Echogenicity of the Substantia Nigra

Association With Increased Iron Content and Marker for Susceptibility to Nigrostriatal Injury

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Background: Patients with Parkinson disease characteristically exhibit an increased echogenicity of the substantia nigra (SN) on transcranial sonography, a new neuroimaging technique. The same echo feature of the SN can be identified in 9% of healthy adults.

Objective: To evaluate the relevance of the echogenic SN in healthy adults.

Design: In the first part of the study, 10 healthy subjects younger than 40 years with a distinct SN hyperechogenicity underwent extensive neurological, motor, neuropsychological, and fluorine 18-dopa positron emission tomographic ([18F]-dopa PET) examinations. Results were compared with those of 10 subjects with a low echogenic SN. In the second part of the study, the postmortem brains of 20 patients without extrapyramidal disorders during their lifetime were sonographically examined with a particular focus on SN echogenicity. Subsequently, one half of the brain was prepared for heavy metal analysis, the other for a histological examination.

Results: Healthy subjects with SN hyperechogenicity exhibited a significant reduction of the [18F]-dopa uptake, especially in the putamen (Wilcoxon matched pair test: left side, P = .006; right side, P = .009), whereas their neuropsychological and motor performance were normal. Postmortem studies showed that the echogenicity of the SN correlated with its iron content.

Conclusions: Increased echogenicity of the SN, characteristically seen in Parkinson disease, is related to a functional impairment of the nigrostriatal system (even in young healthy adults) that can be revealed by [18F]-dopa PET studies. Substantia nigra hyperechogenicity is related to a higher tissue iron level, which is known to enhance the cells’ generation of reactive oxygen specimens. Therefore, we hypothesize that transcranial sonography may identify a susceptibility marker for the development of nigral injury that can be detected early in life, prior to the onset of Parkinson disease.

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SUBJECTS AND METHODS

[18F]-DOPA PET STUDIES OF HEALTHY VOLUNTEERS WITH A HYPERECHOGENIC SN

We identified 10 healthy subjects younger than 40 years with a distinct increase in SN echogenicity (mean ± SD age, 26.7 ± 7.6 years; 3 women and 7 men, all right-handed). Identification and selection of subjects were performed as described previously.3 Subjects were selected from a group of 120 members and students of the University of Würzburg (Würzburg, Germany). Subjects with a hyperechogenic SN with an echogenic signal extension of more than 0.25 cm² on at least 1 side (prevalence, 9%) and no central nervous system disorders were included in this study. Eleven subjects fulfilled these criteria, and 10 of them gave their informed consent according to the Declaration of Helsinki11 to participate in further examinations, including a thorough neurological examination, comprehensive motor and neuropsychological testing, and an [18F]-dopa PET examination. For comparison, we selected 10 healthy controls (3 women, 7 men; mean ± SD age, 40 ± 15.3 years) with areas of low SN echogenicity (area of hyperechogenic signal of the SN < 0.2 cm²) matched for sex and IQ who had already undergone the [18F]-dopa PET protocol in the same nuclear medicine center at the PET Center of the University of Mainz (Mainz, Germany) in the last 12 months. The selection of the control group was performed according to the recommendation of the ethics committee of the University of Würzburg. Following the proposal, we accepted an older age of control subjects because the age-related decline in [18F]-dopa uptake would lessen the differences among a group of subjects with nigral injury and the control group. Control subjects also had an [18F]-dopa PET study, a sonographic examination, and a motor and neuropsychological test battery.

Neuroimaging

For the TCS examination, we used a color-coded phased-array ultrasound system equipped with a 2.5-MHz transducer. The examination was performed through the preauricular acoustic bone window using standard techniques as described previously,13 with the aim of identifying the SN as clearly as possible within the hypoechoic mesencephalic brainstem. Because the signal brightness (echogenicity) is not quantifiable using ultrasound, the area of hyperechogenic signals in the SN region was encircled and measured. All individuals were assessed independently by 2 experienced TCS investigators. The average values of both measurements of each side were used for further analysis. The TCS examiners were blinded to the results of the PET study and the neuropsychological assessments.

Positron emission tomographic scans were performed on an ECAT EXACT 47 tomograph (Siemens, Erlangen, Germany), which provided 47 simultaneous planes with a physical slice thickness of 3.7 mm in a field of view of 16.2 cm. The thickness of the reoriented slice used in the analysis was 6.07 mm. The axial resolution (full with half-maximum) was less than 6 mm, and the in-plane resolution was less than 5.5 mm. Standard techniques as described previously were used for the [18F]-dopa PET scans.3 Activity ratios were calculated as the ratio of the specific [18F]-dopa uptake in the caudate nucleus and putamen divided by the mean uptake in the occipital cortex. In addition, influx constants (Ki) were calculated from the right and left caudate and putamen by using a modified compartment model approach with an occipital nonspecific tissue rather than a plasma input function.3

Motor and Cognitive Examinations

Motor function was assessed using a pegboard examination and a series of foot- and finger-tapping tests for a period of 32 seconds.3,12

In the neuropsychological assessment, standardized psychometric test procedures were used to measure memory (Digit Span Forward and Digit Span Backward of the Wechsler Memory Scale–Revised),13 attention (Alertness and Divided Attention task of the Computerized Neuropsychological Assessment of Attention Deficits),14 and executive functions (S Word Test, H/T Word Test, and the Tower of London Test).13,14 The examiner assessing motor and cognitive performance was unaware of the TCS and PET results.

ANALYSIS OF THE POSTMORTEM SN AND RELATION OF SN ECHOCENICITY TO BIOCHEMICAL AND HISTOLOGICAL PARAMETERS

In the second part of the study, we compared the echo pattern of the SN with histological and neurochemical whether the association between increased SN echogenicity and nigral neural impairment could be identified in this age group. In the second part, reasons for the increase in tissue echogenicity of the SN were investigated by studying postmortem brains and correlating ultrasonographic and neurochemical findings.

RESULTS

[18F]-DOPA PET STUDIES OF HEALTHY VOLUNTEERS WITH AN INCREASED AREA OF SN HYPERECHOCENICITY

In the 10 healthy subjects with SN hyperechogenicity, the median extension of hyperechogenic signals was 0.31
findings to identify the cause of the increased echogenicity. We examined the postmortem brains of 20 patients (6 women and 14 men; median age, 51 years; range, 38–59 years). None of the patients had experienced any extrapyramidal disorder (including PD) prior to death, and no subject had died of a neurological disorder. One patient had a history of epileptic seizures, and another had a history of meningoencephalitis. The average period between death and autopsy was 48 hours (range, 6–144 hours).

After autopsy, an ultrasound examination was performed with the unfixed brains immersed in a vessel filled with isotonic sodium chloride. For this examination we used the same ultrasound system as for the clinical investigations, with a 5-MHz transducer held under the surface of the fluid. For ultrasound system indexes, we chose a penetration depth of 4 to 8 cm and a dynamic range of 50 dB with high persistence. Image brightness and time gain compensation were adapted as needed for each investigation. The brain was placed upside down in the vessel to achieve an optimal picture of the SN. The whole brain was scanned in coronal and axial planes. Attention was focused on the SN region, where hyperechogenic areas were encircled and measured. The average area of hyperechogenic signals of both SNs was related to the neurochemical and histopathological findings.

After the ultrasound examination, all brains were divided midsagitally. The left half was stored at −80°C; the other half was fixed for 10 to 14 days in a solution of 4% paraformaldehyde.

Preparation of Tissue Samples for Trace Metal Analysis

Dissection of the hemisphere was performed when the temperature of the brain had risen to −10°C. The brain was coronally sectioned into slices approximately 10 mm thick. The SN was identified anatomically and dissected. Brain tissue was stored in plastic vessels.

Assessment of Total Iron, Copper, Manganese, Zinc, and Calcium Levels in SN Tissue

Brain specimens of approximately 50 mg were weighed and dried for 2 hours at 105°C. Nitric acid (250 mL, supra-pure) was added, and the mixture was incubated overnight. The mixture was completely digested at 56°C for 2 hours. Diluted samples were measured with a polarized Zeeman atomic absorption spectrophotometer (Z-8100; Hitachi, Tokyo, Japan) according to standard procedures. Concentrations were calculated from external and internal standards in the following ranges: 0.5 to 4 mg/mL (iron), 12 to 100 ng/mL (copper), 1.25 to 10 ng/mL (manganese), 0.25 to 2 mg/mL (calcium), and 1.25 to 10 ng/mL (zinc).

Histopathological Analysis

Dissected areas included the neocortex (Brodmann areas 3, 8, 9, 17, 18, 19, 21, 22, and 40), limbic areas (Brodmann area 24 and the hippocampus), striatum, thalamus, brainstem (SN and pons with the locus coeruleus and medulla), and cerebellum. All paraffin sections were stained with hematoxylin-eosin. Sections of the basal ganglia and SN were stained for iron using the Prussian blue stain. Additionally, selected areas were silver impregnated using the Bielschowsky method and stained for Luxol fast blue.

Immunohistochemistry was performed in selected cortical areas (Brodmann areas 9, 21, 22, 24, and 40), the SN, the locus coeruleus, and the hippocampus according to a standard protocol (peroxidase-antiperoxidase method). These specimens were incubated with phosphorylation-dependent anti-α antibodies (AT8; dilution, 1:800; Immunogenetics, Gent, Belgium), ubiquitin antibodies (dilution, 1:200; DAKO, Glostrup, Denmark), and α-synuclein antibodies (dilution, 1:200; Chemicon, Temecula, Calif). The quality of immunohistochemistry was checked by using control specimens from patients with Lewy body dementia (for α-synuclein antibodies), Alzheimer disease (AT8 and ubiquitin antibodies), and PD.

Biochemical and histological parameters were obtained by investigators blinded to the other results and to those of the ultrasound examination.

STATISTICAL ANALYSIS

Descriptive statistics are given as the median with lower and upper quartiles (25th and 75th percentiles). Intergroup comparison was performed using the U test, Wilcoxon matched pair test, and Kruskal-Wallis test; correlation analysis was done using the Spearman rank correlation and multiple regression analysis. Differences were assumed to be significant at P<.05.

PET Findings

The [18F]-dopa activity ratios for the putamen and the right caudate nucleus were significantly reduced in individuals with SN hyperechogenicity compared with the controls (Table and Figure 2), although controls were older and were therefore supposed to have a lower [18F]-dopa uptake because of age-related nigral cell loss. In addition, the median putamen and caudate Ki values were lower in individuals with enlarged areas of SN hyperchogenicity than in controls (Table 1). Differences were significant for the putamen on both sides (Wilcoxon test: left side, P=.04; right side, P=.03) but not for the caudate nucleus.

Motor and Cognitive Examinations

No significant differences between controls and individuals with more extended areas of SN hyperecho-
Increased echogenicity of the SN is the characteristic ultrasound feature of PD. Our findings indicate that the same echo pattern of the SN detected in young healthy subjects may be associated with an impairment of the nigrostriatal system as revealed by PET. Because the striatal $[^{18}\text{F}]$-dopa uptake is related to the number and metabolism of the nigral dopaminergic neurons, a reduced $[^{18}\text{F}]$-dopa tracer uptake in subjects with SN hyperechogenicity reflects nigral cell damage or a preexisting reduced cell count. These findings corroborate the data from our pilot study. However, subjects included in the present study were even younger. Both the echogenic SN sonographic phenotype and nigral dysfunction can therefore be depicted at a young age. Despite the reduction in striatal $[^{18}\text{F}]$-dopa uptake, results of neurological and neuropsychological examinations of the young subjects in the present study were normal.

Our data do not indicate that subjects with SN hyperechogenicity and reduced $[^{18}\text{F}]$-dopa uptake have preclinical PD because it is unknown whether nigral injury will continue in some of these subjects in the future. However, it may be tempting to consider SN hyperecho-

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**Figure 1.** A. Mesencephalic brainstem with normal findings on transcranial sonography (axial scanning plane). Number 1 indicates the butterfly-shaped mesencephalic brainstem surrounded by the hyperechogenic basal cisterns; arrow, the echogenic border zone of the brainstem raphe; and asterisk, the aqueduct. B. Subject with hyperechogenic substantia nigra on the ipsilateral (arrowheads) and contralateral (encircled) side, depicted within the peduncles of the mesencephalic brainstem (1). Arrow indicates the brainstem raphe; asterisk, the aqueduct.

**POSTMORTEM ANALYSIS OF THE SN AND CORRELATION OF SN ECHOCENICITY WITH TRACE METAL CONTENT AND RELATION TO HISTOLOGICAL PARAMETERS**

Sonographic examination of the SN in the 20 postmortem brains revealed a median extension of the hyperechogenic signals originating from the SN of 0.18 cm$^2$ (range, 0.11-0.25 cm$^2$; left side, 0.17 cm$^2$ [range, 0.11-0.23 cm$^2$]; right side, 0.18 cm$^2$ [range, 0.11-0.26 cm$^2$]). In 3 brains the extension of hyperechogenic signals was much higher than the threshold level of 0.25 cm$^2$ (range, 0.30-0.32 cm$^2$); in another 3 brains it was close to this threshold (range, 0.24-0.27 cm$^2$). The higher frequency of the ultrasound probe results in a higher spatial resolution and, consequently, slightly larger hyperechogenic SN areas compared with the transcranial findings. Analysis of the trace metal and calcium tissue levels of the SN with the extent of echogenic signals of the SN showed a positive correlation for iron (Spearman rank correlation: $R=0.57$; $P=.007$). Subjects with more extended hyperechogenic signals at the SN had higher iron levels than those with less echogenic SNs. **(Figure 3)**. No correlation was found for copper ($R=0.34$; $P=.13$), magnesium ($R=0.31$; $P=.18$), zinc ($R=0.28$; $P=.21$), or calcium ($R=0.43$; $P=.06$).

Histological examination of the SN revealed increased iron staining in subjects with higher iron levels and larger areas of SN hyperechogenicity **(Figure 4A and B)**. In one subject (age 51 years) with a large hyperechogenic SN area (0.3 cm$^2$) and a high SN iron level (752 ppm), histological analysis revealed Lewy bodies in the locus coeruleus (Figure 4C) and SN. Although α-synuclein and ubiquitin immunostaining showed several Lewy bodies in the SN (Figure 4D and 4E), they were even more numerous in the locus coeruleus. Evidence of active cell loss was seen including the presence of extraneuronal melanin and a slight gliosis. Results of the AT8 immunostaining were normal.

No statement regarding a selective reduction of pigmented neurons in the SN could be made because no quantitative morphometric evaluation of the SN was performed. This patient did not have PD or any symptoms attributed to diffuse Lewy body disease during his lifetime; therefore, a case of incidental Lewy body disease might be postulated. In no other patient were we able to identify α-synuclein-positive Lewy bodies in the SN or in any other brain area. In 3 cases, occasional τ-positive nerve cells and neurites in the SN and locus coeruleus were seen. In an additional subject, numerous senile plaques were identified in cortical areas and the hippocampus region. The other cases revealed no major gross or histological changes.

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Increased echogenicity of the SN may be associated with higher tissue iron levels, as seen in the postmortem examination. The finding of a correlation between tissue iron content and echogenicity is supported by an animal experiment revealing a dose-dependent increase in SN echogenicity after the stereotactic injection of various concentrations of iron into the SN. Increased iron content may explain not only the increase in echogenicity but also nigral cell injury as demonstrated by PET findings. From in vitro and in vivo experiments, it is known that iron may catalyze free radical formation, leading to an impairment of cellular function by a cascade of events including damage to the mitochondrial electron transport system, the induction of proteases, and increased membrane lipid peroxidation. However, increased iron content alone cannot be the reason for the observed increased SN echogenicity. Other brain structures with physiologically high amounts of iron, such as the globus pallidus or red nucleus, usually appear as echogenic or slightly echogenic structures on TCS. Furthermore, the physiologically iron-rich SN normally reflects only a slightly echogenic signal. Therefore, factors such as iron-binding proteins may be important in creating increased SN echogenicity.

Additionally, one postmortem study demonstrated an increased SN echogenicity, high SN iron levels, and Lewy bodies in the SN and locus coeruleus, classifying this case as one of incidental Lewy body disease. This single-case observation of an association among SN hyperechogenicity, high iron accumulation, and Lewy body formation must be replicated and confirmed in a larger series, particularly because it contrasts the findings of Dexter et al, who reported normal SN iron levels in patients with incidental Lewy body disease. The prevalence rate of subjects with SN hyperechogenicity is marked with an asterisk. Other brain structures with physiologically high amounts of iron, such as the globus pallidus or red nucleus, usually appear as echogenic or slightly echogenic structures on TCS. Furthermore, the physiologically iron-rich SN normally reflects only a slightly echogenic signal. Therefore, factors such as iron-binding proteins may be important in creating increased SN echogenicity.
imated risk of PD. Therefore, SN hyperechogenicity, which may be related to increased iron content, might be only 1 of several factors playing a role in the initiation of neuronal loss in the SN. Other factors such as endotoxins or exotoxins are probably necessary to induce or accelerate the degenerative process leading to clinical PD. According to the differences in the prevalence rates, it is clear that if at all, only a minority of subjects with SN hyperechogenicity will proceed to PD. However, recent findings provide evidence of an association between SN hyperechogenicity and motor impairment in elderly patients, indicating that the factors causing this echo feature may gain functional relevance during a patient’s lifetime. In a cross-sectional study including 93 subjects older than 60 years without prediagnosed extrapyramidal disorders, subjects with more extended hyperechogenic signals at the SN showed a more severe slowing of motor function even though most of them did not fulfill the diagnostic criteria for PD.

Our findings suggest that an increase in SN echogenicity, which appears to be related to an increase in tissue iron content, may point toward a susceptibility to nigrostriatal injury. This pattern of SN hyperechogenicity, similar to that found in PD, can be detected early in life. To further elucidate the significance of this finding and the relationship between SN hyperechogenicity and PD, follow-up studies with PET should clarify whether the functional impairment of the nigrostriatal system will progress in any of our subjects.

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