Mitochondrial Ferritin in the Substantia Nigra in Restless Legs Syndrome

Amanda M. Snyder, BS, XinSheng Wang, MD, PhD, Stephanie M. Patton, PhD, Paolo Arosio, PhD, Sonia Levi, PhD, Christopher J. Earley, MB, BCh, PhD, FRCPI, Richard P. Allen, PhD, DABSM, FAASM, and James R. Connor, PhD

Abstract

Restless legs syndrome (RLS) is a neurological disorder that is thought to involve decreased iron availability in the brain. Iron is required for oxidative metabolism and plays a critical role in redox reactions in mitochondria. The recent discovery of mitochondrial ferritin (FtMt) provided the opportunity to identify a potential correlation between iron and mitochondrial function in RLS. Human substantia nigra (SN) and putamen autopsy samples from 8 RLS cases and 8 controls were analyzed. Mitochondrial ferritin levels in RLS SN tissue homogenate samples assessed by immunoblots had more FtMt than control samples (p < 0.01), whereas there were no significant differences in FtMt in the putamen samples. By immunohistochemistry, neuromelanin-containing neurons in the SN were the predominant cell type expressing FtMt. Staining in neurons in RLS samples was consistently greater than that in controls. Cytochrome c oxidase staining, which reflects numbers of mitochondria, showed a similar staining pattern to that of FtMt, whereas there was less immunostaining in the RLS cases for cytosolic H-ferritin. These results suggest that increased numbers of mitochondria in neurons in RLS and increased FtMt might contribute to insufficient cytosolic iron levels in RLS SN neurons; they are consistent with the hypothesis that energy insufficiency in these neurons may be involved in the pathogenesis of RLS.

Key Words: Ferritin, Iron, Mitochondrion, Neuron, Restless leg syndrome.

INTRODUCTION

Restless legs syndrome (RLS) is a sensorimotor disorder that may affect 5% to 10% of the population (1) and is more common in women than in men (2). Individuals with RLS report an urge to move the legs usually accompanied by abnormal sensations in their legs, particularly during rest; these symptoms are relieved by voluntary or involuntary movement. There is a circadian rhythm to the symptoms, with sensory symptoms and resulting movements increased in the evening and peak at night (3).

Restless legs syndrome has been closely associated with decreased concentration of iron in the brain and alterations in expression of iron management proteins. Both magnetic resonance imaging and transcranial sonography indicate that there is decreased iron in substantia nigra (SN) of RLS patients (4–6). Moreover, cerebrospinal fluid from RLS patients reflects an iron-deficient profile as demonstrated by decreased ferritin and increased transferrin (7). Indeed, decreased cerebrospinal fluid ferritin has been a consistent finding in early-onset RLS (7–10). In contrast, plasma iron, ferritin, and transferrin levels that reflect systemic iron status are in general within reference ranges in RLS patients (7, 9).

Within the brain, histological examination of the SN in autopsy tissue samples has suggested decreased iron in melanin-containing neurons in RLS compared with controls (8); immunostaining and quantitative analyses have revealed an increase in transferrin and decrease in ferritin and transferrin receptor (8, 10). This pattern of expression of transferrin and ferritin indicates cellular iron deficiency (11). Iron has many roles at the cellular level, but it is an essential component of many mitochondrial enzymes. It is a required cofactor for enzymes of the respiratory chain in complexes I to IV and can also regulate translation of the 75-kd subunit of complex I (12) and complex II (13). Iron is trafficked to the mitochondria by proteins of the adenosine triphosphate-binding cassette family (14). Recently, it has also been suggested that iron loading into the mitochondria can occur through mitoferrin, but this phenomenon has not yet been demonstrated in the brain (15). How iron is subsequently managed within mitochondria (i.e. storage and availability to proteins) is not understood. Inadequately managed iron can lead to oxidative stress through the generation of reactive oxygen species, which are produced by mitochondria themselves because of oxidative phosphorylation within the...
electron transport chain. Reactive oxygen species disrupt mitochondrial function; indeed, a considerable body of the literature has evolved around mitochondrial dysfunction in neurological disorders (16).

The availability of iron in cells is managed by sequestering it in ferritin. Sequestration of iron in ferritin has been shown almost universally among cell types to protect from iron-mediated oxidative stress. The mechanisms of sequestration of iron within the mitochondria are poorly understood. Recently, a mitochondrial ferritin (FtMt) has been identified. This protein has high-sequence homology to H-ferritin, including conservation of the ferroxidase center and activity (17, 18); however, it has a positively charged leader sequence that provides it with a means to access the mitochondria (17). Mitochondrial ferritin has been detected at very low levels in most organs including the brain (17), but levels are high in the testes in which mitochondria are abundant (18, 19). The function of FtMt has not been elucidated, and the iron content of FtMt has not been directly assessed in vertebrates, but in a yeast model, it plays a role in iron sequestration because up to 50% of mitochondrial iron has been associated with this protein (20). Overexpression of FtMt can draw iron from the cytosolic iron pool to the mitochondria and create cellular iron deficiency (21, 22). Moreover, FtMt prevents oxidative stress in a cellular model in which the mitochondrial iron chaperone frataxin is deficient (20). Despite its apparent role in cellular iron homeostasis, the regulation of FtMt differs from the cytosolic ferritins because FtMt does not contain an iron-responsive element in the mRNA (17). Thus, unlike cytosolic ferritin, it is not directly responsive to the cellular labile iron pool. The goal of the present study was to determine if there are differences in FtMt expression in RLS versus controls.

MATERIALS AND METHODS

Immunohistochemistry
Formalin-fixed tissue blocks from the SN of 6 RLS patients (aged 67–86 years) and 6 controls (aged 66–87 years) were obtained from the Harvard Brain Tissue Resource Center, Belmont, MA. All SN samples were from women. Formalin-fixed tissue blocks from the putamen were obtained from the same source; the latter blocks included 12 control samples (9 men; 3 women) and 8 RLS samples (all women). The age ranges of the putamen samples were 49 to 87 years for the controls and 53 to 86 years for the RLS cases. The tissue blocks were embedded in paraffin and sectioned at 10 μm.

Immunohistochemistry was used to detect FtMt, H-ferritin, and cytochrome c oxidase (COX). Briefly, the sections were deparaffinized and then hydrated. Neuromelanin was bleached from the SN sections by incubating the slides for 2 hours in 10% hydrogen peroxide, pH 7.4, according to published methods (23), and then washed with distilled water. The sections then underwent antigen retrieval with 10 mmol/L citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by exposure to methanol/hydrogen peroxide followed by blocking for 1 hour in 2% nonfat milk made in PBS. Tissue was incubated overnight with mouse anti-human FtMt antibody (from Sonia Levi) (22), mouse anti-human monoclonal antibody for H-ferritin (from Paolo Arosio and Sonia Levi) (24), or a polyclonal rabbit anti-COX antibody (ab66739, Abcam, Cambridge, MA) diluted 1:200 in PBS containing 1% nonfat milk and 0.1% of Triton-X. Control staining was performed on sections lacking the primary antibody.

Sections were washed in PBS and then incubated for 1 hour with biotinylated anti-mouse secondary antibody (Vectastain Elite ABC kit, PK-6102, Vector Labs, Burlingame, CA) for FtMt and H-ferritin or biotinylated anti-rabbit secondary antibody (Vectastain Elite ABC kit, PK-6101, Vector Labs) for COX diluted 1:200 in PBS containing 1% nonfat milk and 0.1% of Triton-X. Control staining was performed on sections lacking the secondary antibody. After washing in PBS, sections were incubated with the AB Complex for 1 hour. Sections were then exposed to activated 3,3′-diaminobenzidine (SK-4100, Vector Labs) with nickel chloride enhancement, washed, dehydrated, and coverslipped.

Quantitative Analysis

Fresh-frozen samples of human SN and putamen were obtained from the Harvard Brain Tissue Resource Center. Approximately half of the tissue samples were from the same patients as used for immunohistochemistry. Substantia nigra tissue included samples from 8 RLS patients (aged 53–86 years, all women) and 8 age-matched controls (aged 66–81 years, 5 women, 3 men). Control putamen samples were from 9 men and 3 women; all 8 RLS putamen samples were from women and were from the same patients as the SN samples. The age range of the putamen samples were 49 to 87 years for the controls.

Samples were homogenized on ice in homogenization buffer containing protease inhibitor cocktail with an Ultra-Turrax T25 homogenizer. Each sample was applied in

![Quantification of Mitochondrial Ferritin in SN Homogenates](http://jnen.oxfordjournals.org/)

**FIGURE 1.** Quantification of mitochondrial ferritin in the substantia nigra (SN). Mitochondrial ferritin levels are significantly increased (51%) in restless leg syndrome (RLS) compared with control autopsy SN homogenates (p < 0.01). OD, optical density.
triplicate to nitrocellulose membrane using a slot-blot apparatus (Minifold II, Schleicher and Schuell, Keene, NH) at a concentration of 1 μg/mL and a total volume of 100 μL. The membrane was blocked in 5% nonfat milk made with Tris-buffered saline for 1 hour at room temperature, followed by incubation for 1 hour at room temperature with primary mouse anti-human FtMt (21) diluted 1:1000 in 5% nonfat milk made with Tris-buffered saline with Tween. The membrane was incubated with secondary antibody (anti-mouse IgG conjugated to horseradish peroxidase [NA931V, GE Healthcare UK Limited, Little Chalfont Buckinghamshire, UK]) diluted 1:5000 in 5% nonfat milk made with Tris-buffered saline with Tween for 1 hour and then exposed to chemiluminescent substrate (Western Lightning PLUS ECL, NEL 105001EA, Perkin Elmer, Waltham, MA) for 1 minute and exposed to film.

Statistical Analysis
Optical density (OD) values from the slot blots were obtained using a GS800 Calibrated Densitometer (Bio-Rad, Hercules, CA) and background OD was subtracted. The values from each triplicate sample for each patient were averaged to obtain a mean OD value. These mean OD values were subjected to statistical analysis using the Mann-Whitney U test in GraphPad Prism software (Version 4.03, GraphPad Software, Inc, La Jolla, CA).

RESULTS
Immunoblot Quantification of Human Samples
There was a 51% increase in the concentration of FtMt in the RLS SN compared with controls (p < 0.01) (Fig. 1). Because little is known about sex differences in FtMt expression, men were removed from the control group in a separate analysis. Comparison of the control women to the (all women) RLS group indicated a significant 47% increase (p < 0.01) of FtMt in RLS (not shown). Putamen homogenates were analyzed in a similar manner, and there was no difference between the control and RLS groups (Fig. 2); separate analysis of only female subjects also did not yield a significant difference (not shown).

FtMt Immunohistochemistry
Mitochondrial ferritin immunoreactivity was detected in both the SN and in the putamen (Figs. 3 and 4). In the SN, the reaction product was primarily observed throughout the somata of neuromelanin-containing neurons in both RLS and control tissues (Fig. 3A). The staining intensity was much
less in the controls than in the RLS SN (Fig. 3B). This is consistent with the quantitative data and suggests that neurons account for most of the FtMt expressed. In the putamen, staining was very similar in control and RLS, with no discernible differences in intensity or cellular distribution (Fig. 4).

**COX Immunohistochemistry**

The increase in FtMt in RLS could reflect more mitochondria or more FtMt per mitochondria. To differentiate between these possibilities, staining for the mitochondrial protein COX was performed on SN sections; COX immunostaining intensity should reflect the numbers of mitochondria in the sample (25). The staining intensity for COX was dramatically greater in neuromelanin-containing cells in the RLS tissue compared with control (Fig. 5), suggesting that mitochondrial density is greater in the RLS SN than in the controls.

**H-ferritin Immunohistochemistry**

There is homology between FtMt and H-ferritin. We had previously found a decrease in H-ferritin immunostaining in neuromelanin-containing cells in RLS compared with control (8, 9). Therefore, we immunostained this new set of samples for H-ferritin. Immunostaining for H-ferritin in control tissue was relatively strong in neurons and in some glia (Fig. 6A), whereas in RLS samples, neuron immunostaining was lighter but there seemed to be more immunopositive glia (Fig. 6B).

**DISCUSSION**

We demonstrated that SN FtMt levels are significantly greater in RLS than in control samples and that
FtMt immunoreactivity was predominantly in neuromelanin-containing neurons in the SN. There were no differences between control and RLS in the amount or cellular distribution of FtMt in the putamen. Whether the increase in FtMt in the SN was a result of higher FtMt concentration or mitochondrial proliferation with normal amounts of FtMt could not be determined, but greater immunostaining for COX suggests an increase in mitochondria (25). Thus, greater FtMt immunostaining in the SN in RLS suggests that it is associated with an increase in mitochondria.

Mitochondrial ferritin is reportedly not regulated by cellular iron levels (17). Thus, elevated levels of FtMt in neuromelanin-containing neurons in RLS do not provide direct information on the iron status of these cells. Compelling data, however, suggest that an increase in FtMt would decrease cytosolic iron (21). Therefore, the increase in FtMt coupled with the increased iron demand of more mitochondria would be consistent with our previous report of less stored iron (based on decreased levels of H-ferritin) in neuromelanin-containing cells in RLS compared with controls (8, 10). The demonstration of a decrease in H-ferritin immunostaining in samples not previously examined (Fig. 6) further substantiates the distinction between cytosolic H-ferritin and FtMt and their independent regulation.

A decrease in cytosolic iron availability could affect the production of iron-dependent proteins and be associated with the decrease in the iron-sulfur-containing iron regulatory protein 1 in neuromelanin-containing cells in RLS (10). Indeed, iron deficiency prevents iron-sulfur protein generation and maturation of iron-sulfur-containing mitochondrial proteins (26). Therefore, a decrease in cytosolic iron-sulfur proteins may be a consequence of maintaining mitochondrial functions essential for cell survival, for example, production of tyrosine hydroxylase that seems to be normal or even elevated in the RLS SN (8). The possibility of increased mitochondria in RLS neuromelanin-containing cells also suggests that there is a greater metabolic demand by these cells than in controls. Very little is known about the metabolic activity of the SN in RLS, however, and this seems to be an area that should be investigated further, such as with neuroimaging.

The reasons for increased mitochondriogenesis in RLS are not known. The incidence of RLS is higher in women than men, and increased folate availability has been found to upregulate mitochondriogenesis in brain and other tissues (27). The activation of mitochondrial transcription factors increases mitochondrial proliferation (28), and these transcription factors are regulated by estrogen (29) and estrogen-related receptors (30). Neuronal mitochondrial biogenesis in adults has been reported in response to transient hypoxia (25), and there is some evidence of activation of hypoxia pathways in the neuromelanin-containing cells in RLS (31). In transient hypoxia, mitochondriogenesis resulted from activation of neuronal nitric oxide synthase (nNOS) (25, 32), and genetic variants of nNOS have been reported in RLS patients (33). Although no functional consequences of these mutations have been identified to date, our results suggest that there may be a relationship between mitochondrial density and nNOS mutations.

The specificity of the role of the nigrostriatal pathway has been challenged in RLS pathogenesis (34). One possible area of involvement is A11, the source of the neurons for the descending dopaminergic pathway into the spinal cord (35); however, a recent autopsy study found no change in the volume of tyrosine hydroxylase neurons or gliosis in this region in RLS compared with controls (36). A potential explanation for selective involvement of 1 dopaminergic pathway over another is that mitochondrial size and number are not consistent between dopaminergic cell populations; for example, SN dopaminergic cells have fewer and smaller mitochondria than those in the neighboring ventral tegmental area (A10) (37). There are more than 100 genes that differ in

![FIGURE 6. H-ferritin staining in the substantia nigra after bleaching of neuromelanin. The blue reaction product in the neurons in control tissue (A) is more intense than in the restless leg syndrome (RLS) neurons (B). Brown neuromelanin can be detected in the RLS neurons (B) because of the relative absence of the immunoreaction product for H-ferritin. The numerous small round cells that are stained in the RLS tissue seem to be glia, whereas there are relatively few immunostained glia in the control sample. Original magnification: 20×.](https://jnen.oxfordjournals.org/content/jnen/68/11/1197/F6.large.jpg)
expression level between these 2 dopaminergic cell populations, and genes for metabolism and mitochondrial proteins are substantially elevated in the SN pars compacta compared with the ventral tegmental area (38). The increases in mitochondrial proteins and metabolism strongly suggest that nigral dopaminergic neurons have a higher metabolic rate than other dopaminergic cells. This latter concept is generally offered as the reason why the SN is more sensitive to the mitochondria-damaging agents paraquat, maneb, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and rotenone (39–43). Induction of mitochonndriogenesis in the neuromelanin-containing cells through physiological responses or genetic variation may result in inadequate iron reserves to meet physiological challenges such as diurnal variation in iron availability or other conditions (e.g. pregnancy, anemia, renal dialysis) associated with RLS because of the high metabolic demands of this brain region. The concept that the SN is particularly sensitive to changes in iron status may be consistent with the relatively high levels of iron in this brain region. Iron increase into the SN has long been considered part of the pathological process for diseases involving the SN, although this role of iron in the SN and particularly neuromelanin-containing cells has been questioned (44). In conclusion, the present data indicate that FtMt levels and mitochondrial numbers are increased in the SN in RLS. The augmentation in mitochondria may reflect cellular attempts to correct metabolic insufficiency in these cells, which in turn may lead to cytosolic iron deficiency.

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