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Hypocretin (orexin) cell loss in Parkinson's disease*

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Abstract

It has recently been reported that Parkinson's disease (PD) is preceded and accompanied by daytime sleep attacks, nocturnal insomnia, REM sleep behaviour disorder, hallucinations and depression, symptoms which are frequently as troublesome as the motor symptoms of PD. All these symptoms are present in narcolepsy, which is linked to a selective loss of hypocretin (Hcrt) neurons. In this study, the Hcrt system was examined to determine if Hcrt cells are damaged in PD. The hypothalamus of 11 PD (mean age 79±4) and 5 normal (mean age 77±3) brains was examined. Sections were immunostained for Hcrt-1, melanin concentrating hormone (MCH) and alpha synuclein and glial fibrillary acidic protein (GFAP). The substantia nigra of 10 PD brains and 7 normal brains were used for a study of neuromelanin pigmented cell loss. The severity of PD was assessed using the Hoehn and Yahr scale and the level of neuropathology was assessed using the Braak staging criteria. Cell number, distribution and size were determined with stereologic techniques on a one in eight series.

We found an increasing loss of hypocretin cells with disease progression. Similarly, there was an increased loss of MCH cells with disease severity. Hcrt and MCH cells were lost throughout the anterior to posterior extent of their hypothalamic distributions. The percentage loss of Hcrt cells was minimal in stage I (23%) and was maximal in stage V (62%). Similarly, the percentage loss of MCH cells was lowest in stage I (12%) and was highest in stage V (74%). There was a significant increase (P=0.0006, t=4.25, df=15) in the size of neuromelanin containing cells in PD patients, but no difference in the size of surviving Hcrt (P=0.18, t=1.39, df=14) and MCH (P=0.28, t=1.39, df=14) cells relative to controls.

In summary, we found that PD is characterized by a massive loss of Hcrt neurons. Thus, the loss of Hcrt cells may be a cause of the narcolepsy-like symptoms of PD and may be ameliorated by treatments aimed at reversing the Hcrt deficit. We also saw a substantial loss of hypothalamic

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MCH neurons. The losses of Hcrt and MCH neurons are significantly correlated with the clinical stage of PD, not disease duration, whereas the loss of neuromelanin cells is significantly correlated only with disease duration. The significant correlations that we found between the loss of Hcrt and MCH neurons and the clinical stage of PD, in contrast to the lack of a relationship of similar strength between loss of neuromelanin containing cells and the clinical symptoms of PD, suggests a previously unappreciated relationship between hypothalamic dysfunction and the time course of the overall clinical picture of PD.

Keywords

Parkinson; narcolepsy; sleep; hypocretin; orexin; melanin concentrating hormone

Introduction

Sleep disturbances with a prevalence that ranges from 74% to 98% (Parkkinen et al., 2005; Mochizuki et al., 2006) are major problems in Parkinson's disease (PD), often more disturbing than its motor symptoms. Most PD patients have daytime sleep attacks that resemble narcoleptic sleep attacks and that may be increased with the use of dopaminergic agonists, but that also occur independently of these agents (Frucht et al., 1999; Arnulf et al., 2000, 2002; Frucht, 2002; Arnulf, 2006; Rye, 2006; Savitt et al., 2006). Many PD patients have REM sleep at sleep onset (Arnulf et al., 2002; Onofrj et al., 2003). REM sleep behaviour disorder is common in PD (Schenck and Mahowald, 1992; Gagnon et al., 2002) as are hallucinations, some of which have been found to be linked to REM sleep phenomena (Arnulf et al., 2000; Benbir et al., 2006). Recent work has shown that the sleepiness complaints of PD typically precede the motor symptoms and intensify as the disease progresses (Abbott et al., 2005; Dhawan et al., 2006). All of the above symptoms are also characteristic of narcolepsy, suggesting that these symptoms of narcolepsy and PD may have a common cause.

Other symptoms that are common, but not universal, in narcolepsy are also found in PD. Eighty percent of PD patients experience sleep fragmentation resulting from frequent and prolonged awakenings (Askenasy, 2001). This may be exacerbated by the movement disorders of PD but does not appear to be entirely the result of this symptom (Stocchi et al., 1998; Priano et al., 2003; Barone et al., 2004; Grandas and Iranzo, 2004; Arnulf, 2006). The incidence of major depression is markedly elevated in PD. Other chronic diseases are not accompanied by a similar incidence of depression (Frosh, 2006). Disrupted nighttime sleep and depression are also common in narcolepsy (Aldrich, 1998; Siegel, 1999). One element of narcolepsy that appears to be absent in PD is cataplexy.

Human narcolepsy is caused by a loss of hypocretin (Hcrt) neurons (Peyron et al., 2000; Thannickal et al., 2000a; Thannickal et al., 2000b, 2003). Measurement of Hcrt in the CSF of PD patients has produced inconsistent results. Some studies have reported abnormally low levels, whereas others have reported values in the normal range (Mignot et al., 2002; Overeem et al., 2002; Drouot et al., 2003; Yasui et al., 2006). We have reported that Hcrt levels rise by as much as 100% when dogs or cats play, as compared to levels in quiet

waking (Kiyashchenko et al., 2002; Wu et al., 2002). These findings and other similar findings suggest that any reduction in Hcrt level in PD may be secondary to the reduced movement caused by PD, rather than resulting from primary pathology of the Hcrt system. It has been speculated that the loss of dopamine neurons may be responsible for the sleepiness symptoms of PD (Dzirasa et al., 2006), but this does not appear to explain the early onset of these symptoms nor their striking similarity to those of narcolepsy. Only by examining the Hcrt system directly can we determine if Hcrt cells are damaged in PD.

Materials and methods

The hypothalamus of 11 PD (mean age 79±4) and 5 normal (mean age 77±3) brains was examined (Table 1). Details of the sleep quality of the PD patients and controls were not available, although other reports cited above demonstrate that a high percentage of PD patients have sleep abnormalities. Brains were fixed in 10% buffered formalin containing 0.1M phosphate buffer (pH=7.4). The hypothalamus was cut into 40 μm sections. Sections were immunostained for hypocretin (Hcrt-1), melanin concentrating hormone (MCH), alpha synuclein and glial fibrillary acidic protein (GFAP). The substantia nigra of 10 PD brains and 7 normal brains were used for the study of neuromelanin pigmented cell loss. The severity of Parkinson's disease was assessed using the Hoehn and Yahr scale (Hoehn and Yahr, 2001). The level of neuropathology was assessed using the Braak staging criteria (Braak et al., 2003). Cell number, distribution and size were determined with stereology techniques on a one in eight series. All values are reported as mean and SEM. Comparisons were made using the *t*-test.

Hcrt, MCH and alpha synuclein immunohistochemistry

The sections were treated with 0.5% sodium borohydride in PBS for 30 min and washed with PBS, and then incubated for 30 min in 0.5% H_2O_2 for blocking of endogenous peroxidase activity. For antigen retrieval, sections were heated for 30 min at 80°C in a water bath with 10mM sodium citrate (pH 8.5) solution. The sections were cooled to room temperature in sodium citrate and washed with PBS. Water bath heating produces less tissue damage and more uniform antigen retrieval than other heating techniques (Jiao et al., 1999). After thorough washing with PBS the sections were placed for 2 h in 1.5% normal goat serum in PBS and incubated for 72 h at 4°C with a 1 : 2000 dilution of Hcrt-1 (Orexin-A, Calbiochem, San Diego, CA). Sections were then incubated in a secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA) followed by avidin–biotin peroxidase (ABC Elite Kit; Vector laboratories), for 2 h each at room temperature. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction (Vector laboratories). Adjacent series of sections were immunostained for MCH (1 : 20 000, polyclonal rabbit anti-melanin concentrating hormone, Phoenix Pharmaceuticals, Inc., Belmont, CA). Pretreatment and staining was carried out as described for Hcrt staining. Another series of one in twenty-four sections were used for α-synuclein staining (1 : 10000, mouse anti-alpha synuclein monoclonal antibody, Chemicon International, Temecula, CA). Sections were then incubated in a secondary antibody (biotinylated goat anti-mouse IgG; Vector Laboratories) followed by avidin–biotin peroxidase (ABC Elite Kit; Vector

laboratories), for 2 h each at room temperature. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction (Vector Laboratories).

Double immunolabelling

After antigen retrieval treatment, sections immunochemically stained for orexin and α-synuclein were incubated with a mixture of primary antibodies for orexin-A (1 : 2000) and α-synuclein (1 : 10 000) for 72 h at 4°C. After being rinsed, sections were sequentially incubated in biotinylated goat anti-mouse IgG (Vector Laboratories) for α-synuclein or biotinylated goat anti-rabbit IgG (Vector Laboratories) for Orexin A and followed by avidin–biotin peroxidase (ABC Elite Kit; Vector Laboratories) for 2 h at room temperature. The final product of α-synuclein was visualized with nickel-DAB solution (Vector Laboratories). The color of α-synuclein immunohistochemical products was black. The hypocretin immunohistochemical products were visualized with DAB, which had a yellow–brown colour.

GFAP immunohistochemistry

For GFAP staining, sections were immunostained with a 1:2000 dilution of primary polyclonal rabbit anti- GFAP antibody (DAKO, Carpinteria, CA). Antigen retrieval was not required for GFAP staining. After a hydrogen peroxide treatment and blocking serum, the sections were immunostained with GFAP antibody followed by biotinylated goat anti-rabbit secondary antibody, and an avidin–biotin–HRP complex (Vectastain ABC kit, Vector Laboratories). Incubation times were 24 h (at 4°C) for the primary antibody, 30 min (at room temperature) for the secondary antibody, and 1 h (at room temperature) for the avidin–biotin–HRP complex. Sections were treated with the DAB reaction (Vector Laboratories).

Immunohistochemistry in substantia nigra

Substantia nigra of 10 PD brains and 7 neurologically normal brains were used (Table 1). The substantia nigra were cut into 40-μm thick coronal sections. Haematoxylin and eosin (FD Neurotechnologies Inc, Baltimore, MD) staining were used for the identification of neuromelanin pigmented cells. A one in twenty-four series of sections was stained for GFAP and alpha synuclein immunohistochemistry, with the same procedure used for the hypothalamic sections.

Control sections from each brain were processed without the primary antibody and did not show staining. Brain regions and nuclei were identified using the ‘Atlas of the Human Brain’ (Mai et al., 2004). Digital image acquisition was carried out with a Micro Fire camera (Optronics, Goleta, CA) and imported to the Corel Draw program. Contrast and brightness were corrected.

Quantitative analysis

Hcrt and MCH cell number and distribution were determined with stereological techniques on a one in eight series of sections through the complete hypothalamus. We used a Nikon E600 microscope with three axis motorized stage, video camera, Neurolucida interface and Stereoinvestigator software (MicroBrightfield Corp., Colchester, Vermont). To find

out whether alpha synuclein was colocalized with either Hcrt or MCH cells, we used Neurolucida mapping of the double immunolabelled sections.

The density of GFAP cells in the thalamus and posterior hypothalamus was calculated as the number of cells per unit area (mm^2). After delineating the nucleus, we used 250 × 250 μm as the counting frame size for random sampling with stereological procedures. All values of each nucleus were calculated for each subject. These were pooled to give means and SEM for each region and each group.

To calculate the percentage loss of neuromelanin pigmented cells in the substantia nigra, we used Neurolucida mapping of each section stained with haematoxylin and eosin. The numbers of neuromelanin pigmented cells of PD brains were compared with matching sections of normals and the percentage loss was calculated.

The 'nucleator probe' in the Stereology program was used to estimate the mean cross-sectional area of the Hcrt, MCH and neuromelanin pigmented cells. Neurons with a clear nucleus were chosen for analysis. The nucleator probe was used with the optical fractionator and stereology procedures for systematic random sampling to identify cells (Gundersen, 1988). In the sampling results, the volume estimate associated with each cell was displayed, along with the average volume for the group of cells measured. A total of 606 Hcrt cells from normal (n=5) and 702 cells from PD (n=11) were measured. For MCH a total of 1032 (n=5) from normal and 1109 (n=11) from PD were measured. In the case neuromelanin pigmented cells, 1986 cells from normal (n=7) and 1518 cells from PD (n=10) were measured.

Results

Hcrt and MCH cell loss

We found an increasing loss of hypocretin cells with disease progression (Figs 1 and 2) as measured by the Hoehn and Yahr rating scale (Hoehn and Yahr, 2001). Similarly, there was higher loss of MCH cells with disease severity (Figs 2 and 3). Hcrt and MCH cells were lost throughout the A–P extent of their hypothalamic distributions (Fig. 2C). The percentage loss of Hcrt cells was minimal in stage I (23%) and was maximal in stage V (62%). Similarly, the percentage loss of MCH cells was lowest in stage I (12%) and was highest in stage V (74%). There was a significant increase (P=0.0006, t=4.25, df=15) in the size of neuromelanin containing cells in PD patients as has been reported (Cabello et al., 2002), but no difference in the size of surviving Hcrt (P=0.18, t=1.39, df=14) and MCH (P=0.28, t=1.39, df=14) cells relative to control (Fig. 2B).

Distribution of alpha synuclein, gliosis and neuromelanin pigmented cell loss

Alpha synuclein immunostaining showed a pattern of Lewy body formation in different stages of PD (Fig. 4A). We did not see Lewy bodies in surviving Hcrt (Fig. 4B and D) or MCH cells (Fig. 4C and E), but they were present in surviving neuromelanin containing cells of the substantia nigra (Fig. 4F). We hypothesize that these cells either die by a different mechanism than neuromelanin cells or that they die more rapidly, leaving few in an intermediate state to be observed. There was 50–75% loss of neuromelanin pigmented

cells in the substantia nigra (Fig. 5A) compared to control. In the hypothalamus, we saw increasing levels of GFAP with disease progression in PD (Fig. 5B and C).

Clinicopathological correlations

We used the pathological variables (number of Hcrt, MCH and neuromelanin pigmented cells) and the clinical variables (severity and duration of disease) for the correlation study (Table 2). We found an increasing loss of hypocretin cells with disease progression as measured by the Hoehn and Yahr rating scale (Hoehn and Yahr, 2001). Similarly, MCH cell loss was correlated with disease stage but not with disease duration. In contrast, the loss of neuromelanin pigmented cells was not correlated with disease stage but was with disease duration, extending the conclusions of a recent study which showed that alpha synuclein pathology in neuromelanin cells does not correlate well with PD symptoms (Parkkinen et al., 2005). The Braak stages were correlated with percentage loss of neuromelanin pigmented cells, MCH, Hcrt and the Hoehn and Yahr staging (Table 3).

Discussion

The early loss of Hcrt cells may be related to the early appearance of narcolepsy-like signs in PD patients. This loss is occurring prior to the onset of drug treatment in many PD patients. The loss of Hcrt cells may also explain the orthostatic hypotension reported in PD (Hoehn and Yahr, 2001) which parallels the low BP seen in Hcrt null mutant mice (Kayaba et al., 2003) and the abnormal regulation of body temperature that has been reported in both PD (Elliott et al., 1974) and Hcrt null mutant mice (Mochizuki et al., 2006).

The sleepiness experienced by PD patients may not be solely attributable to the loss of Hcrt neurons. It may be at least partially due to the other neurodegenerative changes in PD, including the loss of dopamine, norepinephrine and serotonin neurons (Braak et al., 2003, 2004), all of which have alerting properties (Siegel, 1990; Wisor et al., 2001; Aston-Jones and Cohen, 2005; Siegel, 2005). The role of the loss of MCH cells reported here in the sleepiness of PD is unclear. In contrast to the maximal activity of Hcrt cells in waking (Lee et al., 2005; Mileykovskiy et al., 2005), MCH cells appear to be maximally active in sleep and are reciprocally connected with Hcrt neurons (Verret et al., 2003; Alam et al., 2005; Modirrousta et al., 2005; Torterolo et al., 2006). The loss of MCH neurons in PD may therefore alter the expression of symptoms produced by loss of Hcrt neurons, which are selectively lost in narcolepsy.

If the loss of Hcrt cells is responsible for the symptoms common to both disorders, PD's narcoleptic like symptoms may respond to the same treatments found effective in narcolepsy. Especially promising would be treatment with hypocretin or hypocretin analogs (Stocchi et al., 1998; John et al., 2000, 2003; Siegel and Boehmer, 2006). The significant correlations that we find between the loss of Hcrt and MCH neurons and the clinical stage of PD, in contrast to the lack of a relationship of similar strength between loss of neuromelanin containing cells and the clinical symptoms of PD, suggests a previously unappreciated relationship between hypothalamic dysfunction and the time course of the overall clinical picture of PD (Langston and Forno, 1978; Kremer and Bots, 1993). The demonstrated relation between Hcrt release and mood (Kiyashchenko et al., 2002; Wu et al., 2002; Siegel,

2004; Mileykovskiy et al., 2005; Siegel and Boehmer, 2006) encourages the investigation of therapies targeted at reversing Hcrt dysfunction to treat depression in PD.

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Abbreviations:

GFAP	glial fibrillary acidic protein
Hcrt	hypocretin
MCH	melanin concentrating hormone
PD	Parkinson's disease

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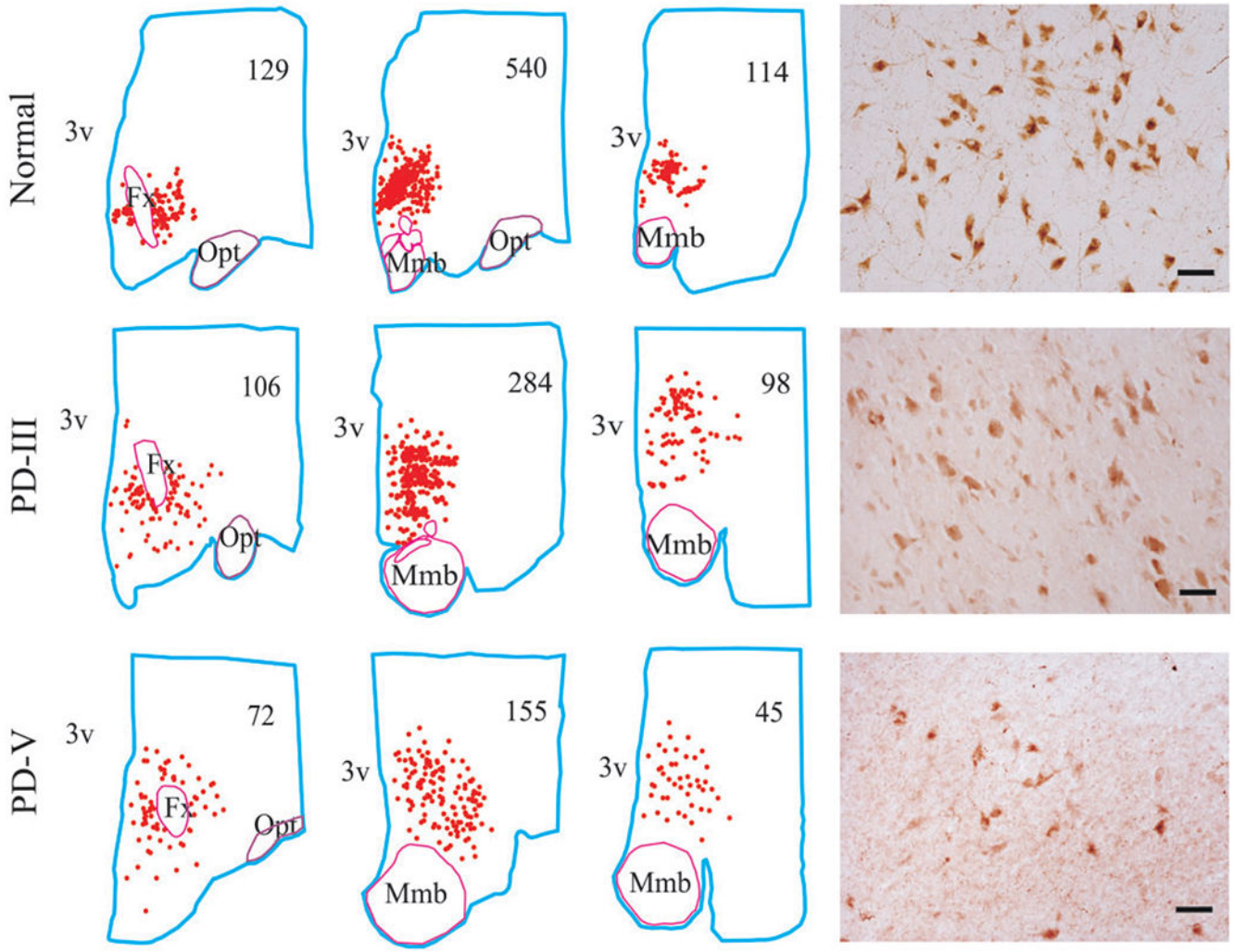

Fig. 1.

Distribution of Hcrt cells in normal and across PD stages. The clinical stages of PD are based on Hoehn and Yahr criteria. The cell distribution and count from a section of anterior, middle and posterior part of the hypothalamus were mapped from a normal, stage III and stage V of PD brains. The cell counts are listed for each section. The number of Hcrt cells is decreased with severity of the disease. 3v—third ventricle, Fx—fornix, Mmb—mammillary body, Opt—optic tract. Scale bars—50 μm.

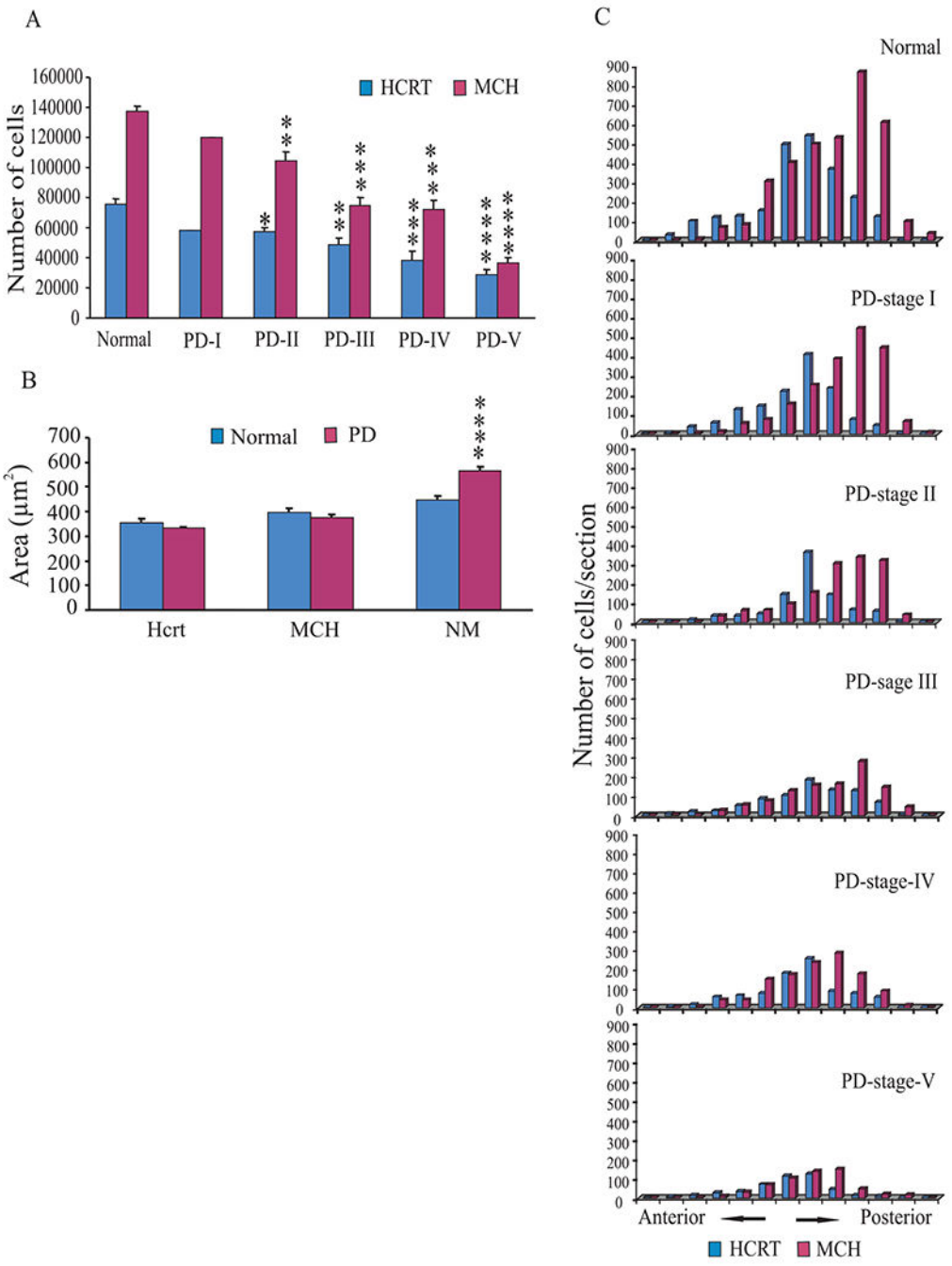

Fig. 2.

Hcrt and MCH pathology in different stages of PD. (**A**) The total number of Hcrt and MCH cells in normal and PD-I, PD-II, PD-III, PD-IV and PD-V. The values are compared to cell numbers in the normal brains. (**B**) The size of the Hcrt, MCH and neuromelanin pigmented cells estimated by nucleator method. Hcrt and MCH cells in PD did not differ in size from those in normal brains. Neuromelanin pigmented cells showed hypertrophy (27%) compared with normal cells. (**C**) Hcrt and MCH cells were mapped in individual sections from anterior to posterior hypothalamus with 1200 μm section interval. One brain from a normal and

one from each stage (Hoehn and Yahr, I–V) of PD were used for Neurolucida mapping. There was a generalized loss of Hcrt and MCH cells with severity of the disease. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Student's t-test.

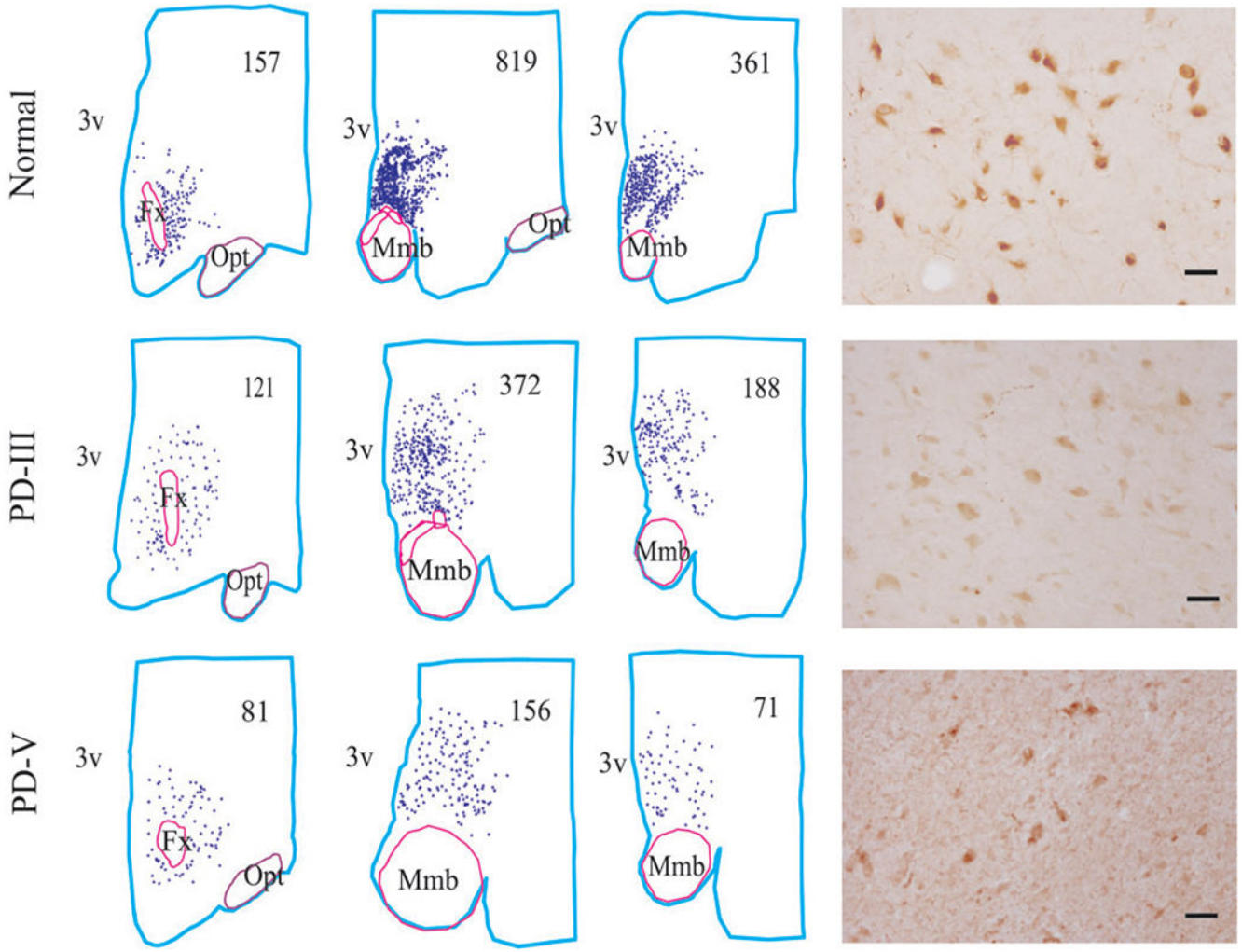

Fig. 3.

Distribution of MCH cells in normal and Parkinson stages. Cell counts are listed in each section. The number of MCH cell was decreased with severity of the disease. The abbreviations are same as in Fig. 1. Scale bars—50 μm.

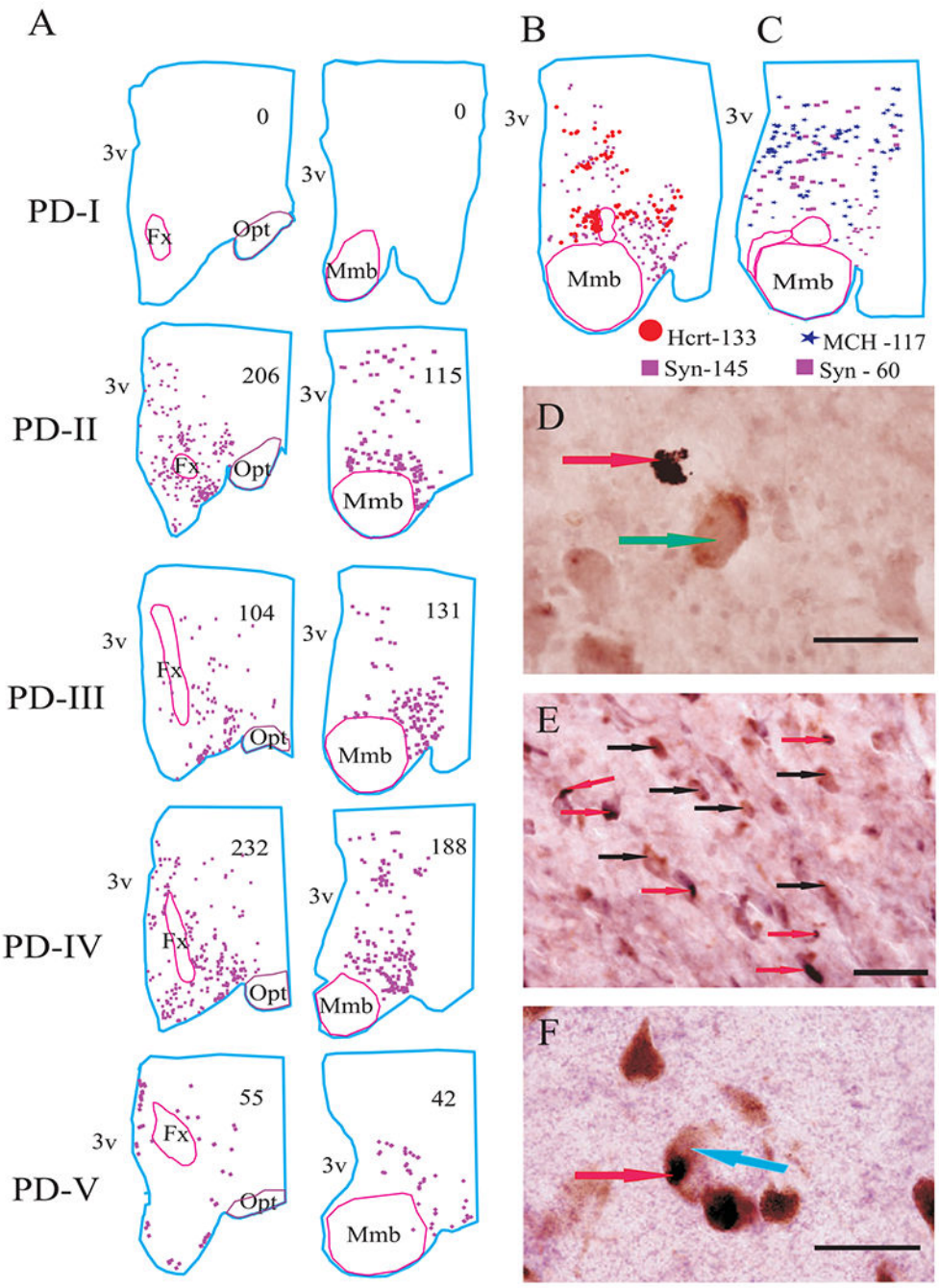

Fig. 4.

Distribution of alpha synuclein in the hypothalamus in different stages of PD. (**A**) Neurolucida mapping of alpha synuclein in PD stages with single immunostaining. (**B**) Mapping of Hcrt and alpha synuclein in double-labelled section. (**C**) Mapping of MCH and alpha synuclein in double-labelled section. Alpha synuclein was not colocalized with Hcrt and MCH cells (**D** and **E**), but it was colocalized with neuromelanin pigmented cells in substantia nigra (**F**). Arrows: red—alpha synuclein, green—Hcrt cell, black—MCH cells and blue—neuromelanin pigmented cell. Scale bars—50 μm.

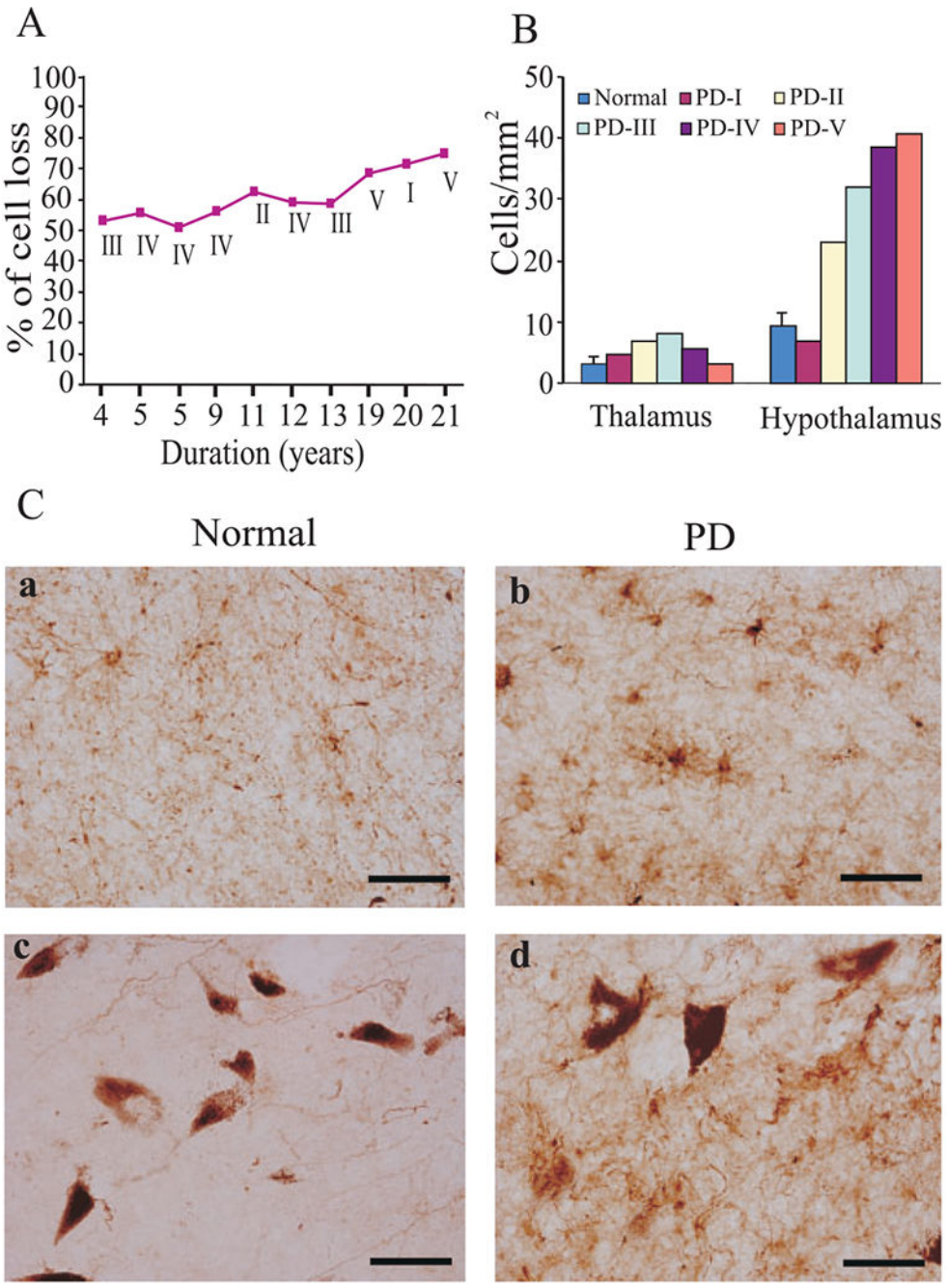

Fig. 5.

Gliosis and neuromelanin pigmented cell loss in PD. (**A**) The percentage loss of neuromelanin pigmented cell loss in the substantia nigra was correlated with duration of the disease. (**B**) The number of glial fibrillary acidic protein-labelled astrocytes (GFAP) in the thalamus and posterior hypothalamus. (**C**) GFAP in the hypothalamus of normal (a) and PD (b). GFAP density in the substantia nigra of normal (c) and PD (d) brain. The number of GFAP-labelled astrocytes were increased with severity of the disease. Scale bars—50 μm.

Table 1

Clinical data of Parkinson's and control subjects, and characteristics of Hcrt and MCH cells

Subjects	Age	Sex	No of Hcrt cell	Hcrt cell size area (μm^2)	No of MCH cells	MCH cell size area (μm^2)	Clinical diagnosis				
Controls											
C–A	61	M	65833	408.48	131616	409.15	Pneumonia, testicular tumor				
C–B	78	M	68932	312.41	123332	407.95	Non neuronal				
C–C	81	F	83330	355.67	138425	388.65	Cerebrovascular				
C–D	85	F	79219	309.71	130840	346.69	Cancer — adenocarcinoma				
C–E	82	M	80632	379.42	137325	436.34	Cerebrovascular				
C–F	73	F	only substantia nigra				Breast cancer				
C–G	80	M	only substantia nigra				Cancer – renal				
Parkinson's								Clin. Stage (Hoehn and Yahr, 2001)	Path. Stage (Braak et al., 2003)	Duration (years)	Medications
PD-A	85	F	58124	340.07	120250	338.30	PD	I	3	20	n/a-
PD-B	68	M	59728	347.44	98924	336.85	PD, dysphagia, dementia	II	4	23	Sinemet, bromocriptine, dopamine.
PD-C	77	M	55505	318.68	110555	392.45	PD, Alzheimer's	II	3	18.5	bromocriptine, Sinemet, Parlodel.
PD-D	70	F	53374	347.39	79582	440.46	PD	III	3	4	Sinemet
PD-E	81	M	44266	306.61	69732	373.01	PD	III	4	13	Sinemet, Parlodel
PD-F	62	M	46800	304.49	71466	335.61	PD	IV	3	5	n/a
PD-G	90	M	45600	360.45	77600	413.50	PD, Alzheimer's	IV	4	5	Sinemet
PD-H	97	F	39642	297.49	62329	330.46	PD, basilar vasculature	IV	4	12	Sinemet
PD-I	62	M	29716	335.98	78571	341.44	PD	IV	4	9	Sinemet, Permax
PD-J	103	F	25866	342.41	32400	401.08	PD	V	6	21	n/a
PD-K	67	M	31742	345.47	40176	415.48	PD, strokes	V	5	19	Sinemet, Eldepryl, Requip, Mirapex, Provigil
PD-L	86	F	only substantia nigra				PD	II	n/a	11	Sinemet, Permax, Eldepryl

Note: Clin.=clinical, Path.=pathological.

Table 2

Correlation analysis of Hcrt, MCH and Neuromelanin pigmented cell loss in PD with clinical stages (Hoehn and Yahr), duration and % cell loss

Correlations	*r*	*P*
% loss of cells versus PD stages		
Hcrt cells and PD stages	0.87	0.0005
MCH cells and PD stages	0.96	0.0001
NM cells and PD stages	0.03	0.94
% loss of cells versus PD duration		
Hcrt cells and PD duration	0.35	0.28
MCH cells and PD duration	0.04	0.90
NM cells and PD duration	0.92	0.002
% loss of cells versus% loss of cells		
Hcrt cells and NM cells	0.45	0.60
MCH cells and NM cells	0.25	0.58
Hcrt cells and MCH cells	0.83	0.001

Note: *r*=correlation; *P*=significance; NM=neuromelanin pigmented cells.

Table 3

Correlation analysis of pathological stages (Braak *et al.*) in PD with Hcrt, MCH and Neuromelanin pigmented cell loss and duration

Correlations	***r***	***P***
% loss of Hcrt cells and pathological stages	0.56	0.06
% loss of MCH cells and pathological stages	0.78	0.004
% of NM cells and pathological stages	0.86	0.001
Duration and pathological stages	0.53	0.09
Duration and clinical stages	0.09	0.78
Pathological stages and clinical stages	0.71	0.01

Note: *r*=correlation; *P*=significance; NM=neuromelanin pigmented cells.