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Microstructural changes in the substantia nigra of asymptomatic agricultural workers

Guangwei Du, M.D., Ph.D.¹, Mechelle M. Lewis, Ph.D.^{1,2}, Nicholas W. Sterling, B.S.¹, Lan Kong, Ph.D.⁷, Honglei Chen, Ph.D.⁸, Richard B. Mailman, Ph.D., Ph.D.^{1,2}, and Xuemei Huang, M.D., Ph.D.^{1,2,3,4,5,6}

¹Department of Neurology, Pennsylvania State University-Milton S. Hershey Medical Center, Hershey PA 17033

²Department of Pharmacology, Pennsylvania State University-Milton S. Hershey Medical Center, Hershey PA 17033

³Department of Radiology, Pennsylvania State University-Milton S. Hershey Medical Center, Hershey PA 17033

⁴Department of Neurosurgery, Pennsylvania State University-Milton S. Hershey Medical Center, Hershey PA 17033

⁵Department of Kinesiology, Pennsylvania State University-Milton S. Hershey Medical Center, Hershey PA 17033

⁶Department of Bioengineering, Pennsylvania State University-Milton S. Hershey Medical Center, Hershey PA 17033

⁷Department of Public Health Sciences, Pennsylvania State University-Milton S. Hershey Medical Center, Hershey PA 17033

⁸National Institute of Environmental Health Sciences, Research Triangle Park, NC 27705

Abstract

Parkinson's disease (PD) is marked by the loss of dopamine neurons in the substantia nigra (SN). Although the exact etiology is unknown, sporadic PD is hypothesized to be a result of genetic susceptibility interacting with environmental insult. Epidemiological studies suggest that pesticide exposure is linked to higher PD risk, but there are no studies demonstrating SN changes with chronic pesticide exposure in human subjects. Thus, high resolution T2-weighted magnetic resonance imaging (MRI) and diffusion tensor (DTI) images were obtained from 12 agricultural workers with chronic pesticide exposure, 12 controls, and 12 PD subjects. Neither controls, nor

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Corresponding Author: Xuemei Huang MD, PhD, Professor of Neurology, Pennsylvania State Hershey Medical Center, 500 University Dr., H-037, Hershey, PA 17033-0850, Voice: 717-531-1530 Fax: 717-531-0226, xuemei@psu.edu.

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pesticide-exposed subjects, had any parkinsonian symptoms. Exposure history to pesticides was assessed by a structured questionnaire. DTI measures in the SN, including fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD), were obtained for all subjects and compared among groups. Compared to controls, PD patients showed the expected significant changes in all DTI measurements in the SN. The pesticides-exposed subjects, compared to controls, had significantly lower FA values ($p=0.022$, after multiple comparisons correction), but no significant differences in RD, MD, or AD measures. The study is the first to demonstrate microstructural changes in the SN of human subjects with chronic pesticide exposure. The changes detected by MRI may mark “one of the hits” leading to PD, and underlie the increased risk of PD in pesticide users found in epidemiological studies. Further human studies assisted by these imaging markers may be useful in understanding the etiology of PD.

Keywords

Parkinson's disease (PD); substantia nigra (SN); diffusion tensor imaging (DTI); pesticides; paraquat; magnetic resonance imaging (MRI)

1. Introduction

Parkinson's disease (PD) is marked primarily by the loss of dopaminergic neurons in the substantia nigra (SN) (Fearnley and Lees, 1991; Dickson *et al.*, 2009). The fact that identical twins frequently are discordant for PD suggested that environmental factors may be associated with the etiology of sporadic PD through mechanisms such as oxidative stress, neuroinflammation, and/or cytotoxicity (Tanner *et al.*, 1999; Wirdefeldt *et al.*, 2004). Recent epidemiologic data support that pesticide exposure is associated with increased risk of PD (McCormack *et al.*, 2002; Tanner *et al.*, 2011; Pezzoli and Cereda, 2013). In addition, a number of pesticides (e.g., paraquat, rotenone, maneb) have been shown to cause direct dopaminergic toxicity in animal models (Uversky, 2004; Desplats *et al.*, 2012). There has been no direct evidence, however, suggesting that chronic pesticide exposure leads to SN changes in human subjects due to lack of *in vivo* markers for the pathological process occurring in PD.

Recently, magnetic resonance imaging (MRI) has been explored extensively to study PD-related pathological changes in human subjects (Chan *et al.*, 2007; Martin *et al.*, 2008; Vaillancourt *et al.*, 2009). In particular, diffusion tensor imaging (DTI), by measuring microstructural disorganization due to dopamine cell loss, has shown promise as a tool of PD-related changes (Chan *et al.*, 2007; Peran *et al.*, 2010; Du *et al.*, 2011). In the MPTP mouse model, DTI changes have been associated with dopamine neuron loss in the SN (Boska *et al.*, 2007). Furthermore, several human studies have demonstrated reduced FA values in the SN of early PD patients, consistent with the notion that DTI changes may be able to detect nigral changes *in vivo* (Vaillancourt *et al.*, 2009; Du *et al.*, 2012).

The current study is the first to investigate potential DTI changes in the SN of asymptomatic pesticide-exposed subjects, testing the hypothesis that chronic pesticide exposure may lead to microstructural changes in the SN, possibly explaining the increased risk of PD in pesticide users.

2. Methods

2.1. Subjects

Twelve male subjects with chronic pesticide exposure were recruited using IRB-approved flyers. Pesticide exposure was evaluated by using a structured questionnaire from the

Agriculture Health Study (<http://www.niehs.nih.gov>) for both pesticide-exposed and control subjects. The 12 idiopathic PD subjects and 12 healthy controls came from the male subgroups of ongoing cohorts at our center that had been recruited either from our tertiary movement disorders clinic and/or using IRB approved flyers (see Table 1 and Table 2 for detailed demographic information). The control and PD subjects were selected impartially from the existing cohorts by matching for age with the pesticide-exposed group using a greedy matching algorithm (<http://mayoresearch.mayo.edu/mayo/research/biostat>).

The control subjects had essentially no history of pesticide exposure, and all of the control and pesticide-exposed subjects were free of any subjective or objective PD symptoms or signs. PD diagnosis was made by a movement disorder specialist (XH) according to published criteria (Hughes *et al.*, 1992; Hughes *et al.*, 2001). All subjects were assessed using the Hoehn & Yahr staging, Unified Parkinson's Disease Rating Scale (UPDRS) sections I-III, and Grooved Pegboard Test (GPT). For PD subjects, disease duration from time of diagnosis was obtained from subject history. All subjects were free of major and acute medical issues such as liver, kidney, or thyroid abnormalities and deficiencies of B12 and folate, and had Mini Mental Status Exam (MMSE) scores >24. All subjects gave written informed consent that was reviewed and approved by the Penn State Hershey Institutional Review Board and was consistent with the Declaration of Helsinki.

2.2. Magnetic resonance imaging (MRI) data acquisition

All subjects were scanned with a 3.0 Tesla MRI system (Trio, Siemens Magnetom, Erlangen, Germany, 8-channel phased array head coil) with high-resolution T2-weighted and diffusion tensor imaging (DTI) sequences. A fast-spin-echo sequence was used to obtain T2-weighted images with TR/TE=2500/316, FOV=256 mm × 256 mm, matrix=256 × 256, slice thickness=1 mm (with no gap), and slice number=176. For DTI, acquisition parameters were as follows: TR/TE=8300/82 ms, b value=1000 s/mm², diffusion gradient directions=42 and 7 b=0 scans, FOV=256 mm × 256 mm, matrix=128 × 128, slice thickness=2 mm (with no gap), and slice number=65.

2.3. Image processing and analysis

2.3.1. Segmentation of the SN—The SN was defined manually on T2-weighted images using ITK-SNAP (Yushkevich *et al.*, 2006) by an investigator blinded to subject diagnosis. The region of interest was defined as a hypointense band between the red nucleus and cerebral peduncle in axial sections (Vaillancourt *et al.*, 2010; Massey and Yousry, 2010), as illustrated in Figure 1. The most superior extent of the SN segmentation was defined at one slice inferior to the axial section of the red nucleus showing the largest radius (Figure 1). A total of four to six slices (from the rostral to caudal part of the SNpc) were used as the SN region of interest (ROI) (Yushkevich *et al.*, 2006). The right and left ROIs of the SN were summed in order to obtain an average measurement from both sides. Another set of ROIs also was obtained by a second rater (GD) using the same segmentation protocol, thus providing an estimate of the inter-rater reliability using intra-class correlation coefficients.

2.3.2. DTI estimation—DTI image quality control and tensor reconstruction was performed using DTIPrep (Neuro Image Research and Analysis Laboratory, University of North Carolina, Chapel Hill, NC USA) that first checks DWI images for appropriate quality by calculating the inter-slice and inter-image intra-class correlation, and then corrects for the distortions induced by eddy currents and head motion (Liu *et al.*, 2010). Diffusion tensor images then were estimated via weighted least squares (Salvador *et al.*, 2005). DTI is thought to reflect the diffusion of water molecules in tissues and thus is an indicator of the structural organization in a region. Several measures typically are used, including fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD).

For the current study, FA, MD, AD, and RD maps all were generated for subsequent analysis. FA is a non-linear combination of three eigenvalues of the tensor matrix calculated by the following equation to represent the local degree of diffusion anisotropy:

$$FA = \sqrt{\frac{1}{2} \frac{\sqrt{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_3 - \lambda_1)^2}}{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}}$$

where λ_1 , λ_2 , λ_3 and represent the degree of diffusion in the three main axes of the tensor (Basser and Pierpaoli, 1996). It can range in value from 0-1, with 0 meaning water is free to move in any direction (no structural integrity/boundaries) and 1 reflecting all water molecules aligned in the same direction (e.g., along an axon). MD is a simple average of the three eigenvalues ($MD = (\lambda_1 + \lambda_2 + \lambda_3)/3$) representing local mean diffusivity. AD ($AD = \lambda_1$) and RD ($RD = (\lambda_2 + \lambda_3)/2$) separate spatial diffusivity into a main component along the axis of maximum diffusion and a remaining diffusivity component for the other two directions. These two measures help yield a better understanding of local diffusivity changes that contribute to FA and/or MD changes.

After manual segmentation, the SN ROIs were mapped to DTI maps (FA, MD, AD, and RD) by co-registering the T2-weighted images to the B0 images of DTI data using an affine registration followed by a b-spline non-rigid registration implemented in 3D Slicer (www.slicer.org) (Rueckert *et al.*, 1999). Mean DTI (FA, MD, AD, and RD) values in the SN of each subject were calculated and used for subsequent statistical analyses.

2.4. Inter-rater reliability validation of DTI measurements

There was strong agreement between the two raters regarding the segmentation and resulting extraction of DTI measures in the SN. The intra-class correlation coefficients (ICC) were 0.97 for FA, 0.93 for MD, 0.91 for AD, and 0.93 for RD values. The independent analysis of the ROIs from the second rater yielded similar results (data available upon request) to those from the first rater that are reported below.

2.5. Statistical analyses

The mean values of demographic factors (age, UPDRS, and GPT) were compared among the three groups by one-way analysis of variance (ANOVA) followed by post-hoc comparisons with Tukey-Kramer multiple comparison correction to probe for specific differences between the groups. SN DTI measures (FA, MD, AD, and RD) were compared among the three groups by one-way analysis-of-covariance (ANCOVA) with adjustment for age and significant results also probed for specific group differences using the Tukey-Kramer multiple comparison correction. We explored the association between pesticide exposure (in the pesticide group), motor function (UPDRS and GPT in both the pesticide and PD groups), and SN DTI measures via Spearman correlation coefficients. All statistical analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Demographics

The demographic and clinical data are summarized in Table 1. All subjects were male, and there were no significant differences in age between pesticide exposed, PD, and control groups ($p=0.772$). Detailed exposure information for pesticide-exposed subjects, including exposure time, number of chemicals and occupation, are summarized in Table 2. All

pesticide-exposed subjects had a minimum of eight years history of chronic application of multiple pesticides, all control and pesticide-exposed subjects were free of any subjective PD symptom, and had UPDRS scores less than six. All PD subjects were at Hoehn & Yahr stage I or II in the “off” drug state, indicating early and mild PD. There were significant differences in UPDRS and GPT scores among the three groups. Post-hoc tests revealed, however, that only PD subjects performed significantly worse on these measures compared to controls and pesticide-exposed subjects, and that there were no significant differences between control and pesticide-exposed subjects (see Table 1).

3.2. Comparison of DTI measures among groups

As shown in Figure 2, there were significant differences in the SN FA [$F_{2,32} = 8.30$, $p < 0.001$], MD [$F_{2,32} = 5.77$, $p = 0.003$], and RD [$F_{2,32} = 6.02$, $p = 0.002$] values, with a trending difference in the AD [$F_{2,32} = 2.81$, $p = 0.055$] value among the three groups. *Post hoc* comparisons showed that, compared to controls, PD subjects had significantly lower FA, higher MD and RD values, and a trend for a higher AD value in the SN [$p < 0.001$ for FA, $p = 0.004$ for MD, $p = 0.003$ for RD, and $p = 0.059$ for AD].

Compared to controls, pesticide-exposed subjects showed a significantly lower FA value ($p = 0.022$, after correction for multiple comparison) in the SN, but no significant differences in MD, RD, or AD measures (Figure 2). The FA change in pesticide-exposed subjects most likely was driven primarily by an increased RD value and not by a change in the AD value. Compared to PD subjects, pesticide exposed subjects showed no significant difference in FA ($p = 0.307$), yet were significantly lower on the other three DTI measures ($p = 0.017$ for MD, $p = 0.046$ for AD, and $p = 0.037$ for RD).

3.3. Clinical and behavioral correlations of DTI measurements

Among either pesticide-exposed subjects or PD patients, there were no correlations between DTI measures and motor functional measurements (data available upon request). Similarly among the pesticide-exposed group, we did not find any significant correlations between DTI measurements, duration of exposure to pesticides, or whether the exposure was as a farmer or applicator (see Table 2).

4. Discussion

Sporadic PD is probably a result of genetic susceptibility interacting with environmental insult. One such environmental factor has been pesticide exposure (Kasten *et al.*, 2007; Cannon and Greenamyre, 2011), and consistent with these epidemiologic data, animal studies have demonstrated that a number of pesticides may be toxic to dopamine neurons (Richardson *et al.*, 2005). The current study is the first to demonstrate directly the microstructural changes in the SN of human subjects having had chronic, low-dose exposure of a diverse group of pesticides. The changes detected by MRI may mark “one of the hits” leading to PD. Moreover, our study demonstrated that DTI could be a potential *in vivo* marker for microstructural changes caused by potential environmental risk factors of PD.

In the current study, we recruited a group of agricultural workers who had extensive histories of chronic, multiple pesticide exposure, several of whom were professional pesticide applicators (Table 2). During our recruitment, we purposefully insured that this entire group had a history of paraquat exposure. This was done because there is strong epidemiological data linking paraquat exposure to PD risk (Hatcher *et al.*, 2008; Ritz *et al.*, 2009; Elbaz *et al.*, 2009; Weisskopf *et al.*, 2010; Tanner *et al.*, 2011), and because of the fact that paraquat is both structurally similar to the dopamine cytotoxicant 1-methyl-4-phenylpyridinium (MPP⁺) and can cause dopamine neuron toxicity in animal models

(Richardson *et al.*, 2005; Cui *et al.*, 2009; Rappold *et al.*, 2011), despite that the toxicity of paraquat in animal models still is a controversial topic (Breckenridge *et al.*, 2013). Although this was a part of the experimental design, the current study was neither designed to, nor was powered adequately, to assess if these PD-like SN changes were primarily due to any specific pesticide.

Recently, dopamine neuron loss in a mouse model has been correlated with DTI changes (Boska *et al.*, 2007), and such DTI changes also have been reported in early stage PD subjects (Chan *et al.*, 2007; Vaillancourt *et al.*, 2009; Peran *et al.*, 2010; Du *et al.*, 2011). The present study is the first to apply DTI in asymptomatic human subjects and demonstrate SN microstructural changes associated with chronic pesticide exposure. The exact mechanism and implications of the DTI changes are unknown. Given the evidence from animal and epidemiological studies, it is plausible that the microstructural changes detected by DTI reflect dopaminergic cell loss similar to that in PD except with a milder magnitude. In recent years, there is a growing realization that PD may be caused by dual or multiple hits (Hawkes *et al.*, 2007; Sulzer, 2007). Thus, the microstructural changes detected by DTI in the current study may mark one of the “hits” leading to PD in pesticide users as predicted by the cited epidemiological studies. Namely, the SN changes do not have to represent PD pathology *per se*, but rather a proximal event that may make exposed individuals more susceptible to a “next” hit that will cumulatively lead to PD.

Diffusion tensor imaging, as a measure of local water diffusivity and microstructure integrity, has shown high sensitivity to detect mild structure alternations in multiple neurological disorders including PD (Vaillancourt *et al.*, 2009; Du *et al.*, 2012). The nonlinear nature of the FA measurement and small sample size, however, limit our ability to detect correlations between brain microstructural changes and exposures. Furthermore, previous research has indicated that FA values are affected by age and factors such as diet and socio-economic status (Salat *et al.*, 2005; Vaillancourt *et al.*, 2010; Wu *et al.*, 2011). The small sample size in our study did not allow us to control for confounders other than age. Nevertheless, the current study does strongly suggest the value of *in vivo* neuroimaging to investigate the roles of environmental factors in the etiology of PD.

In summary, we demonstrate that subtoxic, chronic pesticide exposure may lead to microstructural changes in the SN, and may represent the consequence of pesticide-induced toxicity to nigral circuitry. These findings are consistent with the previously proposed role of pesticide exposure in initiating or accelerating pathological processes that are similar to those occurring in PD. More importantly, our study demonstrated that DTI could detect microstructural changes in the SN possibly caused by pesticides. This approach may be useful in determining if other environmental chemicals alter brain microstructure, and/or can assist in focusing on specific chemicals that affect PD susceptibility. Because of the potential impact, replication of our results using larger cohorts and longitudinal designs is warranted.

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Highlights

- Chronic pesticide exposure is associated with microstructural changes in midbrain.
- Asymptomatic agricultural workers had similar, but less severe, changes than in PD.
- MRI may be useful as a structural marker for potential changes caused by pesticides.

Figure 1. Illustration of region of interest (ROI) definition of the substantia nigra. A, B, and C represent axial (A), sagittal (B) and coronal (C) views of the midbrain in T2-weighted images. The yellow arrows in A and C indicate the substantia nigra (SN), red nucleus (RN), cerebral peduncle (CP), and subthalamic nucleus (STN).

Figure 2.

Comparison of DTI values in the substantia nigra for Control (C), pesticide-exposed, and Parkinson's disease (PD) subjects. Error bars represent the standard deviation. FA: Fractional anisotropy; MD: mean diffusivity; AD: axial diffusivity; RD: radial diffusivity. Significant differences between groups are represented as: +: p<0.1; *: p<0.05; **: p<0.01; ***: p<0.001.

Table 1
Demographics of control, pesticide-exposed, and PD subjects

	Controls	Pesticide-exposed	PD	P-values ^a
Number	12	12	12	-
Age (y)	53.9±11.9	55.8±11.7	57.1±9.1	0.772
Pesticide exposure (y)	0	19.4±6.1	n/a	-
Parkinson's evaluation:				
Hoehn & Yahr stage (number at I/II)	-	-	5/7	-
Disease duration (years)	-	-	4.4(4.7)	-
UPDRS scores:				
Part I	2.3±3.4	4.3±4.3	9.3±4.9 ^b	0.003
Part II	0.1±0.3	0.7±0.9	7.9±6.1 ^b	0.001
Part III	1.0±1.7	3.5±1.8	25.9±13.7 ^b	<0.001
Grooved pegboard test:				
Dominant	79.5±18.3	90.8±20.5	125.3±40.8 ^b	0.001
Non-dominant	88.3±26.4	89.2±18.3	208.3±188.6 ^b	0.016

All values are means ±SD.

^a p-value is calculated from one-way ANOVA.

^b Significantly different from Control and PD-exposed subjects.

Table 2
Pesticide exposure information

ID	Occupation	Exposure (years)	# of Pesticides
Subject 1	Farmer	25	7
Subject 2	Commercial Pesticide Applicator	15	6
Subject 3	Commercial Pesticide Applicator	15	5
Subject 4	Commercial Pesticide Applicator	25	10
Subject 5	Farmer	25	12
Subject 6	Farmer	15	11
Subject 7	Farmer	15	9
Subject 8	Farmer	25	21
Subject 9	Commercial Pesticide Applicator	25	16
Subject 10	Farmer	8	7
Subject 11	Farmer	25	16
Subject 12	Farmer	15	10
Mean (SD)	n/a	19.4 (6.1)	10.8 (4.8)