Supporting Data

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Signs of Chronic Hypoxia Suggest a Novel Pathophysiological Event in α-Synucleinopathies

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ABSTRACT: Background: Multiple system atrophy (MSA) and Parkinson’s disease (PD) patients develop respiratory and cardiovascular disturbances including obstructive sleep apnea, orthostatic hypotension, and nocturnal stridor. We hypothesized that, associated with these respiratory and cardiovascular disturbances, hypoxic events may occur in MSA and PD brains that may play a role in disease progression.

The objective of this study was to evaluate the presence of hypoxia in nonneurological controls and PD and MSA patients.

Methods: Molecular levels of hypoxia markers were measured in postmortem brain tissue from controls and PD and MSA cases.

Results: MSA brain showed signs of chronic hypoxia characterized by the significant accumulation of the hypoxic marker HIF2α as compared to PD patients and controls. We detected no differences between MSA subtypes. Signs of hypoxia were also observed in PD patients with a clinical presentation similar to the MSA cases.

Conclusions: The results obtained from this study suggest a new alternative pathway associated with α-synucleinopathies that may contribute to the pathogenesis of these disorders. © 2020 The Authors. Movement Disorders published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: multiple system atrophy; Parkinson’s disease; α-synucleinopathies; hypoxia

Multiple system atrophy (MSA) is a fatal progressive atypical parkinsonian disorder leading to severe motor disability and death a few years after symptom onset.1 Based on its clinical presentation, MSA is subdivided in 2 main variants: parkinsonian (MSA-P) and cerebellar (MSA-C).1 Postmortem neuropathological analysis defines the final diagnosis of MSA by proving α-synuclein-positive oligodendroglial inclusions and selective neurodegeneration. Based on the pattern of neurodegeneration, MSA can also be classified into 3 subtypes: MSA with striatonigral degeneration (MSA-SND), with olivopontocerebellar atrophy (MSA-OPCA), and a combination of the 2, referred to as mixed.2 One of the early signs of MSA is the appearance of respiratory and cardiovascular autonomic disturbances, including orthostatic hypotension (OH).3 The drop in blood pressure in patients produces a reduction in blood flow that could lead to a fall of oxygen supply to the brain. Moreover, OH when associated with nocturnal hypertension has been described as a risk factor for cerebral microangiopathy (end-organ damage), generating events of brain hypoxia/ischemia followed by reperfusion.4,5 Furthermore, with the progression of the disease, most of the MSA patients develop sleep and respiratory disorders, like obstructive sleep apnea6 and nocturnal stridor,7 which lead to a reduction in oxygen saturation by recurrent collapse of the upper airways during inspiration. Autonomic involvement is common in Parkinson’s disease (PD) but is more variable in severity than in MS.8

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Based on all these findings, we therefore hypothesize that, in association with the pronounced respiratory and cardiovascular autonomic disturbances throughout a disease course of 6 to 8 years, MSA patients are likely to suffer events of hypoxia in the brain, which could lead to an aggravation of disease progression as compared with PD.

**Material and Methods**

**Human Samples**

Fresh-frozen substantia nigra and visual cortex samples were provided by the Queen Square Brain Bank (QSBB) at University College London. The brain donation program and protocols have received ethical approval for donation and research by the NRES Committee London – Central, and tissue is stored for research under a license issued by the Human Tissue Authority (No. 12198). Samples from 18 MSA patients (clinical diagnosis before postmortem evaluation: 8 MSA-P, 10 MSA-C; postmortem pathological evaluation: 3 MSA-SND, 10 MSA-OPCA, 5 MSA-mixed), 12 PD patients, and 10 nonneurological controls (C) were included in this study. Demographical and clinical information was kindly provided by the QSBB (Table 1; the case list including all the clinical and pathological details is available in Supplementary Table 1). No personal information could be extracted from these data. The utilization of postmortem human samples was approved by the corresponding biobank ethics committees and by the Ethics Committee of the Medical University of Innsbruck (AN2016-0012 358/4.2 359/4.1).

**Molecular Analyses**

Total RNA and proteins were obtained from frozen samples using Trizol Reagent (Thermofisher) according to the manufacturer’s instructions. No differences were observed in RNA quality between MSA, PD, and C in the different brain regions, with A260/280 ratios within the expected range (substantia nigra: C, 1.93 ± 0.06; PD, 1.91 ± 0.04; MSA, 1.92 ± 0.05; visual cortex: C, 1.98 ± 0.01; PD, 1.97 ± 0.01; MSA, 1.96 ± 0.03). qRT-PCR was performed using standard procedures. GAPDH, 18S, ACTB mRNA were used as housekeeping genes. All samples were run in duplicate, and results were analyzed using the 2−ΔCt method9 and presented as relative gene expression normalized to the average cycle threshold for the 3 housekeeping genes. Protein quantification and Western blot were performed using standard procedures. Primary antibodies included anti-HIF2α (Cell Signaling; 7096), anti-HIF1α (BD; 610959), anti-GAPDH (Sigma; G9545), anti-α-Tubulin (Abcam; ab7750), and anti-β-tubulin (Sigma; T4026) as loading controls. Signal detection was performed using horseradish peroxidase–conjugated antirabbit (Cell Signaling; 7074) or antimouse (GE Healthcare; NA931) antibodies. Images were acquired using the Fusion FX system for Western blot and gel imaging. Relative protein levels were measured by densitometry using FUSION CAPT V16.09b software (Vilber Lourmat) and normalized to the average densitometric value for the 3 housekeeping proteins.10 A reference sample was loaded in all gels for gel-to-gel normalization. All gels were run, transferred, incubated, and developed in parallel.

**TABLE 1.** Demographic and clinical data of patients with MSA, patients with PD, and C

|                          | C (n = 10) | PD (n = 12) | MSA (n = 18) | Post hoc analysis |
|--------------------------|-----------|------------|-------------|------------------|
| Age (years), mean ± SDd  | 83.1 ± 4.63 | 77.42 ± 5.16 | 65.11 ± 6.18 | MSA vs C     |
| Female, % (n)b           | 60 (6)     | 41.7 (5)   | 61.1 (11)   | MSA vs PD     |
| Disease duration (years), | NA         | 17 ± 7.39  | 7.39 ± 2.62 | PD vs C      |
| mean ± SDd               |            |            |             |                |
| HIF2A mRNA levels in substantia nigra, a.u., mean ± SDd | 0.021 ± 0.011 | 0.018 ± 0.005 | 0.022 ± 0.01 | <0.0001 |
| HIF2x protein levels in substantia nigra, a.u., mean ± SDd | 0.011 ± 0.004 | 0.014 ± 0.007 | 0.023 ± 0.008 | 0.0002 |
| HIF2A mRNA levels in visual cortex, a.u., mean ± SDd | 0.010 ± 0.008 | 0.009 ± 0.005 | 0.014 ± 0.01 | 0.2197 |
| HIF2x protein levels in visual cortex, a.u., mean ± SDd | 0.012 ± 0.012 | 0.017 ± 0.012 | 0.021 ± 0.008 | 0.09 |

NA, not applicable; SD, standard deviation; a.u., arbitrary units.

A more detailed table including all cases and the clinical and pathological information can be found in Supplementary table 1.

Kruskal-Wallis with post hoc Dunn’s test.

Chi-square test.

Unpaired t test, 2-tailed.

One-way ANOVA with post hoc Bonferroni’s test.
**Statistical Analyses**

Most statistical analyses and all graphs were performed in GraphPad Prism version 8.0 (GraphPad Inc.). Data are expressed as mean ± SD; *P* ≤ 0.05 was considered statistically significant. All individual measurements constituted biological replicates. Samples were evaluated for normal distribution using D’Agostino and Pearson’s omnibus normality test. Comparisons were done with 1-way analysis of variance (ANOVA) with Bonferroni’s test for HIF2α protein, HIF2A gene expression, and postmortem interval (PMI). A 2-tailed *t* test was used to analyze disease duration differences between PD and all MSA cases and ANOVA with Bonferroni’s test for PD and MSA variants. The Kruskal-Wallis test with Dunn’s test and the chi-square test were used to evaluate age and sex differences, respectively, between groups. Univariate analysis of variance for HIF2α with covariate age and fixed factors diagnosis and sex was performed with SPSS version 24 (IBM) to evaluate the possible confounding effect of age. Correlations were studied using linear regression analysis.

**Results**

**MSA Brains Show Signs of Chronic Hypoxia**

The hypoxia-inducible factors (HIFs) are key in the cellular adaptation to low oxygen levels. These transcription factors are DNA-binding heterodimers consisting of alpha and beta subunits. Genes coding for HIFα subunits are constitutively transcribed and translated in normal oxygen conditions, but the resultant proteins are degraded by the proteasome through their consecutive hydroxylation by prolyl-hydroxylases (PHDs) and ubiquitination by von Hippel-Lindau ligase in an oxygen-dependent manner. However, when oxygen availability is scarce, PHDs reduce their activity, and HIFα proteins are stabilized and transported to the nucleus. There HIFα proteins heterodimerize with HIFβ subunits and activate a plethora of different transcriptional programs that are cell specific (Fig. 1A). HIF1α and HIF2α constitute the 2 main factors driving the cellular adaptation to hypoxia, in which HIF1α is primarily active during the acute phase of hypoxic adaptation, and HIF2α dominates during later, more chronic phases of hypoxia.

Therefore, to evaluate the presence of hypoxia in human brains, we analyzed the protein levels of HIF1α and HIF2α (Fig. 1B). In agreement with the main hypothesis of the present study, Western blot analyses indicated the presence of a hypoxic environment in the substantia nigra of MSA patients compared with PD patients and controls, characterized by the significant accumulation of the hypoxia marker HIF2α (MSA vs C, *P* = 0.0003; MSA vs PD, *P* = 0.0084; Fig. 1B,C and Table 1). The differences between groups remained significant after performing univariate analysis including age as a covariate (MSA vs C, *P* = 0.021; MSA vs PD, *P* = 0.05) and confirmed that changes in HIF2α levels were determined by diagnosis (*P* = 0.022) but not age (*P* = 0.496) or sex (*P* = 0.502), discarding a possible confounding effect. No differences in HIF2α protein levels were observed between MSA variants defined according to clinical symptoms (MSA-P vs MSA-C, *P* > 0.9999; Supplementary Fig. 1A and Supplementary Table 2) or postmortem pathological subtype (MSA-SND vs MSA-OPCA, *P* > 0.9999; MSA-SND vs MSA-mixed, *P* > 0.9999; MSA-OPCA vs MSA-mixed, *P* > 0.9999; Supplementary Fig. 1B and Supplementary Table 3). Interestingly, 2 of the PD patients with higher levels of HIF2α were originally misdiagnosed as MSA-P, suggesting a possible association between the presence of hypoxia and the clinical presentation (Fig. 1C and Supplementary Fig. 1A,B). Furthermore, gene expression analysis showed no differences in HIF2A (also known as EPAS1) mRNA levels between groups (Table 1, Supplementary Fig. 1C–E, and Supplementary Tables 2–3). This finding indicated that the increase of HIF2α protein was not because of upregulation of the HIF2A gene (HIF2α vs HIF2A: *r*² = 0.01613, *P* = 0.4473), but of protein stabilization under hypoxic conditions in MSA. In addition, we evaluated molecular signs of hypoxia in the visual cortex, a brain region less affected in α-synucleinopathies, in which we observed a numerical increase of HIF2α protein in MSA cases compared with controls, although not significantly (MSA vs C: *P* = 0.0892); see Table 1 and Supplementary Figure 2A,B. As in the substantia nigra, higher HIF2α protein was not associated with upregulation of the HIF2A gene (HIF2α vs HIF2A: *r*² = 0.05844, *P* = 0.1329; Supplementary Fig. 2C). We did not detect HIF1α protein in the tissue samples of patients or controls (Fig. 1B and Supplementary Fig. 2A).

That HIF2α levels were significantly higher in MSA cases compared with controls in areas more affected by the pathology may suggest a possible association between hypoxia and disease progression. Therefore, we hypothesized that hypoxia may also have an impact on disease duration in α-synucleinopathies. However, correlation analysis did not show a significant relationship between disease duration and hypoxia level in MSA (*r*² = 0.1919, *P* = 0.069; Fig. 1D) or in PD (*r*² = 0.1211, *P* = 0.2677; Fig. 1D). Nevertheless, the small number of cases per group and the presence of a similar disease duration in most MSA cases may reduce the statistical power of the correlation analysis. A significant negative relationship between hypoxia and disease duration was only observed when cases from both synucleinopathies were pooled together (*r*² = 0.1385,
suggesting that the presence of hypoxia in MSA may at least partly contribute to the faster progression compared with PD. However, the latter may artificially result from the lack of significant change of HIF2α in PD cases, together with a much longer disease duration in PD than MSA.

Finally, correlation analyses of PMI and HIF2α protein levels in the substantia nigra or visual cortex demonstrated that the presence of hypoxia in MSA brains was independent of the time elapsed between death and the freezing of the sampled tissue (substantia nigra: $r^2 = 0.03268; P = 0.2643$; visual cortex: $r^2 = 0.001221$; $P = 0.2677$).
mittent hypoxia, whereas HIF1 response to chronic hypoxia and being degraded by inter-
oxic conditions11,13–16 (Fig. 1A), our results suggest that MSA patients and controls. Based on HIF2α driving the response to chronic hypoxia and being degraded by intermittent hypoxia, whereas HIF1α being primarily active in acute hypoxia and being destabilized under chronic hypo-
oxic conditions11,13–16 (Fig. 1A), our results suggest that MSA patients may suffer from chronic hypoxic events.

The effects of hypoxia in the brains of humans and animal models have been extensively described. Hypoxia has a major effect on oligodendrocytes, inhibiting the proliferation of progenitors, reducing maturation and myelination,17,18 and directly inducing oligoden-
drocyte cell loss and demyelination.19 Moreover, in vitro and in vivo studies have shown hypoxia induces brain mitochondrial dysfunction,20 oxidative stress,21 neuroinflammation,22–24 and production of reactive oxygen species and proinflammatory cytokines by microglial cells.25,26 In summary, in the central ner-

vous system hypoxia induces detrimental effects in pro-
cesses that are also involved in MSA pathogenesis such as neuroinflammation, neurodegeneration, or demyelin-
ation and especially in some particular cell populations affected in this α-synucleinopathy, that is, oligodendro-
cytes, microglial cells, or neurons. Therefore, the pres-
ence of hypoxia in the brains of MSA patients could lead to an acceleration and aggravation of the pathol-
ogy acting like a double hit that could explain to some extent the severity and rapid disease course of this fatal neurodegenerative disorder (Supplementary Fig. 3).

Further in vitro and in vivo experiments combining the exposure to hypoxia with the presence of α-syn will clarify the pathophysiological consequences of hypoxia in α-synucleinopathy and the cellular and molecular mechanisms underlying these events. We must acknowl-
edge the lack of information regarding the agonal state of all cases included in the study, which could contrib-
ute to the generation of hypoxia. However, we would not expect major differences between the groups con-
idering that nonneurological controls suffered from advanced stages of cancer. We also have to acknowl-
edge that whether the hypoxic environment observed in MSA patients is cause or consequence of the disease remains unclear. The use of postmortem samples from end-stage MSA cases constitutes a limitation because we cannot extract from the current data if hypoxia is present throughout the entire disease course, even at early stages. Nevertheless, based on (1) differences in hypoxia levels between MSA cases and controls being significantly higher in the substantia nigra than in the visual cortex, one of the most affected brain regions in α-synucleinopathies versus an area less affected; (2) PD cases with a similar clinical presentation to MSA showing signs of hypoxia; and (3) hypoxia levels in the sub-
stantia nigra correlating with shorter disease duration (rapid progression) when both α-synucleinopathies are considered, we concluded that the presence of chronic hypoxia may be associated with the neurodegenerative process underlying α-synucleinopathies.

Our results suggest a new alternative pathway to under-
stand the pathogenic events underlying α-synucleinopathies that could provide novel targets for disease modification, especially in MSA. In this regard, the use of supplemental oxygen may constitute a potential therapeutic strategy to mitigate symptoms or even slow the progression of this dev-
astating disease.

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Recovery of Impaired Endogenous Pain Modulation by Dopaminergic Medication in Parkinson’s Disease

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ABSTRACT: Background: Of patients with Parkinson’s disease (PD), 30% to 85% report pain. However, mechanisms underlying this pain remain unclear. In line with known neuroanatomical impairments, we hypothesized that pain in PD is caused by alterations in emotional-motivational as opposed to sensory-discriminative pain processing and that dopamine recovers the capacity for endogenous emotional-motivational pain modulation in patients with PD.

Methods: A total of 20 patients with PD played a random reward paradigm with painful heat stimuli in addition to assessments of pain sensitivity once with and once without levodopa.

Results: Levodopa increased endogenous pain inhibition in terms of perceived pain intensity and un/pleasurableness compared with a medication off state. Higher clinical pain was associated with higher increases in pain inhibition. Levodopa did not affect heat pain threshold, tolerance, or temporal summation.

Conclusion: Patients with PD seem to be predominately impaired in emotional-motivational as opposed to sensory-discriminative pain processing. A differential pain modulation of pain processing may explain the pain variability among patients with PD.

Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

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