J Neural Transm (2010) 117:1287–1292
DOI 10.1007/s00702-010-0504-6

MOVEMENT DISORDERS - SHORT COMMUNICATION

Microglia activation is related to substantia nigra echogenicity

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Received: 20 July 2010 / Accepted: 5 October 2010 / Published online: 6 November 2010
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Abstract Hyperechogenicity of the substantia nigra (SN) is a sensitive marker for Parkinson's disease (PD). Previously, a relation between SN echogenicity and iron as well as neuromelanin content could be described in 60 human brains. In the present study on a subset of 33 brains, SN echogenicity was found to be correlated with microglia activation ($\rho = 0.46$, $p = 0.008$) after correction for iron and neuromelanin content. These findings strengthen the hypothesis of a close pathophysiological connection between SN hyperechogenicity and PD pathology.

Keywords Microglia · Ultrasound · Substantia nigra · Parkinson's disease

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Introduction

Pathophysiological mechanisms that have been discussed to contribute to the development of Parkinson's disease (PD) are among others accumulation of iron (Berg et al. 2001), intra- and extracellular deposits (Kazantsev and Kolchinsky 2008), oxidative stress and inflammatory processes mediated for example by extended microglia activation (Tansey et al. 2007).

Hyperechogenicity of the substantia nigra (SN) as measured by transcranial B-mode sonography (TCS) is a very sensitive and fairly specific diagnostic marker for PD, allowing a correct diagnosis in more than 90% of subjects even in very early disease stages. (Berg et al. 2008; Gaenslen et al. 2008). Previous post-mortem studies have shown that increased cellular iron content correlates with increased SN echogenicity, in human as well as in animal studies (Berg et al. 1999, 2002; Zecca et al. 2005). Moreover, also a small but significant negative correlation between neuromelanin, which is known to bind iron (Ben-Shachar et al. 1991; Tribl et al. 2009), and SN echogenicity could be found (Zecca et al. 2005). Additionally, the contribution of activated microglia to SN hyperechogenicity seems to be interesting since epidemiological, animal model and cell culture studies consistently support the essential role of activated microglia for the development of PD (McGeer and McGeer 2008) and microglia is also known to contain significant amounts of ferritin, the main iron storage protein (Connor et al. 1994).

Therefore, in this study we investigated in addition to the already published examinations concerning the relation of SN echogenicity, iron and neuromelanin content (Berg et al. 2002; Zecca et al. 2005), the association between SN echogenicity and activated microglia in post-mortem brains of subjects with no history of CNS diseases.

Subjects and methods

Sixty post-mortem brains from individuals with no history of psychiatric or neurological disease were obtained from the local Brain Bank in Würzburg. The use of post-mortem human brain tissue was approved by the Ethics Committee of the University Hospital of Würzburg. In addition to the examinations described previously (Berg et al. 2002; Zecca et al. 2005), a subset of 33 brains (20 men, 13 women; mean age 65.1 ± 11.5 years) was examined for activated microglia (Table 1). These included all brains in which iron and alpha-synuclein stainings and sections next to these for microglia staining were available. Brains with aggregates of hyperphosphorylated microtubule-associated protein tau in the substantia nigra visualized by antibody clone AT-8 ($n = 4$) were excluded. The average post-mortem time was 25.4 ± 6.4 h.

The ultrasound examination, brain dissection, photometric measurement of neuromelanin content and assessment of iron content by atomic absorption spectroscopy have been described previously (Zecca et al. 2005).

Determination of activated microglia

Staining for activated microglia was performed in the SN of both hemispheres in 17 subjects; in 16 subjects only the SN of one hemisphere was available for staining, resulting in a total of 50 analyzed SN. The tissue was fixed with formalin, embedded in paraffin and pre-treated with microwaves for antigen retrieval. Ki-M1P antibody, an in-house antibody provided from the University of Kiel (Germany), was used for staining of activated microglia. Ki-M1P is a monoclonal antibody raised against supernatants of detergent solubilized human lymph node tissue. The recognized antigen is composed of five proteins with molecular masses of 60, 92, 98, 124, and 150 kDa found in blood monocytes. In macrophages only the 60 kDa protein is recognized. (Radzun et al. 1991).

Immunohistochemistry was carried out on 3 μm thick paraffin sections using a biotinylated second antibody and a streptavidin-peroxidase detection system (StrAviGen MultiLink Kit; BioGenex, San Ramon, CA, USA) with H_2O_2 as a substrate and 3-amino-9-ethylcarbazole as a chromogen (BioGenex) according to the protocol given by the manufacturer. The degree of Ki-M1P staining was assessed semiquantitatively on a five-point scale by a person, blinded to the results of the ultrasound examination. Grade "1" was given for no specific Ki-M1P staining, "2" for sporadic staining, "3" for slight staining, "4" for moderately strong staining, and "5" for strong Ki-M1P staining (Fig. 1).

Statistical analysis

For statistical analysis we used SPSS 17.0 (SPSS, Chicago, IL, USA). The raw data is given in Table 1. Descriptive statistics are given as median and range, since data of SN echogenicity, SN iron content and SN neuromelanin content were not normally distributed. For analysis of correlations between demographical factors (age, sex, post-mortem time) and other parameters we used Spearman rank correlations. For further analysis partial correlations were used in order to correct for post-mortem time. Results were assumed to be significant at $p < 0.05$.

Results

Descriptive statistics

Four cases of Lewy body pathology were found in the general histopathological workup (Table 1). The median SN area of echogenicity was 0.20 (range 0.05–0.41) cm^2. Median tissue SN iron content was 0.16 (range 0.05–0.27) μg/mg wet tissue. Median SN neuromelanin content was 2.06 (range 1.01–2.97) μg/mg wet tissue. The extent of Ki-M1P staining was classified as grade 1 in 4 (8%) of the examined SN, grade 2 in 11 (22%), grade 3 in 9 (18%), grade 4 in 16 (32%) and grade 5 in 10 (20%) of the examined SNs.

Demographical factors

SN echogenicity, SN iron content, and microglia activation were neither correlated with age and sex, nor with post-mortem time ($p = 0.10$–0.91). Only SN neuromelanin content showed a significant correlation with post-mortem time in this subset of SNs ($\rho = 0.53$, $p < 0.001$). Therefore, for further analysis, data was corrected for post-mortem time.

Correlations

Reproducing the results of the previous study (Zecca et al. 2005), a moderately high positive correlation of SN iron content and SN echogenicity could be found ($\rho = 0.41$, $p = 0.002$) in the subset used in this study, as well as a small but significant negative correlation between SN neuromelanin content and SN echogenicity ($\rho = -0.29$, $p = 0.02$).

Moreover, SN echogenicity showed a small but significant correlation with Ki-M1P staining ($\rho = 0.28$, $p = 0.03$). Figure 2 illustrates the association of Ki-M1P staining and SN echogenicity, demonstrating a relation of SN echogenicity and Ki-M1P staining for small degrees of

Table 1 Individual data

No.	Age (years)	Sex	PMT (h)	COD	Side	SN (cm^2)	Ki-M1P (grade)	Iron (µg/mg wet tissue)	NM (µg/mg wet tissue)	LB
1	70	M	36	2	L	0.33	3	0.17	1.40	–
					R	0.34	3	0.15	1.40	–
2	73	M	24	2	L	0.30	3	0.16	1.98	–
3	83	F	31	1	L	0.25	2	0.13	1.51	
4	69	M	30	1	L	0.23	3	0.14	2.23	–
5	71	M	31	2	L	0.12	2	0.12	2.29	+
6	41	M	27	3	L	0.15	3	0.15	1.30	–
					R	0.23	2	0.11	1.27	–
7	73	F	27	1	R	0.09	1	0.15	1.75	–
8	66	F	19	3	R	0.07	1	0.08	2.02	–
9	63	F	33	1	R	0.05	1	0.05	1.68	–
10	85	M	19	1	L	0.17	2	0.17	2.40	–
11	57	M	24	1	L	0.17	2	0.11	1.93	–
					R	0.18	3	0.18	2.55	–
12	67	F	22	1	L	0.21	2	0.12	2.32	–
					R	0.24	3	0.10	1.66	–
13	56	M	32	1	R	0.13	2	0.19	1.85	–
					L	0.13	2	0.10	1.55	–
14	64	M	24	1	L	0.22	2	0.17	2.58	–
					R	0.41	3	0.23	2.29	–
15	71	M	21	1	R	0.21	2	0.09	2.76	+
16	64	M	20	3	L	0.22	3	0.21	1.86	–
17	58	M	23	1	R	0.10	2	0.10	2.13	–
					L	0.10	1	0.09	2.31	–
18	77	M	26	2	L	0.20	4	0.16	2.03	–
19	44	M	40	4	R	0.25	4	0.14	1.70	–
20	79	F	30	1	R	0.20	4	0.12	1.83	–
21	72	F	13	3	R	0.25	4	0.10	1.51	–
22	56	F	21	3	L	0.25	4	0.16	2.54	–
23	78	F	24	1	L	0.19	4	0.21	2.65	+
					R	0.22	5	0.22	2.50	+
24	72	M	24	2	R	0.19	5	0.20	2.44	–
					L	0.13	5	0.17	2.32	–
25	56	M	22	1	R	0.18	5	0.19	2.03	–
					L	0.25	5	0.23	2.31	–
26	74	F	36	1	L	0.21	4	0.22	1.44	–
					R	0.25	4	0.23	1.01	–
27	67	F	24	1	L	0.12	4	0.13	2.60	–
					R	0.14	5	0.12	2.60	–
28	43	F	36	4	R	0.25	4	0.16	1.53	–
					L	0.28	4	0.26	1.51	–
29	80	M	23	1	R	0.12	5	0.12	2.76	–
					L	0.21	5	0.21	2.00	–
30	58	M	24	2	R	0.12	4	0.18	1.63	–
					L	0.15	4	0.22	2.11	–
31	70	M	24	2	R	0.21	5	0.26	2.58	–
					L	0.20	5	0.27	2.97	+
32	47	F	12	5	R	0.14	4	0.17	2.58	–
33	64	M	20	4	R	0.15	4	0.17	2.12	–
					L	0.24	4	0.22	2.09	–

PMT post-mortem time, *COD* cause of death, *SN* substantia nigra, *NM* neuromelanin, *LB* Lewy body pathology, *1* cardiovascular arrest, *2* acute respiratory failure, *3* sepsis, *4* multiorgan failure, *5* cancer

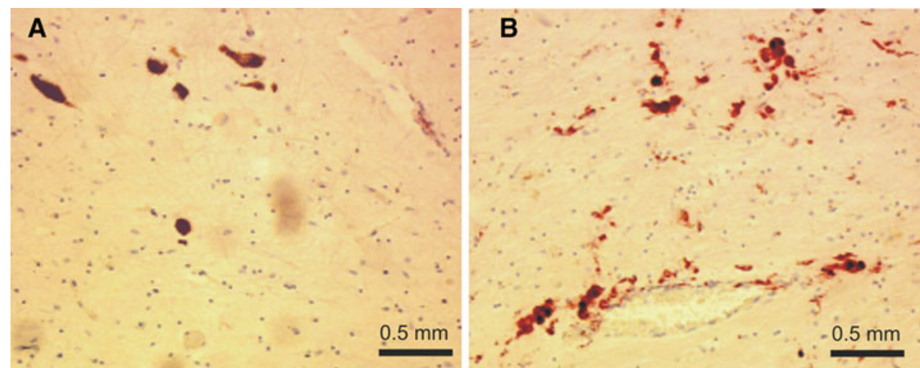

Fig. 1 Staining of activated microglia with KiMP1. Substantia nigra pars reticulata, 20-fold magnification. **a** Sporadic staining (grade 2) and **b** moderately strong staining (grade 4)

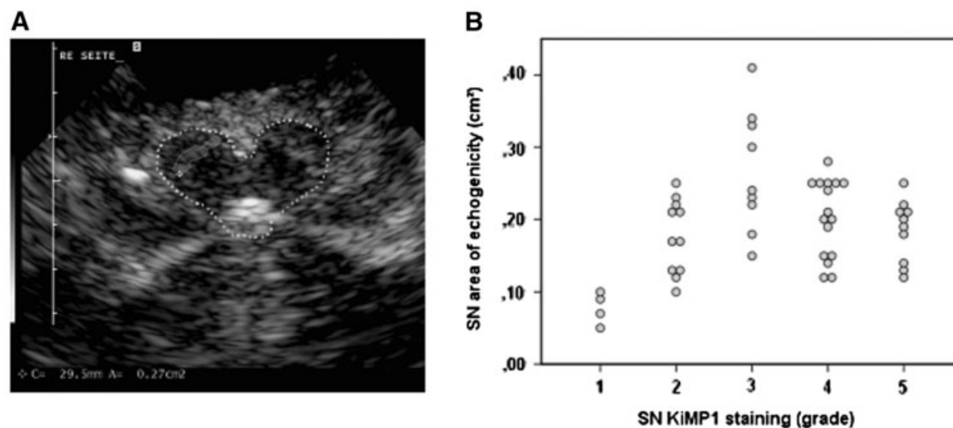

Fig. 2 Association of SN echogenicity and SN microglia activation. **a** Ultrasound image of a post-mortem brainstem. The *butterfly shaped* brainstem is encircled with a *dotted line*, the echogenic area of the SN is encircled with a *continuous line* unilaterally. **b** Correlation of SN echogenicity and Ki-M1P staining (grade 1–5), higher grades of Ki-M1P staining reflect larger amounts of activated microglia. Raw values only show a relation of SN echogenicity and small degrees of microglia activation (grade 1–3, $\rho = 0.74$, $p < 0.001$). After correction for neuromelanin and iron content (partial correlations), all degrees of Ki-M1P staining showed a moderately high correlation to SN echogenicity ($\rho = 0.46$, $p = 0.008$) (data not shown in this figure)

microglia activation (grade 1–3, $\rho = 0.74$, $p < 0.001$) which could not be found for extended microglia activation (grade 4–5, $p = 0.30$). However, Ki-M1P staining was highly correlated with SN iron content ($\rho = 0.61$, $p < 0.001$) and to a lesser degree also with neuromelanin content ($\rho = 0.28$, $p = 0.03$), which both are correlated to SN echogenicity (see above). After correction for neuromelanin and iron content (partial correlations), Ki-M1P staining showed a moderately high correlation to SN echogenicity ($\rho = 0.46$, $p = 0.008$).

Discussion

The aim of this study was to assess the impact of activated microglia on SN echogenicity in post-mortem brains of healthy humans. Indeed, we could demonstrate an association between SN echogenicity and activated microglia for small degrees of microglia activation, which became significant also for extended microglia activation after correction for SN iron and neuromelanin content. This suggests that all three parameters iron content, neuromelanin content and microglia activation independently contribute to alterations of SN echogenicity in healthy human brains. Increased SN iron content and increased amounts of activated microglia are associated with SN hyperechogenicity, whereas increased SN neuromelanin content seems to be related to reduced SN echogenicity.

The reason for activation of microglia in the examined healthy brains is not clear. Microglia activation as a sign of increased cellular distress can be caused by various conditions including oxidative stress, inflammation, apoptosis and hypoxia (McGeer and McGeer 2008) either during agony or clinically unapparent during lifetime. Another explanation might be a regional disruption of the blood brain barrier, resulting in inflammation and iron overload caused by presence of peripheral proteins such as albumin (Oestreicher et al. 1994).

According to our data, increased neuromelanin content seemed to reduce the effect of iron and microglia activation

on SN echogenicity. Partially, this effect might be due to complexion of iron in neuromelanin, where it is partially bound to ferritin (Tribl et al. 2009). Ferritin, in contrast to ferric iron, has been demonstrated not to alter SN echogenicity in rodent brains (Berg et al. 1999). These findings implicate that neuromelanin may preserve normal SN echogenicity by reducing excess ferric iron (Zecca et al. 2005). However, also activated microglia contains larger amounts of ferritin (Kaneko et al. 1989), although it appears to be related to increased rather than decreased SN echogenicity. Therefore, besides regulation of the content of ferric iron, also other factors which are not yet identified are likely to contribute to the formation of SN hyperechogenicity.

One may conclude from this data, that the relation of SN iron load, neuromelanin content and extent of microglia activation seems to be of great importance for the formation of sonographic hyperechogenicity of this region. All of these factors have been shown to be altered in conditions of oxidative stress, inflammation and apoptosis, which all have also been attributed to PD pathology. However, it seems not justified to generally apply results of this study to a PD population, since on the one hand in PD patients the equilibrium of iron, neuromelanin and activated microglia is known to be altered (Berg et al. 2001) and on the other hand factors such as atrophy or deposition of alpha-synuclein (Braak et al. 2003) may additionally influence SN echogenicity.

A number of cross-sectional studies indicate that SN echogenicity seems to be a stable marker in adulthood, which is especially interesting in terms of application as a preclinical marker for PD (for review see Berg et al. 2008) (Berg et al. 2008). Data of this study raises some concerns about the assumption of SN echogenicity as a stable biological marker. Although cellular iron homeostasis is kept rather stable in the SN, neuromelanin content and density increase during lifetime (Fedorow et al. 2006; Zecca et al. 2001). Moreover, cellular distress resulting in microglia activation may occur any time during lifetime. Since all of these factors are associated with SN echogenicity, it might be concluded, that at least in neurodegeneratively healthy subjects SN echogenicity could be a dynamic parameter.

In summary, SN echogenicity constitutes a surrogate sonographical feature, which is differentially influenced by tissue iron and neuromelanin content as well as by microglia activation. These findings may form a basis for a better understanding of pathophysiological mechanisms underlying the characteristic increase of SN echogenicity in Parkinson's disease.

Acknowledgment This manuscript is dedicated to Georg Becker, who pioneered the method of transcranial sonography and was one of the leading characters to initiate the study presented here. His sudden death in a sports accident in 2003 is a great loss to the scientific community.

The study was funded by a research grant from the Deutsche Forschungsgemeinschaft.

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