Published in final edited form as:

Neurodegener Dis. 2020 ; 20(2-3): 65–72. doi:10.1159/000510387.

An MRI study: Mutant G2019S-LRRK2 induces abnormalities in arteriolar cerebral blood volume in mouse brains

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Statement of Ethics

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. Protocol Number: M017M375 Title: Pathogenesis and therapeutic investigation of Parkinson's disease and other neuropsychiatry diseases

Disclosure Statement

None of the authors have conflicts of interest to declare.

Financial Disclosure/Conflict: All authors stated that there are no relevant conflicts

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Abstract

Background: Parkinson's disease (PD) is the second most common neurodegenerative disease and the most common movement disorder characterized by motor impairments resulting from midbrain dopamine (DA) neuron loss. Abnormalities in small pial arteries and arterioles, which are the primary pathways of local delivery of nutrients and oxygen in brain tissue, have been reported in many neurodegenerative diseases including PD. Mutations in LRRK2 cause genetic PD and contribute to sporadic PD. The most common PD-linked mutation LRRK2 G2019S contributes 20–47% of genetic forms of PD in Caucasian population. The human LRRK2 G2019S transgenic mouse model displays PD-like movement impairment and was used to identify novel LRRK2 inhibitors, which provides a useful model for studying microvascular abnormalities in PD.

Objectives: To investigate abnormalities in arteriolar cerebral blood volume (CBVa) in various brain regions using the inflow-based vascular-space-occupancy (iVASO) MRI technique in LRRK2 mouse models of PD.

Method: Anatomic and iVASO MRI scans were performed in 5 female and 7 male non-transgenic (nTg), 3 female and 4 male wild type (WT)-LRRK2, and 5 female and 7 male G2019S-LRRK2 mice of 9-month age. CBVa was calculated and compared in the substantia nigra (SN), olfactory cortex and prefrontal cortex.

Results: Compared to nTg mice, G2019S-LRRK2 mice showed decreased CBVa in the SN, but increased CBVa in olfactory and prefrontal cortex in both male and female groups, whereas WT-LRRK2 mice showed no change in CBVa in the SN (male and female), the olfactory (female) and prefrontal (female) cortex, but a slight increase in CBVa in the olfactory and prefrontal cortex in the male group only.

Conclusions: Alterations in the blood volume of small arteries and arterioles (CBVa) were detected in the G2019S-LRRK2 mouse model of PD. The opposite changes in CBVa in the SN and the cortex indicate that PD pathology may have differential effects in different brain regions. Our results suggest the potential value of CBVa as a marker for clinical PD studies.

Keywords

LRRK2; substantia nigra; blood vessel; perfusion; iVASO; MRI

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder of multiple functional systems and of multiple neurotransmitter systems [1]. It is typically characterized by motor impairments such as bradykinesia, rigidity, and resting tremor resulting from midbrain dopamine (DA) neuron loss in the substantia nigra [2, 3]. Currently, there is no effective therapy to cure the disease largely due to the lack of effective biomarkers and disease-modifying drugs. To this end, the study of pathophysiological changes in the brain in PD using noninvasive MRI techniques may help to improve our understanding of the pathology

of PD, and may aid the development of biomarkers for disease prediction and treatment evaluation in PD.

The health of the microvasculature is closely associated with brain functioning as it is the main regulator for the supply of oxygen and nutrients to neurons and other cells in brain tissue. Impairment in small blood vessels has been reported in PD in several brain regions such as the substantia nigra [4–12]. However, to date, most studies on microvascular abnormalities in the brain measured total cerebral blood flow (CBF) and volume (CBV). These quantities include contributions from different segments of the microvasculature (arteriolar and venous blood vessels), which may have distinct functions and physiology and thus be affected differentially in diseases. Neurophysiological studies have shown that the arterioles are the primary regulator of local tissue perfusion [13–15], and thus may be more sensitive to metabolic disturbances in the brain [16–20]. To measure the blood volume within small pial arteries and arterioles (CBVa), our group has developed an inflow-based vascular-space-occupancy (iVASO) MRI approach [21–26], a non-invasive MRI technique that does not need an exogenous contrast agent. It exploits the difference signal between an arterial blood nulled image and a second image without blood nulling to quantify CBVa [21–26]. Using this method, CBVa abnormalities could be detected in several brain disorders [27], including stroke [24], brain tumors [28, 29], Alzheimer's disease [30], Huntington's disease [31], and schizophrenia [32].

Mutations in Leucine-rich repeat kinase 2 (LRRK2) are the most common cause of familial PD [33]. LRRK2 is a large multidomain protein with GTPase and kinase activities that plays a critical role in PD pathogenesis [34]. We and others have found that inhibition of GTP binding and kinase activities suppresses mutant LRRK2-induced neurodegeneration and other related pathology [35–37]. The most prevalent mutation, G2019S-LRRK2 results in elevated kinase activity and causes about 1% of idiopathic PD in North America and 5% of familial PD [34, 33]. We recently developed a new LRRK2 transgenic mouse model of PD that displays PD-like phenotypes including neurodegeneration and impairment of motor activity [38], which provides a useful model for studying pathophysiological abnormalities in PD.

In this study, we used iVASO MRI to investigate potential CBVa abnormalities in the brain in G2019S-LRRK2 mouse model of PD. Animal models provide unique values for such studies, as rich methodology for studying genetic, pathologic, and therapeutic conditions are available in a tightly controlled environment; MRI measures can be validated with subsequent studies using invasive techniques; and longitudinal study can be performed in a relatively short time period. We focused on three key regions that are known to be affected in PD: the substantia nigra (SN), the olfactory cortex and the prefrontal cortex. The SN is one of the earliest brain regions affected in PD [2, 3]. Olfactory deficits are prevalent in early PD patients [39–43]. The prefrontal cortex is believed to be affected in a relatively later stage in PD. Therefore, we hypothesized that CBVa in these regions may be affected differentially by the PD disease process. In this pilot study, potential CBVa abnormalities were examined in a relatively late stage in the animal model used, which is considered an important first step for our subsequent longitudinal study.

METHODS

Animals

Homozygous human wild type (WT)-LRRK2, and G2019S-LRRK2 transgenic mice at 9 months of age were generated as described previously [38] and served as subjects for this study. In these mice, human wild type LRRK2 and PD-linked G2019S-LRRK2 mutant proteins expressed in brains under mouse prion protein (PrP)-promoter, respectively. The mice were in a B6C3F1 background. Age matched non-transgenic (nTg) mice served as control mice. The genotype of experimental LRRK2 transgenic mice were identified by PCR (LRRK2 primer: ATGATGACTGCACTGGGTTCCCTGAAGAATG; PrP sense primer: GGGACTATGTGGACTGATGTCGG; PrP antisense primer: CCAAGCCTAGACCACGAGAATGC). We scanned 5 female and 7 male nTg, 3 female and 4 male WT-LRRK2, and 5 female and 7 male G2019S-LRRK2 mice at 9-months of age. Mice were housed in the Center for Comparative Medicine at Johns Hopkins University School of Medicine, and exposed to a 12 h light/dark cycle with food and water provided *ad libitum*. Handling procedures for MRI scanning were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committees of the Johns Hopkins School of Medicine.

Rotarod testing

Transgenic mice and age matched non-transgenic controls at 9 months of age were tested for rotarod performance. Mice were trained before testing. A single trial was performed in the training session. The speed in the training session was set to 4 rpm, and the mice were continuously placed back on the rod for 5 minutes. For the testing sessions, mice were on a rotating rod (Columbus Instruments, Columbus, OH) at speeds increasing from 4 to 40 rpm (0.1 rpm increase per second). The testing was repeated for 3 consecutive days. The latency to fall from the rod (number of falls / number of trials %) was recorded.

MRI procedure

In vivo studies were performed on a horizontal bore 11.7 T Bruker Biospec system (Bruker, Ettlingen, Germany) equipped with a physiological monitoring system. A 72 mm quadrature volume resonator was used as a transmitter, and a four-element (2×2) phased array coil was used as a receiver. Anatomical images were acquired using a multi-slice Rapid Acquisition with Relaxation Enhancement (RARE) sequence: echo time (TE)/repetition time (TR)=30/3000 ms, resolution= $0.1 \times 0.1 \times 0.5 \text{ mm}^3$, RARE factor=8. A multi-slice iVASO sequence [32] with RARE MRI readout covering the entire brain was used with parameters optimized for typical blood flow and vascular transit times in mice [44] and relaxation times at 11.7T [45]: TR/inversion time (TI) = 1924/800, 1636/700, 1365/600, 1109/500, 867/400, 750/350, 636/300, 525/250, 415/200 ms, TE = 4.6 ms, resolution= $0.15 \times 0.15 \times 0.8 \text{ mm}^3$, RARE factor=16. The total imaging time was about 40 min per mouse. Note that we used a relatively long iVASO scan to enhance sensitivity in this pilot animal study. Our standard iVASO scan for human studies is approximately 5–10 minutes on clinical 3 T human MRI scanners [31, 32, 30]. Anesthesia was induced with 2% isoflurane in medical air, followed by 1% isoflurane in oxygen and air (1:3 ratio) for maintenance during the MRI scan, applied via a vaporizer and a facial mask. During the MRI scans, the mouse head was positioned

using a bite bar and two ear pins, and the animal was placed on a water-heated bed equipped with temperature and respiratory controls. Respiration rate and body temperature were monitored constantly by an animal monitoring system (SAII, Stony Brook, NY, USA) during MRI. Animals were ventilated to maintain stable physiologic conditions (respiratory rate 50–55 breaths/min).

Data analysis

The statistical parametric mapping (SPM) software package (Version 8, Wellcome Trust Centre for Neuroimaging, London, United Kingdom; <http://www.fil.ion.ucl.ac.uk/spm/>) and other in-house code programmed in Matlab (MathWorks, Natick, MA, USA) were used for image analysis. The iVASO images were motion corrected and the surround subtraction method [46] was used to calculate the difference signal from the nulling and control iVASO images. Partial volume effects on the iVASO difference signal were corrected based on cortical thickness obtained from the anatomical scan [47]. Whole-brain CBVa maps were numerically fitted from the iVASO difference signals using the iVASO theory [26]. Voxels with a signal-to-noise ratio (SNR) one standard deviation below the mean SNR were excluded from further analysis. Three regions-of-interest (ROIs) were manually delineated in each scan: 1) the substantia nigra (SN), 2) the olfactory cortex, and 3) the prefrontal cortex. Each region was identified on all slices of images covering the target structure. Average CBVa values were calculated in each ROI.

Statistics

The Kruskal–Wallis test by ranks with post hoc testing using the Wilcoxon rank-sum test was performed to examine group differences in rotarod testing results and for CBVa values in each ROI. Given the small sample size, non-parametric tests were chosen for this pilot study. Multiple comparisons were corrected using Holm–Bonferroni method [48]. CBVa in male and female mice was analyzed separately. Effect size was estimated with Cohen's *d*.

RESULTS

G2019S-LRRK2 induced movement impairment at the age of 9 months

Consistent with our previous findings [38], mutant G2019S-LRRK2 mice at the age of 9 months displayed severe movement impairment in the accelerating rotarod testing (Figure 1). In addition, we also found in this study that the WT-LRRK2 transgenic mice had only a trend of very subtle decrease of performance that did not differ significantly from non-transgenic controls. Thus, we used 9-month old LRRK2 transgenic mice subsequently to assess whether G2019S-LRRK2 induces neurovascular abnormalities in the brain using iVASO MRI. The human WT-LRRK2 transgenic mice were used as a control to determine whether overexpression of WT or mutant G2019S-LRRK2 could alter CBVa in the brain differently.

CBVa values in the substantia nigra (SN) decreased in G2019S-LRRK2 mice

Figure 2 shows typical raw iVASO images and CBVa maps in the brain of a G2019S-LRRK2 mouse acquired using iVASO MRI. The quantitative results are demonstrated in Figure 3 and are summarized in Tables 1 and 2. The CBVa values in nTg mice were in the

normal range of CBVa reported for mice in the literature [27]. In the SN, CBVa significantly decreased for both sexes in G2019S-LRRK2 mice compared to nTg mice; CBVa did not show significant changes in WT-LRRK2 compared to nTg mice for both sexes. In female mice, CBVa showed a decreasing trend in G2019S-LRRK2 compared to WT-LRRK2 mice, but did not reach significance.

CBVa values in the olfactory cortex and prefrontal cortex increased in G2019S-LRRK2 mice

In the olfactory cortex, CBVa significantly increased for both sexes in G2019S-LRRK2 mice compared to nTg mice; CBVa did not show significant changes in female WT-LRRK2 compared to nTg mice, but increased significantly in male mice; CBVa did not show significant changes in G2019S-LRRK2 compared to WT-LRRK2 mice for both sexes.

In the prefrontal cortex, CBVa significantly increased for both sexes in G2019S-LRRK2 mice compared to nTg mice; CBVa did not show significant changes in female WT-LRRK2 compared to nTg mice, but significantly increased in male mice; CBVa did not show significant changes in female G2019S-LRRK2 compared to WT-LRRK2 mice, but significantly decreased in male G2019S-LRRK2 compared to WT-LRRK2 mice.

DISCUSSION

The main finding in this study is that CBVa decreased significantly in the substantia nigra, but increased in the olfactory and prefrontal cortex in G2019S-LRRK2 mice compared to nTg mice. The decreased CBVa in SN may indicate microvascular impairments in this region in PD, in line with some previous studies [4–12]. On the other hand, the increased CBVa in the olfactory and prefrontal cortices may imply a compensatory mechanism in the microvasculature of these brain regions. Our results provide new information regarding LRRK2-linked cerebrovascular changes, which may be used as an indicator for PD pathogenic processes.

Mutations in LRRK2 are a well-known cause for the genetic form of PD and have been shown to also contribute to sporadic PD. Many studies including ours demonstrate that PD-linked LRRK2 mutants induce neurodegeneration in cell culture. Studies in LRRK2 transgenic or knock-in mouse models display progressive and mild movement impairment with subtle dopaminergic neuronal loss [49, 50]. Consistent with previous studies, G2019S-LRRK2 mice at 9 months of age displayed locomotor impairment (decreasing rotarod performance) compared with non-transgenic control mice at 9 months of age [38]. Meanwhile, there was significant decrease of CBVa in the SN in G2019S-LRRK2 mice compared with WT-LRRK2 and nTg mice. On the other hand, WT-LRRK2 mice only had slightly lower performance (not statistically significant) in rotarod testing compared with nTg mice. Meanwhile, there was slightly lower CBVa (not statistically significant) in the SN in male WT-LRRK2 mice compared with nTg control mice. The SN is known to be one of the first brain regions affected in PD. Postmortem pathological studies have demonstrated that there are loss of dopaminergic neurons and presence of Lewy bodies in the SN at an early stage of the disease. This seems to be congruent with our CBVa results, which showed a significant decrease in CBVa in the SN in G2019S-LRRK2 mice. These findings suggest

that G2019S-LRRK2 induced CBVa decrease might be an indicator for disease severity. Subsequent longitudinal studies in LRRK2 mice are warranted to explore whether CBVa can be a useful biomarker for PD. CBVa values in all three regions in nTg mice were in the normal range of CBVa reported in the literature [27], which is approximately 0.5–1 ml/100ml, but may vary depending on many factors (such as species, anesthesia, brain region, spatial resolution and technical considerations in different methods as described in [27]). Subcortical regions (e.g. SN) usually have smaller CBVa than cortical regions. Interestingly, in the olfactory cortex (not olfactory bulb) and prefrontal cortex, CBVa in G2019S-LRRK2 mice significantly increased compared to nTg mice. CBVa in these brain areas in WT-LRRK2 male mice even increased more than those of G2019S-LRRK2 male mice. We speculate that this increase of CBVa might represent a compensatory mechanism in the microvasculature of these brain regions. In a line with this notion, one previous positron emission tomography (PET) study of human subjects reported that there is significant increase of cholinergic activity in the brain in LRRK2 mutation carriers without PD compared with healthy controls, and in LRRK2 mutation carriers with PD compared with individuals with idiopathic PD [51]. This increase of cholinergic activity might represent early and sustained attempts to compensate for LRRK2-related dysfunction, or alteration of acetylcholinesterase in non-neuronal cells [51]. Another PET study also reported that human subjects with LRRK2 mutations exhibit elevations in serotonin terminal density before the clinical onset of disease, suggesting potential adaptive monoamine changes to compensate for early dopamine neuron loss [52]. A recent functional MRI study of healthy human LRRK2 mutation carriers showed that there are increased activities in the ventral attention system in the brain compared with non-carrier controls [53], which also supports a neural compensatory mechanism in asymptomatic mutation carriers [53]. Our previous human studies found increased CBVa in some cortical regions in the early stages of Alzheimer's disease [30] and Huntington's disease [31]. Taking together, these findings indicate that LRRK2 mutations may result in increases of CBVa in certain brain regions, and activities of other non-dopaminergic systems to overcome or balance the dopamine system deficit. These compensatory mechanisms could either be protective, or cause non-motor symptoms in LRRK2-linked PD cases.

We also observed sex differences in our data. In all three regions, male mice showed more dramatic changes in CBVa compared to female mice in the same groups. This seems to be in line with many clinical studies showing that PD-related symptoms such as rigidity and rapid eye movement (REM) sleep behavior disorder are often more prevalent in men than women [54]. This sex difference and its mechanism warrant further investigation.

Although significant effects were detected in our data, we believe that the cross-sectional design comparing changes at a single time point during the lifespan remains a fundamental limitation of the current study. We chose to perform this study in mice at an age of 9 months, which is considered a relatively late stage of the disease in this mouse model. We are currently performing a follow-up study with larger cohorts and longitudinal measures across the lifespan of the mice especially at early stages of the disease to validate whether CBVa changes can be an indicator for disease progression in PD.

In summary, differential changes in CBVa were detected in the substantia nigra and cortical regions in the brains of LRRK2 mouse PD models. Mutant G2019S-LRRK2 mice displayed impaired small pial arteries and arterioles in the substantia nigra. Expression of LRRK2 may induce the compensatory microvascular alterations in the cortex. These findings provide insights for the pathophysiology of PD and may assist the development of useful biomarkers for monitoring progression and therapies in PD.

Acknowledgments

The authors thank Kazi Akhter (F.M. Kirby Research Center) for experimental assistance.

Funding Sources:

This study was supported by the National Institutes of Health (NIH) grants: R21 NS104480 to J.H., and R01NS093383 to W.W.S.

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Figure 1.

Motor impairment in G2019S-LRRK2 mice. Rotarod testing was performed in nTg, WT-LRRK2 and G2019S-LRRK2 mice at the age of 9 months. The testing was repeated for each mouse for 3 consecutive days. The latency to fall from the rod (number of falls / number of trials %) was recorded and compared. *** $P < 0.001$.

Figure 2.
Typical raw iVASO images and CBVa maps (ml/100ml) in the brain acquired with iVASO MRI in a G2019S-LRRK2 mouse.

Figure 3. Comparison of CBVa values in the substantia nigra (SN), the olfactory cortex, and the prefrontal cortex in nTg, WT-LRRK2 and G2019S-LRRK2 mice. * $P < 0.05$.

Table 1:

CBVa (mL/100mL) in different brain regions and overall difference among three groups.

Mouse Group	n	Sex	Substantia Nigra		Olfactory Cortex		Prefrontal Cortex	
			mean	std	mean	std	mean	std
nTg	5	female	0.51	0.04	0.68	0.05	0.87	0.10
WT-LRRK2	2		0.42	0.12	0.71	0.06	0.92	0.09
G2019S-LRRK2	5		0.37	0.05	0.92	0.06	1.15	0.11
P ^a			0.04		0.02		0.03	
nTg	6	male	0.47	0.05	0.43	0.05	0.73	0.03
WT-LRRK2	4		0.48	0.05	1.03	0.16	1.37	0.08
G2019S-LRRK2	5		0.33	0.03	0.73	0.11	1.11	0.03
P			0.009		0.003		0.002	

^aP value from the Kruskal–Wallis test by ranks.

Table 2:

Pairwise comparisons of CBVa values between mouse groups.

Mouse Group	Sex	Substantia Nigra			Olfactory Cortex			Prefrontal Cortex		
		Relative Change (%) ^a	Effect Size ^b	P ^c	Relative Change (%)	Effect Size	P	Relative Change (%)	Effect Size	P
WT-LRRK2 vs. nTg	female	-18.98	-1.53	0.87	5.52	0.70	0.57	6.17	0.57	0.57
G2019S-LRRK2 vs. nTg		-28.01	-3.35	0.02	36.87	4.59	0.02	32.24	2.71	0.05
G2019S-LRRK2 vs. WT-LRRK2		-11.14	-0.69	0.87	29.71	3.62	0.19	24.56	2.14	0.19
WT-LRRK2 vs. nTg	male	3.57	0.36	0.76	140.97	5.86	0.02	89.09	11.15	0.02
G2019S-LRRK2 vs. nTg		-28.75	-3.34	0.01	71.21	3.83	0.01	52.12	11.30	0.01
G2019S-LRRK2 vs. WT-LRRK2		-31.21	-3.85	0.03	-28.95	-2.28	0.06	-19.55	-4.46	0.02

^aRelative change was defined as 100 * (mean CBVa in group 1 – mean CBVa in group 2) / (mean CBVa in group 2) %.^bEffect size was estimated with Cohen's d = (mean CBVa in group 1 – mean CBVa in group 2) / s, where s is the pooled standard deviation of the two groups.^cAdjusted P value after correcting for multiple comparisons.