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Absence of heparan sulfate proteoglycans in Lewy bodies and Lewy neurites in Parkinson's disease brains

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Abstract. α -Synuclein is the major constituent of Lewy bodies and Lewy neurites in Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Relatively little is known about the exact mechanism of α -synuclein deposition and fibrillization in these α -synucleinopathies. In order to better understand the pathogenesis of α -synucleinopathies it is important to identify molecules that regulate the fibrillization of α -synuclein. Since it has been demonstrated that heparan sulfate proteoglycans (HSPGs) and glycosaminoglycans (GAGs) promote the conversion of non-fibrillar amyloid β -protein (A β) into neurotoxic fibrillar A β in Alzheimer's disease, they might also be involved in α -synuclein aggregation. It was the aim of our study to examine the distribution pattern of these macromolecules in PD brains and the possible association with Lewy bodies and Lewy neurites. Although HSPGs clearly colocalized with senile plaques, we were unable to identify HSPGs or GAGs in Lewy bodies and Lewy neurites and therefore concluded that it is likely that α -synuclein fibrillization and stabilization occurs independently of the presence of HSPGs or GAGs.

Keywords: Lewy body, lewy neurites, parkinson's disease, heparan sulfate proteoglycan, glycosaminoglycan

1. Introduction

The neuropathological diagnosis of both Parkinson's disease (PD) and dementia with Lewy bodies (DLB) relies on identification of intraneuronal Lewy bodies and Lewy neurites [8]. These inclusions occur exclusively in specific types of neurons of select regions of the human nervous system [2,31]. It was demonstrated that α -synuclein is the major constituent of Lewy bod-

ies and Lewy neurites, and that anti- α -synuclein antibodies immunostain Lewy bodies and Lewy neurites in PD and DLB brains [1]. α -Synuclein is also the main component of the glial and neuronal inclusions of multiple system atrophy [13].

α -Synuclein was first isolated and purified from the electric organ of *Torpedo californica* [17]. It was suggested that α -synuclein plays a role in neuronal plasticity because its expression in songbirds increased during learning [10]. In normal functioning neurons, α -synuclein is synthesized in the perikaryon and transported to presynaptic terminals. α -Synuclein is usually localized in synaptic boutons, typically in close proximity of, or bound to synaptic vesicles [17]. The native protein likely plays a role in maintenance and stabi-

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lization of fully matured synapses [18]. However, its normal localization seems to be disturbed in PD/DLB, where reallocation occurs resulting in accumulation of α -synuclein in the cell body and in more proximal parts of the neurites. Monomeric α -synuclein can form fibrils in solution [10,12]. During this fibrillization process, native unfolded α -synuclein undergoes conformational changes resulting in partially folded intermediates and toxic protofibrils. It has been reported that heparan sulfate proteoglycans (HSPGs) and glycosaminoglycans (GAGs) promote the conversion of non-fibrillar $A\beta$ into neurotoxic fibrillar $A\beta$ [3,5,23] and may protect $A\beta$ against proteolytic degradation [5,22]. Furthermore, HSPGs/GAGs have consistently been found in all kinds of amyloid, regardless of the specific type of amyloidogenic protein deposited [23,26–30]. Therefore, it might be expected that these complex macromolecules play a similar role in α -synuclein fibrillogenesis and stabilization. It was the aim of our study to examine the distribution pattern of HSPGs and GAGs in PD brains in order to collect evidence for a specific *in vivo* association of HSPGs/GAGs with α -synuclein.

2. Materials and methods

2.1. Autopsy material

Brain tissue from patients with clinically diagnosed and neuropathologically confirmed PD (with or without dementia) was obtained at autopsy. All brains displayed the presence of α -synuclein-immunoreactive Lewy bodies and Lewy neurites, while at the same time being free of intracytoplasmic inclusions related to non-PD α -synucleinopathies. After the routine autopsy procedure, tissue samples from the anterior cingulate cortex were obtained and snap frozen in liquid nitrogen. Cingulate cortex sections of 10 PD patients (7 male, 3 female) were investigated for the presence of HSPGs and GAGs (age 74.8 ± 7.9 years; post mortem delay 20.1 ± 4.2 hours). This study was conducted with due regard for the restrictions of the Dutch legislation on the use of human tissue material for research purposes (informed consent was obtained of a legal representative of the patient). Samples were anonymized leaving only clinical data accessible.

2.2. Antibodies

Mouse monoclonal antibody synuclein Ab-2 (syn211, NeoMarkers, Fremont, CA), a highly spe-

cific mAb for α -synuclein that does not cross-react with β - and γ -synuclein, was used as primary antibody. Polyclonal antibody 40-4, directed against $A\beta$ (generous gift of Dr. W.E. van Nostrand, Stony Brook, NY [7]) was used to demonstrate the presence of senile plaques. Additionally, specific antibodies directed against heparinase-digested HSPGs (3G10), heparan sulfate side-chains (JM13, JM403), agrin core protein (JM72) and glypican-1 core protein (S1) were used to study the expression of these molecules in PD brains. The characterization of these antibodies has been described in previous reports [6,24]. Anti-glypican-1 (S1) and anti-agrin (JM72) antibodies were selected because both mAbs strongly stain a variety of amyloidogenic lesions such as neurofibrillary tangles, senile plaques and amyloid-laden vessels. We used biotin-labeled horse anti-mouse as secondary antibody with the exception of primary incubations with JM13 and JM403, in which case biotin-labeled goat anti-mouse IgM was used. All secondary antibodies and the avidin-biotin-peroxidase complex (ABC) were obtained from Vector (Burlingame, CA).

2.3. Immunohistochemistry

For immunohistochemical double-staining, 4 μ m cryosections were air-dried, fixed in acetone for 5 minutes, subsequently for 5 minutes in acetone containing 0.15% H_2O_2 to block endogenous peroxidase activity, and preincubated for 30 minutes with 20% normal horse serum with the exception of staining with JM13 and JM403 in which case we used 20% normal goat serum. Sections were incubated overnight at 4°C with either 3G10, JM13, JM403, S1 or JM72 at concentrations of 10 μ g/ml. In case of staining with 3G10, sections were pretreated with 50 mU heparitinase (heparitinase III, EC 4.2.2.8; Sigma Chemical Co., St Louis, MO) diluted in 10 mM HEPES and 2 mM $CaCl_2$ (pH 7.0) at 37°C for 1 hour. Subsequently, sections were incubated with either biotin-labeled horse anti-mouse antibody in case of primary staining with 3G10, JM72 and S1 or biotin-labeled goat anti-mouse IgM in case of primary staining with JM13 and JM403, for 30 minutes at room temperature and with avidin-labeled Texas Red for 45 minutes. Between incubation steps, sections were thoroughly washed with phosphate buffered saline (PBS). After the last rinse in PBS, sections were first incubated with a mixture of rabbit anti-mouse alkaline phosphatase and rabbit anti-mouse horse radish peroxidase for 30 minutes (dilution 1:20), to block residual free binding sites on the mouse antibodies from the

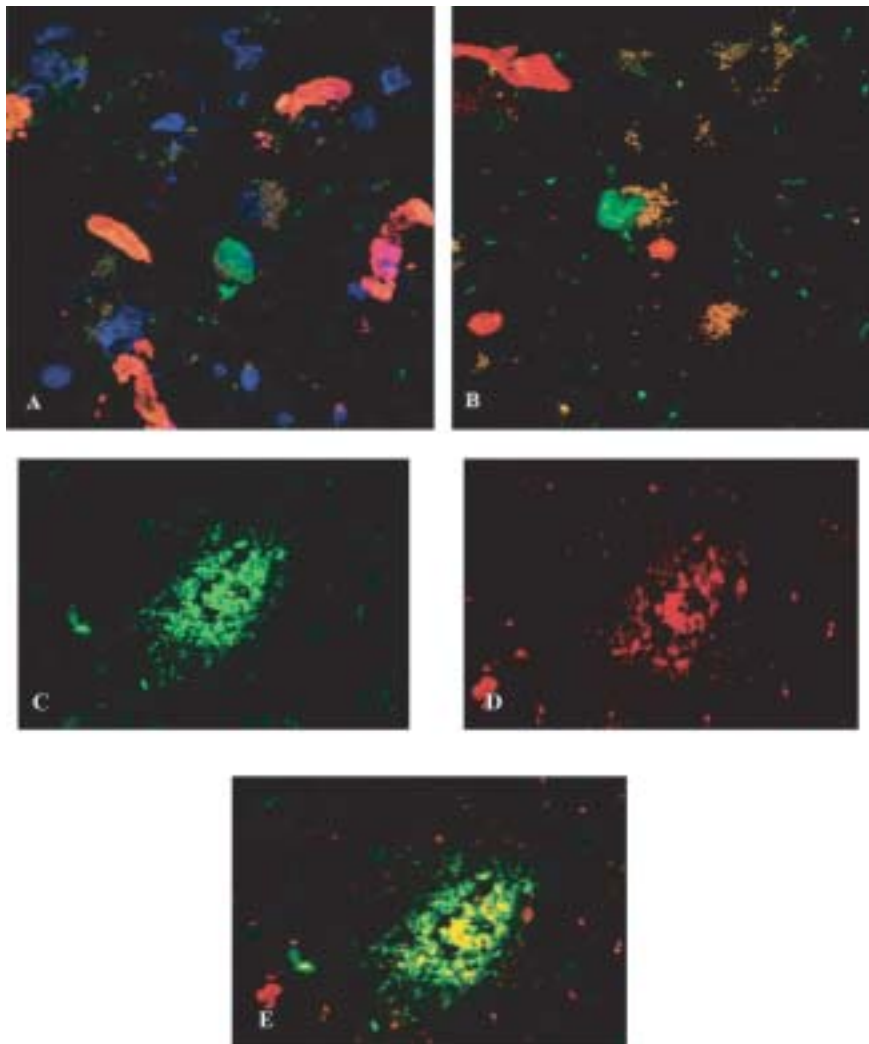


Fig. 1. Immunofluorescence staining of Lewy bodies, Lewy neurites and senile plaques in sections from PD brains for α -synuclein (mAb Ab-2, green) (A, B), HSPG core protein (3G10, red) (A and D), HSGAG (JM403, red) (B) and A β (pAb 40-4, green) (C) In A nuclei were stained blue. Cortical Lewy bodies and Lewy neurites are stained by the anti- α -synuclein antibody. Both 3G10 and JM403 strongly stained the cerebral (micro)vasculature. Neither HSPG core-protein nor HSGAGs colocalized with α -synuclein (A, B), whereas HSPG core-protein was present in A β -positive senile plaques (E). Original magnification 630 x.

first cycle. The success of this blocking procedure was verified by omitting secondary antibodies from the first cycle. Then, sections were washed with PBS and incubated with either anti- α -synuclein Ab-2 or anti-A β 40-4 at a concentration of 10 μ g/ml. Finally, sections were incubated with FITC-labeled sheep anti-mouse or swine anti-rabbit antibodies, respectively, for one hour at room temperature. Selected sections were also incubated with ToPro-3 for nuclear staining (dilution 1:1000, Molecular probes). Between incubation steps, sections were thoroughly washed with PBS and finally mounted in Vectashield (Vector, Burlingame, CA). All antibodies were diluted in PBS containing 0.1% bovine

serum albumin, which also served as a negative control. Sections were examined using a confocal laser-scanning microscope (Leica, Wetzlar, Germany).

3. Results

Ab-2, a monoclonal antibody directed against α -synuclein, clearly stained Lewy bodies and Lewy neurites throughout the cingulate cortex (Fig. 1A and B). Antibodies directed against HS side-chains (JM13, JM403) and heparinase-digested HSPGs (3G10) in-

tensely stained both parenchymal and leptomeningeal vessels. However, no colocalization with α -synuclein in Lewy bodies and Lewy neurites was observed (Fig. 1A and B). Senile plaques were observed in only one PD patient using anti-A β antibody 40-4 (Fig. 1(C)). In contrast to the absence of HSPGs and GAGs in Lewy bodies and Lewy neurites, senile plaques were clearly decorated with both JM 403 (data not shown) and 3G10 (Fig. 1D and E). Anti-glypican-1 (S1) and anti-agrin (JM72) antibodies did not stain Lewy bodies or Lewy neurites (data not shown). However, JM 72 clearly stained the basement membranes of cerebral vessels, indicating that the absence of Lewy bodies and Lewy neurites staining was not due to technical problems.

4. Discussion

Both Lewy bodies and neurofibrillary tangles are associated with cytoskeletal changes leading to accumulation of altered or misfolded proteins. Lewy bodies and Lewy neurites are characterized by inclusions of aggregated α -synuclein, whereas neurofibrillary tangles and dystrophic neurites are composed of microtubule-associated tau protein. Several studies demonstrated that heparan sulfate proteoglycans and heparan sulfate are present in both senile plaques and tangles, promote A β aggregation *in vitro*, and are involved in the stabilization of these amyloid deposits by providing protection against proteolytic degradation and subsequent removal [14, 15, 19, 23, 30]. A large number of components, also observed in association with senile plaques, including ubiquitin, neurofilaments and α B-crystallin have been identified in Lewy bodies. It is unclear, however, if this is a passive association or if these proteins affect α -synuclein fibrillogenesis. It has been described that some pesticides and metals may accelerate the rate of formation of α -synuclein fibrils *in vitro* [24]. Recently, it was found that tissue transglutaminase, by cross-linking α -synuclein, contributed to the generation of α -synuclein aggregates [16].

Relatively little is known about the role of heparan sulfate proteoglycans in α -synuclein fibrillogenesis [21]. Recently, Cohlberg and colleagues demonstrated that glycosaminoglycans, in particular heparan sulfate, heparin and other highly sulfated components, stimulated the formation of α -synuclein fibrils *in vitro* [4]. Apart from its catalytic function, heparin was also incorporated into the fibrils. Based on the binding of basic fibroblast growth factor to hepari-

nase sensitive sites in Lewy bodies, it was suggested that Parkinson disease-associated Lewy bodies contain heparan sulfate proteoglycans [19]. Additionally, several chondroitin sulfate proteoglycan subtypes accumulated in Lewy bodies [9]. We were unable to confirm the accumulation of heparan sulfate proteoglycans/glycosaminoglycans in Lewy bodies and Lewy neurites, suggesting that these sulfated molecules, in contrast to their role in AD pathology, are not involved in α -synuclein fibrillization and stabilization, suggesting that the binding of basic fibroblast growth factor to Lewy bodies may be based on a different type of interaction than the binding to heparan sulfate proteoglycans. In line with our previous observations that heparan sulfate proteoglycans are observed in senile plaques in AD brains (reviewed in [29]), we demonstrated that heparan sulfate proteoglycans and heparan sulfate colocalized with extracellular A β in senile plaques in PD brains. Furthermore, we previously have demonstrated that heparan sulfate proteoglycans accumulate in intracellular tangles [30]. Given the absence of heparan sulfate proteoglycans in Lewy bodies, the present study suggests that heparan sulfate proteoglycans are not always associated with any type of intracellular protein aggregation. In this respect, the pathogenesis of α -synuclein aggregation seems to differ from that of tau aggregation.

Although we cannot exclude that heparan sulfate proteoglycans and heparan sulfate are involved in early stages of Lewy bodies formation, such an involvement was not observed in post-mortem material as a clear colocalization, indicating that either Lewy body formation is independent of the presence of heparan sulfate proteoglycans and heparan sulfate, or that the early stages of Lewy body formation are not represented in our material. Therefore, future research, e.g. using animal models, is warranted to provide more insight into the exact mechanism of α -synuclein fibrillization and a possible role of heparan sulfate proteoglycans and glycosaminoglycans.

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