



Increased synphilin-1 expression in human elderly brains with substantia nigra Marinesco bodies

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Abstract:

The aim of the present study was to examine the expression of synphilin-1, α -synuclein, and tyrosine hydroxylase in human elderly brains and the incidence of Marinesco bodies (MBs, intranuclear inclusions) in the neuromelanin-containing substantia nigra neurons. The brains of twenty-two individuals without clinical signs and symptoms of parkinsonism and dementia and an additional two parkinsonian patients were dissected and subjected to histopathological examination and western blotting. Ubiquitin-positive and α -synuclein-negative MBs were found in 0.84–9.45% of the nigral neurons from brains of 15 healthy individuals and both parkinsonian patients. The frequency of pigmented nigral neurons containing MBs was positively correlated with age. The levels of tyrosine hydroxylase in the caudate nucleus and putamen decreased with age, and were inversely correlated with the MB frequency. The level of synphilin-1 in the caudate nucleus was positively correlated both with age and the MBs. Additionally, the MB appearance was correlated with synphilin-1 level in the substantia nigra. No significant correlation between α -synuclein expression and age or MBs was found.

Our results suggest that synphilin-1 expression increases with aging. Further studies on expression of this protein in elderly brains are warranted.

Key words:

aging, Marinesco bodies, tyrosine hydroxylase, synphilin-1, α -synuclein, substantia nigra, striatum, western blot

Abbreviations: LBs – Lewy bodies, MB – Marinesco bodies, NFT – neurofibrillary tangles, PD – Parkinson's disease, RT – room temperature, TH – tyrosine hydroxylase

Introduction

The accumulation of proteins, as well as the presence of intracellular inclusions in the surviving dopaminer-

gic, neuromelanin-containing neurons of the substantia nigra and in other affected regions of the central nervous system has been observed in several age-dependent neurodegenerative diseases. α -Synuclein and a number of other proteins have been detected in the insoluble intracytoplasmic inclusions known as Lewy bodies (LBs), which are present in the substantia nigra and locus coeruleus in Parkinson's disease (PD) and other synucleinopathies [10, 13, 17]. The

synphilin-1 protein is known to interact directly with α -synuclein and is found in LBs. Immunoreactivity for synphilin-1 has also been detected in glial cytoplasmic inclusions [3, 19, 30, 44, 45].

Both α -synuclein and synphilin-1 are presynaptic, vesicle-binding, cytosolic proteins [39, 44] (for review, see [17, 19]). Several pieces of evidence indicate that α -synuclein influences the functioning of dopaminergic neurons by reducing dopamine synthesis and release [37]. Moreover, the relationship between gene mutations of this protein and early-onset familial PD has strongly suggested its critical role in this disease (for review see [14]). Furthermore, several recent studies have shown that α -synuclein oligomers are toxic [2, 7, 24]. Overexpression of this protein leads to its aggregation *in vitro* [38], as well as to its accumulation in dopaminergic neurons in the substantia nigra, reduction of axon terminals in the striatum [29], or even degeneration of these neurons *in vivo* [18, 47].

Synphilin-1 is a protein of unknown physiological function [19]. *In vitro* studies have shown, however, that this protein reduces dopamine release [31]. Additionally, the Arg621Cys polymorphic form of synphilin-1 can create cytoplasmic inclusions in transfected cells, which become more susceptible to apoptosis than those expressing the wild-type protein [28]. However, the role of synphilin-1 in intracellular inclusion formation, brain degeneration, or age-dependent neuronal alterations in humans is not thoroughly understood.

Marinesco bodies (MBs) are eosinophilic intranuclear inclusions originally described in pigmented neurons in the substantia nigra and locus coeruleus of the human brain [27]. MBs are immunohistochemically positive for ubiquitin [4, 8, 36, 43, 46, 48]. MBs are found in normal brains of elderly individuals and their frequency increases with age [4, 48]. Also, they are more abundant in dementia with LBs and trinucleotide repeat neurodegenerative diseases, such as type 3 spinocerebellar ataxia and myotonic dystrophy [4, 33, 34]. A recent study has shown that the frequency of these inclusions significantly and inversely correlates with the striatal concentration of dopamine transporter and tyrosine hydroxylase, the dopaminergic terminal markers [4]. Therefore, it has been suggested that MBs may be involved in the age-related loss of nigral dopaminergic neurons [4].

The presence of ubiquitin aggregates in MBs suggests disturbances in the ubiquitin-proteasome system, which may be the common mechanism involved

in the formation of both MBs and LBs [4, 8, 36, 46]. Having revealed that α -synuclein and synphilin-1, the above-mentioned main constituents of LBs, are substrates of ubiquitination [6, 26], it is important to examine whether the expression of these proteins is altered in brains with MBs.

Therefore, we compared the expression of tyrosine hydroxylase (a marker of dopaminergic neurons), α -synuclein, and synphilin-1 with the presence of MBs in elderly human brains and in two parkinsonian brains, particularly in regions with the predilection for LB formation.

Materials and Methods

The studies were performed on the brains of 22 individuals, ages 23–95 (mean age = 63.7), who died of various causes, but did not exhibit the clinical signs and symptoms of parkinsonism or dementia, and two brains from PD patients, ages 70 and 75 (mean age = 72.5). All patients were recruited to the study from the University Hospital, Collegium Medicum in Kraków. Post-mortem examination of patients' tissues was conducted after the next of kin provided the written consent. Clinical evaluation, including a detailed and structured history and physical examination, was performed longitudinally. The diagnosis of PD was established according to the United Kingdom Parkinson's Disease Society Brain Bank criteria [12] in conjunction with substantia nigra LBs. Patients were clinically defined as "definitely affected" if they showed at least three of the four cardinal motor signs (resting tremor, bradykinesia, rigidity, and postural instability) with no evidence of pyramidal, cerebellar, and sensory involvement, disturbance of eye movements, early dementia, or severe early autonomic dysfunction. Disease severity was measured with the Unified Parkinson's Disease Rating Scale, and cognitive function was evaluated with the Mini-Mental State Examination (MMSE).

Tissue sectioning

The same qualified group of neuropathologists always collected the fresh tissue samples. Sectioning was performed 8–18 h after death. Samples from the left side of the brain were taken for western blotting,

while those from the right side were subjected to neuropathological examination.

Samples for the western blotting were as follows: 1) the most rostral part of the substantia nigra pars compacta lying anterior to the oculomotor (III) nerve outlet (level XXXII F.p. 3 – according to the stereotaxic atlas of Schaltenbrand and Bailey [40]) (weights of samples: 0.004–0.54 g), 2) the most anterior part (head) of the caudate nucleus located in close proximity to the lateral ventricle (level XXVII F.a. 29 – according to the stereotaxic atlas of Schaltenbrand and Bailey [40]) (weights of samples: 0.065–1.16 g), and 3) medial part of the putamen (level XXVII F.a. 18 – according to the stereotaxic atlas of Schaltenbrand and Bailey [40]) (weights of samples: 0.024–1.05 g).

Samples for the neuropathological examination were as follows: mesencephalon (containing the substantia nigra), dorsal pons (containing n. locus coeruleus), anterior and middle part of the basal ganglia (globus pallidus, caudate nucleus, putamen), hippocampal gyrus with entorhinal cortex, amygdala, and neocortical areas (frontal, parietal, temporal).

Neuropathological examination

The immunohistochemical methods were applied with two goals in mind. First, ubiquitin and α -synuclein were used to detect and differentiate MBs and LBs. Second, β -amyloid, tau-protein, and ubiquitin were used to detect any other pathology that could indicate “senile” or neurodegenerative processes like senile plaques, neurofibrillary tangles (NFTs), dystrophic neurites, and other changes frequently associated with Alzheimer’s diseases and other forms of neurodegeneration.

The samples were fixed in 4% formalin solution, embedded in paraffin blocks, and cut into 6 μ m thick sec-

tions. Series of 8 consecutive sections were taken with 400 μ m intervals between the series. After mounting on glass slides, the neighbouring sections were stained both immunohistochemically and with hematoxylin-eosin. Details of the immunohistochemical procedures are shown in Table 1. All sections used for immunohistochemical staining were slightly counterstained with hematoxylin, for visualization of cell nuclei.

Quantification of Marinesco bodies (MBs)

MBs were counted in the substantia nigra pigmented neurons, which were immunohistochemically stained for ubiquitin in all 24 cases. Sections were examined using a Nikon light microscope equipped with an optic device for morphometry. A magnification objective of 20 \times and 10 \times ocular was used for counting. In every case, the percentage of pigmented cells in the substantia nigra with intranuclear MBs was calculated, according to the method described by Beach et al. [4]. Briefly, MB-containing neurons in each brain were counted in 10 sections representing the anterior (rostral), middle, and posterior (caudal) parts of the substantia nigra. Moreover, this structure in each section was arbitrarily divided into three parts representing its medial, central, and lateral regions. The number of neurons with MBs was counted separately for each part in each section. In each area of the substantia nigra (as described above), a minimum of 50 pigmented neurons were assessed for the presence or absence of MBs, and the calculation of the percentage of cells with MBs was made in relation to all the pigmented neurons within the structure. The results of the assessment of the percentage of MBs were averaged separately for each part and region of the substantia nigra and then for the whole structure for each individual.

Tab. 1. Immunohistochemical methods

Antibody	Source	Titer	Incubation	Visualization	Antigen retrieval
α -Synuclein	Novocastra	1:30	24 h, 4°C	ABCComplex	MV 6 min 600 W + f.a.*, citrate buffer pH = 6.0
Ubiquitin	DAKO	1:100	24 h, 4°C	PAP-	
β -Amyloid	DAKO	1:100	30 min, RT	Envision HRP	MV 6 min 600 W citrate buffer pH = 6.0
Tau protein	DAKO	1:100	24 h, 4°C	Envision HRP	–

* Formic acid digestion 3 min, HRP – horseradish peroxidase, MV – microwaving, RT – room temperature

Western blotting

Antibodies and dilutions

The following antibodies and dilutions were used:

1. Mouse monoclonal anti-human tyrosine hydroxylase antibody (Novocastra, England, dilution 1:200).
2. Rabbit polyclonal anti-synphilin-1 antibody (concentration 4 µg/ml) was a generous gift from Professor Virginia Lee [30].
3. Rabbit polyclonal anti- α -synuclein antibody (Chemicon, USA, dilution 1:1,000).
4. Mouse monoclonal anti- β -actin antibody (Sigma-Aldrich, Germany, dilution 1:8,000).

Procedure

After dissection of the left substantia nigra pars compacta, caudate nucleus, and putamen, the structures were immediately frozen on dry ice and stored at -80°C until analysis. The tissue was homogenized on ice in a 2% sodium dodecyl sulfate-containing mixture of protease inhibitors (Pierce), denatured for 10 min at 95°C , and centrifuged for 10 min at $10,000 \times g$ at 4°C . Protein concentration in the supernatants was determined using a bicinchoninic acid protein assay kit (Pierce). Afterwards, the samples containing 10 µg or 20 µg of total protein were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously by Laemmli [21], and processed in order to detect tyrosine hydroxylase and synphilin-1.

Samples containing 20 µg of protein and fractionated by 15% SDS-PAGE were processed to detect α -synuclein. Proteins from resolved gels were then transferred to nitrocellulose membranes (Sigma). Non-specific binding sites were blocked for 2 h at room temperature (RT) by 2.5% BSA and 2.5% non-fat dry milk in Tris-buffered saline with 0.5% Tween 20 (TBS-T). The membranes were incubated overnight with appropriate antibodies diluted in 0.5% BSA, 0.5% non-fat dry milk in TBS-T at 4°C . After four subsequent washes in TBS-T, membranes were processed according to the standard BM Chemiluminescence western blotting kit protocol (Roche Molecular Biochemicals). Following immunoblot visualization, membranes were blocked with 5% non-fat dry milk in TBS for 10 min at RT and dried on absorbent filter paper. Afterwards, blots were erased in 62.5 mM TrisCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol for 30 min at 50°C , washed twice with TBS, and blocked

overnight with 5% non-fat dry milk in TBS at 4°C . As a control for protein level normalization, erased blots were processed with anti- β -actin antibodies, as described above.

The amounts of protein per lane, as well as antibody concentrations, were optimized in pilot studies so that threefold differences in protein content were linearly reflected on the immunoblots.

The signals were visualized and quantified by densitometric analysis with the FUJI-LAS 1000 system and Fuji Image Gauge v.4.0 software. Results are presented as a percentage of the control of the analyzed protein: β -actin ratio \pm SEM.

Statistics

To determine relationships between analyzed parameters (age, MBs frequency, tyrosine hydroxylase, synphilin-1, α -synuclein), Pearson's correlation was used.

Results

Clinical reports

The 22 patients without parkinsonian symptoms had no history of parkinsonism, dementia, or depression. Five of the patients died of myocardial infarction, and another four died of massive internal organ damage. Cardiac arrest was the cause of death of three patients. Respiratory failure was the cause of death of four patients. Pneumonia was the cause of death of two patients. Two patients died of cardiovascular failure: one due to hemorrhage arcus aortal, the other of a pulmonary embolism.

The two parkinsonian patients can be characterized as follows.

Patient 1 (idiopathic PD, **PD1**).

This 70-year-old man showed a right hand tremor at the age of 61. Shortly afterwards, bradykinesia developed in the right upper extremity. PD was diagnosed a year later. He received levodopa therapy and his motor function improved significantly. His disease progressed slowly. Two years later, he developed slowness of movement, his speech became softer and monotonous. Fluctuations in motor performance de-

Tab. 2. Evaluation of the percentage of pigmented neurons of the substantia nigra pars compacta (SNpc) containing Marinesco bodies (MBs)

No. of case	Age	MBs in whole SNpc (%)	Senile changes***	No. of case	Age	MBs in whole SNpc (%)	Senile changes***
1.	31	0.0	–	13.	79	2.3	++
2.	24	0.39	–	14.	81	2.61	+
3.	53	9.43	+	15.	77	2.29	+
4.	58	3.52	–	16.	95	0.0	+
5.	80	7.06	+	17.	69	4.1	+
6.	67	0.0	–	18.	61	1.45	–
7.	60	2.1	–	19.	73	2.14	–
<i>g^{PD}</i>	70	1.4	+	20.	90	1.43	+
9.	68	0.84	–	21.	78	4.46	–
10.	69	0.83	–	22.	23	0.21	–
11.	58	0.0	–	<i>23^{PD}</i>	75	3.6	+
12.	68	3.25	–	24	40	0.23	–

*** Senile changes are senile plaques and other Alzheimer's disease-related neuropathological changes; case no. 13 marked with ++ is the case that showed relatively strong Alzheimer-type changes, however, did not fulfil CERAD criteria. ^{PD} – Lewy bodies in substantia nigra and locus coeruleus confirmed Parkinson's disease

veloped over time. No pyramidal or cerebellar dysfunction, sensory disturbances, eye movement abnormalities, or dementia have been found (MMSE score, 27 of 30 points). During the following years, his condition deteriorated further, with more bradykinesia and postural instability. He was dependent in some daily activities. He died of pneumonia.

Patient 2 (idiopathic PD, PD2)

This 75-year-old male patient presented at the age of 65 with a left-sided coarse 5–7 Hz resting tremor affecting his arm and subsequently his leg. Rigidity and bradykinesia, although present, were not prominent clinical features. Five years into his disease, similar symptoms began to affect his right side. Disease progression was slow and he started levodopa therapy 6 years after the onset of symptoms. He displayed a good response to levodopa with the relief of bradykinesia and tremor. No cognitive decline, autonomic dysfunction, or other atypical signs were observed (MMSE score, 28 of 30 points). Over the past 3 years, he had developed wearing-off effects and dyskinesias. He remained relatively independent until his death from myocardial infarction.

Neuropathological alterations in the brains of non-neurological individuals and parkinsonian patients

Senile changes

NFT and senile plaques were observed in the entorhinal cortex and hippocampus in 10 brains (Tab. 2). No senile changes were observed in the remaining brains.

Marinesco bodies

In the series of normal individuals, ubiquitin-positive and α -synuclein-negative MBs were found in pigmented neurons of the substantia nigra in 80% of brains studied (Fig. 1, Tab. 2). MB frequency increased with advancing age (Fig. 2). A significant positive correlation was found between the percentage of nigral neurons containing MBs and age ($r = 0.44$; $p < 0.05$; Fig. 2). There was no correlation between the frequency of substantia nigra MBs and neurodegenerative changes of the senile type (Tab. 2). A medium number of MBs have been found in the neurons of substantia nigra in parkinsonian patients.

According to neuropathological and clinical examinations, brains were divided into: 1) control brains without or with MBs appearing only sporadically (**MB(-)**, 0–0.39% of nigral neurons containing MBs,

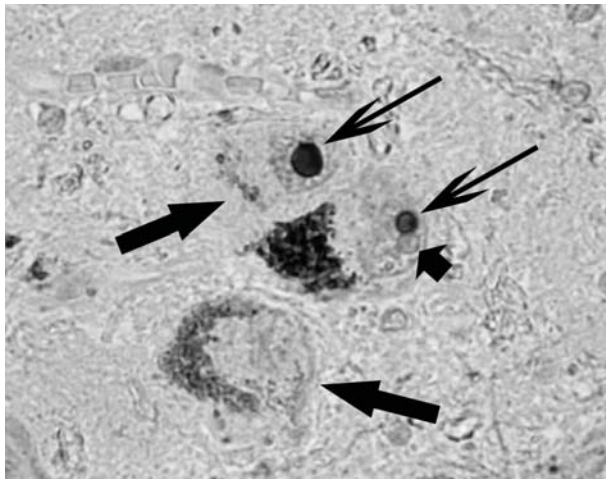


Fig. 1. A photomicrograph showing Marinesco bodies in dopaminergic neurons in a frontal section of the substantia nigra pars compacta of an elderly, non-neurological individual. Bold arrows – dopaminergic, neuromelanin-containing neurons. Thin arrows – Marinesco bodies in hematoxylin-stained nuclei. Arrowhead – nucleolus. Magnification: 280×

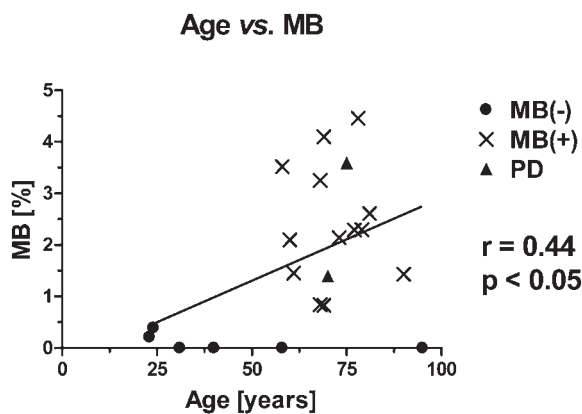


Fig. 2. Correlation between age and the Marinesco bodies (MB) frequency in the substantia nigra pars compacta. Abscissa – age in years; ordinate – the percentage of nigral neurons containing MBs. MB(-) – individuals without MBs, MB(+) – individuals with MBs, PD – Parkinson's disease patients

n = 7, mean age = 48 ± 10); 2) brains with low to medium frequency of MBs (MB(+), 0.84–9.45% of nigral neurons containing MBs, n = 15, mean age = 71 ± 2.6); and 3) parkinsonian brains (PD; 1.4 and 3.6% of nigral neurons containing MBs, n = 2, age = 70 and 75 years).

Lewy bodies

In the two parkinsonian patients, LBs were present in the substantia nigra pars compacta and locus co-

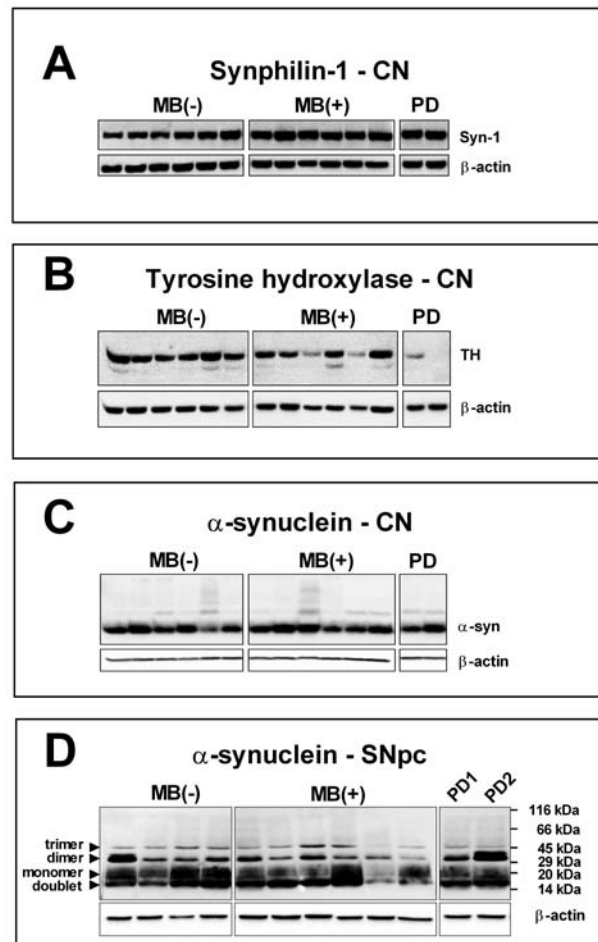


Fig. 3. Representative western blots of synphilin-1 (Syn-1, ca. 90 kDa) (A), tyrosine hydroxylase (TH, ca. 60 kDa) (B), α -synuclein (α -syn, ca. 17 kDa) (C), and β -actin (ca. 42 kDa) in the caudate nucleus (CN). (D) A representative western blot of α -synuclein (monomer, doublet ca. 17 kDa; dimer – ca. 34 kDa; trimer ca. 51 kDa) and β -actin (ca. 42 kDa) in the substantia nigra pars compacta (SNpc). MB(-) – individuals without MBs, MB(+) – individuals with MBs, PD – Parkinson's disease patients

eruleus, as well as the Lewy neurites. In the brains of individuals without neurodegenerative diseases, neither LBs nor Lewy neurites were observed in any case.

Protein levels in the brains of non-neurological individuals with Marinesco bodies and parkinsonian patients (western blot analysis)

Tyrosine hydroxylase

Tyrosine hydroxylase (TH) in the substantia nigra pars compacta, caudate nucleus, and putamen was visualized as a single band with a molecular weight of approximately 60 kDa (Fig. 3B). β -Actin, which

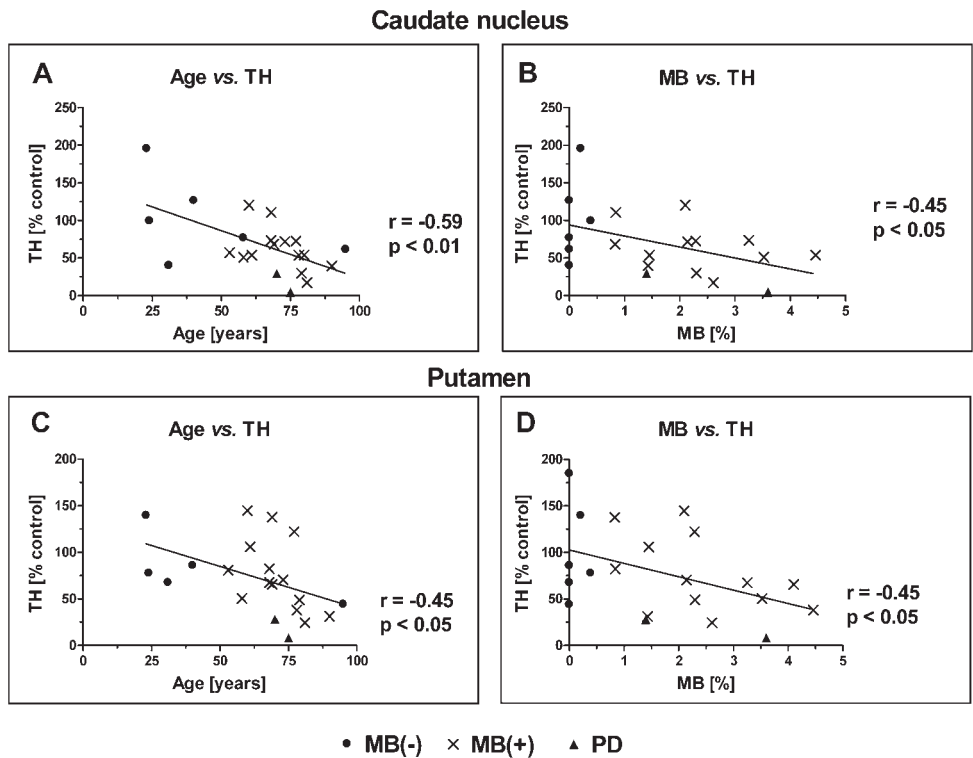


Fig. 4. Correlations between age or frequency of Marinesco bodies (MBs) in the substantia nigra pars compacta and levels of tyrosine hydroxylase (TH) in the caudate nucleus and putamen of non-neurological individuals and parkinsonian patients. Levels of TH were normalized by calculation the ratios of TH/ β -actin (western blot analysis). In control brains of individuals without MBs [MB(-)], the means of normalized levels of proteins were accepted as 100%. Abscissas – age in years (A and C), or percentage of nigral neurons containing MBs (B and D), ordinates – TH (% of control). MB(+) – individuals with MBs, PD – Parkinson's disease patients

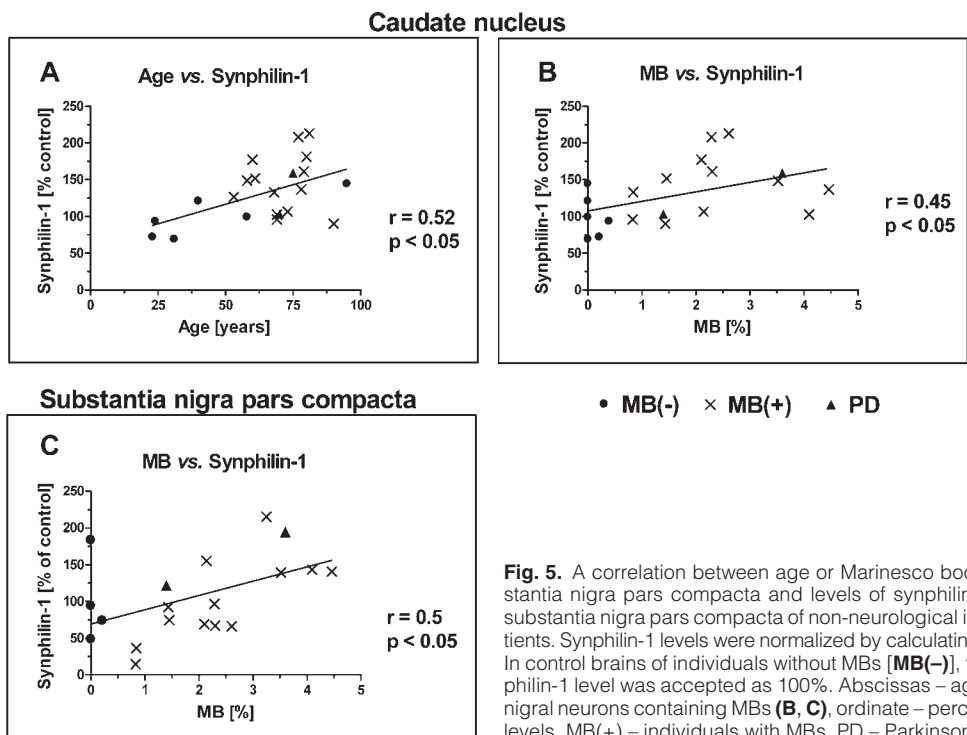


Fig. 5. A correlation between age or Marinesco bodies (MB) frequency in the substantia nigra pars compacta and levels of synphilin-1 in the caudate nucleus and substantia nigra pars compacta of non-neurological individuals and parkinsonian patients. Synphilin-1 levels were normalized by calculating the ratios of synphilin-1/ β -actin. In control brains of individuals without MBs [MB(-)], the mean of the normalized synphilin-1 level was accepted as 100%. Abscissas – age in years (A), or percentage of nigral neurons containing MBs (B, C), ordinate – percentage of the control synphilin-1 levels. MB(+) – individuals with MBs, PD – Parkinson's disease patients

served as a control of protein content in each sample, was seen as a band with a molecular weight of approximately 42 kDa (Fig. 3). In a group of control brains [MB(-)], a mean normalized TH level (TH/ β -actin ratio) was accepted as 100%.

Significant negative correlations were found between: (i) TH levels in the caudate nucleus and age ($r = -0.59$; $p < 0.01$; Fig. 4A); (ii) TH levels in the caudate nucleus and the percentage of nigral neurons containing MBs ($r = -0.45$; $p < 0.05$; Fig. 4B); (iii) TH levels in the putamen and age ($r = -0.45$; $p < 0.05$; Fig. 4C); and (iv) TH levels in the putamen and the percentage of nigral neurons containing MBs ($r = -0.45$; $p < 0.05$; Fig. 4D).

Synphilin-1

Synphilin-1 in the substantia nigra pars compacta, caudate nucleus, and putamen was visualized as a single band with a molecular weight of approximately 90 kDa (Fig. 3A). In a group of control brains [MB(-)], a mean normalized synphilin-1 level (Synphilin-1/ β -actin ratio \pm SEM) was accepted as 100%.

Positive correlations were observed between: (i) synphilin-1 levels in the caudate nucleus and age ($r = 0.52$; $p < 0.05$; Fig. 5A); (ii) synphilin-1 level in the caudate nucleus and the percentage of nigral neurons containing MBs ($r = 0.45$; $p < 0.05$; Fig. 5B); and (iii) synphilin-1 level in the substantia nigra pars compacta and the percentage of nigral neurons containing MBs ($r = 0.50$; $p < 0.05$; Fig. 5C).

α -Synuclein

α -Synuclein in the caudate nucleus and putamen was shown to be present in a monomeric form (a single band) with a molecular weight of ca. 17 kDa (Fig. 3C). In contrast, this protein appeared as a monomer, doublet, dimer and trimer in the substantia nigra pars compacta (Fig. 3D).

In a group of control brains [MB(-)], a mean normalized α -synuclein level (α -synuclein/ β -actin ratio \pm SEM) was accepted as 100%.

No statistically significant correlation between α -synuclein content in the caudate nucleus, putamen, and substantia nigra pars compacta and age or MB formation was found (data not shown). However, a negative trend ($p = 0.092$, $r = -0.38$) was seen between age and the level of this protein in the substantia nigra pars compacta (data not shown).

Discussion

The present autopsy study has shown the occurrence of intranuclear eosinophilic inclusion MBs in the substantia nigra pars compacta pigmented (neuromelanin-containing) neurons of elderly individuals without neurodegenerative diseases, as well as in two individuals with PD. The number of neurons containing MBs increased with age. These results are in agreement with previous findings of other authors, which showed the positive correlation between aging and occurrence of MBs [4, 48]. As it is known that the mesencephalon neuromelanin is selectively present in dopaminergic neurons, we can conclude that this type of neurons was affected.

Thus far, the role of MBs in aging processes and in the development of neurodegenerative diseases is unclear. Some earlier studies suggested that these inclusions had no pathological significance [9, 23, 48], and might be related to physiological aging processes [9, 15, 23, 48]. However, the frequency of these bodies also increases in hepatic encephalopathy [20, 41], trinucleotide repeat neurodegenerative diseases, e.g., myotonic dystrophy [33, 34], and in dementia with LBs [4], which may indicate their involvement in the pathophysiology of neurodegenerative disorders. Moreover, MB formation may be induced by fatal, severe stress to the central nervous system, which has been documented by their appearance in cases of fatal acute mechanical asphyxiation and drowning [36]. Our study also seems to suggest that despite a positive correlation found between the MB frequency and age of the examined individuals, physiological aging may not be a sufficient explanation for their occurrence. This conclusion is based on the finding that, as in earlier studies [36], the correlation coefficient between these variables was not very high ($r = 0.44$), and no MBs were observed in a 95-year-old man. Furthermore, the MB frequency was not correlated with occurrence of neurofibrillary tangles and senile plaques, typical senile neuropathological alterations. This suggests that physiological aging may be only one factor involved in MB formation in the neurons of the substantia nigra pars compacta.

The present study shows a significant negative correlation between the level of TH in the caudate nucleus or putamen and the age of the examined individuals. This result supports the well-known fact that during physiological aging, the number of dopaminer-

gic neurons in the substantia nigra pars compacta declines, which leads to a drop in all their markers (levels of TH, dopamine, dopamine transporter etc.) in the terminal field of the nigrostriatal pathway – the putamen and caudate nucleus (for review, see [35]). Additionally, we found a negative correlation between the percentage of substantia nigra neurons containing MBs and the TH level in the above-mentioned structures. Similarly to the present study, Beach and co-workers [4] found an inverse correlation between the MB frequency and the levels of TH or dopamine transporter (as markers of the dopaminergic terminals) in the caudate nucleus and putamen. Therefore, the current results may support the earlier suggestion [4] of a relationship between MB formation and the disappearance of dopaminergic neurons in the substantia nigra pars compacta and a loss of dopaminergic terminals in the caudate nucleus and putamen during aging.

The present study shows for the first time that the level of synphilin-1 in the caudate nucleus was positively correlated with age. The level of this protein in the substantia nigra pars compacta and caudate nucleus was also positively correlated with the frequency of MBs in pigmented nigral cells. Thus far, the role of synphilin-1 in the aging processes and MB formation is unknown. To our knowledge, our result pointing at this possible relationship is an innovative one.

Synphilin-1, a presynaptic, vesicle-binding, cytosolic protein of unknown physiological function, is a well-known constituent of LBs in PD [19, 30, 39, 44, 45]. This protein is distributed in a punctate pattern in neuropils throughout normal adult human and animal brains. However, perikarya and their processes also exhibit weak immunostaining for this protein [44]. Since synphilin-1 is not selective for dopaminergic neurons, the question arises as to the significance of its increased expression, shown in the present paper, to age-related functional alterations of these neurons. Animal studies have revealed, however, exceptionally high content of synphilin-1 in the substantia nigra pars compacta dopaminergic neurons [39]. Therefore, it may be supposed that the above-mentioned alterations of this protein in the substantia nigra were confined mainly to these neurons (cell bodies and dendrites). A positive correlation between the expression of synphilin-1 in the substantia nigra and the frequency of MBs seems to support this view.

The role of increased expression of synphilin-1 for the functioning of dopaminergic neurons could be

only a matter of speculation. Synphilin-1 is not toxic, however, its isoform synphilin-1A, expression of which amounts to 15% of synphilin-1, causes neuronal death [11]. Since the antibody used in the present study could not detect the latter toxic isoform, we cannot specify whether its expression also increases with age and parallels MB formation. However, if the latter view is true, such a process may contribute to the loss of dopaminergic neurons and the above-observed losses of TH in these cases.

Several studies have shown that synphilin-1 expression is closely related to the functioning of the ubiquitin-proteasome system. First, synphilin-1 is polyubiquitinated and subsequently degraded by the proteasome [19, 22]. Second, overexpression of both isoforms (synphilin-1, synphilin-1A) has been found to promote formation of aggregates by a process enhanced by the inhibition of the ubiquitin-proteasome system [11, 19, 22, 28, 32]. Moreover, the synphilin-1 overexpression inhibits the activity of the proteasome, which could lead to disturbances in the metabolism of other proteins [16] (for review, see [42]). In fact, inhibition of the proteasome activity was reported to occur during aging (for review, see [5]). Therefore, it may be speculated that the age-dependent increase in synphilin-1 expression contributes to the latter process, which leads to an accumulation of damaged proteins in intranuclear inclusions like MBs.

The present study seems to suggest that, in contrast to synphilin-1, the α -synuclein levels in the human substantia nigra, caudate nucleus, and putamen are independent of aging processes and mechanisms of MB formation. This conclusion is based on our western blot studies, which did not reveal any significant correlation between age or the frequency of MBs and α -synuclein levels in the above-mentioned structures. Recent studies by Li and coworkers [25] and Adamczyk et al. [1] have shown an age-dependent decrease in the α -synuclein mRNA expression in the human substantia nigra and rat striatum, respectively. However, earlier data concerning this protein level were controversial. The former authors [25] found an increase in the level of α -synuclein in the substantia nigra in elderly humans, whereas according to the latter [1], α -synuclein immunoreactivity was decreased in the cortex, hippocampus, and cerebellum, but not in the striatum of aged rats. Our present western blot analysis confirms the above-mentioned animal study [1], at least with regard to the striatum (caudate nucleus and putamen). Moreover, a non-significant trend

($p = 0.092$) concerning an inverse correlation between α -synuclein level in the substantia nigra pars compacta and age was noted.

In summary, the present study seems to suggest that synphilin-1 expression is increased during aging and is correlated with MB occurrence. Further research on the expression of this protein and others detected in intracellular inclusions in elderly brain are warranted. Such studies may provide invaluable insight into the basic mechanisms of aging and degenerative diseases.

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