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Nerve cells expressing heat-shock proteins in Parkinson's disease

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Abstract A distinctive histopathological feature of several neurodegenerative diseases, including corticobasal degeneration, argyrophilic grain disease, progressive supranuclear palsy, and Pick's disease, are achromatic nerve cells that express small heat-shock proteins, such as αB-crystallin or hsp-27, and develop in specific telencephalic cortical areas and subcortical nuclei. Here, we point to the consistent presence of such cells in Parkinson's disease. In this disorder, the neurons under consideration remain immunonegative for phosphorylated neurofilaments or for ubiquitin, thus exhibiting an immunocytochemical profile different from that shown by αB-crystallin-positive neurons in other neurodegenerative disorders. In severe cases of Parkinson's disease, the αB-crystallin-positive neurons are dispersed throughout the cerebral cortex, amygdala, and ventral claustrum. In cases showing relatively mild involvement of the telecephalon, these neurons occur chiefly within the reaches of the anterior temporal and insular mesocortex. These telencephalic predilection sites are nearly identical with those of the α-synuclein pathology. Nevertheless, most of the telencephalic αB-crystallin-immunopositive neurons refrain from developing Lewy bodies and Lewy neurites and, *vice versa*, most of the nerve cells containing Lewy bodies do not accumulate αB-crystallin.

Keywords Anterior mesocortex · Ballooned neurons · Heat-shock proteins · Parkinson's disease

Introduction

Parkinson's disease (PD) is characterized morphologically by intraneuronal inclusion bodies containing α-synuclein (α-SN) known as Lewy bodies (LBs) in nerve cell somata and as Lewy neurites (LNs) in cellular processes [1, 16, 23, 25, 39, 41]. This disease-specific pathology evolves in only a few neuronal types in functionally related cortical areas and subcortical nuclei [6, 7, 8].

Apart from the LBs and LNs that contain α-SN, PD cases almost consistently present a supplementary pathology, which, to our knowledge, has not been described in sufficient detail previously. Because its detection is laborious in sections stained for general overview (H&E), it usually escapes recognition. Immunoreactions, however, for the small heat-shock or stress proteins αB-crystallin and/or hsp-27 unequivocally demonstrate this supplementary pathology.

Many heat-shock proteins (HSPs) function as molecular chaperones in that they prevent deleterious protein-protein interactions and assist in the refolding of denatured proteins [10, 19, 21, 26, 32, 37]. The HSP αB-crystallin is normally expressed solely by macroglial cells and not nerve cells. Up-regulation of HSPs is interpreted as one of many cellular responses to stress [17, 19, 20, 24]. Concentrations of HSPs high enough to be detected in immunoreactions during light microscopy are encountered frequently in activated macroglial cells. Similarly, impressive examples of up-regulated HSPs in neurons rarely are seen, but when they do occur it is only in a few types of telencephalic projection cells located in specific cortical layers and areas, as well as in some subcortical nuclei. The intraneuronal appearance of hsp-27 and/or αB-crystallin is often accompanied by telling changes in both the sizes and shapes of the involved neurons, which eventually display considerably bloated or "ballooned" cell bodies together with a few noticeably altered cellular processes [24, 29, 42].

Such neurons are found in other degenerative diseases, among them Pick's disease (PID), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), and progressive supranuclear palsy (PSP) [5, 11, 26, 27, 29, 31, 33, 34, 38, 40, 43]. In PD, up-regulation of αB-crystallin has been identified heretofore in astrocytes and oligodendrocytes [35]. The present study is aimed at supplement-

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ing this notion by showing that αB-crystallin-immunoreactive neurons (αBCINs) consistently develop in the course of PD.

Material and methods

We examined brains removed at autopsy from 19 individuals with clinically documented and neuropathologically confirmed idiopathic PD (11 females, 8 males, ages 72.8±7.0 years, Hoehn and Yahr stages III–V, concomitant Alzheimer's disease (AD)-related neurofibrillary changes rated at less than stage IV, amyloid load 0-C; Table1). In addition, 8 brains obtained at autopsy from persons lacking a history of neurological disorders were used for control and comparison (4 females, 4 males, ages 62.1±16.4 years, Hoehn and Yahr stage 0, concomitant AD-related neurofibrillary changes rated at less than stage IV, amyloid load 0-C, and tissue virtually free of other pathological changes; Table 1). The clinical and post-mortem neuropathological diagnoses of idiopathic PD were established using standard published criteria: The clinical protocols of all of the PD cases noted the presence of tremor, rigidity, and bradykinesia. Moreover, the brain tissue exhibited nigral LBs, loss of nigral neuromelanin-laden neurons, and an associated gliosis [11, 12]. Concomitant AD-related alterations were classified according to a published staging procedure [4]. Cytoskeletal changes related to AGD were classified according to published criteria [5]. None of the cases exhibited AGD-related lesions with the exception of cases 11 and 15, both of which demonstrated a mild degree of AGD pathology.

Following fixation by immersion in a 4% buffered solution of formaldehyde for at least 3 weeks, one hemisphere was cut in the frontal plane into three blocks. These blocks and the brain stems of all of the cases were embedded in polyethylene glycol (PEG 1000, Merck). Each central block and brain stem was then cut perpendicularly to the intercommissural axis of Forel (central block) or at right angles to Meynert's axis (brain stem) into uninterrupted series of 100 µm sections. Ten sets of free-floating sections, each cut serially 1 mm equidistant from the other, were collected.

The first series of sections was stained for both lipofuscin pigment (aldehyde-fuchsin) and Nissl material (Darrow red) to facilitate topographic orientation and identification of specific neuronal types classifiable by virtue of their respective pigment deposits [3]. The following two collections were processed with (1) a silver-pyridine method [9] for detection of LBs/LNs as well as β-amyloid deposits and neuromelanin granules [2, 36], and (2) a silver-iodide method [15] to assess the possible presence of neurofibrillary tangles (NFTs) and neuropil threads (NTs) [4, 22].

The fourth collection was immunostained for α-SN. Sections were pre-treated according to a standard protocol designed to inhibit endogenous peroxidase and prevent nonspecific binding. Incubation for 48 h in the affinity-purified α-SN antiserum (AFshp)

Table 1 Occurrence of nerve cells immunoreactive for heat-shock proteins hsp-27 and/or αB-crystallin in cases of PD and controls (*PD* Parkinson's disease, *H+Y* Hoehn and Yahr stage, *DSM-III-R* cognitive status of cases according to the Diagnostic and Statistical Manual criteria for dementia, *D* dementia, *ND* no dementia. The severity of the concomitant Alzheimer's disease-related pathology [4] appears in Roman numerals under NFP-AT (cortical neurofibrillary pathology of the Alzheimer type: stages I–VI), and upper case letters refer to β-amyloid deposition (*β-Amy: A–C, 0* no amyloid). *αB-crys* indicates the average density of αB-crystallin-immunoreactive nerve cells in the anterior mesocortex. hsp-27 refers to the average density of hsp-27-immunoreactive neurons (*0* no immunopositivity, *1* presence of a few isolated immunoreactive neurons, *2* moderate numbers, *3* dense accumulation of immunoreactive neurons, *n.e.* not evaluated)

Case	Age	Gender	Diagnosis	H+Y	DSM-III-R	NFP-AT	β-Amy	αB-crys	hsp-27
1	61	M	PD	III	D	I	0	1	n.e.
2	62	F	PD	V	D	I	B	1	1
3	63	F	PD	IV	ND	I	B	1	n.e.
4	67	M	PD	V	D	I	B	1	n.e.
5	68	F	PD	III	D	II	B	1	1
6	68	M	PD	IV	ND	III	C	1	1
7	69	F	PD	IV	D	I	A	1	n.e.
8	70	F	PD	V	D	II	B	3	n.e.
9	75	F	PD	IV	ND	II	C	1	1
10	75	M	PD	V	D	II	0	1	1
11	75	M	PD	III	D	I	B	1	n.e.
12	76	F	PD	IV	ND	II	0	1	n.e.
13	76	M	PD	V	D	II	A	1	1
14	77	F	PD	III	D	II	C	2	1
15	77	M	PD	IV	D	II	B	2	1
16	78	F	PD	IV	D	II	C	2	n.e.
17	79	M	PD	IV	D	II	C	1	n.e.
18	80	F	PD	V	D	II	C	2	n.e.
19	86	F	PD	V	D	III	C	3	2
20	33	F	Control	0	ND	0	0	0	0
21	45	M	Control	0	ND	II	A	0	0
22	59	M	Control	0	ND	I	0	0	0
23	62	F	Control	0	ND	I	0	0	0
24	69	F	Control	0	ND	I	B	0	0
25	69	M	Control	0	ND	II	B	0	0
26	80	F	Control	0	ND	II	B	0	0
27	80	M	Control	0	ND	II	B	0	0

at a dilution of 1:2,000–4,000 followed. This antiserum, which is specific to α-SN, was generated in sheep by W.P. Gai (see Acknowledgments) using a peptide corresponding to the amino acid residues 116–131 of the human α-SN [14]. After processing with biotinylated secondary antibodies (anti-sheep IgG, 2 h), the reactions were visualized with the avidin-biotin-peroxidase complex (ABC, Vectastain, Vector) and 3,3-diaminobenzidine-tetra-HCl/ H_2O_2 (D7679 Sigma). Omission of the primary antiserum resulted in non-staining.

Varying numbers of sections from the fifth collection were used for immunoreactions with antibodies against hsp-27 (1:1,000, StressGen), αB-crystallin (1:2,000, Novocastra), ubiquitin (1:500, DAKO), and phosphorylated neurofilaments (1:5,000, SMI 31, Sternberger). Some of the sections were stained initially for lipofuscin deposits, subsequently immunostained, and then coverslipped or counterstained a second time for Nissl material to permit recognition of immunopositive material in specific cortical layers and to identify select types of nerve cells. Others were silver-stained initially for LB/LNs and then underwent immunoreactions with an antibody against αB-crystallin, and still other sections were immunostained first with an antibody against αB-crystallin and subsequently with a second antibody against α-SN. Finally, all of the sections were cleared and mounted in a synthetic resin (Permount, Fischer).

Results

All of the PD cases examined exhibited LBs/LNs in neuromelanin-laden projection neurons of the substantia nigra and moderate to severe neuronal loss in this nuclear gray. Furthermore, the cases displayed the characteristic extranigral pathology that consistently develops in the course of this disorder. Varying densities of LBs and/or LNs were found within select nuclei in the brain stem, thalamus, hypothalamus, amygdala, and claustrum, as well as in select areas of the hippocampal formation, entorhinal region, meso- and neocortex. The anterior mesocortex, in particular the periallocortical transentorhinal and the proneocortical ectorhinal regions, showed a particular proclivity to develop the PD-specific lesions [6, 8]. All controls were free of this pathology. Many PD cases and controls contained concomitant mild AD-related neurofibrillary alterations corresponding to stages I–III. The PD cases either were devoid of telencephalic β-amyloid precipitates or contained only a few such deposits (Table 1).

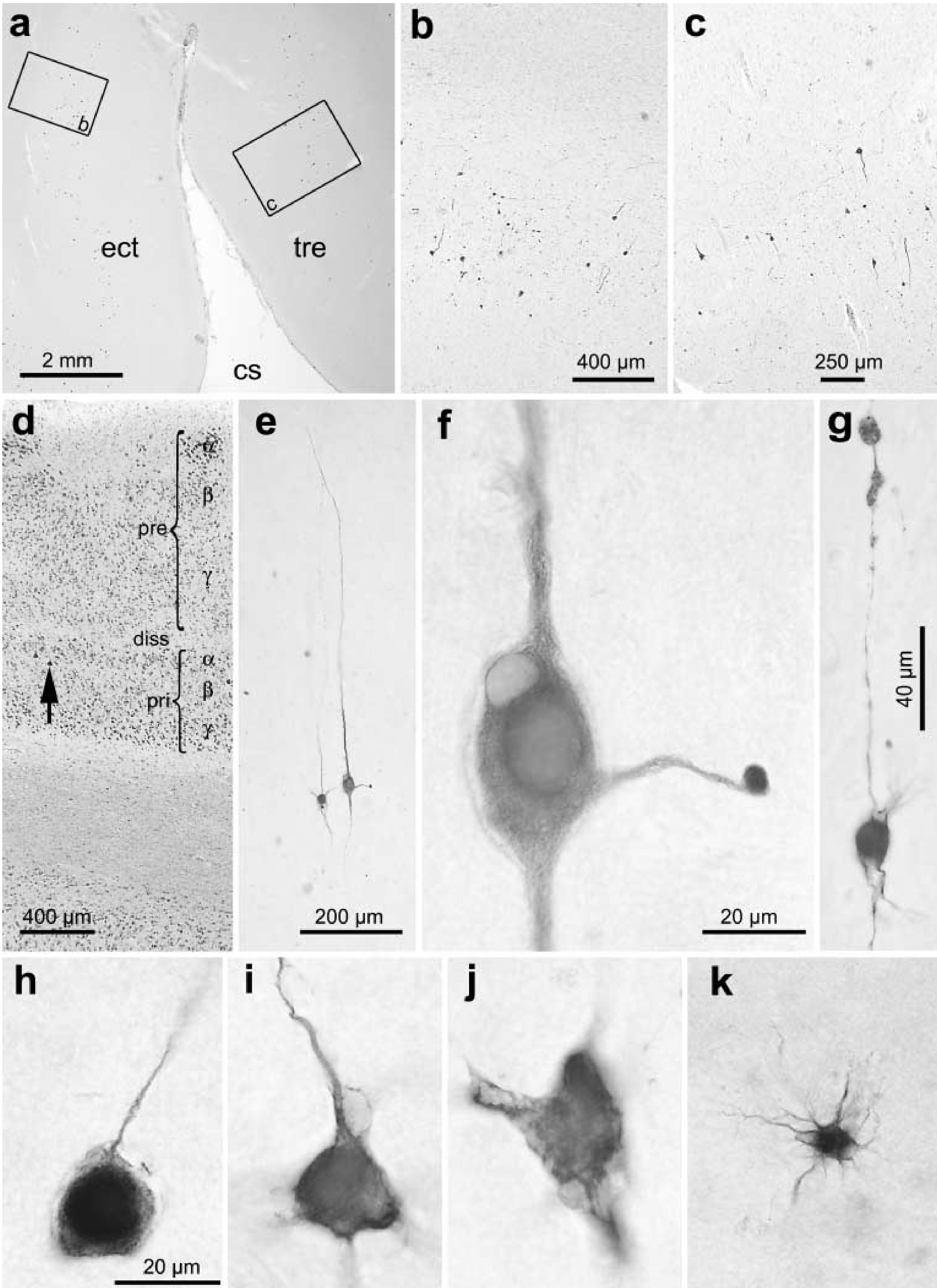
Notably, all of the PD cases displayed the supplementary pathology under consideration here. The PD-associated αBCINs usually elude detection light microscopically in sections stained with conventional techniques or with the Gallyas silver-staining method but were easily identified in immunoreactions directed against αB-crystallin or hsp-27 (Fig.1). The severity of this pathology varied among cases. None of the controls contained αBCINs (Table 1).

The PD-associated αBCINs were dispersed preferentially throughout the deep layers V–VI of the anterior temporal and insular neo- and mesocortex. Only on rare occasion were a few isolated α-BCINs seen in layer IIIc. Isolated or small groups of αBCINs were also encountered in both the amygdala and the ventral claustrum but not in other subcortical nuclear grays. The involvement of the hippocampal formation and entorhinal cortex was comparably mild. Allocortical predilection sites were the first Ammon's horn sector and layer pri-α of the entorhinal cortex (Fig. 1d). The highest density of αBCINs was usually attained in the anterior mesocortex, i.e., within a stretch of cortex encompassing, among other areas, the periallocortical transentorhinal region, the proneocortical ectorhinal region (Fig. 1a–c), and the insular mesocortex. Cases in which the anterior mesocortex was very severely involved likewise displayed remarkably high densities of αBCINs in the amygdala and ventral claustrum. Cortical αBCINs gradually decreased in number and maintained higher intervals from each other in an imaginary line extending from the anterior mesocortex into the neocortical prefrontal areas or temporal sensory association areas.

All of the neurons under consideration were intensely immunoreactive with antibodies against αB-crystallin (Fig. 1). A subset of these nerve cells also displayed a slightly less pronounced immunoreaction with antibodies directed against hsp-27 (Table 1). Notably, immunoreactions for the possible presence of phosphorylated neurofilaments within the somatodendritic compartments of the αBCINs proved negative, and the abnormal material contained in these neurons was also ubiquitin negative. Most of the αBCINs contained no α-SN-immunoreactive inclusion bodies and most of the immediately surrounding non-ballooned nerve cells containing LBs/LNs or AD-related NFTs/NTs lacked immunocytochemically detectable amounts of hsp-27 or αB-crystallin. In addition, no obvious relationship existed between αBCINs and extracellular deposits of β-amyloid protein. The overall packing density and topographic distribution of the αBCINs, however, reflected the overall severity and distribution pattern of both the PD-specific LBs/LNs and the AD-related NFTs/NTs.

Despite severe distortion of their somata and the cellular processes, αBCINs could be readily distinguished from activated astrocytes containing HSPs chiefly owing to their rounded and sharply drawn contours (compare Fig. 1e–j with k). In general, the αBCINs exhibited conspicuously bloated cell bodies. Such cells usually have reduced numbers of distorted cellular processes that appear to be considerably reduced in length. Often, two stout cellular processes emerge from the cell body at opposite poles and, in cortical αBCINs, these neurites are aligned at right angles to the surface. Similarly, the main neurites of amygdalar or claustral αBCINs tended to run counter to each other but with their longitudinal axes lying criss-cross or in every which direction.

Immunoreactions for αB-crystallin either revealed homogeneous somatodendritic distributions of the protein (Fig. 1e, f, i) or exhibited a fine, granular substance with globular and very intensely immunoreactive condensation of the material (Fig. 1h). Occasionally, clear vacuoles of varying sizes and shapes were seen scattered in the neuronal perikarya and in the proximal portions of their cellular processes, with the pale and cap-like immunonegative nuclei regularly assuming a peripheral position within the cell (Fig. 1i, j). In sections stained for lipofuscin granules and basophilic material, the α-BCINs were remark-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◀ **Fig. 1a–k** Parkinson's disease (case 14, Table 1), anteromedial portion of the temporal lobe cut coronally at the level of the uncus. The micrographs show a portion of the anterior mesocortex, including the proneocortical ectorhinal region and periallocortical transentorhinal region. **a** Considerable numbers of αBCINs are seen in infragranular layers of the anterior mesocortex: *ect* ectorhinal region (proneocortex), *cs* collateral sulcus, *tre* transentorhinal region (periallocortex). The frames indicate the position of the micrographs seen in **b** and **c** at higher magnification. **b, c** Note the relatively high packing density of bloated and achromatic αBCINs in the infragranular layers. **d** Co-staining of intraneuronal lipofuscin granules with aldehyde-fuchsin permits recognition of the layers harboring the αBCINs. The αBCINs, seen in this micrograph, are located in layer pri-α of the entorhinal cortex (*arrow*). The lamination pattern of the entorhinal cortex is indicated at the *right margin*. **e** αBCINs often exhibit two radially aligned dendrites emerging from opposite poles of the cell body. **f** αBCIN with relatively evenly distributed immunoreactive material. Note the club-shaped swelling at the tip of one of the basal dendrites. This cellular process is greatly reduced in size. **g** The cellular processes often show irregularly arranged swellings and constrictions. **h** The immunoreactive material is frequently partially condensed into a globular and centrally placed mass. **i, j** Clear vacuoles often are encountered in the cell body and in the proximal dendrites. **k.** αB-crystallin-immunoreactive astrocyte in neocortical layer III. PEG-embedded material, 100 μm, immunoreaction for αB-crystallin. *Bar* in **h** also applies to **i–k.** (*αBCIN* αB-crystallin-immunoreactive neuron)

ably depleted of their Nissl substance but they contained numerous eccentrically positioned lipofuscin granules.

In addition to the highly altered nerve cells, less severely affected αBCINs occurred, albeit in small numbers. A morphological spectrum could be established ranging from immunoreactive neurons of nearly normal appearance to extremely bizarre-looking cells. Neurons with the mildest changes in size and shape were recognizably pyramid-shaped projection neurons possessing apical and basal dendrites that emerged from the somata with conical stems and gradually tapered off distally. Often, an axon was also identifiable as a thread-like cellular process of unvarying diameter, usually aligned radially and headed directly for the white substance. In sections stained for lipofuscin pigment and Nissl material, such cells showed features closely resembling either those of the large pyramidal cells located in layer Vb or those of the multipolar projection neurons in the claustrum and amygdala.

Discussion

In the course of specific neurodegenerative diseases, a few types of nerve cells in select telencephalic cortical areas and subcortical nuclei register characteristic stress responses by up-regulating small HSPs, such as αB-crystallin and/or hsp-27, in their somata and neuritic processes [24]. None of these reactions occur in healthy neurons. The neurons in question mostly appear in the form of achromatic ballooned cells and have been described in detail in cases of PID, AGD, PSP, and CBD [5, 11, 26, 27, 28, 29, 30, 31, 33, 34, 38, 40, 43]. The present study demonstrates their consistent presence in 19 cases with idiopathic PD.

Immunoreactivity to αB-crystallin is a feature common to such neurons in each of the disorders mentioned above. Immunoreations for phosphorylated neurofilaments have frequently been employed with success to identify αBCINs in PID, PSP, and CBD. Notably, the PD-associated αBCINs do not contain conspicuous amounts of phosphorylated neurofilaments in their somatodendritic compartments. This peculiarity corroborates the notion that αBCINs associated with a spectrum of neurodegenerative diseases react differently to the presence of phosphorylated neurofilaments [40]. PD-associated αBCINs, like those occurring in PID [25] and CBD [17], show the absence of immunoreactions with antibodies directed against ubiquitin [43]. The αBCINs that appear in the tauopathy AGD exhibit remarkably large amounts of abnormally phosphorylated tau protein distributed evenly throughout their somatodendritic compartments [40]. A similar co-occurrence of α-SN aggregates is not regularly found in the PD-associated αBCINs.

Most of the Lewy body-bearing nerve cells seen in the vicinities of αBCINs do not show remarkable up-regulation of the HSPs under consideration here, thus suggesting that the existing pathogenic mechanisms for the development of PD-associated αBCINs differ from those underlying the formation of LBs and LNs. At the same time, however, the overall amounts of αBCINs, together with their regional predilection sites in idiopathic PD, appear to overlap with the severity and predilection sites of the PD-specific LBs/LNs. It should be noted in this context that the PD-related neuronal devastation observed in the substantia nigra is always accompanied by extranigral alterations which, in the telencephalon, usually most severely affect the simply organized frontal, insular, and temporal mesocortical transitional zones. From there, the density of nerve cells containing LBs/LNs and α-BCINs decreases in inverse proportion to the evolutionary trajectories of increasing differentiation and hierarchical refinement on the part of the various neocortical areas [6, 8].

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