficiency in RLS. These data also indicate the need to look beyond the sensorimotor symptoms that typically define the syndrome and its assumed relationship to the dopaminergic system. Understanding the full range of RLS pathology may help us better understand the complex, intermittent nature and diversity of the clinical features of RLS and expand our consideration of treatment options for RLS.

Keywords

myelin proteins; oligodendrocytes; iron; transferrin; ferritin; voxel-based morphometry

Introduction

Brain iron insufficiency is known to occur with restless legs syndrome (RLS), and the documented neuropathology of RLS largely matches that produced by inadequate brain iron levels. A consistent observation associated with brain iron deficiency is hypomyelination. For example, indirect biomarkers of hypomyelination in humans, such as increased latency of auditory brain stem potentials and visual evoked potentials, have been reported in iron-deficient children [1, 2]. In rats, the most frequently used animal model for iron deficiency, there is a decrease in myelin proteins (MBP and PLP), lipids (galactolipids and phospholipids) and cholesterol in iron-deficient animals compared to controls [3, 4]. Furthermore, genetic disruption resulting in decreased expression of the H-ferritin subunit in mice also results in a profile of hypomyelination similar to that seen with iron deficiency [4]. Placing rats on an iron-restricted diet, even after weaning (PNDs 21-63), was associated with a significant decrease in myelin proteins [5]. This latter study indicates that disruption of iron availability can impact myelin formation not only during peak periods of neonatal development but at later ages. Indeed, myelin may be much less static than traditionally considered as genes for myelin proteins and cholesterol synthesis have a clear circadian rhythm [6] and cholesterol synthesis is enriched in myelin and dependent upon iron status. Moreover, there is evidence for continuing development of myelination in the adult human brain [7] suggesting a continued iron requirement for maintenance of adequate myelin in the adult brain.

Myelin is synthesized in the brain by oligodendrocytes and these cells stain more strongly for iron than any other cell type in the brain [8]. Oligodendrocytes store iron predominantly in H-ferritin [9], and they are the only cells in the brain outside of the choroid plexus to make transferrin for mobilizing iron [10]. Unlike transferrin made in hepatocytes, however, transferrin made in oligodendrocytes is not secreted [11]. Enzymes for synthesizing myelin components are enriched in oligodendrocytes, and many of these, such as HMG-CoA reductase (the rate limiting step in cholesterol synthesis), require iron for bioactivity [12]. Considering the importance of iron in the process of myelin synthesis and the evidence for insufficient brain iron in RLS, we hypothesized that there would be decreased myelination in RLS brains.

To test our hypothesis, we performed both a postmortem analysis on myelin from RLS and control brains and voxel-based morphometry (VBM) on living subjects. This latter technique has been widely utilized to characterize cerebral gray and white matter differences associated with normal development, aging and neurological diseases using structural MRI scans. In contrast to methods that frame the search in terms of regions of interest, VBM can detect structural differences throughout the brain.

Materials and Methods

Brain tissue procurement

Frozen tissue from the frontal (Brodmann areas 9, 10) and temporal (Brodmann area 22) cortices was obtained from postmortem samples of RLS (n=11, all females) and age matched controls (n=11, all females). The control group consisted of individuals who lacked any significant neurological history. The tissue was obtained from the Harvard Brain Bank, a collection maintained by the RLS foundation. The clinical diagnosis of RLS was confirmed by a RLS expert board certified in Sleep Medicine who reviewed a detailed diagnostic questionnaire completed by the RLS individuals before their death. The age range of RLS patients at the time of death was 53-91 years, and the age range of controls was 58-87 years.

Isolation of crude myelin

Myelin was extracted from frozen brain tissue according to the method described by Norton and Poduslo [13] with the exception that the protocol was terminated after obtaining crude myelin. The crude myelin fractions were lyophilized and the dry weight of myelin was noted. An aliquot of dry myelin was suspended in ripa buffer (Sigma, Saint Louis, MO), kept on ice for 30 min and then centrifuged for 1hr at 100,000g at 4°C. The supernatant is collected and total Protein concentration was determined using BCA assay (Pierce, Thermochemical, Rockford, IL).

Western blot analysis

SDS-PAGE was performed on equal amounts of total protein (20ug) for quantification of oligodendrocyte specific enzyme 3'5'-cyclic nucleotide phosphohydrolase (CNPase) and myelin-related proteins, Myelin basic protein (MBP) and proteolipid protein (PLP) in the myelin. An equal amount of a pooled sample of myelin was included on each gel as a reference for comparing the relative amounts on each myelin protein. After quantification of the immunoblots for the above mentioned proteins, the intensity of each sample was expressed as percentage of the intensity of pooled sample.

Western blot analysis was also performed for quantitation of transferrin (Tf) and H-Ferritin (H-Frt) in the myelin. Each sample was denatured at 95°C for 5 min and applied to a 4-20% gradient polyacrylamide gel at 100 V for 1.05 h (NuSep gels, Austell, GA). Proteins were transferred from the gel onto 0.2µm pore size nitrocellulose membrane (Pall Corporation, Pensacola, FL), blocked for 1 h with 5% nonfat dry skimmed milk in Tris-buffered saline (TBS), and washed with TBST containing 0.05% Tween. The membranes were incubated overnight with a primary antibody. The primary antibodies used were anti-MBP (abcam, Cambridge, MA), anti-CNPase (abcam, Cambridge, MA), anti-PLP (Chemicon, Temecula, CA), anti-Tf (EY laboratories, San Mateo, CA) and anti H-Frt (Santa Cruz, Santa Cruz, CA). HRP-conjugated secondary antibodies (GE Health Care, Buckinghamshire, UK) were used at 1: 2500 dilution and incubated for 1hr at RT. The immunoblots were then washed with TBST and exposed to enhanced chemiluminescent detection solution (Perkin Elmer, Waltham, MA) for 1 min, scanned and analyzed using the Multigauge program (V3.0) (Fuji Film System).

Human subjects

Twenty three RLS (mean $\pm$ SD; 55.59 $\pm$ 13.16 yrs, 8 males, 15 females) and 23 age- and gender-matched normal subjects (mean $\pm$ SD; 55.97 $\pm$ 13.07 yrs, 8 males, 15 females) were included in this study. The RLS subjects were referred from the internal medicine clinics at Penn State Hershey Medical Center and the diagnosis was made by a board certified neurologist or pulmonologist experienced with RLS following the standard criteria [14].

Subjects were excluded from the normal cohort if they had any history of nervous system diseases or clinical findings, suggesting that RLS may be secondary to another illness. The severity of RLS symptoms was assessed using the International Restless Legs Syndrome Study Group (IRLSSG) Rating Scale [15] by a board-certified movement disorders neurologist on the same day of the MRI study. If subjects were taking medications to treat the symptoms of RLS, the IRLSS and the brain imaging were obtained at least one week after the medications were discontinued. This study was approved by the Institutional Review Board, and written informed consent was obtained from all subjects.

MRI data acquisition

MRI studies were performed on a 3.0 T scanner (Philips Medical, Netherlands) with an 8-channel phased array head coil for reception. Brain T1-weighted images of isotropic 1 mm voxel were acquired with a 3D MPRAGE (magnetization-prepared rapid-acquisition gradient echo) sequence with TR / TE = 9.87 / 4.59 ms, inversion time = 600 ms and flip angle = 8°.

Voxel-based morphometry (VBM)

Image statistical analysis was performed using the VBM5 toolbox (<http://dbm.neuro.uni-jena.de>) implemented in Statistical Parametric Mapping (SPM5, Wellcome Department of Imaging Neuroscience Group, London, UK); running on MATLAB 7.1 (The MathWorks, USA). To minimize potential bias between images of the cohort and anatomical template provided by SPM5, a study-specific template was generated using both RLS and control subjects [16, 17]. First, all images were transformed to standard space by using a 12-parameter affine model without any nonlinear factor to preserve morphology of the subjects [17, 18]. Subsequently, the transformed images were averaged and smoothed with a Gaussian kernel of isotropic 8-mm full-width at half-maximum (FWHM) [16]. The original T1 images were then normalized into MNI space via affine registration of brain image of each subject to the study-specific T1 image template and subsequent nonlinear iterations using 7×9×7 basis functions to account for global nonlinear shape differences [19]. Transformed brain images were resampled to a voxel size of 1×1×1 mm. All normalized images were automatically segmented using a cluster analysis to identify voxels belonging to particular tissue types [20]. Since our aim is to compare brain volumes between two study cohorts, the correction for volume changes from nonlinear registration was made by modulation [17]. Finally, the modulated white matter (WM) partition was smoothed with an 8-mm FWHM Gaussian kernel. WM masks based on the average of the smoothed and normalized WM segmentations were generated and applied prior to statistical analysis.

2.7 Statistical analysis

Statistical analysis of postmortem myelin data was carried out by Mann Whitney U test (one tail/two tail) using Graph Pad Prism 4 software. To minimize confounding effects such as age and gender, a paired *t*-test was used to compare brain morphology for age- and gender-matched pairs from the two groups.

RESULTS

Myelin analysis

The myelin protein profile revealed a significant decrease in the expression of the three proteins analyzed (Fig 1a-1c). The expression of MBP in RLS brains was 23.7% less than control brains ($p < 0.05$). The expression of PLP and CNPase was also significantly decreased (24.6% and 32% respectively; $p < 0.05$) in RLS compared to the controls.

Transferrin and H-Ferritin in the myelin fraction

As an indirect assessment of iron status in the white matter, we determined the concentrations of Tf and H-Frt in the crude myelin fraction of the RLS and control brains. Tf was decreased in the myelin from RLS brains by 26.7% compared to control ($p < 0.010$) and the amount of H-Frt was also significantly decreased (25.4%) in the RLS myelin compared to the controls ($p < 0.05$) (Fig 2a-b).

Voxel-based morphometric analysis

There was no difference in age (t -test; $t = 0.102$, $p = 0.92$) and gender (chi-square test; $p = 0.749589$) between the RLS and control groups. The average severity score of RLS subjects was 24.4 ± 6.1 and average disease duration was 12.6 ± 11 yrs. Eighteen RLS subjects were treatment-naïve and 5 subjects being treated with pramipexole or ropinirole discontinued their medication one week prior to MRI scanning (Table 1). The maximum age difference within the gender- and age-matched pairs used for these analyses was 3 yrs (mean difference $\pm$ SD; -0.4 ± 1.6 yrs). The results of the VBM analysis are summarized in Table 2. Significant regional reductions of WM volumes were observed in RLS compared to controls (paired t -test, corrected $p < 0.005$) in the corpus callosum, anterior cingulum and white matter regions adjacent to precentral gyrus.

Discussion

In this study we confirmed from both autopsy and *in vivo* studies our hypothesis that RLS brains have wide-spread impairment in myelin consistent with that seen with experimentally-induced brain iron deficiency in animals. The *in vivo* imaging studies used voxel-based analyses to reveal volume reductions in the corpus callosum and white matter regions adjacent to precentral gyrus. Brodmann area 9, in particular, showed significant volumetric reduction in the RLS which coincided with the brain area where tissue samples were taken for postmortem analysis. A decrease in volume in the areas near the precentral gyrus was also reported by Unrath et al [21].

Another area in which diminished white matter signal was detected in RLS brains was the dorsal medial corpus callosum. The corpus callosum is highly organized with fibers connecting specific cortical areas traveling through distinct callosal regions. The dorsal medial corpus callosum consists of myelinated fibers connecting primary motor and somatosensory motor [22]. It is not clear that these changes would have any clinical significance but recent human cortical stimulation studies, demonstrating that select areas of the parietal cortex interact with the presupplementary motor area to produce a compulsory “urge to move” and then subsequent “involuntary” movement [23, 24], may be relevant to our findings. The essential core feature of RLS symptoms is this overwhelming “urge to move,” which will produce an involuntary movement if the patient tries to resist moving [14]. Is it possible that changes in myelination along these interactive pathways might lower the threshold for expression of this symptom from these cortical sites? It may also be relevant to our imaging data that similar findings of a smaller mid-sagittal area of the corpus callosum have been reported for ADHD patients [25]. Similar to RLS, ADHD has also been found associated with reduced iron status with lower serum ferritin levels [26] and clinical improvement with iron supplementation [27]. The iron compromise in these disorders may overlap enough to produce some common neuropathology and possibly some common symptoms. The relationship of our data to the reports of increased incidence of RLS in multiple sclerosis [28-31] raises the interesting possibility that loss of myelin in some way contributes to expression of RLS symptoms.

The loss of integral myelin proteins as found in the postmortem study provides compelling evidence for impaired myelin in RLS. Moreover, the decrease in both Tf and H-ferritin, which are found in white matter predominantly in oligodendrocytes [9, 32], coupled with a decrease in the oligodendrocyte specific marker CNPase further indicate a compromise in iron acquisition and oligodendrocyte function. The decrease in Tf, ferritin and CNPase could occur for either or both of the following two reasons: first, there could be fewer oligodendrocytes in the white matter in RLS. A decrease in the number of oligodendrocytes in the white matter tracts following iron deficiency has been previously reported in the rat model of iron deficiency [33]. Second, the iron-deficient oligodendrocytes will have less H-ferritin and CNPase. The effects of cellular iron deficiency on Tf in oligodendrocytes are not known. In other tissues Tf would be expected to increase, but oligodendrocytes do not secrete Tf [11]. Decreased intracellular iron status in these cells might be expected to lead to the observed decreased Tf.

The overall picture of decreased expression of the integral myelin proteins MBP and PLP in white matter (isolated from the cerebral cortices) and the decrease in the CNPase, along with the decrease in H-ferritin and Tf, indicate a wide-spread alteration in myelin composition and oligodendrocyte function in the RLS brain. These results are consistent with iron deficiency effects in animals [4], providing further support that significant brain iron-deficiency occurs with RLS. The mechanism underlying the interrelationship between iron requirements of oligodendrocytes and myelin has yet to be definitively elucidated, but is likely a combination of iron requirements for synthesis and catabolism of myelin rich components such as cholesterol and lipids as well as the energy requirements of maintaining a membrane that has been estimated at 100X the weight of the cell body [9].

The results are particularly notable for decreased myelin in the RLS brains reported herein given the areas examined. The frontal and temporal cortices were chosen because they have no known relevance to the dopaminergic pathways considered important for RLS symptoms. Although the clinical significance of these findings is not clear, the myelin deficit in these regions demonstrates the very pervasive nature of the neuropathology of RLS. These myelin analyses argue that although some specific brain regions or specific pathology in a given brain region may relate more directly to certain symptoms or phenotypes, understanding the full range of RLS pathological may help us better understand the complex, intermittent nature and diversity of the clinical features of RLS and expand our consideration of treatment options for RLS. In particular, these data—indicating not only a widespread myelin deficit, but also an iron deficit—argue that we need to look beyond the sensorimotor symptoms that typically define the syndrome and its assumed relationship to the dopaminergic system.

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Western blot analyses of myelin-related proteins in RLS and control brain autopsies. MBP (1a), PLP (1b) and CNPase (1c) are significantly decreased in RLS. Solid line represents the median value and the error bars represent the SEM (*p<0.05).

Figure 2. Western blot analysis of transferrin and H-ferritin in the myelin fraction of RLS and control brains. The amounts of transferrin (2a) and H-ferritin (2b) in the myelin were significantly decreased in RLS compared to the control myelin (* $p < 0.05$).

Figure 3. Brain white matter areas (clusters) showed significant volume reduction detected by VBM in RLS group compared to the control group ($p < 0.001$, uncorrected) overlaid on axial T1-weighted images. T-score of clusters are color-coded in the image.

Table 1

Age (average, SD and range), gender, IRLS and medication history (the later for RLS only)

Subjects with MRI evaluations		
	Controls	RLS
Sample size	23	23
Mean age $\pm$ SD	55.5 $\pm$ 13.1	55.9 $\pm$ 13.0
Gender F:M	15:8	15:9
Mean IRLS $\pm$ SD		24.4 $\pm$ 6.1
(max-min IRLS)		Sep-36
number not previously treated		17

TABLE 2
Volumes of cluster with significant volume decrease in RLS compared to age- and gender-matched controls.

Cluster volume		Voxel level	Cluster location in MNI coordinate (mm)			Brain region (BA)	Side
(mm ³)	P (corrected)		x	y	z		
123	0.005	4.17	5	9	24	Corpus callosum	
181	0.002	4.34	10	-5	36	Adjacent to anterior cingulum (BA24)	R
386	0.005	4.40	-7	-4	34	Adjacent to anterior cingulum (BA24)	L
211	0.002	4.71	-40	-4	40	Adjacent to precentral gyrus (BA6)	L
354	0.001	5.77	47	11	9	Adjacent to precentral gyrus (BA44)	R
301	0.001	4.46	46	0	21	Adjacent to inferior frontal gyrus (BA9)	R
78	0.003	3.77	-22	-34	44	Adjacent to cingulate gyrus (BA31)	R

Each reported anatomical location exceeded a voxel-wise statistical threshold of $P < 0.001$ (uncorrected) and an extent threshold of $P < 0.05$ (corrected for repeated measures). MNI coordinates refer to the Montreal Neurological Institute space and the anatomical areas were denoted by Brodmann area (BA); R, Right; L, Left